Exploring the functional interactions between geminivirus and host

Tesis Doctoral

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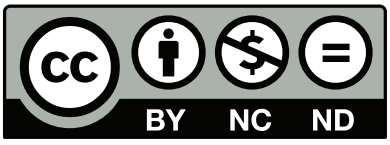

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Que Doña Tábata Victoria Rosas Díaz, ha realizado bajo nuestra dirección y supervisión, en la Universidad de Málaga, el trabajo que bajo el título "Exploring the functional interactions between geminivirus and host" presenta en esta memoria, la que constituye su tesis doctoral para aspirar al grado de Doctor en Biología.

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A mi familia,

A mis almas gemelas

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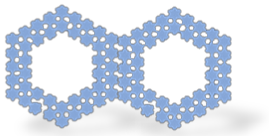
General Introduction



GEMINIVIRUSES: LIMITED INFORMATION WITH HUGE IMPACT

Distinguishing features of geminiviruses

Geminiviruses are insect-transmitted plant viruses with circular, single-stranded (ss) DNA genomes that cause some of the most economically important diseases in vegetable and field crops worldwide. By causing huge losses on food and cash crops, such as cassava, tomatoes, grain legumes, vegetables, maize and cotton, geminiviruses represent a new threat to global food security and sustainability (Figure 1A) (Mansoor et al., 2003; Rojas et al., 2005; Briddon, 2009).

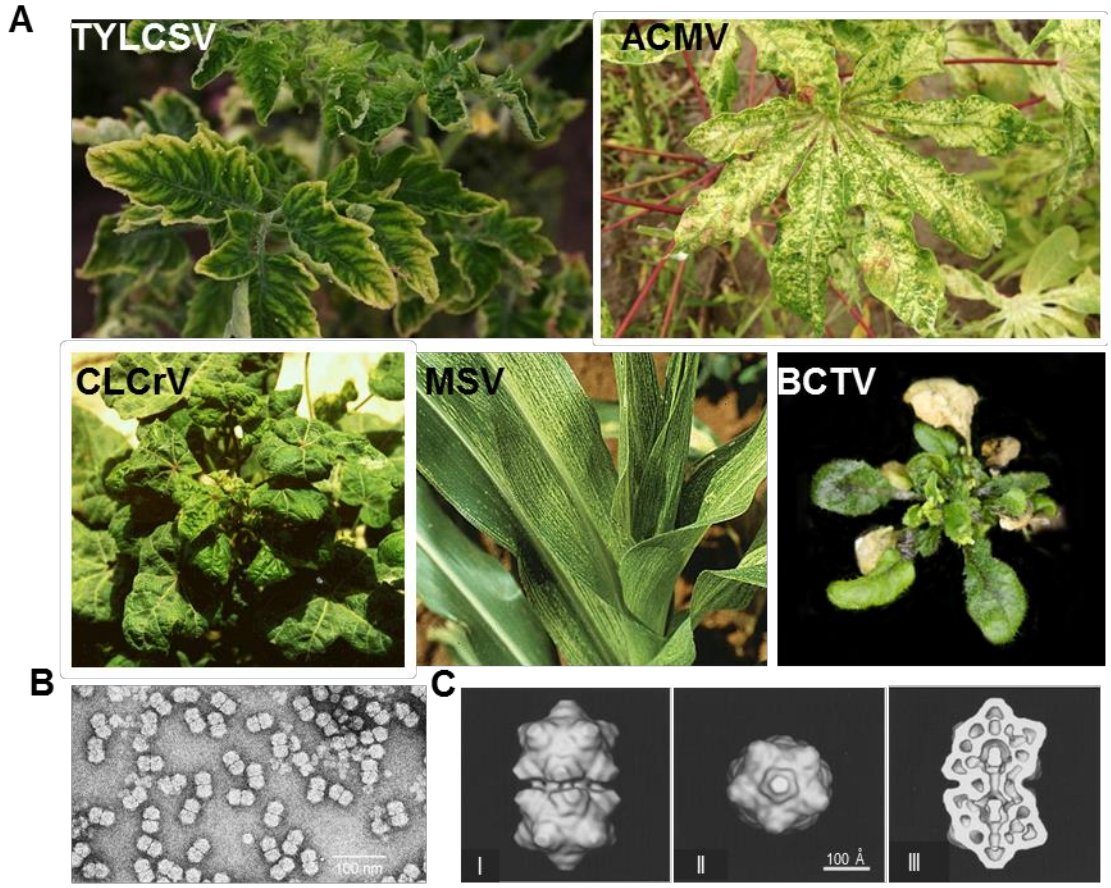


Figure 1. Geminivirus symptoms and geminiviral virion particles. A) Symptoms of geminiviruses infection in different hosts: *Tomato yellow curl leaf Sardinia virus* (TYLCSV*)* in tomato, *African cassava mosaic virus* (ACMV) in cassava, *Cotton leaf crumple virus* (CLCrV) in cotton, *Maize streak virus* (MSV) in maize and *Beet curly top virus* (BCTV) in *Arabidopsis* plants. **B)** Electron micrograph of Geminivirus (MSV) virion particles (taken from ICTVdB). **C)** Models resembling the typical structure of a geminiviral virion particle. These models were generated *in silico* from cryo-electron microscopy. Longitudinal (I) and transversal (II) views, and longitudinal section (III). (taken from Zhang et al., 2001)

Geminiviruses possess a genome comprised of one or two circular ssDNA molecules, each of which is ~2.5–3.0 kb. This is among the smallest known genome for an independently replicating virus (Rojas et al., 2005). A distinguishing feature of geminiviruses is their twinned icosahedral virions (diam. ~18 nm, length 30 nm) from which the family derives its name (from the Latin "geminus", meaning twin) (Figure 1B, 1C) (Zhang et al., 2001; Bottcher et al., 2004; Krupovic et al., 2009).

Members of the Geminiviridae family utilize bidirectional transcription and overlapping genes for efficient coding of proteins. All geminiviruses also carry one or more intergenic regions (IRs), one of which contains the origin of replication and the signature stem-loop structure containing an invariant nonanucleotide motif involved in rolling circle replication (Figure 2). Geminiviruses encode only six to eight proteins, and this limited 'armoury' has to be enough to infect the host plant. For this, they rely heavily on host cellular machineries and interact with a wide range of plant proteins and processes during infection. Geminiviral proteins are small in size, ranging approximately from 80 to 360 amino acids. Moreover, these proteins are multifunctional and, at the same time, different proteins encoded by the same viral genome may affect similar processes. For example, geminiviruses encode multiple silencing suppressors that interfere with plant small interfering RNA (siRNA) production and alter plant DNA methylation and microRNA (miRNA) pathways, often causing developmental abnormalities (reviewed in (Raja et al., 2010; Hanley-Bowdoin et al., 2013)).

In fundamental research, geminiviruses have become popular instruments to analyze the molecular biology of plant gene regulation and cell–to-cell communication. Due to their small genome size, studying DNA replication, transcription, mRNA processing, protein expression, and gene silencing using geminiviruses as models is easier than with their host counterparts (Jeske, 2009).

Family Geminiviridae and its genomic organization

In addition to the basic properties mentioned above, members of the family Geminiviridae exhibit diversity in terms of their genome structure, sequence, host range, tissue tropism, and insect vectors. Based on these properties, geminiviruses have been classified into seven genera—*Begomovirus, Mastrevirus, Curtovirus, Becurtovirus, Eragrovirus, Topocuvirus and Turncurtovirus* (Table 1) (Brown JK, 2012; Varsani et al., 2014). This level of diversity suggests a long evolutionary history and a remarkable flexibility in genome evolution. Among the seven genera three of them encompass most of the viral known species: *Mastrevirus, Curtovirus* and *Begomovirus.* Members of the Mastreviruses are transmitted by leafhoppers, have a single genome component, infect both monocotyledonous and dicotyledonous plants, and are found primarily in the Old World.

Curtoviruses are also transmitted by leafhoppers and have one genomic DNA, but infect only dicots in the New World. Begomoviruses, which constitute the largest genus, are transmitted by whiteflies and are found in the Old and New World; they can have monopartite or bipartite genomes. A large number of monopartite Begomoviruses have been shown to be associated with ssDNA satellites known as betasatellites, which are pathogenicity-determinant molecules completely dependent of the geminivirus for their replication, encapsidation, and transmission (Navas-Castillo et al., 2011).

The following section will be focused on the *Begomovirus* and *Curtovirus* genera, due to the relevance of these two genera for this thesis.

Genus	Type member	Host range	Vector	Genome
Mastrevirus	*Maize streak virus* (MSV)	Monocots (and few dicots)	Leafhopper (Fam. Cicadellidae)	Monopartite
Curtovirus	*Beet curly top virus* (BCTV)	Dicots	Leafhopper (Fam. Cicadellidae)	Monopartite
Begomovirus	*Bean golden mosaic virus* (BGMV)	Dicots	Whiteflies (*Bemisia tabaci*)	Mono- or bipartite
Topocuvirus	*Tomato pseudo-curly top virus* (TPCTV)	Dicots	Treehopper (Fam. Membracidae)	Monopartite
Becurtovirus	*Beet curly top Iran virus* (BCTIV)	Dicots	Leafhopper (*Circulifer haematoceps*)	Monopartite
Turncurtovirus	*Turnip curly top Virus* (TCTV)	Dicots	Leafhopper (*Circulifer haematoceps*)	Monopartite
Eragrovirus	*Eragrostis curvula streak virus* (ECSV)	Monocots	Unknown	Monopartite

Table 1. Currently recognized genera of the Geminiviridae family, their type members and properties.

Begomoviruses

Begomoviruses have either monopartite or bipartite genomes (Figure 2, Table 1), are transmitted primarily by the whitefly *Bemisia tabaci*, and infect a wide range of dicots in the Old and New World. The bipartite *Begomovirus* genome is composed of two DNA molecules of similar size (DNA-A and DNA-B) that share no sequence similarity, except for a highly conserved common region (CR) of approximately 200 nucleotides. This CR that corresponds to the IRS, contains the regulatory sequences needed for replication and transcription. The DNA-A component contains four open reading frames (ORFs), encoding proteins required at least for replication *AL1* (also named *Rep* or *AC1*), *C3* (also named *REn*, *AL3* or *AC3*), transcription *C2* (also named *Trap*, *AL2*, or *AC2*) and encapsidation (CP). DNA-B contains two ORFs, a viral movement nuclear shuttle protein (NSP) and movement protein (MP), which mediate viral DNA movement into and out of the nucleus and between cells. DNA-A and DNA-B components are individually encapsidated and both of them are

essential for the accomplishment of the infection (Rojas et al., 2005; Jeske, 2009). The monopartite *Begomovirus* genome is homologous to the DNA-A component of the bipartite members. The complementary-sense strand contains four genes, *C1* (or *Rep*), *C2* (or *TrAP*), *C3* (or *REn*) and *C4,* while two ORFs are contained in the virion-sense strand, the precoat or *V2* and the capsid protein (*CP*).

The CP forms the viral capsid and mediates vector transmission (Briddon et al., 1990; Wartig et al., 1997; Noris et al., 1998). Consistent with its role in encapsidation of virion particles, the CP is localized to the nucleus, via an N-terminal nuclear localization signal (NLS) and the interaction with the importin-alpha machinery (Kunik et al., 1998; Kunik, 1999). The CP has been involved in viral movement and determines the specificity for insect vector transmission (Rojas et al., 2001) (Rojas et al., 2005).

The V2 protein functions as a post-transcriptional gene silencing (PTGS) suppressor (Glick et al., 2008; Luna et al., 2012; Zhang et al., 2012); together with CP, V2 also provides the movement function (Poornima Priyadarshini CG, 2011). On the other hand, V2 is a determinant of virulence and hypersensitive response (Mubin et al., 2010; Sharma and Ikegami, 2010).

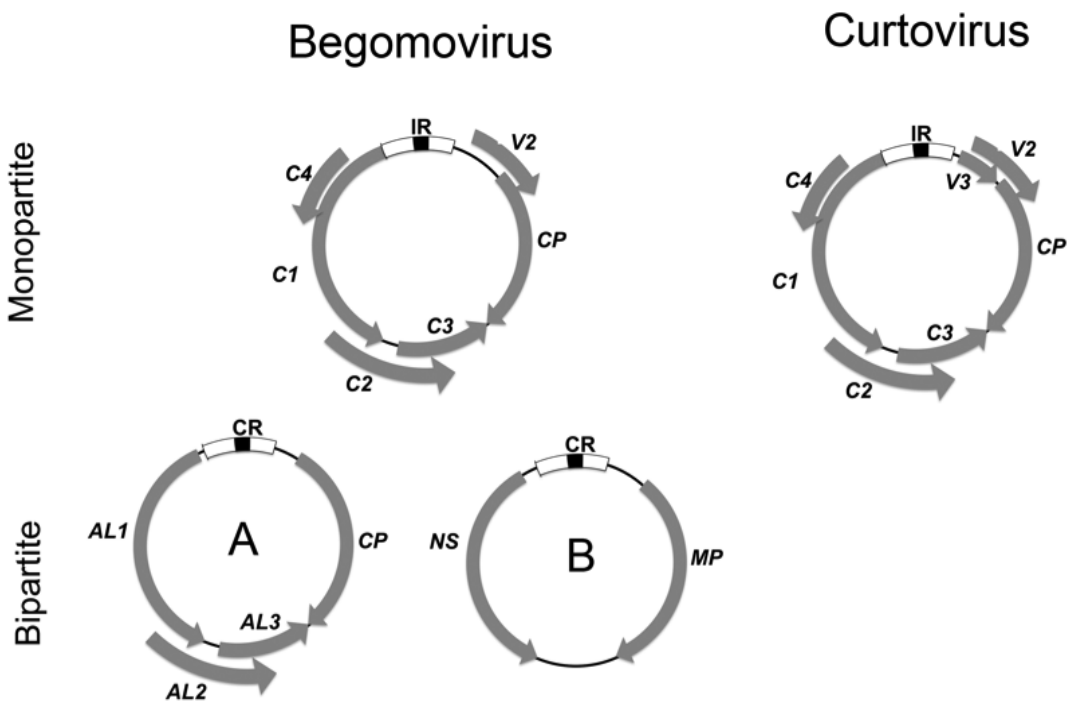


Figure 2. Genome organization of begomoviruses and Curtoviruses.

Rep is a multifunctional protein that initiates and terminates the virus-strand replication, binding to a DNA sequence motif located in the stem loop of the IR, and has been assigned DNA helicase activity (Clerot and Bernardi, 2006; Choudhury et al., 2006). It has been proposed that Rep mediates replication not only by binding viral DNA, but also by inducing a cellular environment suitable for this process, most likely through the interaction with cellular components. Besides interacting with itself and C3 (Settlage et al., 1996), remarkably C3 interaction with Rep enhance the Rep mediated ATPase activity (Pasumarthy et al., 2010), Rep interacts with a plethora of host proteins, including the proliferating-cell nuclear antigen, PCNA (Castillo et al., 2003; Bagewadi et al., 2004), the plant homologue to retinoblastoma, pRBR (for retinoblastoma-related; (Xie et al., 1995), the replication factor C (Luque et al., 2002), the histone 3 (Kong and Hanley-Bowdoin, 2002), a mitotic kinase (Kong and Hanley-Bowdoin, 2002), the GRIK kinase (Selth et al., 2005), and the SUMO-conjugating enzyme (SCE1), a regulator of the cellular homeostasis (Castillo et al., 2004; Sánchez-Durán et al., 2011). More recently, it has been described that Rep protein is a transcriptional gene silencing (TGS) suppressor that modifies the plant epigenome, demonstrating that this pathogenicity factor plays a role in the infection process that goes beyond DNA replication (Rodriguez-Negrete et al., 2013).

C2 is a nuclear multifunctional protein (van Wezel et al., 2001; Sharma et al., 2010) that appears to function in transactivation: C2 acts as a transcription factor required for the expression of viral genes needed at late times in infection, like the CP (Sunter and Bisaro, 1991; 1992), and also triggers transactivation of host genes (Trinks et al., 2005). Moreover, C2 is also a pathogenicity factor and a PTGS and TGS (Transcriptional gene silencing) suppressor (reviewed in (Raja et al., 2010; Hanley-Bowdoin et al., 2013)) and compromises the ability of the CSN (COP9 signalosome), a protein complex that functions in the ubiquitin–proteasome pathway, to modify Cullin-1 (CUL1), which is an essential component of the SCF (SKP1, CUL1/CDC53, F box proteins) ubiquitin E3 ligase complex. C2-COP9 interaction alters the cellular processes regulated by SCF complexes, including jasmonate (JA) signalling (Lozano-Durán et al., 2011a).

C3 is also a nuclear protein (Nagar et al., 1995; Selth et al., 2005) , which has been described as an enhancer of viral replication, although it is not essential for this process. As previously mentioned, C3 interacts with Rep, most likely promoting viral replication (Settlage et al., 1996; Pasumarthy et al., 2010). In addition, C3 also binds pRBR and PCNA, consistent with a role in replication and cell cycle modification (Settlage et al., 2001; Castillo et al., 2003).Furthermore, C3 also has been shown to interact with SlNAC1, a tomato member of the plant NAC domain superfamily of transcription factors (Selth et al., 2005).

C4 is a pathogenicity determinant localized to the plasma membrane (Sharma *et al.*, 2010) that has been assigned a role in viral movement (Rojas et al., 2001) and suppression of PTGS (Gopal et al., 2007; Dogra et al., 2009; Raja et al., 2010; Xie et al., 2013).

Curtoviruses

The curtoviruses have a monopartite genome, are transmitted by the beet leafhopper *Circulifer tenellus*, and are found in the Old and New World (Table 1). Unlike begomoviruses, most curtoviruses have evolved the capacity to infect a wide range of hosts. Their genome contains seven ORFs (CP, V2 and V3 in the virion-sense strand and Rep, C2/L2, C3/L3 and C4/L4 in the complementary-sense strand) and an IR carrying the origin of replication (Figure 2).

As in other geminiviruses, the CP is a multifunctional protein with structural function further implicated in systemic infection and insect transmission (Rojas et al., 2005). V2 modulates levels of ss-and ds-DNA and mutants have highly attenuated symptom phenotypes; it remains to be established whether this reflects a role in replication or movement (Rojas et al., 2005). V3, a protein specific to *Curtovirus* genus, is involved in viral movement (Rojas et al., 2005). A genetic analysis of *Beet curly top virus* (BCTV) genes showed that the V3 encodes the MP (Hormuzdi and Bisaro, 1995), which forms vesicle-like structures that co-localize with the endoplasmic reticulum (ER) and are trafficked intracellularly from the nucleus to the cell periphery (Soto, 2001). The significance of these vesicles remains to be established; however, ER-associated cytoplasmic inclusions and vesicle-like structures were described in pioneering ultrastructural studies of curtovirus infected cells (Esau and Magyarosy, 1979).

As in begomoviruses, Rep is essential for viral DNA replication, whereas C3/L3 functions as an enhancer (Jeske, 2009). C2/L2 has been described to act as a pathogenicity determinant and a suppressor of TGS (Wang et al., 2005; Baliji et al., 2007; Raja et al., 2010). Additionally curtovirus C2/L2 promotes viral replication, probably by restoring the DNA replication competency of the infected cells (Caracuel et al., 2012).

C4/L4 localizes to the plasma membrane (Piroux et al., 2007) and is a major symptom determinant: it alters plant development, probably through the disruption of multiple hormonal pathways (Mills-Lujan and Deom, 2010), and leads to hyperplasia of the phloem cells (Latham, 1997), most likely through the induction of RPK, a RING ubiquitin E3 ligase (Lai et al., 2009). Additionally, C4/L4 is a suppressor of gene silencing (Raja et al., 2010) and is involved in movement (Teng et al., 2010). Interestingly, even though both begomovirus and curtovirus C4/L4 elicit a disease phenotype when expressed ectopically in plants, they exhibit little sequence similarity, which has led to the hypothesis that they are analogues rather than homologues. The mode of action by which C4

induces cell division has yet to be determined; however, it may serve a critical role in amplifying the number of permissive cells available for replication (Rojas et al., 2005). Another consequence would be enhanced delivery of infectious DNA into the phloem for long-distance movement and vector acquisition (Rojas et al., 2005).

Geminivirus life cycle

A number of key evolutionary developments opened the door for the adaptation of geminiviruses to their plant hosts. This process involved several steps, of which three were of paramount importance: replication, movement, and suppression/evasion of host defence responses. In the following sections, recent developments in these areas are examined in terms of how geminiviruses found and exploited “chinks in the plant’s armory”.

Replication

After injection into phloem cells by the insect’s stylet, geminivirions can move inside the sieve elements; however, they are not able to replicate therein, because these cells do not contain nuclei. For further propagation geminiviruses need to enter nucleus-containing phloem cells, like companion and phloem parenchyma cells (reviewed in (Jeske, 2009)).

After having entered the nucleus, geminiviruses establish viral replication. First the ssDNA molecule must be replicate to form dsDNA by an unknown mechanism. Next, geminiviruses replicate by a rolling circle replication (RCR) mechanism that involves dsDNA replicative form (RF) intermediates, although some recombination-dependent replication (RDR) also occurs (reviewed in (Jeske, 2009)). To initiate the RCR, the begomovirus Rep binds to the Rep complex binding site, which contains a directly repeated sequence between the TATA box and the transcription start site (Fontes et al., 1992). The dsDNA RF molecules are templates for replication and transcription and become associated with cellular histone proteins to form viral minichromosomes (Pilartz and Jeske, 1992; 2003), which could carry marks associated with repressive or active chromatin (Raja et al., 2008). Viral minichromosomes remain extrachromosomal and serve as templates for transcription, further replication and finally the production of progeny ssDNA that is encapsidated by the CP again (Jeske, 2009). The infection is propagated inside the plant by the movement of viral DNA out of the nucleus and into the next cell or the phloem through the action of two viral movement proteins (Figure 3) (reviewed in (Rojas et al., 2001; Hanley-Bowdoin et al., 2013)).

Similarly to many other small DNA viruses, geminiviruses do not encode their own DNA polymerases and instead depend on host polymerases and associated factors (together termed the host replisome) for viral DNA synthesis during the elongation step (Hanley-Bowdoin et al., 2004). In

healthy plants, the availability of the host replisome is tightly regulated by cell cycle and developmental controls, which must be reprogrammed before geminiviruses can replicate their genomes. Rep, the only viral protein that is essential for replication, is likely to have a key role in the recruitment and assembly of the viral replisome, a complex that includes viral proteins and host factors involved in DNA replication, repair and other nuclear functions (reviewed in (Hanley-Bowdoin et al., 2013)). The viral replication enhancer protein C3/L3, which greatly enhances begomovirus and curtovirus DNA accumulation and interacts with Rep and host replication factors (Settlage et al., 2005), is also likely to be part of the viral replisome.

Geminiviruses spread: movement

Once geminiviruses gained control over the requisite DNA replication machinery, the next challenge involved moving out of the initially infected cells. Plant cells are usually connected by cytoplasmic bridges through plasmodesmata (PD). As a consequence of their limited coding capacity, geminiviral-encoded transport-mediating proteins have to interact with a variety of plant factors involved in macromolecular trafficking to overcome cellular boundaries and transfer viral DNA (vDNA) from a nucleus through the cytoplasm and via PD into an adjacent cell and into the nucleus of that cell (Figure 3).

As mentioned above, bipartite begomoviruses encode two proteins, NSP and MP, which mediate the viral transport processes (Rojas et al., 2005). Several studies provided evidence that NSP facilitates trafficking of vDNA into and out of the nucleus, and that MP serves as a membrane adaptor and mediates cell-to-cell transfer via PD as well as long-distance spread through the phloem (Rojas et al., 2005; Krichevsky et al., 2006; Wege and Pohl, 2007; Jeske, 2009). Two models are currently suggested for the role of NSP and MP during cell-to-cell transport of bipartite geminiviruses. The “couple-skating” model suggests that MP binds the NSP/vDNA complex at the cytoplasmic side of plasma membranes or microsomal vesicles, and transfers the nucleoprotein complex into the next cell either along the plasma membrane or via the ER that spans the PD (Sanderfoot and Lazarowitz, 1995; Zhang et al., 2002; Hehnle et al., 2004; Frischmuth et al., 2007). In contrast, the “relay race” model predicts that after NSP-mediated nuclear export the vDNA is taken over by MP, which then transports the vDNA into the adjacent cell (Noueiry et al., 1994; Rojas et al., 1998; Rojas et al., 2001). Less is known about the movement proteins of monopartite geminiviruses, in which CP acts as the NSP, whereas MP function is mediated by V2 alone or in a complex with C4 (Rojas et al., 2001).

Independent of the transport model, the begomoviral MPs have to mediate multiple functions during intra and intercellular trafficking. The identification of three phosphorylation sites in the begomoviral

MP, which have an impact on symptom development and/or vDNA accumulation (Kleinow et al., 2009), indicates regulation of diverse MP functions by yet unknown host kinases. Currently, three interacting host factors of begomoviral MPs have been identified: a histone H3 (Zhou *et al*., 2011), a synaptotagmin, SYTA (Lewis and Lazarowitz, 2010), and a chaperone, the heat shock cognate 70 kDa protein cpHSC70-1 (Krenz et al., 2012) .

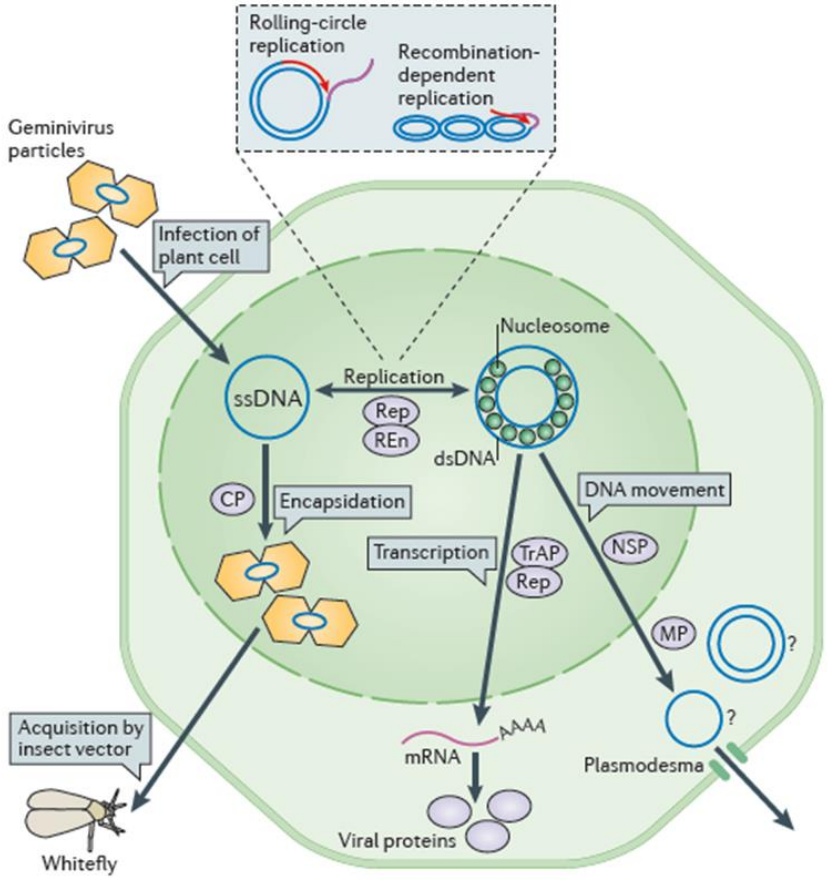


Figure 3. The begomovirus life cycle. Infection begins in a plant cell when viral single-stranded DNA (ssDNA) is released from virions and copied to generate double-stranded DNA (dsDNA). The dsDNA, which assembles with nucleosomes, is transcribed by host RNA polymerase II, allowing production of replication initiator protein (Rep). Rep initiates rolling-circle replication by introducing a nick into a viral dsDNA molecule to generate a free 3′-hydroxyl end that primes ssDNA synthesis, leading to displacement of the parental strand (inset). The released ssDNA is converted to dsDNA to re-enter the replication cycle. Viral replication transitions to recombination-dependent replication, which is initiated by homologous recombination between a partially replicated ssDNA and a closed, circular dsDNA to form a looped molecule that serves as a template for both ssDNA and dsDNA synthesis (inset). Later in infection, Rep represses its own transcription, leading to activation of transcriptional activator protein (TrAP) expression, which in turn activates coat protein (CP) and nuclear shuttle protein (NSP) expression. Circular ssDNA can then be encapsidated by CP into virions, which are available for whitefly acquisition. NSP binds to viral DNA and moves it across the nuclear envelope, where movement protein (MP) traffics it across a plasmodesma. It is not known whether viral DNA moves as ssDNA versus dsDNA or as a linear versus a circular molecule (taken from Hanley-Bowdoin et al., 2013).

Geminiviruses hijack their host cells

Host DNA replication machinery

As mentioned above, both Rep and C3 bind to proliferating cell nuclear antigen (PCNA) (Castillo et al., 2003; Bagewadi et al., 2004), the processivity factor for host DNA polymerase-δ. PCNA is highly conserved across eukaryotes and interacts with a variety of proteins involved in cell cycle regulation, DNA replication and DNA repair. Rep binds to RAD54, which is involved in homologous recombination and might have a role in viral replication mediated by recombination-dependent replication (Kaliappan et al., 2012). In addition, begomovirus Rep (Ach et al., 1997; Kong et al., 2000) and the Replication-associated protein A (RepA), which is translated from a Rep transcript in mastreviruses (Xie et al., 1996; Horvath et al., 1998; Liu et al., 1999), bind to plant homologues of the cell-cycle regulator retinoblastoma (plant retinoblastoma-related, pRBR). By analogy with mammalian DNA viruses (Weinberg, 1995), these interactions may bypass a pRBR phosphorylation requirement for cell-cycle entry and G1 progression during geminivirus infection. Rep also interacts with the replication factor C, a subunit of the heteropentameric RFC clamp loader which stimulates the loading of the replication machinery to the template, and directs the binding of this RFC subunit to the Rep binding site (Luque et al., 2002). RFC components, including the replication factor C, are subjected to cell-cycle regulation, with a peak of expression during S-phase (Luque et al., 2002). According to its expression pattern, Rep must be also promoting the expression of this gene in infected, terminally differentiated cells.

Host transcription

Another example of geminiviral manipulation of the host cell is the activity of C2 in activating the expression of both viral and host genes. In begomoviruses, C2 has been described to act as a transcription factor needed for the expression of late viral genes (Sunter and Bisaro, 1991; 1992). C2, however, does not possess transcription factor activity per se, since it does not bind DNA either strongly or in a sequence-specific manner (reviewed in (Fondong, 2013)). It was demonstrated that C2 is able to interact with the plant transcription factor PEAPOD2 (PPD2), which binds the DNA sequences responsible for activation of the CP expression in mesophyll cells (Lacatus and Sunter, 2009). In infected cells, a C2/PPD2/CP promoter complex would form, leading to the expression of the viral CP. In conclusion, this finding indicates that C2 is able to address itself to its target DNA sequence through the interaction with a plant protein, highlighting the potential of C2 to manipulate plant proteins to exert its viral functions. Since this interaction cannot explain all the transactivation functions of C2, it would be expectable that new interactions with other elements of the transcriptional machinery remain to be discovered. Furthermore, C2 also activates transcription of

certain genes in the host cell (Trinks et al., 2005), suggesting a role in creating a cell environment favorable for the virus, but the mechanism underlying this effect is still elusive.

Ubiquitination and ubiquitination-like pathways

Protein modifications by ubiquitin and ubiquitin-like proteins are post-translational modification (PTMs) that modulate protein function and regulate many plant processes, including development, cell cycle and responses to abiotic and biotic stresses (reviewed in (Castro et al., 2012; Marino et al., 2012)). Ubiquitin is covalently linked to lysine residues in the target protein through an enzymatic cascade comprising an E1 ubiquitin activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase, which binds to the substrate and confers specificity. Sumoylation, which conjugates small ubiquitin-like modifier peptide (SUMO), requires its own set of related E1, E2 and E3 enzymes. Polyubiquitination targets proteins to the proteasome for degradation, whereas monoubiquitination or sumoylation can alter protein activities, subcellular localization and/or interaction partners. Interestingly, some viral proteins can be modified by ubiquitin and ubiquitin-like proteins, and some can function as enzymes in the ubiquitination pathway (reviewed in (Alcaide-Loridan and Jupin, 2012)).

Geminiviruses alter the ubiquitin and ubiquitin-like protein machineries to achieve a full infection. Infection is impaired when there is a reduction in the expression of several ubiquitination-related genes, such as ubiquitin-like modifier-activating enzyme 1 (UBA1), RING-H2 group F2A (RHF2A, which is an E3 ubiquitin ligase), S-phase kinase-associated protein 1 (ASK2; also known as SKP1-like 2) or COP9 signalosome 3 (CSN3 a subunit of from the Constitutive Photomorphogenic 9 – COP9/CSN - complex) (Lozano-Durán et al., 2011b). Also, infection protects some unstable host proteins from degradation, including GRIK and S-adenosyl methionine decarboxylase 1 (SAMDC1) (Shen and Hanley-Bowdoin, 2006; Zhang et al., 2011).

The physical association between geminiviral proteins and components of the ubiquitin pathway has also been reported (reviewed in (Lozano-Durán and Bejarano, 2013)). One example is the interaction between the βC1 protein (which is encoded in a satellite βDNA) from *Cotton leaf curl Multan virus (*CLCuMV) and the tomato ubiquitin E2 enzyme SlUBC3 (Eini et al., 2009). Transgenic expression of βC1 in tobacco led to a reduction in the level of polyubiquitinated proteins, suggesting that βC1 is interfering with ubiquitination in a general, non-specific manner, probably through its interaction with the SlUBC3 homologue. The results obtained with CLCuMV indicate that ubiquitination could be globally detrimental for the infection by geminiviruses, and therefore a preferred target for viral effectors. In agreement with this hypothesis, it has been shown that silencing of the ubiquitin-activating enzyme UBA1 in *Nicotiana benthamiana* results in enhanced

Tomato yellow leaf curl Sardinia virus (TYLCSV) infection (Lozano-Durán et al., 2011b). Interestingly, the tomato UBA1 has been found to interact with TYLCSV C2 in yeast (Hericourt et al., in preparation), which raises the idea that C2 could be inhibiting UBA1 during the viral infection as a virulence strategy. Along the same lines, a negative impact of geminiviral C2 on ubiquitination has been described, at least for TYLCSV, BCTV and *Tomato yellow leaf curl virus* (TYLCV). These three C2 proteins interact with CSN5, catalytic subunit of the CSN complex, which normally removes the ubiquitin-like protein RUB from Cullin 1 (CUL1), and thereby C2 might redirect ubiquitination by collectively targeting a broad range of E3 ligases SCF complex (Skp1-Cullin-F-box protein complex) through modification of their rubylation status. Given that SCF ligases are key regulators of many cellular processes, the capacity of geminiviruses to hijack these complexes represents a powerful strategy for modulating host function (Lozano-Durán et al., 2011a). All these observations highlight the functional importance of interactions with the ubiquitin pathway for geminivirus infection.

SUMOs and ubiquitin are highly similar structurally, despite being diverse in sequence, and the enzymatic cascade leading to SUMO conjugation also resembles that of ubiquitination. Like for others PTMs, sumoylation is essential in eukaryotes, reversible, and seems to regulate an abundance of cell processes such as cell cycle, transcription or subcellular trafficking (reviewed in (Ulrich, 2009; Castro et al., 2012)). The importance of sumoylation in geminivirus-plant interactions is illustrated by the finding that the essential Rep protein interacts with the host SUMO-conjugating enzyme E1 (Castillo et al., 2004; Sánchez-Durán et al., 2011). Viral replication was strongly reduced by altered expression of SUMO, either positively or negatively (Castillo et al., 2004), suggesting that the accumulation of this peptide needs to be carefully fine-tuned for successful geminiviral replication, therefore highlighting its importance in this process. This idea is further supported by the fact that silencing of SUMO-conjugating enzyme SCE1 in *N. benthamiana* results in suppression of TYLCSV infection (Castillo et al., 2007).

Hormonal signalling pathways

Finally, geminiviruses have been described to alter diverse plant hormone signalling pathways, such as those triggered by salicylic acid (SA), ethylene (ET), jasmonate (JA) , brassinosteroids and cytokinin (Ascencio-Ibánez et al., 2008; Chen et al., 2010; García-Neria and Rivera-Bustamante, 2011; Lozano-Durán et al., 2011a; Miozzi et al., 2014). It has been reported that geminiviruses activate SA and ET pathways, which both participate in the host defence response, and plants with increased SA levels or higher expression of components in this pathway are resistant to infection by geminiviruses (Ascencio-Ibánez et al., 2008; Chen et al., 2010; García-Neria and Rivera-Bustamante, 2011). JA-responsive genes are generally suppressed during infection (Ascencio-

Ibánez et al., 2008). Ectopic expression of some viral proteins can inhibit the JA pathway, but the biological relevance of these changes is not known (Yang et al., 2008; Lozano-Durán et al., 2011a). Geminiviruses also interact with the cytokinin and auxin pathways, which promote cell proliferation and modulate differentiation in plants (Park et al., 2004; Miozzi et al., 2014). C2 has been shown to interact with and inhibit the plant adenosine kinase (ADK), and this inhibition seems to promote the expression of cytokinin-responsive genes (Baliji et al., 2010). C4 has been described to interact with the shaggy-related protein kinase AtSKeta, a component of the brassinosteroids signalling pathway (Piroux et al., 2007); additionally, ectopic expression of C4 renders the plant hypersensitive to gibberellins and cytokinins (Mills-Lujan and Deom, 2010) (Mills-Lujan and Deom, 2010). Transgenic expression of V2 or C2 makes *Arabidopsis* plants less sensitive to jasmonates, and suppresses the expression of JA-responsive genes (Luna *et al.*, unpublished; (Lozano-Durán et al., 2011a)). More recently, a transcriptomic analysis of tomato plants infected with TYLCSV showed an induction of a series of hormone responses, including gibberellin and acid abscisic; however, changes in JA-responsive genes were not observed (Miozzi et al., 2014).

In conclusion, multiple phytohorme pathways seem to be altered during geminivirus infection, but the molecular mechanisms underpinning these changes and the associated biological relevance remain largely elusive. One main problem is that the data currently available derive from different combinations of geminivirus species and host plants, and therefore drawing general conclusions is difficult. Additionally, transcriptomic analyses have used whole infected plants, while geminiviruses are only present in a minority of the plant cells; as a consequence, changes linked to the infection can get diluted, and local and systemic responses are indistinguishable. A qualitative change in resolution will be required for understanding the interplay between geminivirus disease complexes and hormonal pathways in a host-dependent manner.

A GLIMPSE AT ANTI-VIRAL DEFENCE MECHANISMS

Resistance to viruses is not always genetically predetermined and can be highly adaptive in nature (Carr et al., 2010). RNA silencing and the ubiquitin/26S proteasome pathway have long been established as an antiviral defence mechanism in plants (Waterhouse et al., 2001; Baulcombe, 2004; Smalle and Vierstra, 2004). The plant response to viral infection includes also the induction of localized plant cell death (associated with the hypersensitive response, HR) and the up-regulation of resistance against many types of pathogen throughout the plant (systemic acquired resistance, SAR). Unfortunately, it is still poorly understood how virus infection is inhibited and

restricted during the HR and in plants exhibiting SAR. In this section, some general concepts of basic defence mechanisms against plant viral pathogens will be reviewed.

Resistance conditioned by *R* genes

The study of dominant *R* gene-regulated responses has proved to be the richest seam for extracting information on induced resistance to viruses and the signals responsible for its establishment (Carr et al., 2010). Over the past decade, several *R* genes that mediate resistance against viruses have been identified (reviewed in (Collier and Moffett, 2009; Gururani M.A., 2012; de Ronde et al., 2014)). All of the R proteins encoded by ‘‘antiviral’’ resistance genes that have been isolated from diverse crop and model plants belong to the nucleotide-binding site-leucine-rich repeat (NBS–LRR) class and are localized intracellularly (reviewed in (de Ronde et al., 2014)). These proteins can be further subdivided depending on whether they have a Toll/interleukin receptor-1 (TIR)-like domain (e.g., the *N* protein; (Whitham et al., 1994)) or a coiled-coil (CC)-like domain within the N-terminus (e.g., the Rx protein; (Moffett et al., 2002)).

The HR is one of the most common plant resistance reactions to any type of pathogenic organism, including viruses. During an incompatible viral infection a HR response is initiated by the interaction of a pathogen virulence factor (called in this context an avirulence (Avr) factor, since its recognition triggers the onset of resistance), and the plant defence protein called an R (resistance) protein (Avr/R). HR induces the production of nitric oxide (NO), and the accumulation of reactive oxygen species (ROS), such as O^{2-} and hydrogen peroxide; it also triggers changes in the levels of the hormones SA and JA, both in the infected and non-infected tissues (Culver and Padmanabhan, 2007; Carr et al., 2010; Pallas and Garcia, 2011; Mandadi and Scholthof, 2012). At the cellular level, HR affects calcium (Ca^{2+}) ion homeostasis and alters membrane potential and permeability (Mur et al., 2008). The first HR report was by Holmes in 1938 (Holmes, 1938), working with *Tobacco mosaic virus* (TMV) infection of *Nicotiana glutinosa*, where he described a resistance response associated with a gene, *N* (for necrotic lesion response). Holmes moved the *N* gene from *N. glutinosa* to economically important tobacco (*Nicotiana tabacum*) and became the first scientist to demonstrate that a dominant gene was associated with the resistance response against TMV infection. After Holmes’ description, many other works reported HR-mediated resistance during plant viruses-host responses (Bendahmane et al., 1999; Chu et al., 2000; Ishibashi et al., 2007) . Interestingly, a case of resistance to the geminivirus *Bean dwarf mosaic virus* (BDMV) in beans associated with development of HR in vascular (phloem) tissues has been described (Seo et al., 2004). At the molecular and biochemical level, several genetic signalling cascades are activated during HR to induce multiple proteins, including mitogen-activated protein (MAP) kinases. Downstream of these primary signalling cascades, expression of several defence-related proteins,

such as glucanases, chitinases, defensins, and the pathogenesis-related protein family, are up-regulated (Mur et al., 2008). Current studies of plant defence are uncovering a complex network of interactions between basal *R*-mediated and systemic defence responses. Recent works have specifically led to modifications of the original gene-for-gene model, according to which the recognition of the pathogen's *Avr* gene product by the *R* gene product leads to resistance. One of these modification is the 'guard' hypothesis (Dangl and Jones, 2001), according to which R-mediated resistance is not necessarily initiated by a direct interaction between the Avr factor and its cognate R protein. Instead, R proteins might be 'surveying' the status of Avr targets, detecting the pathogen factors through their effect on the plant cell. The more recently proposed 'decoy model' suggests the existence of a host decoy protein that mimics effector targets to trap the pathogen into a recognition event (van der Hoorn and Kamoun, 2008). Additionally, a growing body of evidence points to cross-talk and shared signalling elements between basal and R-mediated defences

Systemic acquired resistance (SAR)

Similar to HR, SAR is triggered during an incompatible interaction involving Avr and R proteins in the primary infected cells. However, the resistance is transduced to the non-infected distant tissues (Durrant and Dong, 2004). Although the exact mechanisms of SAR are not defined, it is initiated through a local interaction among Avr and R proteins and results in accumulation of phytohormones such as SA and JA in the distant tissues. In contrast with the HR, SAR results in a broader and long-lasting resistance to diverse pathogen types simultaneously (Vlot et al., 2008). It has been suggested that SAR likely involves interaction among multiple SAR signals, such as methyl salicylate (MeSA), lipid-transfer proteins, and glycerolipids (Liu et al., 2011).

Defence-promoting phytohormones: jasmonates and salycilic acid

Phytohormones are small molecules that are essential for the regulation of plant growth, development, reproduction and survival. These molecules play important roles in signalling networks involved in plant responses to a wide range of biotic and abiotic stresses (reviewed in (Pieterse et al., 2009)). Several hormones have long been known for their roles in tuning plant responses to pathogens, such as SA, JA and ET. More recently, reports have indicated that other hormones such as abscisic acid, auxin, gibberellic acid, cytokinin, brassinosteroids and peptide hormones are also implicated in plant defence signalling pathways, but their role in plant defence is less well studied (Bari and Jones, 2009; Pieterse et al., 2009; Santner et al., 2009; Denance et al., 2013).

Remarkably, some pathogens (bacterial and fungal species) produce hormone mimics such as coronatine (COR, produced by the bacterium *Pseudomonas syringae*), a mimic of the bioactive JA

hormone, JA-isoleucine (JA-Ile) (Bender et al., 1999; Fonseca et al., 2009). Pathogen-synthesized hormones can promote virulence via suppression of host defences; another possibility can be that pathogens modify their host's hormone balance using specific effectors (reviewed in (Robert-Seilaniantz et al., 2011)).

Infection of plants with diverse pathogens, including viruses, results in changes in the level of various phytohormones (Adie et al., 2007; Robert-Seilaniantz et al., 2007). Likewise, exogenous application of plant hormones can have an impact on viral infection, and consequently there have been attempts to induce resistance to, or to control, virus infection in plants by the application of plant hormones or other chemicals, including JA and SA (reviewed in (Alazem and Lin, 2014)). Due to the importance for this thesis we will focus on the implication of SA and JA signalling in defence against plant pathogens.

Salicylic acid

Salicylic acid plays a major role in disease resistance signalling (Vlot et al., 2009) and is generally involved in the activation of defence responses against biotrophic and hemi-biotrophic pathogens as well as the establishment of SAR as mentioned above (Grant and Lamb, 2006). Signalling downstream of SA is largely controlled by the regulatory protein NONEXPRESSOR OF PR GENES 1 (NPR1), which upon activation by SA acts as a transcriptional co-activator of a large set of defence related genes, including pathogenesis related (*PR*) genes (Dong, 2004). Shortly, in the absence of SA, NPR1 is localized in the cytoplasm, where it forms multimers. SA treatment or pathogen attack induce a redox change in the cell leading to the dissociation of the NPR1 complex and migration of NPR1 monomers into the nucleus. Once inside the nucleus, NPR1 binds to TGA transcription factors, enhancing their binding to SA-responsive promoters (Despres et al., 2000; Dong, 2004). A work by Wu and colleagues (Wu et al., 2012) revealed that NPR1 is the SA receptor in *Arabidopsis,* whereas Fu and colleagues (Fu et al., 2012) demonstrated that the NPR1 paralogues NPR3 and NPR4 are actually the SA receptors.

SA levels increase in pathogen-challenged tissues of plants, and exogenous applications result in the induction *PR* genes and enhanced resistance to a broad range of pathogens, including RNA and DNA viruses (Whitham et al., 2006; Ascencio-Ibánez et al., 2008). Among the best-characterized *PR* genes is *PR-1*, which is often used as a robust marker for SA-responsive gene expression (van Loon et al., 2006; Pieterse et al., 2012).

Jasmonate

Jasmonic acid and its metabolites, collectively known as jasmonates (JAs), are important signalling molecules mediating response to both biotic and abiotic stresses and aspects of growth and development (Wasternack, 2007). Jasmonic acid is an oxygenated fatty acid (oxylipin) involved in resistance to necrotrophic pathogens and insect infestation (Thaler et al., 2004). The response to JAs involves several signal transduction events: the perception of the primary wound or stress stimulus and transduction of the signal locally and systemically; the perception of this signal and induction of JA biosynthesis; the perception of JA and induction of responses; and finally, integration of JA signalling with outputs from the SA, ET, and other signalling pathways (Wasternack and Hause, 2013).

Generally, JAs are believed to reallocate plant resources to either defence or growth, depending on the environmental cues. JAs are synthesized rapidly via the oxylipin biosynthesis pathway upon pathogen or insect attack (Gfeller et al., 2010). In the absence of JAs, JA-induced gene expression is repressed by a family of transcriptional repressors named JASMONATE-ZIM-DOMAIN (JAZ) proteins. The JAZ family consists of twelve members in *Arabidopsis* that have emerged as central modulators of JA signalling (Chini et al., 2007; Thines et al., 2007). JAZ repressors act together with the transcriptional repressor TOPLESS (TPL), a common element with the auxin signalling, through the interaction with NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). In the presence of JAs, JAZ proteins are recognized and ubiquitinated by the ubiquitin E3 ligase SCF complex (SKP1, CUL1/CDC53, F box proteins) assembled with the F-box protein CORONATINE-INSENSITIVE 1 (COI1), SCF^{COI}. JAZ proteins directly interact with the well characterized transcription factor MYC2, known to modulate JA-dependent transcription (Chini et al., 2007); degradation of JAZ proteins in response to JAs would allow MYC2 to activate or repress downstream target genes (Figure 4). JAZ proteins have also been shown to interact with MYC3, a close relative to MYC2 (Pauwels et al., 2010). Though protein–protein interactions have been required to elucidate the JA sensing mechanism, the COI1 protein was identified through the study of mutants. One of the first JA signalling mutant, *coi1-1* (Feys et al., 1994), was identified in a screen for *Arabidopsis* seedlings resistant to root growth inhibition by COR (Fonseca et al., 2009). *coi1-1* mutants were male sterile, resistant to root growth inhibition by JA, and deficient in all JA-related responses (Feys et al., 1994; Xie et al., 1998).

The role of JA in plant development processes such as seed germination, trichomes formation, seedling development, root growth, flower development, seed development, tuber formation and senescence has been intensively studied (reviewed in (Wasternack and Hause, 2013)). However, how JA distinctly regulates development and defence responses is not fully elucidated.

Interestingly, a recent work has described *JAV1*, a key gene in the JA pathway, which functions as a negative regulator to control plant defence but does not play a detectable role in plant development (Hu et al., 2013).

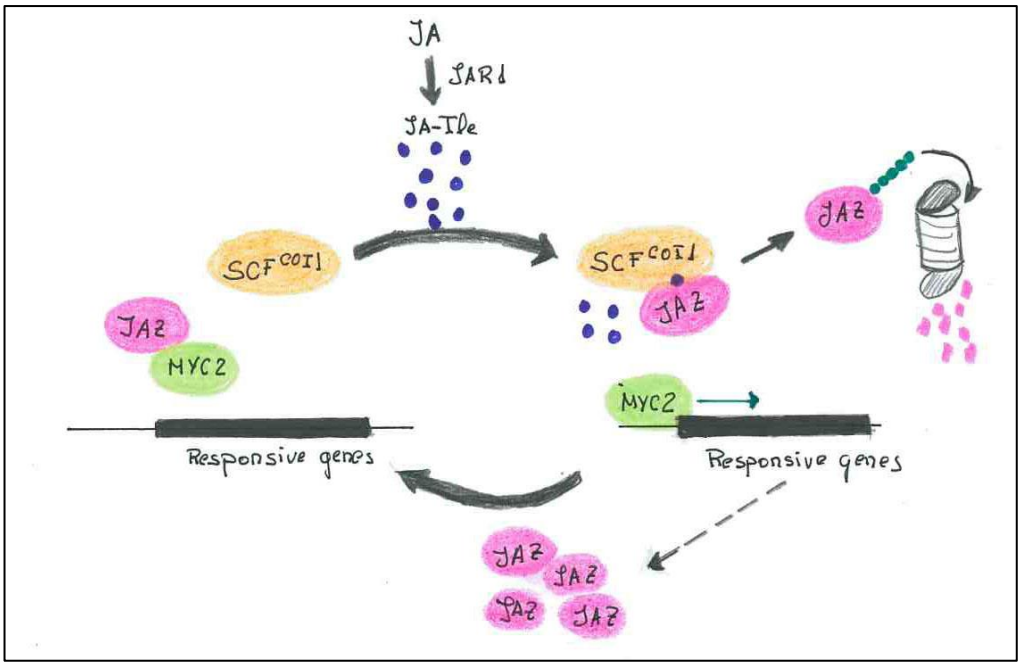


Figure 4. Model for the negative feedback loop regulation of JA responses in *Arabidopsis*. In an uninduced situation, MYC2 activity is repressed by the direct binding of JAZ proteins.) Upon a stimulus, synthesis of JA–Ile by JAR1 promotes the interaction of JAZ repressors with SCF^{COI1}. Ubiquitination by SCF^{COI1} promotes JAZ-protein degradation by the 26S proteasome releasing MYC2 and probably other TFs. Activation of MYC2 induces transcription of early JA-responsive genes including the JAZs. De novo synthesis of JAZ proteins restores MYC2 repression and turns the pathway off.

Pathway crosstalk to fine-tune defence

In nature, plants often deal with simultaneous or subsequent invasion by multiple aggressors as well as beneficial microorganisms, which can influence the primary induced defence response of the host plant (Stout et al., 2006). Plants need regulatory mechanisms to effectively and efficiently adapt to changes in their complex environment. Crosstalk between hormonal signalling pathways provides the plant with such a powerful regulatory potential and may allow the plant to tailor its defence response to the invaders encountered (Reymond and Farmer, 1998; Pieterse et al., 2009; Verhage et al., 2010). For instance, in *Arabidopsis,* transcriptome analyses of wild-type and mutant plants challenged with different attackers revealed complex antagonistic and synergistic regulatory relationships between SA and JA signalling sectors of the plant immune signalling network (Glazebrook et al., 2003; De Vos et al., 2005; Sato et al., 2010). Such hormonal crosstalk is thought to optimize the immune response against single attackers that stimulate both the SA and the JA pathway or to prioritize one pathway over the other when plants are simultaneously or sequentially

attacked by different enemies (Pieterse et al., 2012; Thaler et al., 2012). Several other hormones, such as ET, abscisic acid, gibberellic acid, and auxin, antagonistically or synergistically interact with the SA and JA pathways (Robert-Seilaniantz et al., 2011), adding yet another layer of complexity to the plant immune signalling network. Interestingly, successful pathogens and insect herbivores have been demonstrated to hijack hormone signal integration, either through the production of plant hormones, hormone mimics, or effectors that target hormone signalling components to manipulate the plant immune signalling network for their own benefit (Dangl and Jones, 2001; Walling, 2008; Pieterse et al., 2012). For a comprehensive picture of one of the best studied examples of defence-related signal crosstalk in the antagonistic interaction between the SA and JA pathways, there are several excellent reviews (Koornneef and Pieterse, 2008; Pieterse et al., 2009; Thaler et al., 2010; Pieterse et al., 2012).

The SA and JA signalling sectors often act antagonistically. For instance, the JA-mimicking phytotoxin COR, produced by virulent *P. syringae* bacteria, promotes virulence by suppressing effectual SA-dependent defences in *Arabidopsis* and tomato (*Solanum lycopersicum*) (Brooks et al., 2005; Zheng et al., 2012). Conversely, many studies have demonstrated that endogenously accumulating SA antagonizes JA-dependent defences, thereby prioritizing SA-dependent resistance over JA-dependent defence (Pieterse et al., 2012). Pharmacological experiments with *Arabidopsis* revealed that JA responsive marker genes, such as *PLANT DEFENSIN 1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN* 2 (*VSP2*) are highly sensitive to suppression by SA (van Wees et al., 1999; Spoel et al., 2003). Although many reports describe an antagonistic interaction between the SA and JA pathways with corresponding trade-offs in disease and pest resistance, neutral and synergistic interactions have been described as well (Schenk et al., 2000; van Wees et al., 2000; Mur L.A., 2006). Clearly, the kinetics of hormone production and signalling during the interaction of a plant with its enemies is highly decisive in the final outcome of the defence response (Koornneef et al., 2008; Leon-Reyes et al., 2010).

As mentioned before, the defence regulatory protein NPR1 was identified as a key signalling node in the regulation of SA/JA crosstalk because in mutant *npr1-1* plants, the antagonistic effect of SA on *PDF1.2* and *VSP2* transcription was completely abolished (Spoel et al., 2003; Leon-Reyes et al., 2010). Several other molecular players in SA/ JA crosstalk have been identified, including the mitogen-activated protein kinase MPK4 (Petersen et al., 2000), the lipase-like proteins ENHANCED DISEASE SUSCEPTIBILITY 1 and PHYTOALEXIN- DEFICIENT 4 (Brodersen et al., 2006), the fatty acid desaturase SUPPRESSOR OF SA INSENSITIVITY 2 (Kachroo et al., 2003), glutaredoxin GRX480 (Ndamukong et al., 2007; Zander et al., 2010), and class II TGA and WRKY transcription factors (Liu et al., 2004b; Mao et al., 2007; Ndamukong et al., 2007; Leon-Reyes et al., 2010;

Zander et al., 2010). More recently, it has been reported that SA pathway inhibits JA signalling downstream of the SCF^{COI1}-JAZ complex by targeting GCC-box motifs in JA-responsive promoters via a negative effect on the transcriptional activator OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF domain protein 59 (ORA59; (Van der Does et al., 2013)).

VIRUS-INDUCED GENE SILENCING AS A TOOL IN REVERSE GENETICS

Reverse genetics is the search for gene functionality, starting from a gene sequence. To knockout or reduce gene expression, traditional approaches for high-throughput reverse genetic gene function screening include chemical-induced mutagenesis, random mutagenesis, and T-DNA insertional mutagenesis. However, these methods may be hindered in the studies of non-model plants because of their usually large genome size, low transformation efficiency, and/or lack of a clear genetic background (Huang et al., 2012). A powerful approach to functional genomics, and an alternative to the massive generation of transgenic plants, is the use of the extensively used virus induce gene silencing (VIGS) tool which utilizes virus-derived vectors to knock down a gene of interest.

Virus-induced gene silencing (VIGS) is based on a silencing mechanism that regulates gene expression by the specific degradation of RNA by PTGS (reviewed in (Benedito et al., 2004; Burch-Smith et al., 2004; Robertson, 2004; Carrillo-Tripp et al., 2006)). As mentioned above, one of the roles of RNA silencing is to defend plants against viruses, and viruses are both triggers and targets of this mechanism. Briefly, VIGS methodology is based in a recombinant viral vector (VIGS vector), carrying a host-derived target gene sequence; when this virus infects a plant, viral double-stranded RNAs are synthesized, leading to the activation of the antiviral RNA silencing pathway and the subsequent knockdown of the endogenous host gene (Bachan and Dinesh-Kumar, 2012) . In addition to local RNA degradation, a mobile silencing signal is produced that brings the instructions for specific degradation far away from the inoculation point (Dunoyer et al., 2010).

As a tool for reverse genetics, VIGS has many advantages over other common ways to study gene function because of the ability of viruses to replicate and move systemically within a plant. VIGS can generate a phenocopy of a mutant without all the troubles of traditional methods of mutagenesis. For example, viral vectors have the advantage over transgenes that are introduced directly into the nuclear genomes of host plants in that they are extrachromosomal entities, and therefore the variation in gene expression caused by positional effects is avoided (Timmermans MCP, 1994; Burch-Smith et al., 2004). Additionally, VIGS analyses can be carried out in species

that are recalcitrant to transformation. Moreover, high-throughput VIGS studies can be done relatively quickly. Finally, VIGS provides the ability to work with genes whose knockout mutants would be hard to generate because the mutations are lethal, for example genes that are involved in development (Peele et al., 2001). Another feature that makes VIGS a very powerful tool is that it provides the possibility of silencing multiple genes from the same family to overcome functional redundancy (Burch-Smith et al., 2004).

Despite the valuable advantages of VIGS approach, there are also limitations. One of the most important ones is that complete loss-of-function by VIGS might not be achieved. Generally between a 75 and 90% down-regulation in the expression level of the targeted gene is accomplished. Unfortunately the remaining low level of gene expression can be enough to produce functional protein, which would mask the phenotype of the silenced plant (reviewed in (Unver and Budak, 2009)). Something to keep in mind is that some viral infections can cause symptoms on the plant that might mask the phenotype caused by the silencing of the desired gene. This problem might be minimized by using the *Tobacco rattle virus* (TRV)–based VIGS system because of the mild symptoms triggered by this particular virus (Ratcliff et al., 2001).

One of the most common vectors currently used is based on TRV (Liu et al., 2002; Brigneti et al., 2004; Robertson, 2004). This method uses a bipartite vector system designed between left and right borders of the *Agrobacterium* Ti plasmid. TRV 1 contains the RNA-dependent RNA polymerase (RdRp) and the MP components of the virus whereas TRV 2 contains multiple cloning sites (MCS) and the CP sequences (Figure 5). The bipartite plasmids are flanked by the 35S Cauliflower mosaic virus promoter and a Nopaline synthase gene terminator. The MCS in TRV 2 allows ligation of DNA target sequences that will induce PTGS in the plant upon delivery by agroinoculation (Ratcliff et al., 2001). The multiplication of the vector in the plant tissue triggers the cleavage of target sequence resulting in loss of expression (Liu et al., 2002). TRV-based VIGS vectors have been used to silence genes in a number of plants, among others, Solanaceous plant species, including *N. benthamiana,* tomato, pepper (*Capsicum annuum)*, potato (*Solanum tuberosum)* and petunia (*Petunia hybrida*) (Ratcliff et al., 2001; Liu et al., 2002; Brigneti et al., 2004; Chung et al., 2004; Chen et al., 2005). One distinct advantage of using TRV for VIGS is the ability of the virus to infect the meristem of its hosts (Ratcliff et al., 2001), and it has been used to study flowering in *N. benthamiana* (Liu et al., 2004a) and petunia (Chen et al., 2005), in addition to fruit development in tomato (Fu et al., 2005). Among other features, TRV-based VIGS has been extensively used to dissect the genetics of floral development and scent production (Spitzer et al., 2007), water deficit stress tolerance (Senthil-Kumar et al., 2008), embryogenesis, chlorophyll

biosynthesis and disease resistance (Burch-Smith *et al.*, 2004), and protective acyl sugars in trichomes (Schilmiller et al., 2012) .

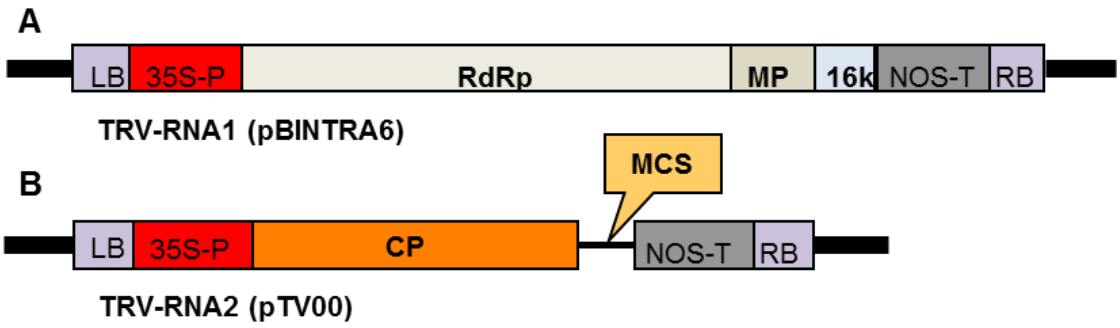


Figure 5. Genomic organization of TRV vector. (A) The T-DNA organization of pBINTRA6, a plant binary transformation plasmid containing a cDNA clone of TRV RNA 1. **(B)** The T-DNA organization of pTV00, a plant binary transformation plasmid containing a cDNA clone derived from TRV RNA 2. The cDNA clones are positioned between the left and right border (LB and RB) of the T-DNA, and between CaMV 35S promoters (35S-P) and Nopaline synthase terminator (NOS-T). The TRV open reading frames correspond to the RNA-dependent RNA polymerase (RdRp), movement protein (MP), 16k protein (16k), Coat Protein (CP). The multiple cloning site (MCS) introduced during the cloning of RNA 2, unique sites in MCS (5' to 3') are *Spe*I, *Sma*I, *Xma*I, *Hind*III, *BspD*I, *Cla*I, *Acc*I, *Apa*I(modified from Ratcliff *et al.*, 2001).

Remarkably, VIGS can silence either an individual gene or multiple genes in a single plant and can also be used in a high-throughput manner to silence genes in multiple plants, making VIGS an attractive alternative instrument in functional genomics. The high-throughput techniques for gene discovery and expression analysis, such as whole genome sequencing and microarrays, demand efficient procedures to unravel gene functions, in order to make them useful for fundamental and applied purposes. The information generated by high-throughput technologies can be combined with PTGS approaches to probe gene function in an efficient and fast fashion. Indeed, some high- or medium-throughput works have been reported using VIGS in *N. benthamiana* plants in order to elucidate genes related to pathogen response and/or defence (Lu et al., 2003; Lozano-Durán et al., 2011b; Yuan et al., 2011; Czosnek et al., 2013; Senthil-Kumar et al., 2013; Xu et al., 2014).

THE VESICLE TRAFFICKING MACHINERY

Concepts of vesicle trafficking in plants

The plant endomembrane system encompasses a series of compartments which provide specialized surfaces and segregated areas in membrane-delimited compartments for the production and storage of biomolecules. These intracellular compartments are not static: they exchange proteins and lipids continuously in a directional and regulated manner (Bassham, 2009). The exchange of material (cargoes) between compartments is mostly conducted by coated transport vesicles that bud from one membrane and fuse with another. Transport vesicles are hence essential for maintaining organelle identity and lipid homeostasis and for the secretion of proteins. The formation of transport vesicles is mediated by cytosolic coat proteins. These proteins can bind each other as well as the membrane of a compartment and can interact with cargoes. To form a transport vesicle, the coat proteins must collect cargo, must induce membrane bending to form a coated bud, must coordinate membrane scission to release a vesicle, and must then disassemble to allow fusion of the vesicle with the target membrane (Faini et al., 2013).

The three best-characterized types of vesicular carrier involved in intracellular trafficking are distinguished by their different coat proteins and their different trafficking routes. Clathrin-coated vesicles act in the late secretory pathway and in the endocytic pathway, COPII-coated vesicles export proteins from the endoplasmic reticulum (ER), and COPI-coated vesicles shuttle within the Golgi organelle and from the Golgi back to the ER. Despite having different compartment specificities and different structural components, the mechanisms of their formation follow similar rules (Figure 6) (Paul and Frigerio, 2007; Faini et al., 2013). The time and place at which vesicle formation occurs are most often regulated by small GTP-binding proteins. In these cases, vesicle formation is initiated by activation of a small GTPase, stimulated by specific guanine exchange factors, ARF1 (ADP-RIBOSYLATION FACTOR 1) and SAR1 for COPI and COPII respectively (Faini et al., 2013). Consistent with these expanded roles, the ARF-family is large in most eukaryotes, and may have as many as twenty-one members in *Arabidopsis* (Paul and Frigerio, 2007). Due to the importance for the present thesis the COPI complex will be further described.

COPI complex: making a vesicle

Cytoplasmic vesicles containing a COPI coat are best known for their involvement in retrograde transport of cargo from the Golgi apparatus to ER (Thompson and Brown, 2012). COPI coats may also be involved in forming coated vesicles at the ER and endosomal compartments, as well as within the Golgi (Kirchhausen, 2000; Beck et al., 2009). In plants, retrograde transport includes two

types of protein: escaped ER residents which need to be returned to the ER, e.g., chaperones, and proteins which continually cycle between the two compartments, e.g., p24 proteins or SNAREs (Robinson D. , 2007).

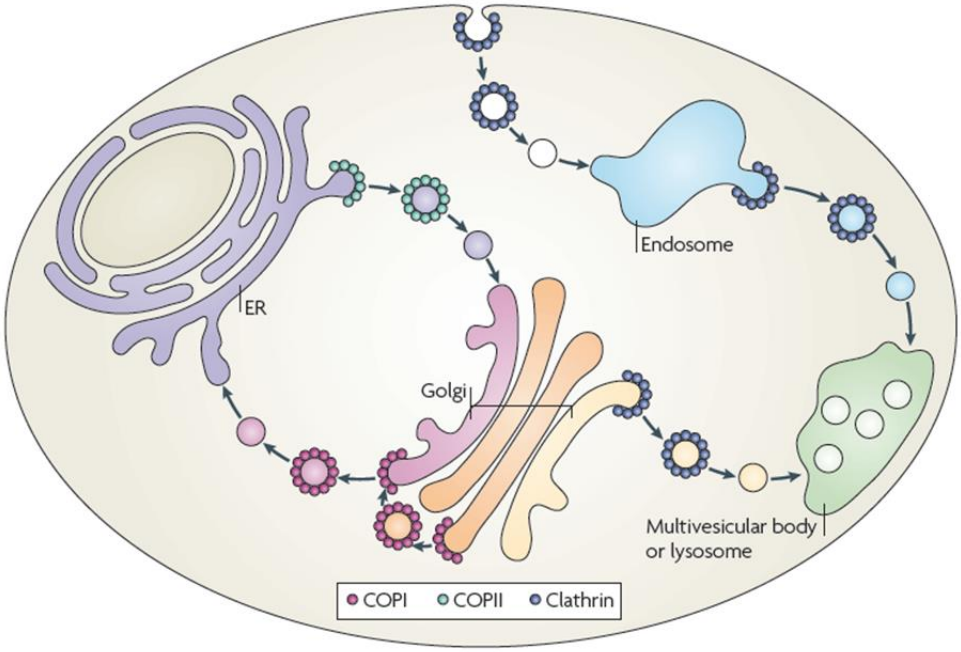


Figure 6. Pathways of vesicular transport by the better characterized coat proteins. Coat protein II (COPII; green) forms vesicles for anterograde transport from the endoplasmic reticulum (ER) to the Golgi, whereas COPI (red) forms vesicles for both intra-Golgi transport and retrograde transport from the Golgi to the ER. Clathrin (blue) forms multiple complexes based on its association with different adaptor proteins (APs). clathrin that is associated with AP1 and AP3 forms vesicles for transport from the *trans*-Golgi network to the later endosomal compartments, and also for transport that emanates from the early endosomal compartments. clathrin that is associated with AP2 forms vesicles from the plasma membrane that transport to the early endosomes (taken from Hsu et al., 2009).

The coat protein COPI consists of a protein complex known as coatomer together with the GTPase ARF1. The COPI complex is composed of seven subunits (α, β, β', γ, δ, ε, and ζ) that form a cage-like structure (Boehm et al., 2001; Lee and Goldberg, 2010). The complex is organized in two subcomplexes: a trimer composed of α/β'/ε-COP, and a tetramer of β/γ/δ/ζ-COP (Popoff et al., 2011). With the exception of γ-COP and δ-COP, the *Arabidopsis* genome encodes for two or more isoforms of each COPI protein. The significance of these multiple copies is unclear, but might indicate the existence of different classes of COPI-vesicles in plants (Robinson D. , 2007).

In the cytosol, ARF1 is present in its GDP form (GDP-bound), and is recruited to the surface of Golgi membranes by a Sec7 type guanine exchange factor (GEF) which converts it into the GTP form (GTP-bound). Coatomer then attaches to ARF1-GTP. ARF1 appears to have multiple roles in

plant cells. It has been shown that ARF1 is distributed to the Golgi and to post-Golgi compartments that bud from the Golgi apparatus, but may also be involved in endocytosis (Xu and Scheres, 2005) (Stefano et al., 2006; Matheson et al., 2007).

The coatomer formation begins when ARF1-GDP is activated by a Golgi localized exchange factor (Peyroche et al., 1996) to replace GDP with GTP, triggering a conformational change in ARF1 whereby the myristoylated and amphipathic N-terminal α helix is displaced from a surface groove of the G-protein and embedded in the bilayer (Amor et al., 1994; Antonny et al., 1997; Goldberg, 1998). Next, ARF1-GTP recruits coatomer through a direct, GTP-dependent interaction (Serafini et al., 1991). Membrane-associated coatomer then binds to cargo molecules and self-assembles to form a polyhedral cage that molds the membrane into a COPI-coated bud (Figure 7) (Bremser et al., 1999). Recently, using mammalian coatomer proteins, showed that β/δ/γ/ζ-COP interacts with two molecules of ARF1-GTP, which bind to quasi-equivalent sites on the large β-COP and γ-COP subunits with ARF1-GTP (Yu et al., 2012).

Proteins are directed into COPI vesicles by various mechanisms based on direct or indirect binding to the coat. Membrane proteins to be included into COPI vesicles can also be recognized directly by coatomer through sorting motifs present in their sequence. Proteins found in the lumen of the Golgi complex that need to be transported to the lumen of the ER contain the signal peptide KDEL. Well-characterized cargo proteins of COPI vesicles, in mammals, include the KDEL receptors, certain p24 family members and SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) (reviewed in (Hsu et al., 2009)). All of these COPI cargo proteins interact directly not only with coatomer but also with ARF-activating protein 1 (ARFGAP1) (Lee et al., 2005). An exception is p23, a member of the p24 family of cargo proteins, which interacts only with coatomer and not with ARFGAP1 (Lee et al., 2005).

In eukaryotic cells transmembrane proteins contain sorting signals to exit from Golgi and return to the ER. These sorting signals, or motifs, typically contain the amino acid sequence KKXX present at the extreme carboxyl terminus of membrane proteins (Nilsson et al., 1989). It has been reported that KKXX motifs interact with a specific domain of α-COP, and KXKXX binds to a similar domain within β'-COP (Eugster et al., 2004). Furthermore, arginine-based motifs that conform to the consensus sequence (Φ/Ψ/R) RXR (where Φ/Ψ is an aromatic or bulky hydrophobic residue) (Zerangue et al., 2001) are recognized by coatomer subunits β- and δ-COP (Michelsen et al., 2007). Additional sorting motifs are based on aromatic residues. A "δL" motif confers binding to δ-COP and retrieval to the ER in yeast (Cosson et al., 1998). However, not all the membrane proteins

transported within COPI vesicles carry coatomer-interacting motifs. Notably, this is the case of glycosylation enzymes with their tails lacking any known sorting signal (Popoff et al., 2011).

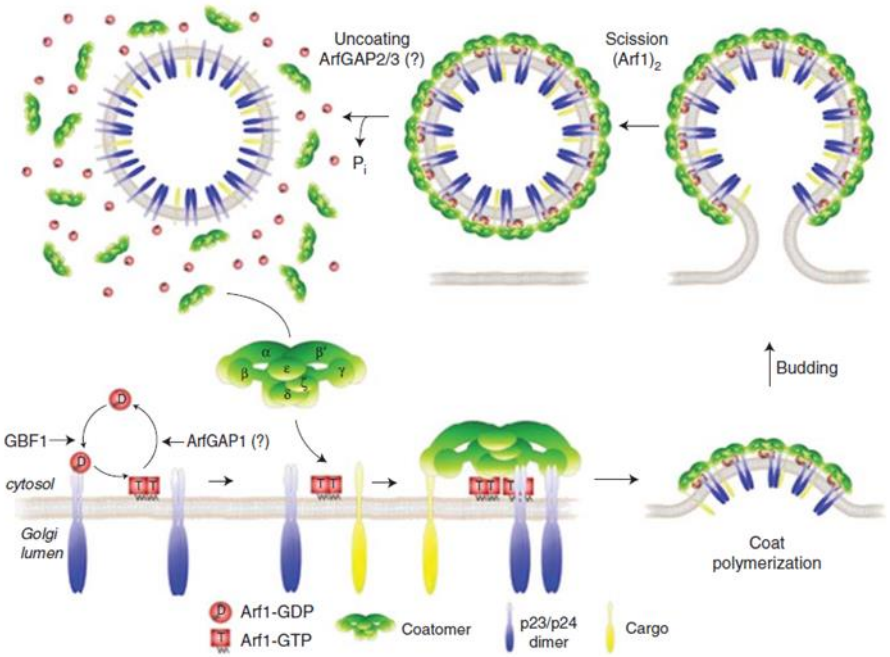


Figure 8. Individual steps in the formation of a COPI vesicle (taken from Popoff *et al.*, 2011).

REFERENCES

- Ach, R.A., Durfee, T., Miller, A.B., Taranto, P., Hanley-Bowdoin, L., Zambryski, P.C., Gruissem, W., 1997. RRB1 and RRB2 encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. Molecular and cellular biology 17, 5077-5086.

- Adie, B.A., Perez-Perez, J., Perez-Perez, M.M., Godoy, M., Sanchez-Serrano, J.J., Schmelz, E.A., Solano, R., 2007. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. The Plant cell 19, 1665-1681.

- Alazem, M., Lin, N.S., 2014. Roles of plant hormones in the regulation of host-virus interactions. Molecular plant pathology.

- Alcaide-Loridan, C., Jupin, I., 2012. Ubiquitin and plant viruses, let's play together! Plant physiology 160, 72-82.

- Amor, J.C., Harrison, D.H., Kahn, R.A., Ringe, D., 1994. Structure of the human ADP-ribosylation factor 1 complexed with GDP. Nature 372, 704-708.

- Antonny, B., Huber, I., Paris, S., Chabre, M., Cassel, D., 1997. Activation of ADP-ribosylation factor 1 GTPase-activating protein by phosphatidylcholine-derived diacylglycerols. The Journal of biological chemistry 272, 30848-30851.

- Ascencio-Ibánez, J.T., Sozzani, R., Lee, T.J., Chu, T.M., Wolfinger, R.D., Cella, R., Hanley-Bowdoin, L., 2008. Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant physiology 148, 436-454.

- Bachan, S., Dinesh-Kumar, S.P., 2012. Tobacco rattle virus (TRV)-based virus-induced gene silencing. Methods in molecular biology 894, 83-92.

- Bagewadi, B., Chen, S., Lal, S.K., Choudhury, N.R., Mukherjee, S.K., 2004. PCNA interacts with Indian mung bean yellow mosaic virus rep and downregulates Rep activity. Journal of virology 78, 11890-11903.

- Baliji, S., Lacatus, G., Sunter, G., 2010. The interaction between geminivirus pathogenicity proteins and adenosine kinase leads to increased expression of primary cytokinin-responsive genes. Virology 402, 238-247.

- Baliji, S., Sunter, J., Sunter, G., 2007. Transcriptional analysis of complementary sense genes in Spinach curly top virus and functional role of C2 in pathogenesis. Molecular plant-microbe interactions : MPMI 20, 194-206.

- Bari, R., Jones, J.D., 2009. Role of plant hormones in plant defence responses. Plant molecular biology 69, 473-488.

- Bassham, D.C., 2009. Function and regulation of macroautophagy in plants. Biochimica et biophysica acta 1793, 1397-1403.

- Baulcombe, D., 2004. RNA silencing in plants. Nature 431, 356-363.

- Beck, R., Rawet, M., Wieland, F.T., Cassel, D., 2009. The COPI system: molecular mechanisms and function. FEBS letters 583, 2701-2709.

- Bendahmane, A., Kanyuka, K., Baulcombe, D.C., 1999. The Rx gene from potato controls separate virus resistance and cell death responses. The Plant cell 11, 781-792.

- Bender, C.L., Alarcon-Chaidez, F., Gross, D.C., 1999. Pseudomonas syringae phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiology and molecular biology reviews : MMBR 63, 266-292.

- Benedito, V.A., Visser, P.B., Angenent, G.C., Krens, F.A., 2004. The potential of virus-induced gene silencing for speeding up functional characterization of plant genes. Genetics and molecular research : GMR 3, 323-341.

- Boehm, M., Aguilar, R.C., Bonifacino, J.S., 2001. Functional and physical interactions of the adaptor protein complex AP-4 with ADP-ribosylation factors (ARFs). The EMBO journal 20, 6265-6276.

- Bottcher, B., Unseld, S., Ceulemans, H., Russell, R.B., Jeske, H., 2004. Geminate structures of African cassava mosaic virus. Journal of virology 78, 6758-6765.

- Bremser, M., Nickel, W., Schweikert, M., Ravazzola, M., Amherdt, M., Hughes, C.A., Sollner, T.H., Rothman, J.E., Wieland, F.T., 1999. Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. Cell 96, 495-506.

- Briddon, R.W., Pinner, M.S., Stanley, J., Markham, P.G., 1990. Geminivirus coat protein gene replacement alters insect specificity. Virology 177, 85-94.

- Briddon, R.W.a.S.J., 2009. Geminiviridae. John Wiley & Sons, Ltd.

- Brigneti, G., Martin-Hernandez, A.M., Jin, H., Chen, J., Baulcombe, D.C., Baker, B., Jones, J.D., 2004. Virus-induced gene silencing in Solanum species. The Plant journal : for cell and molecular biology 39, 264-272.

- Brodersen, P., Petersen, M., Bjorn Nielsen, H., Zhu, S., Newman, M.A., Shokat, K.M., Rietz, S., Parker, J., Mundy, J., 2006. Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. The Plant journal : for cell and molecular biology 47, 532-546.

- Brooks, D.M., Bender, C.L., Kunkel, B.N., 2005. The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. Molecular plant pathology 6, 629-639.

- Brown JK, F.C., Briddon RW, Zerbini M, Moriones E, Navas-Castillo J, 2012. Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses. Academic press, London.

- Burch-Smith, T.M., Anderson, J.C., Martin, G.B., Dinesh-Kumar, S.P., 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. The Plant journal : for cell and molecular biology 39, 734-746.

- Caracuel, Z., Lozano-Duran, R., Huguet, S., Arroyo-Mateos, M., Rodriguez-Negrete, E.A., Bejarano, E.R., 2012. C2 from Beet curly top virus promotes a cell environment suitable for

efficient replication of geminiviruses, providing a novel mechanism of viral synergism. The New phytologist 194, 846-858.

- Carr, J.P., Lewsey, M.G., Palukaitis, P., 2010. Signaling in induced resistance. Adv Virus Res 76, 57-121.

- Carrillo-Tripp, J., Shimada-Beltran, H., Rivera-Bustamante, R., 2006. Use of geminiviral vectors for functional genomics. Current opinion in plant biology 9, 209-215.

- Castillo, Morilla G., Lozano R., Collinet D., Perez-Luna A., and, K.A., E.R., B., 2007. Identification of Plant Genes Involved in TYLCV Replication, Tomato Yellow Leaf Curl Virus Disease.

- Castillo, A.G., Collinet, D., Deret, S., Kashoggi, A., Bejarano, E.R., 2003. Dual interaction of plant PCNA with geminivirus replication accessory protein (Ren) and viral replication protein (Rep). Virology 312, 381-394.

- Castillo, A.G., Kong, L.J., Hanley-Bowdoin, L., Bejarano, E.R., 2004. Interaction between a geminivirus replication protein and the plant sumoylation system. Journal of virology 78, 2758-2769.

- Castro, P.H., Tavares, R.M., Bejarano, E.R., Azevedo, H., 2012. SUMO, a heavyweight player in plant abiotic stress responses. Cellular and molecular life sciences : CMLS 69, 3269-3283.

- Clerot, D., Bernardi, F., 2006. DNA helicase activity is associated with the replication initiator protein rep of tomato yellow leaf curl geminivirus. Journal of virology 80, 11322-11330.

- Collier, S.M., Moffett, P., 2009. NB-LRRs work a "bait and switch" on pathogens. Trends in plant science 14, 521-529.

- Cosson, P., Lefkir, Y., Demolliere, C., Letourneur, F., 1998. New COP1-binding motifs involved in ER retrieval. The EMBO journal 17, 6863-6870.

- Culver, J.N., Padmanabhan, M.S., 2007. Virus-induced disease: altering host physiology one interaction at a time. Annual review of phytopathology 45, 221-243.

- Czosnek, H., Eybishtz, A., Sade, D., Gorovits, R., Sobol, I., Bejarano, E., Rosas-Diaz, T., Lozano-Duran, R., 2013. Discovering host genes involved in the infection by the Tomato Yellow Leaf Curl Virus complex and in the establishment of resistance to the virus using Tobacco Rattle Virus-based post transcriptional gene silencing. Viruses 5, 998-1022.

- Chen, H., Zhang, Z., Teng, K., Lai, J., Zhang, Y., Huang, Y., Li, Y., Liang, L., Wang, Y., Chu, C., Guo, H., Xie, Q., 2010. Up-regulation of LSB1/GDU3 affects geminivirus infection by activating the salicylic acid pathway. The Plant journal : for cell and molecular biology 62, 12-23.

- Chen, J.C., Jiang, C.Z., Reid, M.S., 2005. Silencing a prohibitin alters plant development and senescence. The Plant journal : for cell and molecular biology 44, 16-24.

- Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., Solano, R., 2007. The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448, 666-671.

- Choudhury, N.R., Malik, P.S., Singh, D.K., Islam, M.N., Kaliappan, K., Mukherjee, S.K., 2006. The oligomeric Rep protein of Mungbean yellow mosaic India virus (MYMIV) is a likely replicative helicase. Nucleic acids research 34, 6362-6377.

- Chu, M., Desvoyes, B., Turina, M., Noad, R., Scholthof, H.B., 2000. Genetic dissection of tomato bushy stunt virus p19-protein-mediated host-dependent symptom induction and systemic invasion. Virology 266, 79-87.

- Chung, E., Seong, E., Kim, Y.C., Chung, E.J., Oh, S.K., Lee, S., Park, J.M., Joung, Y.H., Choi, D., 2004. A method of high frequency virus-induced gene silencing in chili pepper (Capsicum annuum L. cv. Bukang). Molecules and cells 17, 377-380.

- Dangl, J.L., Jones, J.D., 2001. Plant pathogens and integrated defence responses to infection. Nature 411, 826-833.

- de Ronde, D., Butterbach, P., Kormelink, R., 2014. Dominant resistance against plant viruses. Frontiers in plant science 5, 307.

- De Vos, M., Van Oosten, V.R., Van Poecke, R.M., Van Pelt, J.A., Pozo, M.J., Mueller, M.J., Buchala, A.J., Metraux, J.P., Van Loon, L.C., Dicke, M., Pieterse, C.M., 2005. Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Molecular plant-microbe interactions : MPMI 18, 923-937.

- Denance, N., Sanchez-Vallet, A., Goffner, D., Molina, A., 2013. Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. Frontiers in plant science 4, 155.

- Despres, C., DeLong, C., Glaze, S., Liu, E., Fobert, P.R., 2000. The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. The Plant cell 12, 279-290.

- Dogra, S.C., Eini, O., Rezaian, M.A., Randles, J.W., 2009. A novel shaggy-like kinase interacts with the Tomato leaf curl virus pathogenicity determinant C4 protein. Plant molecular biology 71, 25-38.

- Dong, X., 2004. NPR1, all things considered. Current opinion in plant biology 7, 547-552.

- Dunoyer, P., Schott, G., Himber, C., Meyer, D., Takeda, A., Carrington, J.C., Voinnet, O., 2010. Small RNA duplexes function as mobile silencing signals between plant cells. Science 328, 912-916.

- Durrant, W.E., Dong, X., 2004. Systemic acquired resistance. Annual review of phytopathology 42, 185-209.

- Eini, O., Dogra, S., Selth, L.A., Dry, I.B., Randles, J.W., Rezaian, M.A., 2009. Interaction with a host ubiquitin-conjugating enzyme is required for the pathogenicity of a geminiviral DNA beta satellite. Molecular plant-microbe interactions : MPMI 22, 737-746.

- Esau, K., Magyarosy, A.C., 1979. Nuclear abnormalities and cytoplasmic inclusion in Amsinckia infected with the curly top virus. Journal of ultrastructure research 66, 11-21.

- Eugster, A., Frigerio, G., Dale, M., Duden, R., 2004. The alpha- and beta'-COP WD40 domains mediate cargo-selective interactions with distinct di-lysine motifs. Molecular biology of the cell 15, 1011-1023.

- Faini, M., Beck, R., Wieland, F.T., Briggs, J.A., 2013. Vesicle coats: structure, function, and general principles of assembly. Trends in cell biology 23, 279-288.

- Feys, B., Benedetti, C.E., Penfold, C.N., Turner, J.G., 1994. Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. The Plant cell 6, 751-759.

- Fondong, V.N., 2013. Geminivirus protein structure and function. Molecular plant pathology 14, 635-649.

- Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C., Solano, R., 2009. (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nature chemical biology 5, 344-350.

- Fontes, E.P., Luckow, V.A., Hanley-Bowdoin, L., 1992. A geminivirus replication protein is a sequence-specific DNA binding protein. The Plant cell 4, 597-608.

- Frischmuth, S., Wege, C., Hulser, D., Jeske, H., 2007. The movement protein BC1 promotes redirection of the nuclear shuttle protein BV1 of Abutilon mosaic geminivirus to the plasma membrane in fission yeast. Protoplasma 230, 117-123.

- Fu, D.Q., Zhu, B.Z., Zhu, H.L., Jiang, W.B., Luo, Y.B., 2005. Virus-induced gene silencing in tomato fruit. The Plant journal : for cell and molecular biology 43, 299-308.

- Fu, Z.Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., Mohan, R., Spoel, S.H., Tada, Y., Zheng, N., Dong, X., 2012. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486, 228-232.

- García-Neria, M.A., Rivera-Bustamante, R.F., 2011. Characterization of Geminivirus resistance in an accession of Capsicum chinense Jacq. Molecular plant-microbe interactions : MPMI 24, 172-182.

- Gfeller, A., Dubugnon, L., Liechti, R., Farmer, E.E., 2010. Jasmonate biochemical pathway. Science signaling 3, cm3.

- Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T., Katagiri, F., 2003. Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. The Plant journal : for cell and molecular biology 34, 217-228.

- Glick, E., Zrachya, A., Levy, Y., Mett, A., Gidoni, D., Belausov, E., Citovsky, V., Gafni, Y., 2008. Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. Proceedings of the National Academy of Sciences of the United States of America 105, 157-161.

- Goldberg, J., 1998. Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. Cell 95, 237-248.

- Gopal, P., Pravin Kumar, P., Sinilal, B., Jose, J., Kasin Yadunandam, A., Usha, R., 2007. Differential roles of C4 and betaC1 in mediating suppression of post-transcriptional gene silencing: evidence for transactivation by the C2 of Bhendi yellow vein mosaic virus, a monopartite begomovirus. Virus research 123, 9-18.

- Grant, M., Lamb, C., 2006. Systemic immunity. Current opinion in plant biology 9, 414-420.

- Gururani M.A., V., Upadhyaya C.P., Nookaraju A., Pandey S.K , Park W., 2012. Plant disease resistance genes: Current status and future directions. Physiological and Molecular Plant Pathology 78, 51-65.

- Hanley-Bowdoin, L., Bejarano, E.R., Robertson, D., Mansoor, S., 2013. Geminiviruses: masters at redirecting and reprogramming plant processes. Nature reviews. Microbiology 11, 777-788.

- Hanley-Bowdoin, L., Settlage, S.B., Robertson, D., 2004. Reprogramming plant gene expression: a prerequisite to geminivirus DNA replication. Molecular plant pathology 5, 149-156.

- Hehnle, S., Wege, C., Jeske, H., 2004. Interaction of DNA with the movement proteins of geminiviruses revisited. Journal of virology 78, 7698-7706.

- Holmes, 1938. Inheritance of resistance to tobacco mosaic disease in tobacco. Phytopathology 28, 553-561.

- Hormuzdi, S.G., Bisaro, D.M., 1995. Genetic analysis of beet curly top virus: examination of the roles of L2 and L3 genes in viral pathogenesis. Virology 206, 1044-1054.

- Horvath, G.V., Pettko-Szandtner, A., Nikovics, K., Bilgin, M., Boulton, M., Davies, J.W., Gutierrez, C., Dudits, D., 1998. Prediction of functional regions of the maize streak virus replication-associated proteins by protein-protein interaction analysis. Plant molecular biology 38, 699-712.

- Hsu, V.W., Lee, S.Y., Yang, J.S., 2009. The evolving understanding of COPI vesicle formation. Nature reviews. Molecular cell biology 10, 360-364.

- Hu, P., Zhou, W., Cheng, Z., Fan, M., Wang, L., Xie, D., 2013. JAV1 controls jasmonate-regulated plant defense. Molecular cell 50, 504-515.

- Huang, C., Qian, Y., Li, Z., Zhou, X., 2012. Virus-induced gene silencing and its application in plant functional genomics. Science China. Life sciences 55, 99-108.

- Ishibashi, K., Masuda, K., Naito, S., Meshi, T., Ishikawa, M., 2007. An inhibitor of viral RNA replication is encoded by a plant resistance gene. Proceedings of the National Academy of Sciences of the United States of America 104, 13833-13838.

- Jeske, H., 2009. Geminiviruses. Current topics in microbiology and immunology 331, 185-226.

- Kachroo, A., Lapchyk, L., Fukushige, H., Hildebrand, D., Klessig, D., Kachroo, P., 2003. Plastidial fatty acid signaling modulates salicylic acid- and jasmonic acid-mediated defense pathways in the Arabidopsis ssi2 mutant. The Plant cell 15, 2952-2965.

- Kaliappan, K., Choudhury, N.R., Suyal, G., Mukherjee, S.K., 2012. A novel role for RAD54: this host protein modulates geminiviral DNA replication. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 26, 1142-1160.

- Kirchhausen, 2000. Three ways to make a vesicle. Nature Reviews Molecular Cell Biology 1, 187-198.

- Kleinow, T., Nischang, M., Beck, A., Kratzer, U., Tanwir, F., Preiss, W., Kepp, G., Jeske, H., 2009. Three C-terminal phosphorylation sites in the Abutilon mosaic virus movement protein affect symptom development and viral DNA accumulation. Virology 390, 89-101.

- Kong, L.J., Hanley-Bowdoin, L., 2002. A geminivirus replication protein interacts with a protein kinase and a motor protein that display different expression patterns during plant development and infection. The Plant cell 14, 1817-1832.

- Kong, L.J., Orozco, B.M., Roe, J.L., Nagar, S., Ou, S., Feiler, H.S., Durfee, T., Miller, A.B., Gruissem, W., Robertson, D., Hanley-Bowdoin, L., 2000. A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. The EMBO journal 19, 3485-3495.

- Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Den Otter, F.C., Van Loon, L.C., Pieterse, C.M., 2008. Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. Plant physiology 147, 1358-1368.

- Koornneef, A., Pieterse, C.M., 2008. Cross talk in defense signaling. Plant physiology 146, 839-844.

- Krenz, B., Jeske, H., Kleinow, T., 2012. The induction of stromule formation by a plant DNA-virus in epidermal leaf tissues suggests a novel intra- and intercellular macromolecular trafficking route. Frontiers in plant science 3, 291.

- Krichevsky, A., Kozlovsky, S.V., Gafni, Y., Citovsky, V., 2006. Nuclear import and export of plant virus proteins and genomes. Molecular plant pathology 7, 131-146.

- Krupovic, M., Ravantti, J.J., Bamford, D.H., 2009. Geminiviruses: a tale of a plasmid becoming a virus. BMC evolutionary biology 9, 112.

- Kunik, T., Mizrachy, L., Citovsky, V., and Gafni, Y., 1999. Characterization of a tomato karyopherin alpha that interacts with the Tomato Yellow Leaf Curl Virus (TYLCV) capsid protein. Journal of experimental botany 50, 731-732.

- Kunik, T., Palanichelvam, K., Czosnek, H., Citovsky, V., Gafni, Y., 1998. Nuclear import of the capsid protein of tomato yellow leaf curl virus (TYLCV) in plant and insect cells. The Plant journal : for cell and molecular biology 13, 393-399.

- Lacatus, G., Sunter, G., 2009. The Arabidopsis PEAPOD2 transcription factor interacts with geminivirus AL2 protein and the coat protein promoter. Virology 392, 196-202.

- Lai, J., Chen, H., Teng, K., Zhao, Q., Zhang, Z., Li, Y., Liang, L., Xia, R., Wu, Y., Guo, H., Xie, Q., 2009. RKP, a RING finger E3 ligase induced by BSCTV C4 protein, affects geminivirus infection by regulation of the plant cell cycle. The Plant journal : for cell and molecular biology 57, 905-917.

- Latham, J.R., 1997. Induction of plant cell division by beet curly top virus gene C4. The Plant journal : for cell and molecular biology 11, 1273-1283.

- Lee, C., Goldberg, J., 2010. Structure of coatomer cage proteins and the relationship among COPI, COPII, and clathrin vesicle coats. Cell 142, 123-132.

- Lee, S.Y., Yang, J.S., Hong, W., Premont, R.T., Hsu, V.W., 2005. ARFGAP1 plays a central role in coupling COPI cargo sorting with vesicle formation. The Journal of cell biology 168, 281-290.

- Leon-Reyes, A., Van der Does, D., De Lange, E.S., Delker, C., Wasternack, C., Van Wees, S.C., Ritsema, T., Pieterse, C.M., 2010. Salicylate-mediated suppression of jasmonate-responsive gene expression in Arabidopsis is targeted downstream of the jasmonate biosynthesis pathway. Planta 232, 1423-1432.

- Lewis, J.D., Lazarowitz, S.G., 2010. Arabidopsis synaptotagmin SYTA regulates endocytosis and virus movement protein cell-to-cell transport. Proceedings of the National Academy of Sciences of the United States of America 107, 2491-2496.

- Liu, L., Saunders, K., Thomas, C.L., Davies, J.W., Stanley, J., 1999. Bean yellow dwarf virus RepA, but not rep, binds to maize retinoblastoma protein, and the virus tolerates mutations in the consensus binding motif. Virology 256, 270-279.

- Liu, P.P., von Dahl, C.C., Klessig, D.F., 2011. The extent to which methyl salicylate is required for signaling systemic acquired resistance is dependent on exposure to light after infection. Plant physiology 157, 2216-2226.

- Liu, Y., Nakayama, N., Schiff, M., Litt, A., Irish, V.F., Dinesh-Kumar, S.P., 2004a. Virus induced gene silencing of a DEFICIENS ortholog in Nicotiana benthamiana. Plant molecular biology 54, 701-711.

- Liu, Y., Schiff, M., Dinesh-Kumar, S.P., 2002. Virus-induced gene silencing in tomato. The Plant journal : for cell and molecular biology 31, 777-786.

- Liu, Y., Schiff, M., Dinesh-Kumar, S.P., 2004b. Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to tobacco mosaic virus. The Plant journal : for cell and molecular biology 38, 800-809.

- Lozano-Durán, R., Bejarano, E.R., 2013. Geminiviral Co-option of Post-Translational

- Modification Pathways. Advances in Virus Research.

- Lozano-Durán, R., Rosas-Diaz, T., Gusmaroli, G., Luna, A.P., Taconnat, L., Deng, X.W., Bejarano, E.R., 2011a. Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes and inhibit jasmonate signaling in Arabidopsis thaliana. The Plant cell 23, 1014-1032.

- Lozano-Durán, R., Rosas-Diaz, T., Luna, A.P., Bejarano, E.R., 2011b. Identification of host genes involved in geminivirus infection using a reverse genetics approach. PLoS One 6, e22383.

- Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., Baulcombe, D.C., 2003. Virus-induced gene silencing in plants. Methods 30, 296-303.

- Luna, A.P., Morilla, G., Voinnet, O., Bejarano, E.R., 2012. Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. Molecular plant-microbe interactions : MPMI 25, 1294-1306.

- Luque, A., Sanz-Burgos, A.P., Ramirez-Parra, E., Castellano, M.M., Gutierrez, C., 2002. Interaction of geminivirus Rep protein with replication factor C and its potential role during geminivirus DNA replication. Virology 302, 83-94.

- Mandadi, K.K., Scholthof, K.B., 2012. Characterization of a viral synergism in the monocot Brachypodium distachyon reveals distinctly altered host molecular processes associated with disease. Plant physiology 160, 1432-1452.

- Mansoor, S., Briddon, R.W., Zafar, Y., Stanley, J., 2003. Geminivirus disease complexes: an emerging threat. Trends in plant science 8, 128-134.

- Mao, P., Duan, M., Wei, C., Li, Y., 2007. WRKY62 transcription factor acts downstream of cytosolic NPR1 and negatively regulates jasmonate-responsive gene expression. Plant & cell physiology 48, 833-842.

- Marino, D., Peeters, N., Rivas, S., 2012. Ubiquitination during plant immune signaling. Plant physiology 160, 15-27.

- Matheson, L.A., Hanton, S.L., Rossi, M., Latijnhouwers, M., Stefano, G., Renna, L., Brandizzi, F., 2007. Multiple roles of ADP-ribosylation factor 1 in plant cells include spatially regulated recruitment of coatomer and elements of the Golgi matrix. Plant physiology 143, 1615-1627.

- Michelsen, K., Schmid, V., Metz, J., Heusser, K., Liebel, U., Schwede, T., Spang, A., Schwappach, B., 2007. Novel cargo-binding site in the beta and delta subunits of coatomer. The Journal of cell biology 179, 209-217.

- Mills-Lujan, K., Deom, C.M., 2010. Geminivirus C4 protein alters Arabidopsis development. Protoplasma 239, 95-110.

- Miozzi, L., Napoli, C., Sardo, L., Accotto, G.P., 2014. Transcriptomics of the interaction between the monopartite phloem-limited geminivirus tomato yellow leaf curl Sardinia virus and Solanum lycopersicum highlights a role for plant hormones, autophagy and plant immune system fine tuning during infection. PLoS One 9, e89951.

- Moffett, P., Farnham, G., Peart, J., Baulcombe, D.C., 2002. Interaction between domains of a plant NBS-LRR protein in disease resistance-related cell death. The EMBO journal 21, 4511-4519.

- Mubin, M., Amin, I., Amrao, L., Briddon, R.W., Mansoor, S., 2010. The hypersensitive response induced by the V2 protein of a monopartite begomovirus is countered by the C2 protein. Molecular plant pathology 11, 245-254.

- Mur L.A., K.P., Atzorn R., Miersch O. and Wasternack C., 2006. The Outcomes of Concentration-Specific Interactions between Salicylate and Jasmonate Signaling Include Synergy, Antagonism, and Oxidative Stress Leading to Cell Death. Plant physiology 140, 1249-1262.

- Mur, L.A., Kenton, P., Lloyd, A.J., Ougham, H., Prats, E., 2008. The hypersensitive response; the centenary is upon us but how much do we know? Journal of experimental botany 59, 501-520.

- Nagar, S., Pedersen, T.J., Carrick, K.M., Hanley-Bowdoin, L., Robertson, D., 1995. A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. The Plant cell 7, 705-719.

- Navas-Castillo, J., Fiallo-Olive, E., Sanchez-Campos, S., 2011. Emerging virus diseases transmitted by whiteflies. Annual review of phytopathology 49, 219-248.

- Ndamukong, I., Abdallat, A.A., Thurow, C., Fode, B., Zander, M., Weigel, R., Gatz, C., 2007. SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. The Plant journal : for cell and molecular biology 50, 128-139.

- Nilsson, T., Jackson, M., Peterson, P.A., 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. Cell 58, 707-718.

- Noris, E., Vaira, A.M., Caciagli, P., Masenga, V., Gronenborn, B., Accotto, G.P., 1998. Amino acids in the capsid protein of tomato yellow leaf curl virus that are crucial for systemic infection, particle formation, and insect transmission. Journal of virology 72, 10050-10057.

- Noueiry, A.O., Lucas, W.J., Gilbertson, R.L., 1994. Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. Cell 76, 925-932.

- Pallas, V., Garcia, J.A., 2011. How do plant viruses induce disease? Interactions and interference with host components. The Journal of general virology 92, 2691-2705.

- Park, J., Hwang, H., Shim, H., Im, K., Auh, C.K., Lee, S., Davis, K.R., 2004. Altered cell shapes, hyperplasia, and secondary growth in Arabidopsis caused by beet curly top geminivirus infection. Molecules and cells 17, 117-124.

- Pasumarthy, K.K., Choudhury, N.R., Mukherjee, S.K., 2010. Tomato leaf curl Kerala virus (ToLCKeV) AC3 protein forms a higher order oligomer and enhances ATPase activity of replication initiator protein (Rep/AC1). Virology journal 7, 128.

- Paul, M.J., Frigerio, L., 2007. Coated vesicles in plant cells. Seminars in cell & developmental biology 18, 471-478.

- Pauwels, L., Barbero, G.F., Geerinck, J., Tilleman, S., Grunewald, W., Perez, A.C., Chico, J.M., Bossche, R.V., Sewell, J., Gil, E., Garcia-Casado, G., Witters, E., Inze, D., Long, J.A., De Jaeger, G., Solano, R., Goossens, A., 2010. NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464, 788-791.

- Peele, C., Jordan, C.V., Muangsan, N., Turnage, M., Egelkrout, E., Eagle, P., Hanley-Bowdoin, L., Robertson, D., 2001. Silencing of a meristematic gene using geminivirus-derived vectors. The Plant journal : for cell and molecular biology 27, 357-366.

- Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., Mundy, J., 2000. Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell 103, 1111-1120.

- Peyroche, A., Paris, S., Jackson, C.L., 1996. Nucleotide exchange on ARF mediated by yeast Gea1 protein. Nature 384, 479-481.

- Pieterse, C.M., Leon-Reyes, A., Van der Ent, S., Van Wees, S.C., 2009. Networking by small-molecule hormones in plant immunity. Nature chemical biology 5, 308-316.

- Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., Van Wees, S.C., 2012. Hormonal modulation of plant immunity. Annual review of cell and developmental biology 28, 489-521.

- Pilartz, M., Jeske, H., 1992. Abutilon mosaic geminivirus double-stranded DNA is packed into minichromosomes. Virology 189, 800-802.

- Pilartz, M., Jeske, H., 2003. Mapping of abutilon mosaic geminivirus minichromosomes. Journal of virology 77, 10808-10818.

- Piroux, N., Saunders, K., Page, A., Stanley, J., 2007. Geminivirus pathogenicity protein C4 interacts with Arabidopsis thaliana shaggy-related protein kinase AtSKeta, a component of the brassinosteroid signalling pathway. Virology 362, 428-440.

- Poornima Priyadarshini CG, A.M., Tippeswamy R, Savithri HS., 2011. Functional characterization of coat protein and V2 involved in cell to cell movement of Cotton leaf curl Kokhran virus-Dabawali. PLoS One 6 (11) e26929.

- Popoff, V., Adolf, F., Brugger, B., Wieland, F., 2011. COPI budding within the Golgi stack. Cold Spring Harbor perspectives in biology 3, a005231.

- Raja, P., Sanville, B.C., Buchmann, R.C., Bisaro, D.M., 2008. Viral genome methylation as an epigenetic defense against geminiviruses. Journal of virology 82, 8997-9007.

- Raja, P., Wolf, J.N., Bisaro, D.M., 2010. RNA silencing directed against geminiviruses: post-transcriptional and epigenetic components. Biochimica et biophysica acta 1799, 337-351.

- Ratcliff, F., Martin-Hernandez, A.M., Baulcombe, D.C., 2001. Technical Advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. The Plant journal : for cell and molecular biology 25, 237-245.

- Reymond, P., Farmer, E.E., 1998. Jasmonate and salicylate as global signals for defense gene expression. Current opinion in plant biology 1, 404-411.

- Robert-Seilaniantz, A., Grant, M., Jones, J.D., 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annual review of phytopathology 49, 317-343.

- Robert-Seilaniantz, A., Navarro, L., Bari, R., Jones, J.D., 2007. Pathological hormone imbalances. Current opinion in plant biology 10, 372-379.

- Robertson, D., 2004. VIGS vectors for gene silencing: many targets, many tools. Annual review of plant biology 55, 495-519.

- Robinson D. , H.M.C., Bubeck J., Pepperkok R. and Ritzenthaler C., 2007. Membrane Dynamics in the Early Secretory Pathway. Critical Reviews in Plant Sciences 26

- 199-225.

- Rodriguez-Negrete, E., Lozano-Duran, R., Piedra-Aguilera, A., Cruzado, L., Bejarano, E.R., Castillo, A.G., 2013. Geminivirus Rep protein interferes with the plant DNA methylation machinery and suppresses transcriptional gene silencing. The New phytologist 199, 464-475.

- Rojas, M.R., Hagen, C., Lucas, W.J., Gilbertson, R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annual review of phytopathology 43, 361-394.

- Rojas, M.R., Jiang, H., Salati, R., Xoconostle-Cazares, B., Sudarshana, M.R., Lucas, W.J., Gilbertson, R.L., 2001. Functional analysis of proteins involved in movement of the monopartite begomovirus, Tomato yellow leaf curl virus. Virology 291, 110-125.

- Rojas, M.R., Noueiry, A.O., Lucas, W.J., Gilbertson, R.L., 1998. Bean Dwarf mosaic geminivirus movement proteins recognize DNA in a form- and size-specific manner. Cell 95, 105-113.

- Sánchez-Durán, M.A., Dallas, M.B., Ascencio-Ibanez, J.T., Reyes, M.I., Arroyo-Mateos, M., Ruiz-Albert, J., Hanley-Bowdoin, L., Bejarano, E.R., 2011. Interaction between geminivirus replication protein and the SUMO-conjugating enzyme is required for viral infection. Journal of virology 85, 9789-9800.

- Sanderfoot, A.A., Lazarowitz, S.G., 1995. Cooperation in Viral Movement: The Geminivirus BL1 Movement Protein Interacts with BR1 and Redirects It from the Nucleus to the Cell Periphery. The Plant cell 7, 1185-1194.

- Santner, A., Calderon-Villalobos, L.I., Estelle, M., 2009. Plant hormones are versatile chemical regulators of plant growth. Nature chemical biology 5, 301-307.

- Sato, M., Tsuda, K., Wang, L., Coller, J., Watanabe, Y., Glazebrook, J., Katagiri, F., 2010. Network modeling reveals prevalent negative regulatory relationships between signaling sectors in Arabidopsis immune signaling. PLoS pathogens 6, e1001011.

- Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., Manners, J.M., 2000. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proceedings of the National Academy of Sciences of the United States of America 97, 11655-11660.

- Schilmiller, A.L., Charbonneau, A.L., Last, R.L., 2012. Identification of a BAHD acetyltransferase that produces protective acyl sugars in tomato trichomes. Proceedings of the National Academy of Sciences of the United States of America 109, 16377-16382.

- Selth, L.A., Dogra, S.C., Rasheed, M.S., Healy, H., Randles, J.W., Rezaian, M.A., 2005. A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. The Plant cell 17, 311-325.

- Senthil-Kumar, M., Lee, H.K., Mysore, K.S., 2013. VIGS-mediated forward genetics screening for identification of genes involved in nonhost resistance. Journal of visualized experiments : JoVE, e51033.

- Senthil-Kumar, M., Rame Gowda, H.V., Hema, R., Mysore, K.S., Udayakumar, M., 2008. Virus-induced gene silencing and its application in characterizing genes involved in water-deficit-stress tolerance. Journal of plant physiology 165, 1404-1421.

- Seo, Y.S., Gepts, P., Gilbertson, R.L., 2004. Genetics of resistance to the geminivirus, Bean dwarf mosaic virus, and the role of the hypersensitive response in common bean. TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik 108, 786-793.

- Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R.A., Rothman, J.E., 1991. ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. Cell 67, 239-253.

- Settlage, S.B., Miller, A.B., Gruissem, W., Hanley-Bowdoin, L., 2001. Dual interaction of a geminivirus replication accessory factor with a viral replication protein and a plant cell cycle regulator. Virology 279, 570-576.

- Settlage, S.B., Miller, A.B., Hanley-Bowdoin, L., 1996. Interactions between geminivirus replication proteins. Journal of virology 70, 6790-6795.

- Settlage, S.B., See, R.G., Hanley-Bowdoin, L., 2005. Geminivirus C3 protein: replication enhancement and protein interactions. Journal of virology 79, 9885-9895.

- Sharma, P., Ikegami, M., 2010. Tomato leaf curl Java virus V2 protein is a determinant of virulence, hypersensitive response and suppression of posttranscriptional gene silencing. Virology 396, 85-93.

- Sharma, P., Ikegami, M., Kon, T., 2010. Identification of the virulence factors and suppressors of posttranscriptional gene silencing encoded by Ageratum yellow vein virus, a monopartite begomovirus. Virus research 149, 19-27.

- Shen, W., Hanley-Bowdoin, L., 2006. Geminivirus infection up-regulates the expression of two Arabidopsis protein kinases related to yeast SNF1- and mammalian AMPK-activating kinases. Plant physiology 142, 1642-1655.

- Smalle, J., Vierstra, R.D., 2004. The ubiquitin 26S proteasome proteolytic pathway. Annual review of plant biology 55, 555-590.

- Soto, 2001. Intracellular distribution of the three virion-sense encoded proteins of Beet curly top virus. Phytopathology 91.

- Spitzer, B., Zvi, M.M., Ovadis, M., Marhevka, E., Barkai, O., Edelbaum, O., Marton, I., Masci, T., Alon, M., Morin, S., Rogachev, I., Aharoni, A., Vainstein, A., 2007. Reverse genetics of floral scent: application of tobacco rattle virus-based gene silencing in Petunia. Plant physiology 145, 1241-1250.

- Spoel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., Pieterse, C.M., 2003. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. The Plant cell 15, 760-770.

- Stefano, G., Renna, L., Chatre, L., Hanton, S.L., Moreau, P., Hawes, C., Brandizzi, F., 2006. In tobacco leaf epidermal cells, the integrity of protein export from the endoplasmic reticulum and of ER export sites depends on active COPI machinery. The Plant journal : for cell and molecular biology 46, 95-110.

- Stout, M.J., Thaler, J.S., Thomma, B.P., 2006. Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. Annual review of entomology 51, 663-689.

- Sunter, G., Bisaro, D.M., 1991. Transactivation in a geminivirus: AL2 gene product is needed for coat protein expression. Virology 180, 416-419.

- Sunter, G., Bisaro, D.M., 1992. Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. The Plant cell 4, 1321-1331.

- Teng, K., Chen, H., Lai, J., Zhang, Z., Fang, Y., Xia, R., Zhou, X., Guo, H., Xie, Q., 2010. Involvement of C4 protein of beet severe curly top virus (family Geminiviridae) in virus movement. PLoS One 5, e11280.

- Thaler, J.S., Agrawal, A.A., Halitschke, R., 2010. Salicylate-mediated interactions between pathogens and herbivores. Ecology 91, 1075-1082.

- Thaler, J.S., Humphrey, P.T., Whiteman, N.K., 2012. Evolution of jasmonate and salicylate signal crosstalk. Trends in plant science 17, 260-270.

- Thaler, J.S., Owen, B., Higgins, V.J., 2004. The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. Plant physiology 135, 530-538.

- Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., Browse, J., 2007. JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448, 661-665.

- Thompson, J.A., Brown, J.C., 2012. Role of Coatomer Protein I in Virus Replication. Journal of virology & antiviral research 1.

- Timmermans MCP, D.O., Messing J, 1994. Geminiviruses and their uses as extrachromosomal replicons. . Annu Rev Plant Physiol Plant Mol Biol 45, 79-112.

- Trinks, D., Rajeswaran, R., Shivaprasad, P.V., Akbergenov, R., Oakeley, E.J., Veluthambi, K., Hohn, T., Pooggin, M.M., 2005. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. Journal of virology 79, 2517-2527.

- Ulrich, H.D., 2009. The SUMO system: an overview. Methods in molecular biology 497, 3-16.

- Unver, T., Budak, H., 2009. Virus-induced gene silencing, a post transcriptional gene silencing method. International journal of plant genomics 2009, 198680.

- Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M.C., Rodenburg, N., Pauwels, L., Goossens, A., Korbes, A.P., Memelink, J., Ritsema, T., Van Wees, S.C., Pieterse, C.M., 2013. Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. The Plant cell 25, 744-761.

- van der Hoorn, R.A., Kamoun, S., 2008. From Guard to Decoy: a new model for perception of plant pathogen effectors. The Plant cell 20, 2009-2017.

- van Loon, L.C., Rep, M., Pieterse, C.M., 2006. Significance of inducible defense-related proteins in infected plants. Annual review of phytopathology 44, 135-162.

- van Wees, S.C., de Swart, E.A., van Pelt, J.A., van Loon, L.C., Pieterse, C.M., 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America 97, 8711-8716.

- van Wees, S.C., Luijendijk, M., Smoorenburg, I., van Loon, L.C., Pieterse, C.M., 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with

a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. Plant molecular biology 41, 537-549.

- van Wezel, R., Liu, H., Tien, P., Stanley, J., Hong, Y., 2001. Gene C2 of the monopartite geminivirus tomato yellow leaf curl virus-China encodes a pathogenicity determinant that is localized in the nucleus. Molecular plant-microbe interactions : MPMI 14, 1125-1128.

- Varsani, A., Navas-Castillo, J., Moriones, E., Hernandez-Zepeda, C., Idris, A., Brown, J.K., Murilo Zerbini, F., Martin, D.P., 2014. Establishment of three new genera in the family Geminiviridae: Becurtovirus, Eragrovirus and Turncurtovirus. Archives of virology 159, 2193-2203.

- Verhage, A., van Wees, S.C., Pieterse, C.M., 2010. Plant immunity: it's the hormones talking, but what do they say? Plant physiology 154, 536-540.

- Vlot, A.C., Dempsey, D.A., Klessig, D.F., 2009. Salicylic Acid, a multifaceted hormone to combat disease. Annual review of phytopathology 47, 177-206.

- Vlot, A.C., Klessig, D.F., Park, S.W., 2008. Systemic acquired resistance: the elusive signal(s). Current opinion in plant biology 11, 436-442.

- Walling, L.L., 2008. Avoiding effective defenses: strategies employed by phloem-feeding insects. Plant physiology 146, 859-866.

- Wang, H., Buckley, K.J., Yang, X., Buchmann, R.C., Bisaro, D.M., 2005. Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. Journal of virology 79, 7410-7418.

- Wartig, L., Kheyr-Pour, A., Noris, E., De Kouchkovsky, F., Jouanneau, F., Gronenborn, B., Jupin, I., 1997. Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2, and C2 ORFs in viral pathogenesis. Virology 228, 132-140.

- Wasternack, C., 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Annals of botany 100, 681-697.

- Wasternack, C., Hause, B., 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Annals of botany 111, 1021-1058.

- Waterhouse, P.M., Wang, M.B., Finnegan, E.J., 2001. Role of short RNAs in gene silencing. Trends in plant science 6, 297-301.

- Wege, C., Pohl, D., 2007. Abutilon mosaic virus DNA B component supports mechanical virus transmission, but does not counteract begomoviral phloem limitation in transgenic plants. Virology 365, 173-186.

- Weinberg, R.A., 1995. The retinoblastoma protein and cell cycle control. Cell 81, 323-330.

- Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C., Baker, B., 1994. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. Cell 78, 1101-1115.

- Whitham, S.A., Yang, C., Goodin, M.M., 2006. Global impact: elucidating plant responses to viral infection. Molecular plant-microbe interactions : MPMI 19, 1207-1215.

- Wu, Y., Zhang, D., Chu, J.Y., Boyle, P., Wang, Y., Brindle, I.D., De Luca, V., Despres, C., 2012. The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. Cell reports 1, 639-647.

- Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., Turner, J.G., 1998. COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280, 1091-1094.

- Xie, Q., Sanz-Burgos, A.P., Hannon, G.J., Gutierrez, C., 1996. Plant cells contain a novel member of the retinoblastoma family of growth regulatory proteins. The EMBO journal 15, 4900-4908.

- Xie, Q., Suarez-Lopez, P., Gutierrez, C., 1995. Identification and analysis of a retinoblastoma binding motif in the replication protein of a plant DNA virus: requirement for efficient viral DNA replication. The EMBO journal 14, 4073-4082.

- Xie, Y., Zhao, L., Jiao, X., Jiang, T., Gong, H., Wang, B., Briddon, R.W., Zhou, X., 2013. A recombinant begomovirus resulting from exchange of the C4 gene. The Journal of general virology 94, 1896-1907.

- Xu, J., Scheres, B., 2005. Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. The Plant cell 17, 525-536.

- Xu, L., Zhang, W., He, X., Liu, M., Zhang, K., Shaban, M., Sun, L., Zhu, J., Luo, Y., Yuan, D., Zhang, X., Zhu, L., 2014. Functional characterization of cotton genes responsive to Verticillium dahliae through bioinformatics and reverse genetics strategies. Journal of experimental botany 65, 6679-6692.

- Yang, J.Y., Iwasaki, M., Machida, C., Machida, Y., Zhou, X., Chua, N.H., 2008. betaC1, the pathogenicity factor of TYLCCNV, interacts with AS1 to alter leaf development and suppress selective jasmonic acid responses. Genes & development 22, 2564-2577.

- Yu, X., Breitman, M., Goldberg, J., 2012. A structure-based mechanism for Arf1-dependent recruitment of coatomer to membranes. Cell 148, 530-542.

- Yuan, C., Li, C., Yan, L., Jackson, A.O., Liu, Z., Han, C., Yu, J., Li, D., 2011. A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots. PLoS One 6, e26468.

- Zander, M., La Camera, S., Lamotte, O., Metraux, J.P., Gatz, C., 2010. Arabidopsis thaliana class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. The Plant journal : for cell and molecular biology 61, 200-210.

- Zerangue, N., Malan, M.J., Fried, S.R., Dazin, P.F., Jan, Y.N., Jan, L.Y., Schwappach, B., 2001. Analysis of endoplasmic reticulum trafficking signals by combinatorial screening in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 98, 2431-2436.

- Zhang, J., Dong, J., Xu, Y., Wu, J., 2012. V2 protein encoded by Tomato yellow leaf curl China virus is an RNA silencing suppressor. Virus research 163, 51-58.

- Zhang, S.C., Ghosh, R., Jeske, H., 2002. Subcellular targeting domains of Abutilon mosaic geminivirus movement protein BC1. Archives of virology 147, 2349-2363.

- Zhang, S.C., Wege, C., Jeske, H., 2001. Movement proteins (BC1 and BV1) of Abutilon mosaic geminivirus are cotransported in and between cells of sink but not of source leaves as detected by green fluorescent protein tagging. Virology 290, 249-260.

- Zhang, Z., Chen, H., Huang, X., Xia, R., Zhao, Q., Lai, J., Teng, K., Li, Y., Liang, L., Du, Q., Zhou, X., Guo, H., Xie, Q., 2011. BSCTV C2 attenuates the degradation of SAMDC1 to suppress DNA methylation-mediated gene silencing in Arabidopsis. The Plant cell 23, 273-288.

- Zheng, X.Y., Spivey, N.W., Zeng, W., Liu, P.P., Fu, Z.Q., Klessig, D.F., He, S.Y., Dong, X., 2012. Coronatine promotes Pseudomonas syringae virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. Cell host & microbe 11, 587-596.

AIMS

The main aim of this work is to explore the functional interactions between geminiviruses and their hosts. For the accomplishment of this aim, several approaches have been followed:

1. To study the interaction between the jasmonate signalling pathway and the geminiviral infection.

2. To identify novel host genes involved in geminivirus infection through a functional genomics approach using virus-induced gene silencing in combination with transgenic 2IRGFP *N. benthamiana* plants.

3. To characterize the role of retrograde vesicle trafficking in the geminivirus infection and in other plant-pathogen interactions.

Chapter I

JASMONATE: UNRAVELING THE PHYTOHORMONE SIGNALLING DURING GEMINIVIRUS INFECTION

ABSTRACT

Viruses must create a suitable cell environment and elude defence mechanisms, which likely involves interactions with host proteins and subsequent interference with or usurpation of cellular processes. Geminiviruses are small DNA viruses infecting a wide range of plant species worldwide. Due to limited coding capacity, geminiviruses must rely on the plant machinery for the accomplishment of their infection. C2 is a multifunctional geminiviral protein important for pathogenicity, which has been described to interfere with the function of the ubiquitin E3 ligase SCF complexes in the cell, affecting multiple plant responses. Here, we describe that C2 from the begomovirus *Tomato yellow leaf curl Sardinia virus* (TYLCSV) interferes with jasmonate (JA) signalling when transgenically expressed in *Arabidopsis*. Given that the SCF^{COI1} complex is the JA receptor, it would be feasible to speculate that the C2-mediated inhibition of JA responses is likely due to the effect of C2 on this complex. However, transcriptomic analyses show that C2 is not generally affecting the transcriptional response to jasmonates, but specifically suppressing JA-triggered defence responses and secondary metabolism. Consistently with the inhibition of JA-mediated defences, transgenic plants expressing C2 are more susceptible to biotrophic pathogens. Interestingly, we found that C2 from TYLCSV, but not C2 from the curtovirus *Beet curly top virus* (BCTV), physically interacts with a member of the JAZ family of repressor proteins, and that overexpression of this JAZ protein exerts a negative impact on the infection by begomoviruses in *Arabidopsis*. To further investigate this, we examined the effect of JA-signalling over TYLCSV in tomato. Strikingly, we found that C2 from TYLCSV does not interact with JAZ repressor proteins from tomato, and that exogenous application of jasmonates have opposite effects on the infection by TYLCSV in *Arabidopsis* and tomato. Based on our findings, we propose that C2 from TYLCSV may interfere with the JA response at multiple levels in a host-dependent manner.

INTRODUCTION

Geminiviruses constitute a large family of plant viruses with circular, single-stranded (ss) DNA genomes packaged within geminate particles (Rojas et al., 2005; Seal et al., 2006) which infect a broad range of plants and cause devastating crop diseases (Morales and Anderson, 2001; Mansoor et al., 2003). Based on host range and insect transmission, the family *Geminiviridae* has been classified into seven genera (Brown JK, 2012; Varsani et al., 2014). *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato yellow leaf curl virus* (TYLCV) are members of the *Begomovirus* genus, and are two of the causal agents of the Tomato yellow leaf curl disease (TYLCD). TYLCSV and TYLCV have a monopartite genome, which encodes six proteins and contains an intergenic region (IR) comprising the origin of replication and viral promoters. The open reading frames (ORFs) in the complementary sense orientation encode a replication-associated protein (Rep/C1), a transcriptional activator protein (TrAP/C2/L2/AL2), and a replication enhancer protein (REn/C3); a small ORF, C4, is located within the Rep ORF but in a different reading frame. The virion strand contains two ORFs encoding the coat protein (CP) and a small protein named V2 (Rojas et al., 2005; Jeske, 2009). In spite of their limiting coding capacity, the few proteins encoded by the viral genome are sufficient to complete all processes required for infection, such as viral replication, movement, and suppression or evasion of plant defence mechanisms.

An excellent example of the multifunctionality of geminiviral proteins is C2. C2 is a small protein, around 15 KDa, which localize mainly in the nucleus. C2 proteins contain a NLS in its basic N terminus, a central zinc finger domain, and a C-terminal acidic domain that seems to play a role in transcriptional activation. A comparison of the C2 protein from TYLCSV and TYLCV can be found in Figure 1. C2 has been shown to be required for either viral infection or full infectivity in several cases, suggesting a high-value role during geminivirus infection (Etessami et al., 1988; Sung and Coutts, 1995; Wartig et al., 1997; Lozano-Durán et al., 2011a). Begomovirus C2 has been described as a transcription factor for viral genes (Sunter and Bisaro, 1991; 1992), also able to trigger transcription of host genes (Trinks et al., 2005) and a suppressor of gene silencing, both post-transcriptional (PTGS) and transcriptional (TGS) (Dong et al., 2003; Wang et al., 2003; Vanitharani et al., 2004; Wang et al., 2005; Buchmann et al., 2009; Luna et al., 2012). C2 interacts with one of the subunit of the CSN (COP9 signalosome) complex affecting the ability of the CSN to regulate the SCF-type (SKP1, CUL1/CDC53, F box proteins) ubiquitin E3 ligase complex. C2-CSN interaction impairs the cellular processes regulated by SCF complexes, including jasmonate (JA) signalling, when the viral protein is transgenically expressed in *Arabidopsis* (Lozano-Durán et al., 2011a). These results, together with the extraordinary variety of molecular functions assigned to

this small viral protein to date, makes it an especially intriguing target for both functional and evolutionary studies.

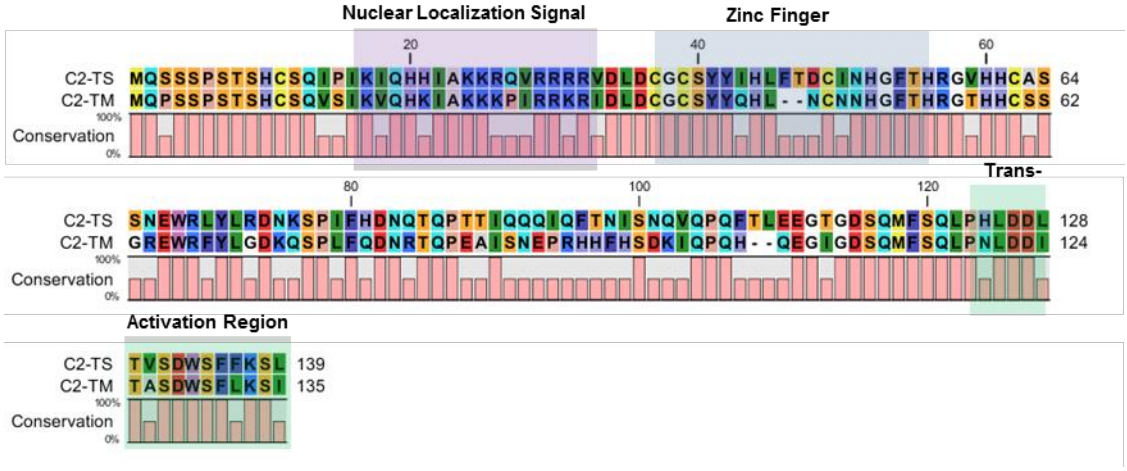


Figure 1. Alignment of C2 from the begomoviruses TYLCSV and TYLCV. The nuclear localization signal, the zinc finger domain and the transactivation region are indicated. C2-TS: C2 from TYLCSV (*Tomato yellow leaf curl Sardinia virus*); C2-TM: C2 from TYLCV (*Tomato yellow leaf curl virus*). The sequence alignment was performed using ClustalW (EBI server; (McWilliam et al., 2013)), and the figure was generated by CLC Main Workbench 7.

Plant innate immunity is based on a surprisingly complex response that is highly flexible in its capacity to recognize and counteract different invaders, including viral infections (reviewed in (Nicaise, 2014)). Besides passive defence, based on the presence of pre-existing barriers like the rigid cell wall, plants can mount a systemic response that establishes an enhanced defensive capacity in tissues distant from the site of primary attack. This systemically induced defence response protects the plant against subsequent invaders. Systemic acquired resistance (SAR) is an example of an inducible defence response that is activated in the distal organs of a plant upon local infection with a pathogen, conferring resistance against subsequent attacks by a wide array of pathogens (viruses, bacteria, fungus and insects) (reviewed in (Durrant and Dong, 2004)). Multiple metabolites may function as systemic signals contributing to long-distance signalling in plant defence against pathogens and herbivores, including among others the plant hormones salicylic acid (SA), ethylene (ET) and JA (Pieterse and Van Loon, 2004; Shah, 2009).

The oxylipin jasmonic acid and its metabolites, collectively known as jasmonates (JAs), are important plant signalling molecules that mediate biotic and abiotic stress responses as well as several aspects of growth and development. Under normal growth conditions, JA levels are low and JA-mediated transcriptional responses are kept in a repressed state by JASMONATE ZIM-DOMAIN (JAZ) proteins. In response to stresses, such as those that result from insect feeding or necrotrophic pathogen infection, an increase in the levels of bioactive jasmonic acid-isoleucine (JA–

Ile) allows the hormone to facilitate interaction between the JAZ repressors and the F-box protein CORONATINE INSENSITIVE 1 (COI1), the recognition component of the JA receptor, the E3 ubiquitin ligase SCF^{COI1}. This interaction targets JAZs for ubiquitination and degradation via the 26S proteasome pathway, allowing for the induction of JA-responsive genes (Chini et al., 2007; Thines et al., 2007). JA-dependent transcriptional reprogramming is regulated by a cascade of transcription factors (TFs), in which MYC2 plays a major role, as indicated by the lower sensitivity to JA displayed by the *jin1* mutant, carrying a mutation in the *MYC2* gene (Boter et al., 2004; Lorenzo et al., 2004). JAZ proteins directly interact with MYC2 in the absence of JA keeping this transcription factor inactive (Chini et al., 2007); degradation of JAZ proteins in response to JA would allow MYC2 to activate or repress downstream target genes. JAZ proteins have also been shown to interact with MYC3 and MYC4 (Pauwels et al., 2010; Fernández-Calvo et al., 2011). Notably, JAZ expression is induced after JA perception or wounding, indicating that JAZ repressors are also JA-responsive genes, as part of the negative feedback loop regulation of JA responses (Chung et al., 2008). The SCF^{COI1} is required for JA perception and subsequent signalling in *Arabidopsis* but also in tomato (Ishiga et al., 2013). Interestingly, the phytotoxin coronatine (COR), which is synthesized and secreted by the plant pathogenic bacterial strain *Pseudomonas syringae* pv *tomato* DC3000 (*Pto* DC3000), activates the JA-signalling pathway by mimicking JA-Ile, the active form of the hormone, and binding the SCF^{COI1} in *Arabidopsis* and tomato, in turn suppressing the stomata-mediated and/or SA-mediated defences (Melotto et al., 2006; Uppalapati et al., 2007; Geng et al., 2014).

Within the signalling cascades that are triggered by JAs, JAZ repressor proteins play a central role. There are twelve JAZ proteins (JAZ1 to JAZ12) in *Arabidopsis* (reviewed in (Wager and Browse, 2012)). Members of the JAZ family exhibit high sequence variability, but generally possess three conserved domains which represent the distinguishing characteristics of the group (Figure 2). The N-terminal (NT) region contains a weakly conserved NT domain, which is involved in a small set of protein–protein interactions and that remains largely uncharacterized (Hou et al., 2010). The ZIM domain, within the central portion of the JAZ peptide sequence, contains a highly conserved TIFY motif (TIF[F/Y]XG; (Vanholme et al., 2007)). The ZIM domain mediates the interaction with an adaptor protein called NOVEL INTERACTOR OF JAZ (NINJA), which functions to recruit the transcription corepressor TOPLESS (TPL) and TPL-related proteins (TPRs) (Chung and Howe, 2009; Chung et al., 2009; Pauwels et al., 2010; Pauwels and Goossens, 2011). The ZIM domain also promotes homo- and heteromeric interactions among the twelve JAZ proteins in *Arabidopsis* (Chini et al., 2009a). The C-terminal Jas domain is strongly conserved across the JAZ family, being identical or with conservative substitutions across all twelve *Arabidopsis* JAZ proteins (Chini et al.,

2007; Thines et al., 2007). The Jas domain interacts, among others, with members of the basic helix-loop-helix (bHLH) (e.g., MYC2) and R2R3 MYB families of transcription factors, which in the absence of JAZ proteins promote the expression of JA-responsive genes (Chini et al., 2007; Melotto et al., 2008; Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Qi et al., 2011; Song et al., 2011). Within the Jas domain a degron signal can be found, which is responsible for the degradation of JAZs in the presence of JA–Ile (Chini et al., 2007; Thines et al., 2007; Melotto et al., 2008), and may play a role in nuclear localization (Thines et al., 2007; Grunewald et al., 2009) (see Figure 4 from general Introduction).

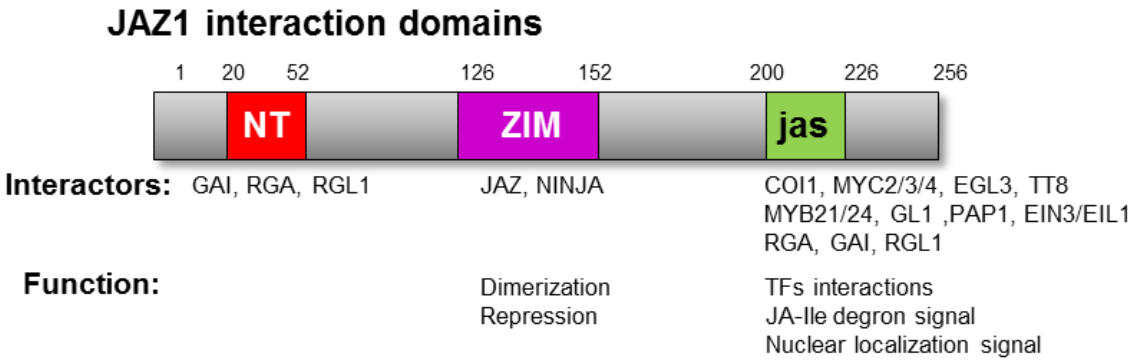


Figure 2. Schematic representation of JAZ1 interaction domains. Colored boxes indicate conserved domains. Known protein interactors and functions are listed below. DELLA proteins: GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GA (RGA), and RGA-LIKE1 (RGL1); JASMONATE ZIM DOMAIN (JAZ); NOVEL INTERACTOR OF JAZ (NINJA); CORONATINE INSENSITVE 1 (COI1); bHLH Transcription factors (TFs): MYC2/3/4, GLABRA (GL), ENHANCER OF GL3 (EGL3), TRANSPARENT TESTA8 (TT8); R2R3 MYB TFs: MYB21/24, GL1 and PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1); TFs involved in other hormonal signalling pathways: ETHYLENE INSENSITIVE 3 (EIN3)/ EIN3-LIKE1 (EIL1) (modified from (Pauwels and Goossens, 2011))

There were several hints at the transcriptional repression by JAZs, but the generation of mutants with the expected JA-hypersensitive phenotype was upset by the obvious redundancy among the JAZ proteins. Only the T-DNA insertional mutant *jaz10-1* and RNAi lines of *JAZ1* and *JAZ10* exhibited enhanced JA sensitivity (Grunewald et al., 2009), whereas other single JAZ mutants and the T-DNA insertion mutant *jaz1-1* did not show such a phenotype (Demianski et al., 2012).

Recently, a dominant repression activity by full length *JAZ8* has been described (Shyu et al., 2012) which is based on increased stability of JAZ8 due to lack of the conserved LPIARR motif, a part of the degron signal. Due to its absence in JAZ8, a strong interaction with COI1 in the presence of JA-Ile is excluded, leading to the increased stability of JAZ8. As a consequence, JAZ8 is stabilized against JA-mediated degradation and, when ectopically expressed in *Arabidopsis*, represses JA-

regulated growth and defence responses (Shyu et al., 2012). However, the consequences of JAZ8 removal from cells are unknown.

Traditionally, JA-mediated defences have been ascribed a role against necrotrophic pathogens and herbivorous insects. A growing body of evidence now points at these hormones as acting also at plant-virus interactions, and the repression of the JA response might be a pre-requisite for the infection by a wide array of RNA viruses: (i) stimulation of JA production in maize tolerant to *Maize rough dwarf virus-Río Cuarto* when infected with the virus suggests that higher JA content may be related to disease tolerance (Vigliocco et al., 2002); (ii) JA treatment induce early local but not systemic resistant defence of potato to *Potato virus Y* (PVY) (Kovak, 2009); (iii) infection with *Tobacco etch virus* (TEV) represses the JA response (Agudelo-Romero et al., 2008); (iv) the *Cucumber mosaic virus* (CMV) 2b protein disrupts JA signalling (Lewsey et al., 2010); (v), the treatment of *N. benthamiana* plants with JA or SA enhances systemic resistance to TMV, and that resistance is further enhanced by pre-treatment with JA followed by SA (Zhu et al., 2014).

On the contrary, there are studies that support a positive role of the JA in viral infections. For example, co-infection with PVY and *Potato virus X* (PVX), or infection with PVY carrying HC-Pro from *Plum pox virus* (PPV) induced oxylipin biosynthesis genes at early stages of infection and PCD-associated symptoms (Pacheco et al., 2012; García-Marcos et al., 2013). Both works showed that knocking down of the SCF^{COI1} F-box protein COI1 accelerated the development of symptoms and the viral accumulation at early stages of the infection, although both symptoms and viral titres were similar to wild-type plants as infection progressed. Interestingly, JA treatment at early stages of PVY–PVX double infection enhanced resistance, but later application increased susceptibility, probably as a result of the antagonistic effect of JA on SA (García-Marcos et al., 2013). Similar studies have shown that JA-responsive genes are modulated at early stages of infection, e.g. in the infection by *Cauliflower mosaic virus* (CaMV) in *Arabidopsis* and by *Panicum mosaic virus* and its satellite virus in the monocot plant *Brachypodium distachyon* (Love et al., 2005; Love et al., 2012; Mandadi and Scholthof, 2012).

Virus-induced gene silencing (VIGS) of the SCF^{COI1} F-box protein COI1 compromises *N* gene–mediated resistance to *Tobacco mosaic virus* (TMV) (Liu et al., 2004); however, a more recent work demonstrated that *N*-mediated resistance to TMV was enhanced in the *NtCOI1-RNAi* line, indicating that COI1 negatively affects resistance, partially as results of elevated SA levels (Oka et al., 2013). Furthermore, it has also been reported that silencing of *AOS* (*ALLENE OXIDE SYNTHASE*), a JA biosynthetic gene, enhanced resistance to TMV, and that exogenous application of JA reduced local resistance to TMV and permitted systemic movement, implying that such treatment abolished *N*-mediated resistance to TMV (Oka et al., 2013).

It has been reported that the two additional branches of the oxylipin biosynthesis pathway, the 9-*lipoxigenase* (LOX) and α-*dioxygenase* (DOX) branches, are also involved in conferring resistance against biotrophic pathogens (Vicente et al., 2012). More recently, it has been described that silencing of different branches of the oxylipin biosynthetic pathway, either the 9-LOX, 13-LOX, or α-DOX-1, attenuates the PCD-associated symptoms caused by co-infection of PVX with PVY or became less susceptible to *Tomato spotted wilt virus* (García-Marcos et al., 2013).

JA signalling is also altered during geminiviral infection. Repression of the JA pathway or JA-responsive genes have been reported in transgenic plants expressing a pathogenicity factor encoded by the DNAβ of *Tomato yellow leaf curl China virus* (TYLCCNV) or in *Arabidopsis* plants infected with *Cabbage leaf curl virus* (CaLCuV) (Ascencio-Ibánez et al., 2008; Yang et al., 2008), and we have reported that plants expressing the C2/L2 protein from the begomoviruses TYLCSV or TYLCV or the curtovirus *Beet curly top virus* (BCTV) display an inhibition of the JA signalling pathway at the transcriptional level, and are less sensitive to exogenously applied JA. Since a malfunction of SCF E3 ubiquitin ligases has been described in these transgenic C2/L2 *Arabidopsis* plants, the lower sensitivity to JA could be linked to a functional impairment of the JA receptor, the SCF^{COI1} complex (Lozano-Durán et al., 2011a).

Interestingly, a negative impact of JA on the geminivirus infection has been observed: exogenous application of JA interferes with BCTV infection in *Arabidopsis*, leading to milder symptoms and lower viral accumulation (Lozano-Durán et al., 2011a). Therefore, we hypothesized that the suppression of the JA-response in the host exerted by geminiviruses could be a virulence mechanism.

Given that geminiviruses are insect-transmitted viruses, the suppression of the JA response might imply an additional potential benefit, since it could enhance viral transmission by the insect vector and thus promote the spread of the disease. For example it has been shown that nymphal development of the whitefly *Bemisia tabaci*, the begomovirus vector, is slowed or disrupted by JA induced defences (Kempema et al., 2007; Valenzuela-Soto et al., 2010). Interestingly, a similar hypothesis has been proposed for the suppression of JA signalling by CMV 2b protein (Lewsey et al., 2010). The authors propose that 2b may be an important, albeit indirect, factor affecting transmission of CMV, since it may create conditions on CMV-infected plants that could promote aphid-mediated viral transmission. Supporting this idea, it has been demonstrated that CMV-infected cucumber plants attract aphids more efficiently than non-infected plants (Mauck et al., 2010). More recently, it has been shown that begomovirus TYLCCNV and beta-satellite coinfection suppresses JA-dependent defences in the plant and thereby contributes to the improved

performance of whiteflies on virus-infected tobacco plants (Zhang et al., 2013); which viral protein(s) is/are involved in this effect is currently unknown.

In this work, we analyse in detail the suppression of the JA response exerted by geminivirus C2 using transcriptomic analyses as well as the effect of C2 on plant defence through pathogen challenge of C2 transgenic plants. Strikingly, C2-expressing plants show a suppression of JA-mediated defence processes as well as JA-dependent secondary metabolism, which may involve additional, specific protein-protein interactions. We also show that C2 is able to interact with and might destabilize AtJAZ8, which exerts a negative impact on the infection by TYLCV in *Arabidopsis*. Also, we have evaluated the effect of JA-signalling over TYLCSV in its natural host, tomato, observing opposite results from those obtained with TYLCV in the non-natural host *Arabidopsis*. Based on our findings, we propose that C2 from TYLCSV may specifically interfere with the JA response at multiple levels in a host-dependent manner.

RESULTS

Transgenic plants expressing C2 from different geminiviruses are less sensitive to exogenous jasmonates and to the bacterial toxine coronatine

(Results presented in figures 3 and 4 were obtained by Rosa Lozano-Durán at the University of Málaga as part of her PhD thesis).

It has been previously described that transgenic *Arabidopsis* plants expressing C2 from geminiviruses TYLSCV (C2-TS) and TYLCV (C2-TM) display a misregulation of the SKP1/CUL1/F-box (SCF) E3-ligase complexes, which can be observed as an alteration of several SCF-regulated hormonal responses (Lozano-Durán et al., 2011a). Strikingly, transcriptomic analysis of C2-TS plants unveiled the jasmonate (JA) response as the main hormonal response affected by expression of C2 (Lozano-Durán et al., 2011a), even though the exact molecular mechanism underlying this specificity was unknown. In order to further characterize the response to exogenous JA applications in the C2-TS and C2-TM plants, we analyzed the inhibition of primary root elongation caused by treatment with increasing concentrations of MeJA (JA: 0, 10, 50 and 100 μM). As expected, the results confirmed that *Arabidopsis* transgenic plants expressing C2-TS and C2-TM were less sensitive to JA than control plants (Figure 3). The differential in sensitivity to jasmonates (JAs) displayed by the C2-TS and C2-TM transgenic plants could be consistent with a malfunction of the SCF^{COI1} complex.

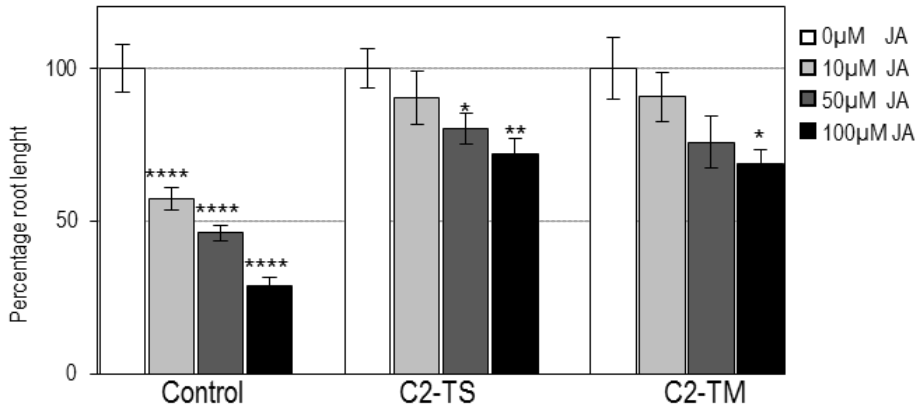


Figure 3. Root growth inhibition assays in C2-expressing plants. Relative root length of wild-type Col-0 (control), C2-TS and C2-TM *Arabidopsis* seedlings in increasing concentrations of MeJA (JA; 0, 10, 50 and 100 μM). The values are the mean of at least ten seedlings. Bars represent standard error. Asterisks indicate samples that are statistically significant different from the 0μM JA sample from each genotype (****,p-value < 0.0001; **, p-value < 0.01; *, p-value < 0.05), according to a Student's t-test. Experiments were repeated three times with similar results; results from one representative experiment are shown.

The SCF^{COI1} complex acts as the JA receptor, but is also the receptor for the bacterial toxin coronatine (COR, an analogue of jasmonoyl-Ile). Consequently, if the activity of the SCF^{COI1} is hindered in the presence of the viral protein, transgenic C2 plants should also be less sensitive to COR. In order to test this, transgenic C2 plants were dip-inoculated with *Pto* DC3000 wild-type (*Pto*) or a deficient strain unable to synthesize COR (COR−) and bacterial growth was measured four days post inoculation (dpi). In the dipping inoculation method, the bacteria are forced to enter the plant tissues through natural openings, such as the stomata. However, following PAMP (pathogen-associated molecular patterns) perception, stomatal closure is triggered to prevent pathogen entry; this closure, nevertheless, can be reverted by the successful pathogen *Pto* DC3000 through the activity of COR inside the plant cell. Therefore, the wild-type bacteria will trigger the re-opening of the stomata after toxin production and its perception by the plant SCF^{COI1} complex, allowing bacterial entry, whereas the COR− deficient bacteria will not, and will thus invade the plant tissues less efficiently (Melotto et al., 2006). Consistently with this model, our results show a reduced growth of the COR− when dip-inoculated into control (wild-type) *Arabidopsis* plants compared to the growth of the wild-type bacteria (Figure 4A). Nonetheless, C2-TS and C2-TM *Arabidopsis* plants showed increased resistance to wild-type bacterial penetration, since the bacterial numbers obtained were significantly lower than in control plants. This difference is due to decreased bacterial penetration and not a decrease in bacterial replication in the apoplast, since we observed that C2-TS and C2-TM plants are more susceptible to wild-type *Pto* DC3000 when the bacteria were infiltrated into the leaves (bypassing the bacterial entry into the plant tissues; Figure 4B). In contrast, results obtained with the COR− strain were not different to those obtained with the wild-type strain in the C2-TM plants when the bacteria are inoculated by dipping, whereas in the C2-TS plants bacterial growth of the COR- strain was slightly lower to that of the wild-type (Figure 4A). A good correlation can be found between symptom severity and bacterial numbers in the dip inoculation experiments (Figure 4C). These results suggest that the COR produced by the wild-type bacteria is not properly exerting its function in the C2 transgenic plants, particularly in those expressing C2 from TYLCV. Even though there is also a slight decrease in the growth of the COR-deficient strain compared to the wild-type bacteria in the C2-TS plants, the difference is considerably lower than that in the control plant. This small difference between the wild-type and the COR-deficient bacteria in the C2-TS plants could be explained by a combination of two observations: (i) The activity of the SCF complexes is not completely impaired in the C2 plants, but rather partially hindered, so the toxin produced by the wild-type bacteria will exert a residual activity; and (ii) In the absence of COR, the bacteria will only be able to enter the plant tissues through open stomata whose PAMP-triggered closure has not been accomplished yet - in the C2

plants, stomata are more efficiently closed as a consequence of ABA hypersensitivity of the guard cells (Lozano-Durán et al., 2011a), so the bacterial entry will be hampered. The results obtained here are consistent with a reduced sensitivity to COR displayed by the C2 plants and, together with the lower sensitivity to JA, support a malfunction of the SCF^{COI1} complex in the presence of C2.

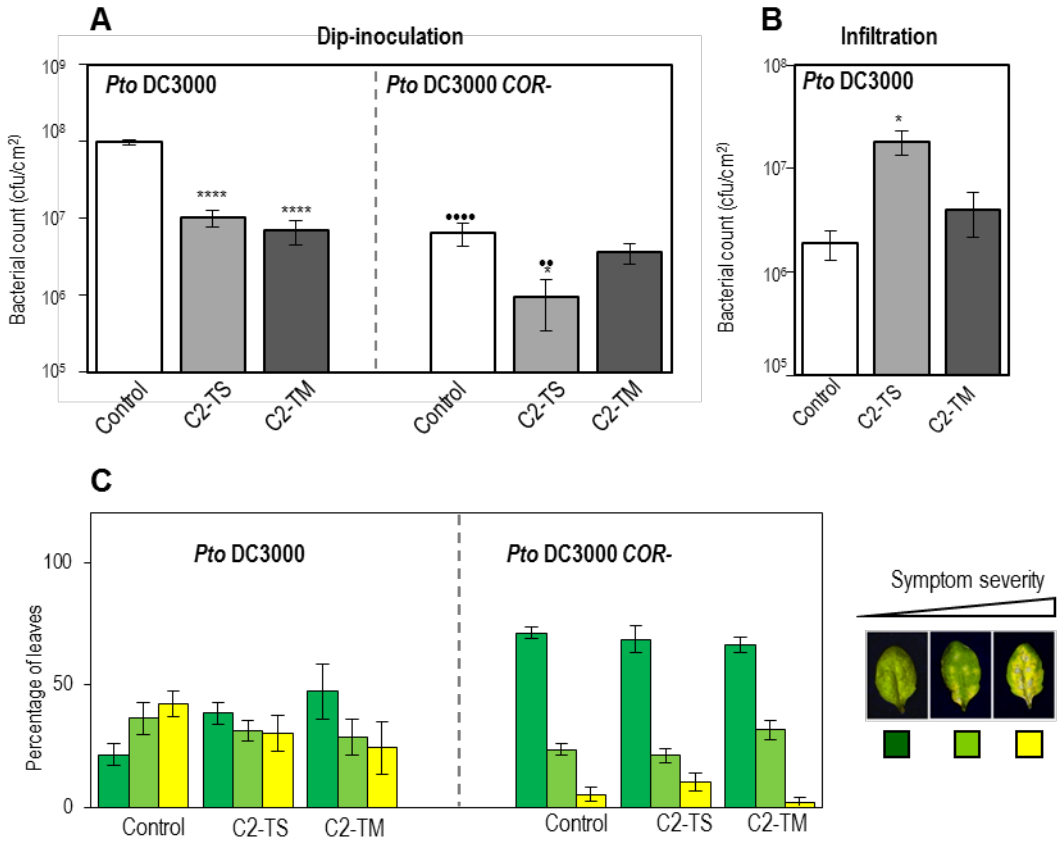


Figure 4. Infections of C2-expressing and control plants with *Pseudomonas syringae* pv *tomato* DC3000. (A) Bacterial growth of wild-type (*Pto* DC3000) or a deficient strain unable to synthesize coronatine (COR-) in wild-type Col-0 (control) or transgenic C2-expressing plants in dip-inoculation experiments. Samples were taken at 4 dpi. Values are the mean of five plants. Bars represent standard error. Asterisks (control vs. C2 plants) or dots (bacterial wild-type vs. COR-) indicate a statistically significant difference compared to the relevant control, (****/••••, p-value < 0.0001; ••, p-value < 0.01; *, p-value < 0.05) according to a Student's t-test. **(B)** Bacterial growth of *Pto* DC3000 in wild-type or C2-expressing plants in infiltration experiments. Samples were taken at 4 dpi. Values are the mean of five plants. Bars represent standard error. Asterisk indicate samples that are statistically different from the control sample (*, p-value < 0.05) according to a Student's t-test. **(C)** Symptoms displayed by dip-inoculated plants. Three different categories are considered: no symptoms, few symptoms or full symptoms, as indicated in the legend. The percentage of leaves in each category is represented. Bars represent standard error. In A and B, results are the mean of three independent biological replicates. In A, B and C, experiments were repeated three times with similar results; results from one representative experiment are shown.

Transcriptomic analysis of C2-TS plants upon exogenous jasmonate application

(Result presented in figure 5 was obtained by Rosa Lozano-Durán at the University of Málaga as part of her PhD thesis).

With the aim of gaining insight into the effect of C2 on the response to JAs, we did a microarray analysis of the *Arabidopsis* transgenic plants expressing TYLCSV C2 (C2-TS) in both basal conditions and after MeJA treatment.

C2-TS or control seedlings were grown on plates with kanamycin for 7 days, and then treated with 50μM MeJA (JA) or mock solution for ten hours; three biological and three technical replicates were used. After the treatment, samples were harvested and total RNA was extracted; the RNA from each technical replicate in the different biological replicates was pooled and subsequently used for the microarray hybridizations.

For the analysis of the transcriptomic data, four comparisons were made: (i) C2-TS versus control plants (mock-treated), (ii) JA-treated control plants versus mock-treated control plants, (iii) JA-treated C2-TS plants versus mock-treated C2-TS plants, and (iv) JA-treated C2-TS plants versus JA-treated control plants. The number of up- and down-regulated genes in each comparison is represented in Figure 5. The expression of C2-TS causes transcriptional changes in the absence of exogenous treatment, especially involving down-regulation of gene expression. Similar results have been reported using a CATMA microarray (Lozano-Durán et al., 2011a).

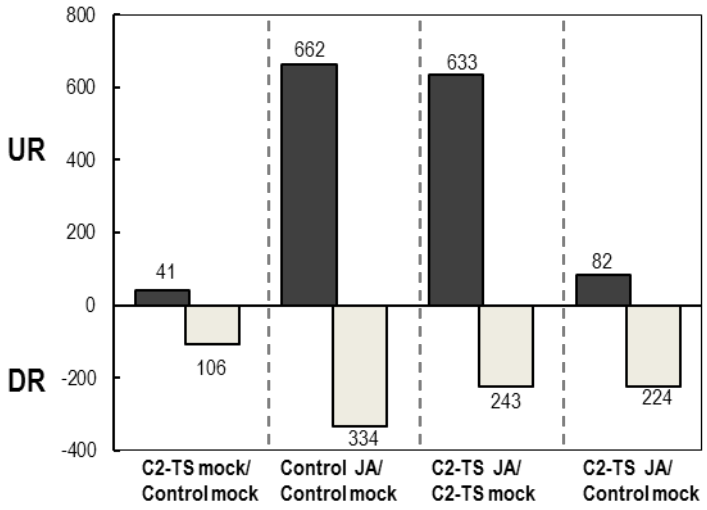


Figure 5. **Number of differentially expressed genes in mock-treated C2-TS transgenic plants and JA-treated C2-TS or control *Arabidopsis* plants**. The p-value cutoff was 0.05. The number of genes is indicated for each bar. UR: up-regulated genes; DR: down-regulated genes.

JA treatment triggers dramatic transcriptional changes in *Arabidopsis,* similar to those observed in previous works (Nemhauser et al., 2006; Jung et al., 2007). Transcriptional changes in JA-treated

C2-TS transgenic plants show a reduced response to the hormone: the number of either up- or down-regulated genes after JA treatment is lower in the C2-TS plants, and the comparison between the JA-treated C2-TS and control plants reveals a subset of genes differentially expressed in response to JA in the presence of C2, most of them being down-regulated, which indicates the existence of a group of JA-responsive genes that do not respond or respond to a lower level in the C2-TS plants (Figure 5).

We sought to explore which functional categories were affected by the expression of TYLCSV C2 and/or JA treatment. To this end, GO functional enrichment analyses were performed using the VirtualPlant BioMaps tool ((Katari et al., 2010); http://virtualplant.bio.nyu.edu/cgi-bin/vpweb2/). Tables 1 to 3 show the non-redundant GO terms over-represented in the up- or down-regulated subsets of genes in the different comparisons.

Functional enrichment of the subset of up-regulated genes only in JA-treated control plants (198 genes; Figure 6A) shows 7 over-represented GO categories exceeding the cut-off α=0.05 (Table 1). These categories represent well-characterized JA-responsive processes, such as the response to wounding and biotic stimulus, response to jasmonic acid and oxidative stress. Functional enrichment of the subset of down-regulated genes (219 genes; Figure 6A) shows 44 GO categories over-represented. Among them, it is noteworthy the presence of processes related to growth, which is known to be repressed by JA.

Table 2 shows GO terms over-represented in the differentially expressed genes exclusively represented in JA-treated C2-TS plants (when compared to mock-treated C2-TS plants). As suggested by the number of genes induced by JA treatment (170 genes; Figure 6A), C2-TS plants respond to JA, but this response is quantitatively and qualitatively different to that of wild-type plants. The subset of down-regulated genes (64 genes; Figure 6A) contains 15 GO categories, versus the 44 present in the same subset of JA-treated control plants. Among these categories, the responses to several stimuli such as responses to virus, cytokinin, ethylene, far red light and defence response to fungus are of special interest, since these processes are not repressed by JAs in the control plants.

The comparison of differentially expressed genes in C2-TS plants and control plants in response to JA is depicted in Venn diagrams in Figure 6B. It is noteworthy that 31% (33/106) of the repressed genes in C2-TS plants (when compared to control plants) are up-regulated in response to JA in control plants (compared to JA-treated control plants).

The functional enrichment of the intersection contains 7 GO categories (Table 3). Among them, it is remarkable the presence of the response to jasmonic acid stimulus, the response to wounding and the lipid transport categories.

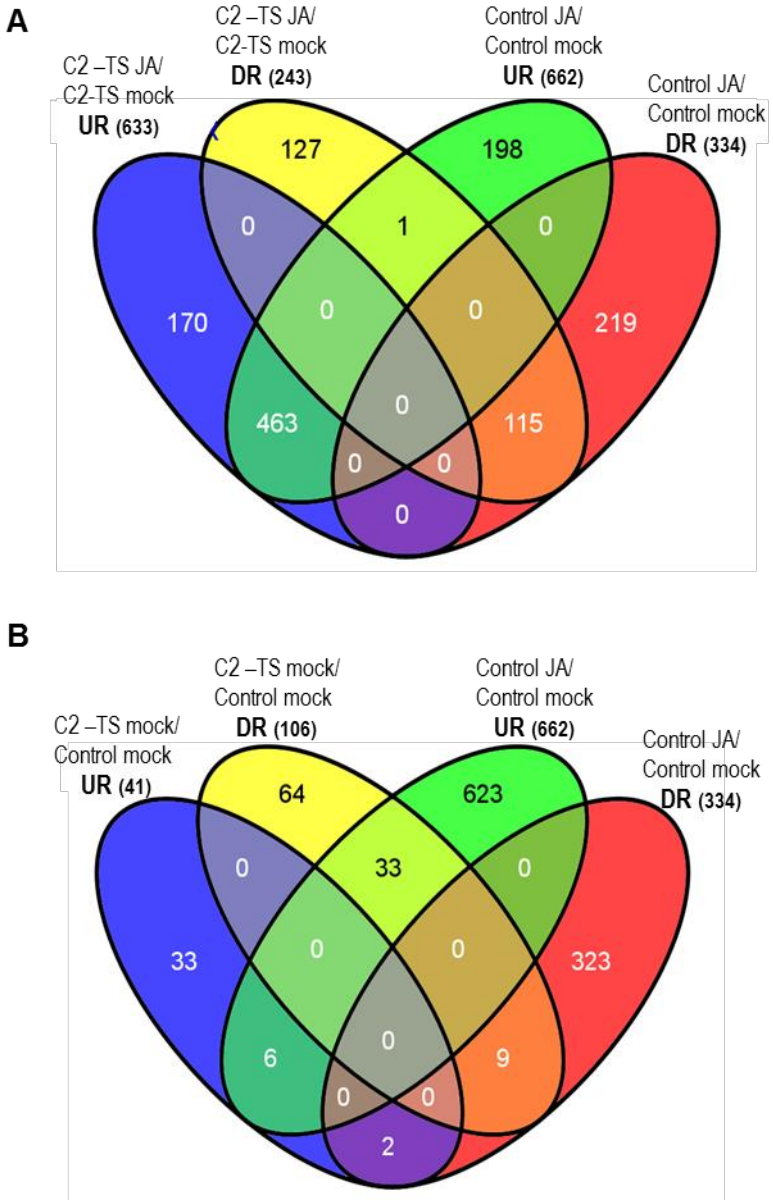


Figure 6. Venn diagrams showing the number of genes up- or down-regulated (UR or DR, respectively) in C2-TS plants, either JA- or mock-treated (in A and B, respectively), and JA-treated control *Arabidopsis* plants. The Venn diagrams were constructed using the software Venny (http://bioinfogp.cnb.csic.es/tools/venny).

GO Term	Description	OF (%)	EF (%)	p-value
UP REGULATED				
Response to stimulus				
GO:0006950	Response to stress	19.8	9.9	0.01
GO:0042221	Response to chemical stimulus	18.6	9	0.01
GO:0050896	Response to stimulus	29.9	17.3	0.01
GO:0006979	Response to oxidative stress	6	1.4	0.01
GO:0009611	Response to wounding	4.2	0.7	0.02
GO:0009753	Response to jasmonic acid stimulus	4.2	0.8	0.03
GO:0009607	Response to biotic stimulus	8.4	3	0.03
DOWN REGULATED				
Cellular organization and growth				
GO:0006949	Syncytium formation	3.2	0.1	7.56E-06
GO:0009828	Plant-type cell wall loosening	3.7	0.2	1.65E-05
GO:0009827	Plant-type cell wall modification	3.7	0.2	6.57E-05
GO:0009664	Plant-type cell wall organization	3.7	0.3	0.000444
GO:0007047	Cellular cell wall organization	3.7	0.4	0.000606
GO:0048646	Anatomical structure formation involved in morphogenesis	3.7	0.4	0.00171
GO:0042545	Cell wall modification	4.2	0.7	0.00307
GO:0070882	Cellular cell wall organization or biogenesis	4.2	0.7	0.00453
GO:0071669	Plant-type cell wall organization or biogenesis	3.7	0.6	0.00539
GO:0009831	Plant-type cell wall modification involved in multidimensional cell growth	2.1	0.1	0.00646
GO:0045229	External encapsulating structure organization	3.7	0.5	0.00216
GO:0042547	Cell wall modification involved in multidimensional cell growth	2.1	0.1	0.0065
GO:0071555	Cell wall organization	4.2	0.8	0.0065
GO:0071554	Cell wall organization or biogenesis	4.7	1.2	0.00998
GO:0016049	Cell growth	4.7	1.2	0.01
GO:0009826	Unidimensional cell growth	4.2	0.9	0.01
GO:0048589	Developmental growth	4.2	1.1	0.02
GO:0060560	Developmental growth involved in morphogenesis	4.2	1	0.02
GO:0040007	Growth	4.7	1.4	0.03
GO:0000902	Cell morphogenesis	4.2	1.2	0.03
GO:0032989	Cellular component morphogenesis	4.2	1.2	0.03
Response to stimulus				
GO:0080167	Response to karrikin	5.8	0.6	1.53E-05
GO:0010033	Response to organic substance	11.6	5.4	0.01
GO:0050896	Response to stimulus	26.3	17.3	0.02
GO:0009733	Response to auxin stimulus	4.2	1.3	0.05
GO:0042221	Response to chemical stimulus	15.3	9	0.05
GO:0071370	Cellular response to gibberellin stimulus	1.6	0.2	0.05
GO:0009628	Response to abiotic stimulus	12.1	6.6	0.05
GO:0009719	Response to endogenous stimulus	8.9	4.3	0.05
GO:0009725	Response to hormone stimulus	18.4	4	0.05
Transport				
GO:0006869	Lipid transport	5.8	0.6	7.56E-06
GO:0071702	Organic substance transport	6.8	1.1	4.91E-05
GO:0006833	Water transport	1.6	0.1	0.0065
GO:0042044	Fluid transport	1.6	0.1	0.0065
GO:0006810	Transport	15.8	8	0.0078
GO:0015840	Urea transport	1.1	0	0.02
GO:0042886	Amide transport	1.1	0	0.03
GO:0080170	Hydrogen peroxide transmembrane transport	1.1	0	0.03
Signalling				
GO:0007167	Enzyme linked receptor protein signalling pathway	3.7	0.7	0.0078
GO:0007169	Transmembrane receptor protein tyrosine kinase signalling pathway	3.7	0.7	0.0078
GO:0007166	Cell surface receptor linked signalling pathway	3.7	0.8	0.01
GO:0009740	Gibberellic acid mediated signalling pathway	1.6	0.2	0.05
Metabolism				
GO:0006808	Regulation of nitrogen utilization	1.1	0	0.03
Others				
GO:0051234	Establishment of localization	15.8	8.1	0.0078

Table 1. Functional enrichment analysis (biological process functional gene ontology) of the subset of up- and down regulated genes only in JA-treated samples in *Arabidopsis* control plants. The percentages of genes belonging to each category are reported for this subset of genes and for the total present in the microarray (observed- (OF) and expected (EF) frequencies). The p-value cutoff was 0.05.

GO Term	Description	OF (%)	EF (%)	p-value
UP REGULATED				
Metabolism				
GO:0019748	Secondary metabolic process	8.9	1.8	0.00202
GO:0006790	Sulfur compound metabolic process	5.2	0.8	0.00844
GO:0016051	Carbohydrate biosynthetic process	5.9	1.2	0.01
GO:0034637	Cellular carbohydrate biosynthetic process	5.2	0.9	0.01
GO:0016143	S-glycoside metabolic process	3	0.2	0.01
GO:0019757	Glycosinolate metabolic process	3	0.2	0.01
GO:0019760	Glucosinolate metabolic process	3	0.2	0.01
GO:0016137	Glycoside metabolic process	3.7	0.5	0.02
GO:0080003	Thalianol metabolic process	1.5	0	0.02
GO:0010683	Tricyclic triterpenoid metabolic process	1.5	0	0.02
GO:0044262	Cellular carbohydrate metabolic process	7.4	2.2	0.02
GO:0045730	Respiratory burst	1.5	0	0.02
GO:0016138	Glycoside biosynthetic process	3	0.3	0.02
GO:0005975	Carbohydrate metabolic process	10.4	4.1	0.02
GO:0016144	S-glycoside biosynthetic process	2.2	0.2	0.03
GO:0019758	Glycosinolate biosynthetic process	2.2	0.2	0.03
GO:0019761	Glucosinolate biosynthetic process	2.2	0.2	0.03
GO:0009098	Leucine biosynthetic process	1.5	0	0.03
GO:0009312	oligosaccharide biosynthetic process	2.2	0.2	0.04
Response to stimulus				
GO:0050896	Response to stimulus	33.3	17.3	0.00202
GO:0009607	Response to biotic stimulus	11.1	3	0.00227
GO:0051707	Response to other organism	9.6	2.7	0.00844
GO:0051704	Multi-organism process	9.6	2.9	0.00938
GO:0002679	Respiratory burst involved in defence response	1.5	0	0.02
GO:0006950	Response to stress	19.3	9.9	0.02
GO:0006952	Defence response	9.6	3.5	0.02
GO:0009624	Response to nematode	3	0.3	0.02
GO:0042221	Response to chemical stimulus	17.8	9	0.02
Transport				
GO:0006857	Oligopeptide transport	3.7	0.3	0.00844
GO:0015833	Peptide transport	3.7	0.3	0.00844
DOWN REGULATED				
Response to stimulus				
GO:0050896	Response to stimulus	38.3	17.3	3.57E-05
GO:0006950	Response to stress	25.2	9.9	0.000421
GO:0042221	Response to chemical stimulus	23.5	9	0.000432
GO:0051707	Response to other organism	11.3	2.7	0.00183
GO:0051704	Multi-organism process	11.3	2.9	0.00251
GO:0009607	Response to biotic stimulus	11.3	3	0.00298
GO:0009628	Response to abiotic stimulus	17.4	6.6	0.0044
GO:0009615	Response to virus	3.5	0.2	0.00738
GO:0010033	Response to organic substance	14.8	5.4	0.00738
GO:0009735	Response to cytokinin stimulus	3.5	0.4	0.03
GO:0050832	Defence response to fungus	4.3	0.6	0.03
GO:0009723	Response to ethylene stimulus	4.3	0.7	0.03
GO:0009639	Response to red or far red light	4.3	0.7	0.05
Metabolism				
GO:0009759	Indole glucosinolate biosynthetic process	1.7	0	0.02
GO:0042343	Indole glucosinolate metabolic process	1.7	0	0.03

Table 2. Functional enrichment analysis (biological process functional gene ontology) of the subset of up- and down- regulated genes only in JA-treated samples in *Arabidopsis* C2-TS transgenic plants. The percentages of genes belonging to each category are reported for this subset of genes and for the total present in the microarray (observed- (OF) and expected (EF) frequencies). The p-value cutoff was 0.05.

GO Term	Description	OF (%)	EF (%)	p-value
DOWN REGULATED C2TS mock/ UP REGULATED Control JA				
Response to stimulus				
GO:0009611	Response to wounding	13.8	0.7	0.00565
GO:0009753	Response to jasmonic acid stimulus	13.8	0.8	0.00565
GO:0009607	Response to biotic stimulus	17.2	3	0.04
GO:0051704	Multi-organism process	17.2	2.9	0.04
GO:0051707	Response to other organism	17.2	2.7	0.04
GO:0050896	Response to stimulus	41.4	17.3	0.04
Transport				
GO:0006869	Lipid transport	10.3	0.6	0.03

Table 3. Functional enrichment analysis (biological process functional gene ontology) of the subset of the intersection between down regulated genes in C2-TS transgenic and up-regulated genes in JA-treated control plants. The percentages of genes belonging to each category are reported for this subset of genes and for the total present in the microarray (observed- (OF) and expected (EF) frequencies). The p-value cutoff was 0.05.

The functional enrichment analysis, together with the comparison of the responses to JA in C2-TS and control plants, suggest that in the C2-TS plants only certain JA-triggered responses are inhibited when compared to treated control plants. C2 from begomoviruses acts as a transcription factor for viral genes, and it also affects transcription in the host (Trinks et al., 2005). It would be feasible that C2 could be affecting the expression of some key element(s) of the JA response, consequently altering this process. The activation of response genes is due to the action of determined transcription factors and degradation/activation of repressors of the JA response acting as part of the signalling cascade. To date, three transcription factors (TFs) have been found to act in the JA response downstream of the SCF^{COI1} and upstream of the responsive genes: MYC2, MYC3 and MYC4 (Chini et al., 2007; Fernández-Calvo et al., 2011). Additionally, there are twelve JAZ transcriptional repressors, and one negative regulator, JAV1, recently described in *Arabidopsis* (Thines et al., 2007; Hu et al., 2013). With the aim of evaluating the expression level of these genes in our C2-TS plants, we retrieved these data from our microarray analysis. It is noteworthy that most of the JAZ repressors and the TFs MYC2 and MYC3 are induced by JA treatment in both C2-TS and control plants. *JAZ8*, however, was induced to a significantly lower level in JA-treated C2-TS plants when compared to control plants in the same condition (Table 4). Interestingly, *JAZ1, JAZ5* and *JAZ9* are down-regulated in C2-TS plants in basal conditions (Table 4). Based on the amplitude and quality of the differences detected between the C2-TS and wild-type plants, it seems unlikely that these transcriptional changes underlie the observed negative effect exerted by C2 on JA signalling.

Gene	Common name	Contol JA/ Control mock	C2-TS JA/ C2-TS control	C2-TS mock/ Control mock	C2-TS JA/ Control JA
F-BOX					
At2g39940	*COI1*	-0.18	-0.10	-0.09	-0.02
TFs					
At1g32640	*MYC2*	2.96	3.39	-0.68	-0.25
At5g46760	*MYC3*	0.94	0.94	-0.55	-0.55
At4g17880	*MYC4*				
Repressors					
At1g19180	*JAZ1*	2.79	2.91	-0.65	-0.53
At1g74950	*JAZ2*	3.11	3.14	-0.07	-0.05
At3g17860	*JAZ3*	2.25	2.62	-0.26	0.11
At1g48500	*JAZ4*	0.06			0.02
At1g17380	*JAZ5*	4.45	4.25	-0.28	-0.48
At1g72450	*JAZ6*	2.34	2.29	-0.25	-0.30
At2g34600	*JAZ7*	3.56	3.02	-0.17	-0.70
At1g30135	*JAZ8*	2.72	1.79	-0.21	-1.14
At1g70700	*JAZ9*	4.50	5.42	-1.10	-0.18
At5g13220	*JAZ10*	5.57	5.87	-0.55	-0.25
At3g43440	*JAZ11*	ND	ND	ND	ND
At5g20900	*JAZ12*	0.92	0.88	0.03	-0.01
At3g22160	*JAV1*	1.16	0.84	-0.48	-0.80

Table 4. Gene expression of JA pathway signalling components. Up- and down-regulation (shown in red and green, respectively) of selected genes in transgenic C2-TS and control plants according to microarray data. The numbers represent the expression value in Log2. The p-value cutoff was 0.05.

To validate the microarray data, we measured the expression of several detected up- and down-regulated genes in the C2-TS transgenic and control *Arabidopsis* plants by real-time PCR. For this purpose, we measured the expression level of the JA-inducible genes *GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1* (*AtASN1*), *ALLENE OXIDE SYNTHASE* (*AtAOS1*), and *GALACTINOL SYNTHASE 1* (*AtGOLS*); and of the JA-repressed genes *XYLOGLUCAN ENDOTRANSGLYCOSYLASE/HYDROLASE* (*AtXTH31*), *BASIC PATHOGENESIS-RELATED PROTEIN 1* (*AtPRB1*), *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR* (*AtTINY*), and *FLAGELLIN-SENSITIVE 2* (*AtFLS2*); as controls, we use *AUXIN-INDUCED IN ROOT CULTURES 1* (*AtAIR1*) and *PATHOGENESIS-RELATED 1* (*AtPR1*), whose expression was not affected by JA treatment. The changes in expression detected in the microarray were confirmed for most genes (Figure 7, Table 5).

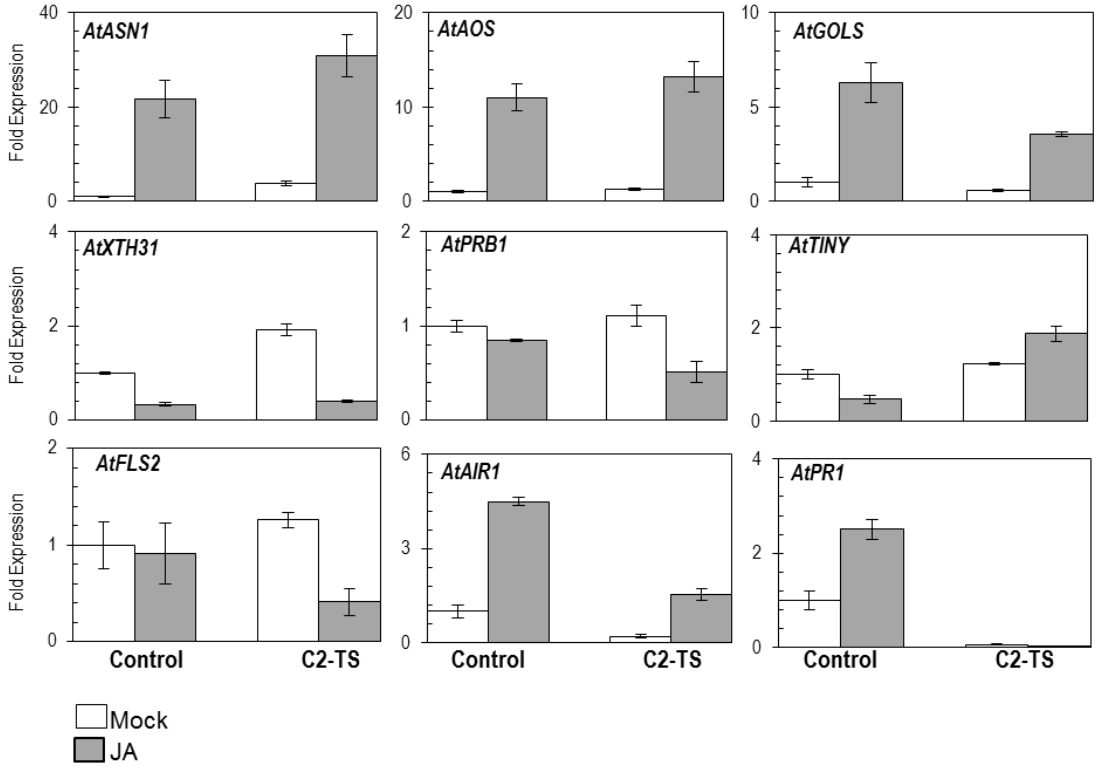


Figure 7. Expression levels of JA-regulated and control genes in *Arabidopsis* transgenic C2-TS and control plants for microarray validation. Relative expression level of *AtASN1* (At3g47340), *AtAOS1* (At5g42650), *AtGOLS* (At2g47180), *AtXTH31* (At3g44990), *AtPRB1*; (At2g14580), *AtTINY* (At5g25810), *AtFLS2*; (At5g46330), *AtAIR1* (At4g12550) and *AtPR1* (At2g14610) genes in transgenic C2-TS and control *Arabidopsis* seedlings, mock- or JA-treated, determined by real-time PCR. Values are the mean of three independent experiments. Bars represent standard error.

		Microarray (Fold-Change)				Real-time PCR			
	e-FP Browser (3h)	Control JA Control mock	C2-TS JA C2-TS mock	C2-TS mock Control mock	C2-TS JA control JA	Control JA Control mock	C2-TS JA C2-TS mock	C2-TS mock Control mock	C2-TS JA Control JA
ASN1	6.17	51.33	15.76	2.91	1	21.62	8.14	3.80	1.43
AOS	13.49	9.29	16.93	1	1	11.01	10.67	1.24	1.20
GOLS	6.49	11.02	7.94	1	0.48	6.30	6.19	0.58	0.57
XTH31	0.46	0.16	0.29	1	1	0.34	0.21	1.91	1.19
PRB1	0.48	1.81	1.27	0.54	0.38	0.85	0.46	1.10	0.60
TINY	0.77	0.25	1	1	1.99	0.47	1.52	1.23	4.01
FSL2	0.93	0.31	0.43	1	1	0.91	0.33	1.26	0.45
AIR	1.06	10.45	14.27	0.18	0.25	4.49	7.59	0.20	0.34
PR1	1.16	5.70	0.93	0.093	0.015	3.17	0.54	0.055	0.009

Table 5. Comparison between microarray data and real-time PCR of JA-regulated and control genes in *Arabidopsis* transgenic C2-TS and control plants. Expression level of *AtASN1* (At3g47340), *AtAOS1* (At5g42650), *AtGOLS* (At2g47180), *AtXTH31* (At3g44990), *AtPRB1* (At2g14580), *AtTINY* (At5g25810), *AtFLS2* (At5g46330), *AtAIR1* (At4g12550) and *AtPR1* (At2g14610) genes in transgenic C2-TS and control *Arabidopsis* seedlings, mock- or JA-treated. Up- and down-regulation are shown in red and green, respectively; lack of differential expression or coincidence between the two methods are shown in grey and white, respectively. e-FP bowser genes expression after 3 hours of JA treatment is also represented (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The numbers represent the expression value in fold change.

Transgenic C2 plants are more susceptible to *Pseudomonas syringae* and *Potato virus X*

(Results presented in figures 8 and 9B were obtained by Rosa Lozano-Durán at the University of Málaga as part of her PhD thesis).

Based on the finding that JA-dependent defence responses are transcriptionally repressed in C2 transgenic plants, we decided to test the susceptibility of the C2 transgenic lines to different pathogens. For this purpose, we infected transgenic plants expressing C2 with *P. syringae*, RNA viruses and geminiviruses. *Arabidopsis* plants (C2-TS and C2-TM) were inoculated with wild-type *Pto* DC3000, a *hrcC* non-pathogenic mutant strain, or a wild-type strain expressing the avirulence effector AvrRpt2. Besides, C2-TS and C2-TM plants were agroinoculated with *Tomato yellow leaf curl virus* (TYLCV); we chose this viral species instead of TYLCSV because of its ability to infect *Arabidopsis* plants, which TYLCSV lacks (Cañizares M.C. et al., 2014). *Nicotiana benthamiana* plants expressing C2-TS were agroinoculated with infectious clones of *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) labeled with GFP. Consistently with the transcriptional repression of the defence response, C2-TS plants are more susceptible to wild-type *Pto* DC3000 than wild-type plants when the bacteria are infiltrated into the leave (bypassing the bacterial entry into the plant tissues) (Figure 8; Figure 4B). No significant differences were found after infiltration with non-pathogenic or avirulent bacteria (Figure 8).

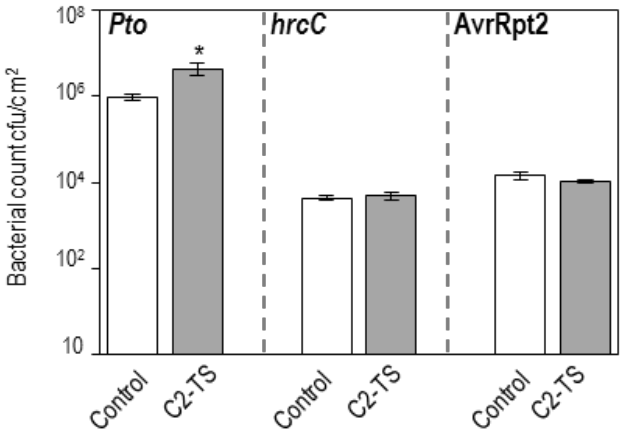


Figure 8. Transgenic C2-TS plants are more susceptible to *Pseudomonas syringae* pv *tomato* DC3000. Bacterial growth of wild-type *Pto* DC3000, a *hrcC* mutant, or a wild-type strain expressing the avirulence effector AvrRpt2 on wild-type or C2-TS-expressing *Arabidopsis* plants. Values represent the average of five plants. Bars represent standard error. Asterisks indicate a statistically significant difference compared to the relevant control, according to a Student's t-test with p-value<0.05. Three independent experiments were performed with similar results; the graph represents one representative repeat.

C2-TS *N. benthamiana* plants were more susceptible to infection with a PVX-GFP clone: the levels of GFP and the viral RNA expression in C2-TS plants were higher than in control plants (Figure 9B). However, no significant changes on viral expression were detected in plants inoculated with TMV-GFP (Figure 9B). Strikingly, none of the C2-expressing *Arabidopsis* plants showed significant changes on TYLCV DNA accumulation (Figure 9A). Altogether, these results suggest that C2 could partially suppress basal defence responses, at least in specific plant-pathogen interactions.

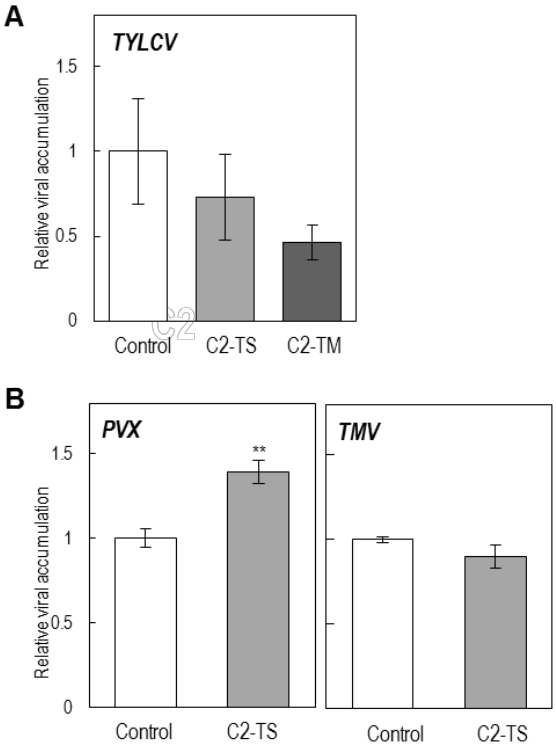


Figure 9. Transgenic C2-expressing plants are more susceptible to *Potato virus X* but not to TYLCV. **(A)** Infection of wild-type Col-0 (control), C2-TS and C2-TM *Arabidopsis* plants with the geminivirus TYLCV. The relative viral DNA accumulation was determined by real-time PCR of total DNA extracted from whole plants at 21dpi. **(B)** Infection of wild-type Col-0 (control) or C2-TS *N. benthamiana* plants with PVX-GFP or TMV-GFP at 10 dpi. Values represent the relative expression of viral RNA estimated by semi-quantitative RT-PCR. In A and B, the values represent the average of ten infected plants. Bars represent standard error. Asterisk indicate samples that are statistically different from the control sample (**,p-value < 0.01) according to a Student's t-test. Two independent experiments were performed with similar results; results from one representative replicate are shown.

C2 interacts with AtJAZ8 and destabilizes this protein *in planta*

Because transcriptomic data showed that C2 is repressing specifically certain JA-induced responses, it is doubtful that this suppression can be through SCF^{COI1} since, if this were the case, the effect would be general. One alternative hypothesis to explain this effect of C2 would be the occurrence of protein-protein interaction(s) between this viral protein and some component(s) of the JA signalling pathway. Feasible targets for such an interaction would be the JAZ family of repressors, which is composed of twelve members in *Arabidopsis*. We tested the interaction between TYLCSV C2 and eleven members of the JAZ family, and found that this viral protein is able to interact with JAZ8 in yeast (Figure 10), suggesting an additional level of interference with the response to JAs. Interestingly, the C2 protein from the curtovirus *Beet curl top virus* (BCTV) does not interact with any member of the JAZ family of repressor proteins in yeast (data not shown). To assess whether C2 from TYLCSV interacts with JAZ8 *in planta*, we conducted a co-immunoprecipitation experiment (Co-IP) using transient expression in *N. benthamiana.* In these experiments, we used binary-based vectors that allow the expression of 3xHA-tagged JAZ8 (JAZ8-HA; (Giménez-Ibánez et al., 2014)) and GFP-tagged C2 (GFP-C2; (Lozano-Durán et al., 2011a)), both proteins under the control of the 35S promoter. Upon immunoprecipitation of GFP-C2 with ***α***-GFP, we could detect co-immunoprecipitated JAZ8, indicating that these two proteins interact *in planta* (Figure 11A). Strikingly, the abundance of JAZ8-HA in the crude of the immunoprecipiation experiments was lower when in the presence of C2 (Figure 11A). In order to determine whether this can be an artefact due to co-expression, we did a new co-expression experiment, using co-expression with 35S:GFP as a control. We observed that, whereas JAZ8 could be detected in all cases, its accumulation was reduced when co-expressed with C2-GFP (Figure 11B), suggesting that C2 could destabilize JAZ8.

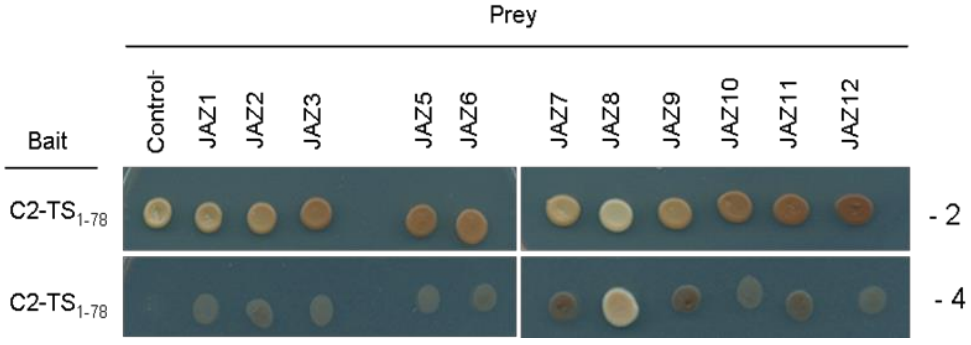


Figure 10. C2-TS interacts with JAZ8 from *Arabidopsis* in yeast. Yeast cells co-transformed with pGBKT7-C2-$TS_{(1-78)}$ (bait) and pGADT7-JAZ (prey) were selected and subsequently grown on medium lacking Leu and Trp (-2), as a co-transformation control, or on selective medium lacking Ade, His, Leu and Trp (-4) to test protein interactions. As a control, pGBTKT7-C2-TS was co-transformed with the pGADT7 vector.

Altogether, our results indicate that C2 from TYLCSV specifically interacts with AtJAZ8 in yeast and *in planta*. This positive interaction could lead to the destabilization of JAZ8 *in planta*. Whether this is a direct or an indirect effect, the biological impact of the destabilization of JAZ8 on JA signalling and/or response, and whether this leads to a lower JAZ8 activity or, on the contrary, is the result of an enhancement of its function, remain to be determined.

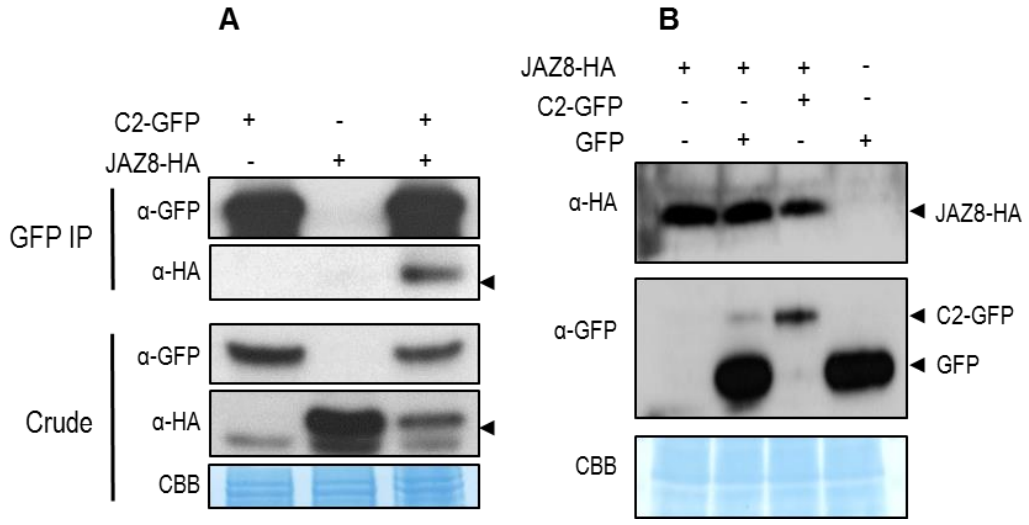


Figure 11. C2-TS interacts with JAZ8 from *Arabidopsis* and destabilizes this protein *in planta*. (A) JAZ8 (indicated by an arrow) co-inmunoprecipates with C2-TS. For inmunoprecipitation experiments, *N. benthamiana* leaves transiently co-expressing GFP-C2 and JAZ8-HA were harvested at 2 days post-infiltration. **(B)** JAZ8 is partially destabilized when expressed in the presence of C2. As control, GFP was co-expressed together JAZ8. *N. benthamiana* leaves co-expressing GFP-C2/GFP and JAZ8-HA were harvested at 2 dpi. Equal loading of samples was confirmed via comassie blue staining (CBB).

Exogenous jasmonate application in *Arabidopsis* negatively impacts infection by geminiviruses

We have previously shown that exogenous JA treatment had a negative effect over BCTV infection in *Arabidopsis* plants (Lozano-Durán et al., 2011a). In order to determine the effect of exogenous JA treatment on the infection by TYLCV, we agroinoculated JA and mock-treated *Arabidopsis* plants with this virus and measured viral DNA accumulation three weeks later. In brief, four to five-week-old *Arabidopsis* Col-0 plants were infected with TYLCV, and after two days post infection (dpi) plants were treated every other day with 50μM MeJA (JA) or mock solution. Samples were taken at 21 dpi. To evaluate the relevance of the hormonal cross-talk between JA and salycilic acid (SA) on TYLCV- infection, we also treated TYLCV-infected plants with 0.5mM SA or mock solution up to 21 dpi as described above. The obtained results show that application of exogenous JA, but not SA, results in a lower viral DNA accumulation (Figure 12). The efficacy of the treatments was

confirmed by measuring the expression level of JA-responsive genes *AtASN1*, *AtJAZ8* and *AtJAZ10* and the SA-responsive gene *PR1* (Figure 13). Our results indicate that the activation of the JA signalling pathway, through exogenous hormone treatment, has a negative effect over the geminivirus infection, and that this effect is most likely independent of the SA-JA crosstalk.

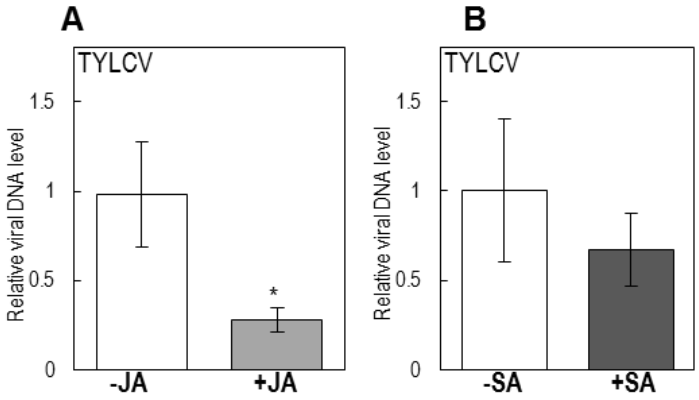


Figure 12. **Exogenous JA application in *Arabidopsis* negatively impacts infection by TYLCV.** Four- to five-week-old *Arabidopsis* Col-0 plants were agroinoculated with TYLCV, and after 2 dpi plants were sprayed every other day with MeJA (JA), SA or mock solutions. **(A)** Relative TYLCV DNA accumulation in plants treated with 50μM JA (+JA) treatment or mock solution (-JA) (0.5% ethanol in water (v/v)). The asterisk indicates statistically significant difference between untreated and treated samples with *p-value < 0.05, according to a Student's t-test. **(B)** Relative viral DNA accumulation in plants treated with 0.5mM SA (+SA) treatment or mock solution (-SA). In A and B, viral DNA was determined by real-time PCR of total DNA extracted from whole plants at 21dpi. The values represent the average of twelve infected plants. Bars represent standard error. Three independent experiments were performed with similar results; results from one representative replicate are shown.

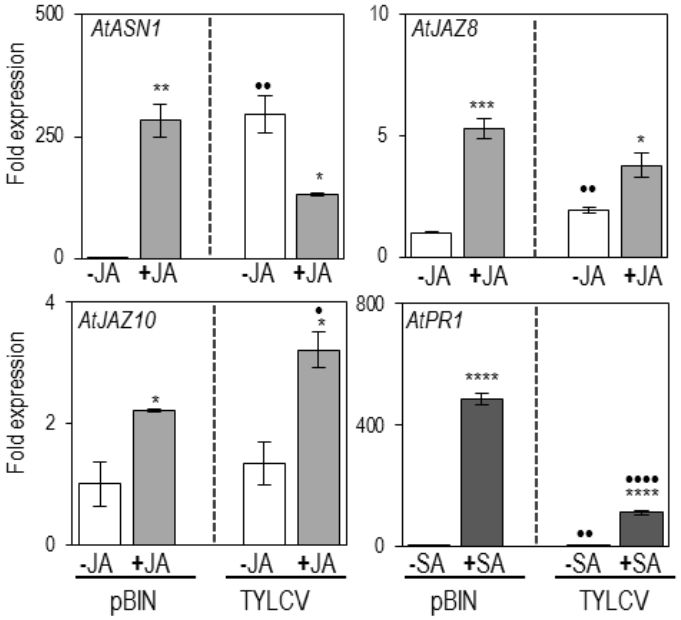


Figure 13. **Expression of JA- and SA-responsive genes upon exogenous treatments in *Arabidopsis* plants.** Mock- (pBIN) and TYLCV-infected plants were treated with 50μM MeJA (+JA) or mock solution (-JA)

(***Figure 13 Legend continued***) (0.5% ethanol in water (v/v)); or 0.5mM SA (+SA) treatment or mock solution (-SA) (water). JA responsive genes *AtASN1* (At3g47340), *AtJAZ8* (At1g30135) and *AtJAZ10* (At5g13220) and SA responsive gene *AtPR1* (At2g14610) were quantified by real-time PCR at 17 days post treatment. The values represent the average of three plants. Bars represent standard error. Asterisks (between –JA vs. +JA and –SA vs.+ SA) or dots (pBIN vs. TYLCV) indicate a statistically significant difference compared to the relevant control (****/••••, p-value < 0.0001; ***, p-value < 0.005; **/••, p-value < 0.01; */•, p-value < 0.05), according to a Student's t-test. Three independent experiments were performed with similar results; results from one representative replicate are shown.

Geminivirus infection of *Arabidopsis* mutants in jasmonate signalling components

Our results raised the idea that the C2-mediated suppression of the JA response might be important for the establishment and/or successful development of geminivirus infection. In agreement with that, exogenous JA treatment negatively affects the infection by TYLCV- (this work) and BCTV (Lozano-Durán et al., 2011a) in *Arabidopsis.* If this were the case, one would expect that the geminiviral infection of a JA-insensitive host would be more successful. To test this hypothesis, we agroinoculated three *Arabidopsis* lines deficient in JA perception or signalling with TYLCV: the *coi1-1* and the *jin1* mutants, a *35S:JAZ8* overexpressor (Shyu et al., 2012), and the JA biosynthetic mutant *aos/dde2* mutant (Park et al., 2002). At 21 dpi, viral accumulation was measured (Figure 14). Strikingly, the viral DNA accumulation in *coi1-1, 35S:JAZ8*, and *aos/dde2* plants was lower to that in the control (Col-0 wild-type), and these differences were statistically significant. No statistically significant differences between viral accumulation of the *jin1* mutant and the wild-type could be detected.

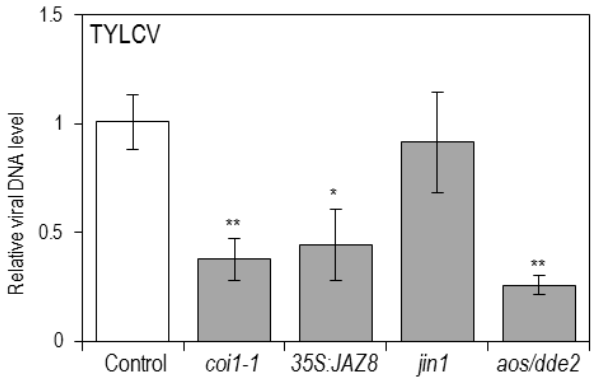


Figure 14. TYLCV infection of *coi1-1*, *35S:JAZ8*, *jin1* and *aos/dde2 Arabidopsis* plants. Four to five-week-old wild-type Col-0 (control), *coi1-1*, *jin1* and *aos/dde2* mutants and *35S:JAZ8 Arabidopsis* plants were agroinoculated with TYLCV. The relative accumulation of viral DNA was determined by real-time PCR of total DNA extracted from whole plants at 21dpi. The values represent the average of fifteen infected plants. Bars represent standard error. Asterisks indicate samples that are statistically different from the control sample (**,p-value < 0.01; *,p-value < 0.05) according to a Student's t-test. Three independent experiments were performed with similar results; results from one representative replicate are shown.

In mounting an effective defence response against bacterial pathogens SA plays an essential role. However, JA can counteract the SA-mediated defence to fine-tune the immune response through signalling crosstalk (Kunkel and Brooks, 2002; Spoel and Dong, 2008; Grant and Jones, 2009). In the COR-insensitive *coi1-1* mutant, SA levels are elevated, and resistance to *P. syringae* is enhanced (Feys et al., 1994; Kloek et al., 2001; Macho et al., 2010). The JA signalling mutant *jin1*, which is less sensitive to COR, also exhibits a similar phenotype (Nickstadt et al., 2004; Laurie-Berry et al., 2006).

Based on these observations, the *coi1-1* and the *jin1* mutants may be able to mount a more effective SA-based defence response, which may have an impact on the *Agrobacterium tumefaciens* (*Agrobacterium*) replication and/or T-DNA transformation and, as a consequence, adversely affect the efficiency of the geminivirus agroinoculation. In order to determine whether this is the case, we tested the ability of *Agrobacterium* to replicate in the *coi1* and *jin1* mutants. For this purpose, we agroinoculated *coi1-1* and *jin1* mutants and wild-type plants with *Agrobacterium* LBA4404 containing a TYLCV infectious clone. Bacterial growth was determined at 3 dpi; as expected, only a minor increase in *Agrobacterium* numbers could be detected in both control and mutant plants (Figure 15). The low bacterial replication could be due to the perception of pathogen-associated molecular patterns (PAMPs) by the plant, leading to a rapid activation of defence mechanisms (Zipfel et al., 2006).

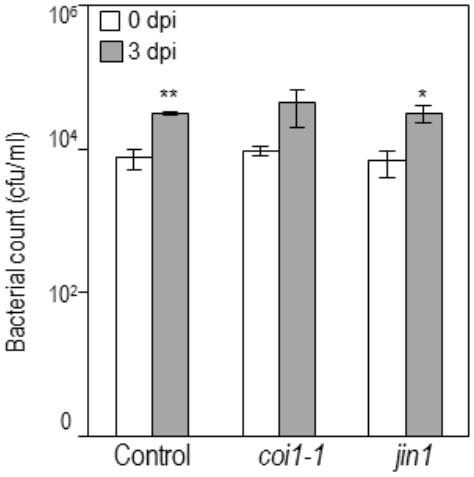


Figure 15. *Agrobacterium* growth in *coi1-1* and *jin1 Arabidopsis* mutant plants. Four- to five-week-old wild-type (control), *coi1-1* and *jin1 Arabidopsis* plants were infiltrated with *Agrobacterium* LBA4404 carrying a TYLCV infectious clone at $5*10^5$ cfu/mL (DO_{600}=0.001). Bacterial counts were obtained at 0 and 3 dpi. Bars represent the mean value of five plants. Asterisk indicate samples that are statistically different from the control sample (**,p-value < 0.01; *,p-value < 0.05) according to a Student's t-test This experiment was repeated at least twice with similar results; results from one experiment are shown.

To evaluate the impact of the different genotypes used previously on the *Agrobacterium*-mediated T-DNA transfer, we tested the transfer of a β-glucuronidase (GUS) transgene. Control (Col-0 wild-type), *coi1-1*, *35S:JAZ8*, *jin1* and *aos/dde2* leaves were injected with the hyper-virulent non-tumorigenic *Agrobacterium* strain LBA4404 containing a binary plasmid with a GUS-intron construct (pBIN19-35S:GUS) (Zipfel et al., 2006) that allows expression in plants but not in bacteria. As a control, we included the *Arabidopsis efr-1* mutant, which has an enhanced susceptibility to *Agrobacterium*, as revealed by a higher efficiency of T-DNA transformation (Zipfel et al., 2006). As shown in Figure 16A, in control leaves only weak GUS staining was detectable at 4 dpi, in accordance with published results (Zipfel et al., 2006), while *efr-1* and *aos/dde2* leaves exhibited more GUS staining. In the *coi1-1* mutant leaves, non-detectable GUS staining could be observed, while *35S:JAZ8*, *jin1* and *aos/dde2* leaves showed GUS staining similar to that of control plants (Figure 16A). These results indicate that JA perception through COI1 affects the efficiency of *Agrobacterium* T-DNA transfer, which renders the TYLCV agroinfection experiments in *Arabidopsis* the *coi1-1* mutant inconclusive. Results obtained with the *35S:JAZ8*, *jin1* and *aos/dde2* lines seem to be reliable, since T-DNA transfer in these plants is comparable to that of the wild-type (Figure 16A). A good correlation can be found between GUS activity and histochemical GUS assays (Figure 16B).

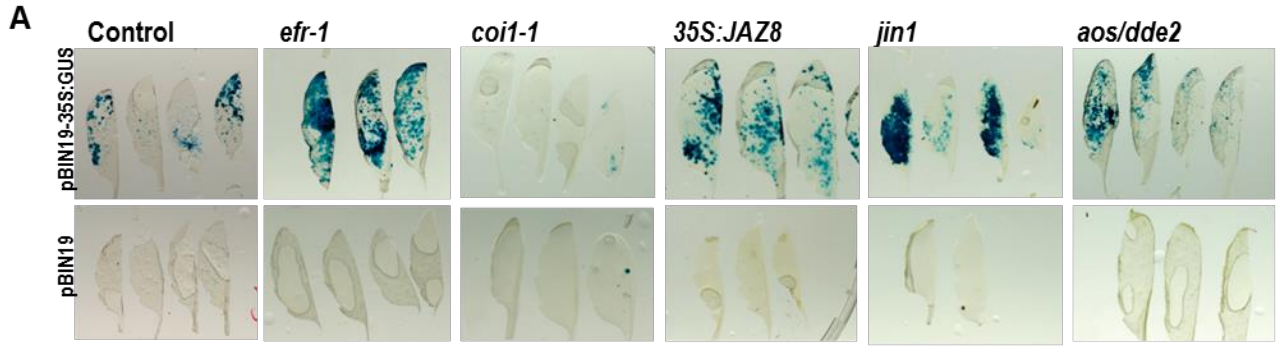


Figure 16. ***Agrobacterium*-mediated T-DNA transfer in JA-related *Arabidopsis* mutants**. **(A)** Staining for GUS activity in leaves of wild-type Col-0 (control), *efr-1*, *coi1-1*, *35S:JAZ8*, *jin1* and *aos/dde2* after infiltration with $2.5*10^7$ cfu/ml (DO_{600}=0.05) *Agrobacterium* LBA4404 carrying pBIN19-35S::GUS (35S::GUS) or pBIN19 as a control. Photographs were taken at 4 dpi. Images are representative of > 48 inoculated leaves per plant genotype. **(B)** Quantitative GUS assays with extracts from leaves of wild-type and *efr-1*,

(***Figure 16 Legend continued***) *coi1-1*, *35S:JAZ8*, *jin1* and *aos/dde2* at 4 dpi with $2.5*10^7$ *Agrobacterium* carrying pBIN19-35S::GUS. Leaves were injected with bacterial suspensions. Values are the means of five plants per genotype (three leaves each) +/- standard error. Experiments were repeated three times with similar results.

In summary, our findings provide compelling evidence of a C2-mediated suppression of the transcriptional JA response; we also show that C2 interacts with JAZ8 at the protein level; and, finally, we detect a negative effect of exogenous JA application on the geminivirus infection in *Arabidopsis*.

Exogenous jasmonate application in tomato positively impacts infection by geminiviruses

Although interesting, the previous results were obtained using *Arabidopsis* plants; as a follow-up, we aimed at assessing whether similar effects can be observed in tomato, which is the natural host of TYLCSV.To investigate whether induction of the JA response, through exogenous JA treatment, has an impact on geminivirus infection in tomato, and whether the potential effect could be due to the JA-SA crosstalk, we tested TYLCSV infection on tomato plants treated with JA, SA and JA in combination with SA (JA+SA). For JA exogenous treatments, six to eight-week-old tomato cv. Moneymayker plants were infected with TYLCSV and after two days post infection (dpi) plants were treated every other day with 50μM MeJA (JA) or mock solution. To evaluate the relevance of the hormonal JA-SA crosstalk we treated TYLCSV infected plants as described above, but this time applying 100μM SA, 50μM JA + 100μM SA or mock solution. Apical leaves from each plant were taken to test the accumulation of TYLCSV in JA, SA, JA+SA and control-treated plants at 17 dpi. Figure 17 shows that exogenous JA and JA+SA treatments result in a significant increase of viral DNA accumulation compared to the level in mock-treated tomato plants. This higher viral accumulation was independent of SA-dependent defences, as SA-treated plants showed viral DNA accumulation similar to that of control. With regards to TYLCSV symptoms in the infected plants, we observed similar severity and time of appearance among treatments and replicates. How JA treatments can enhance TYLCSV accumulation is currently unknown.

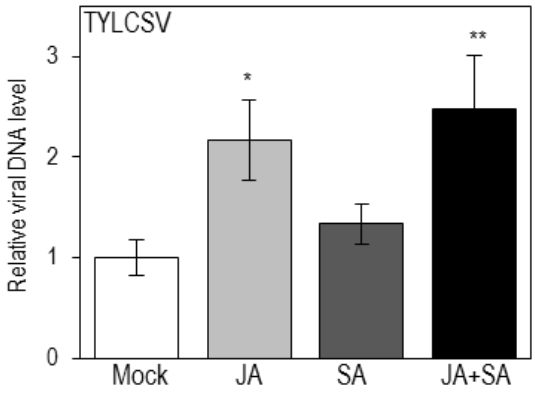


Figure 17. Exogenous JA application in tomato positively impacts infection by geminiviruses. Six- to eight-week-old tomato (*Solanum lycopersicum)* cv. Moneymaker plants were agroinoculated with TYLCSV, and sprayed every other day with mock solution, 50μM MeJA (JA), 100μM SA (SA), or JA+SA at the same concentration. Quantification of viral DNA accumulation was performed in apical leaves from each plant at 17 dpi by real-time PCR. The values represent the average of ten infected plants. Bars represent standard error. Asterisks indicate samples that are statistically different from the control sample (**,p-value < 0.01; *,p-value < 0.05) according to a Student's t-test. Three independent experiments were performed with similar results; results from one representative replicate are shown.

The efficacy of the treatments was confirmed by measuring the expression level of the JA-responsive gene *PROTEINASE INHIBITOR 2* (*SlPIN-2*) and the SA-responsive gene *PATHOGENESIS-RELATED PROTEIN 6* (*SlPR-P6)* (Figure 18).

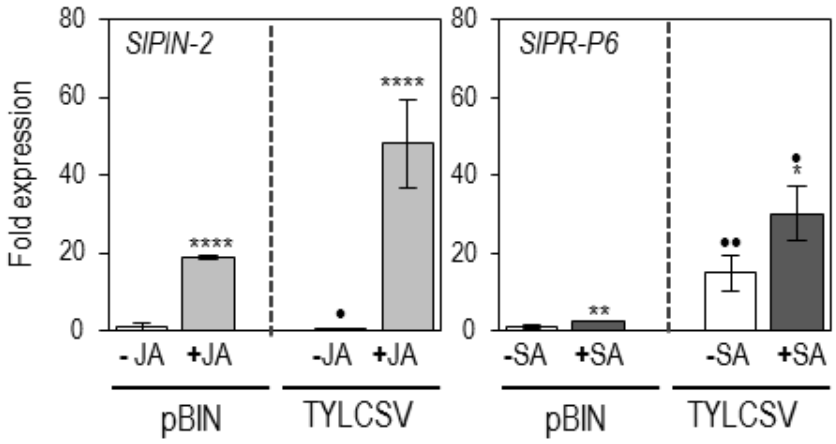


Figure 18. Expression of JA- and SA-responsive genes upon exogenous treatments in tomato plants. Mock- (pBIN) and TYLCSV-infected plants were treated with 50μM MeJA (+JA) or mock solution (-JA) (0.5% ethanol in water (v/v)); and 100 μM SA (+SA) treatment or mock solution (-SA) (water). The JA-responsive gene *SlPIN-2* and the SA-responsive gene *SlPR-P6* were quantified by real-time PCR at 17 dpi. The values represent the average of five plants. Bars represent standard error. Asterisks (between –JA vs. +JA; –SA vs.+ SA) or dots (pBIN vs. TYLCSV) indicate a statistically significant difference compared to the relevant control (****, p-value < 0.0001; **/••, p-value < 0.01; */•, p-value < 0.05), according to a Student's t-test. Two independent experiments were performed with similar results; the graph represents one of the two replicates.

The *jasmonic acid insensitive 1* (*jai1*) mutant is more resistant to TYLCSV infection

Our results show that exogenous JA treatments enhance TYLCSV infection in its natural host, tomato. To further substantiate the effects of the JA signalling pathway on geminiviral infection, we inoculated a tomato mutant *jasmonic acid insensitive 1* (*jai1-1*) (Li *et al*., 2004), defective in JA perception, with TYLCSV. It has been reported that the *jai1-1* mutation is a 6.2-kb deletion in the *COI1* gene from *Solanum lycopersicum* (Li et al., 2004). For this experiment, we agroinoculated tomato cv. Castlemart wild-type (CM: wild-type), *JAI1* (*J/J*; wild-type results from the F2 JAI1/*jai1-1* genotyping) and *jai1-1* mutant (*j/j*; homozygous mutant) with TYLCSV to compare viral DNA accumulation. Strikingly, the viral DNA accumulation in the *jai1-1* mutant was lower than that on both wild-type genotypes at 17 dpi. No statistically significant differences between viral accumulation of the CM and *J/J* wild-type could be detected (Figure 19A). Remarkably, TYLCSV symptoms observed in *jai1-1* mutant seem to be different from those in control wild-type plants (Figure 19B).

To investigate whether down-regulation of the JA signalling pathway in tomato *jai1-1* could elevate the SA levels in the mutant, we measured the expression of the tomato pathogenesis-related *PR-1* gene, which is a marker of the SA response. Figure 19D shows that *PR-1* is highly induced in the *jai1-1* mutant. The increase of SA response could have an important impact on the *Agrobacterium* replication and T-DNA transfer, as previously observed in *Arabidopsis.*

To test this idea, we evaluated *Agrobacterium* bacterial growth on CM, *JAI1* and *jai1-1* genotypes. For this purpose, we agroinoculated each plant with *Agrobacterium* GV3101 containing a TYLCSV infectious clone. At 3 dpi, bacterial growth was determined: as can be observed, there is no statistical difference in the bacterial counts among both wild-types and *jai1* mutant plants (Figure 19C). Whether the *jai1-1* mutation affects the *Agrobacterium*-mediated T-DNA transfer, however, remains to be determined. To overcome *Agrobacterium* limitations we are carrying out TYLCSV infection using its natural vector *Bemisia tabaci*. Our preliminary data from a time course analysis suggest that viral DNA accumulation in the *jai1-1* mutant was lower than that on both wild-type genotypes at 27 dpi but not at earlier times of infection (Figure 20). However, more experiments and a deeper analysis must be completed in order to reach conclusions.

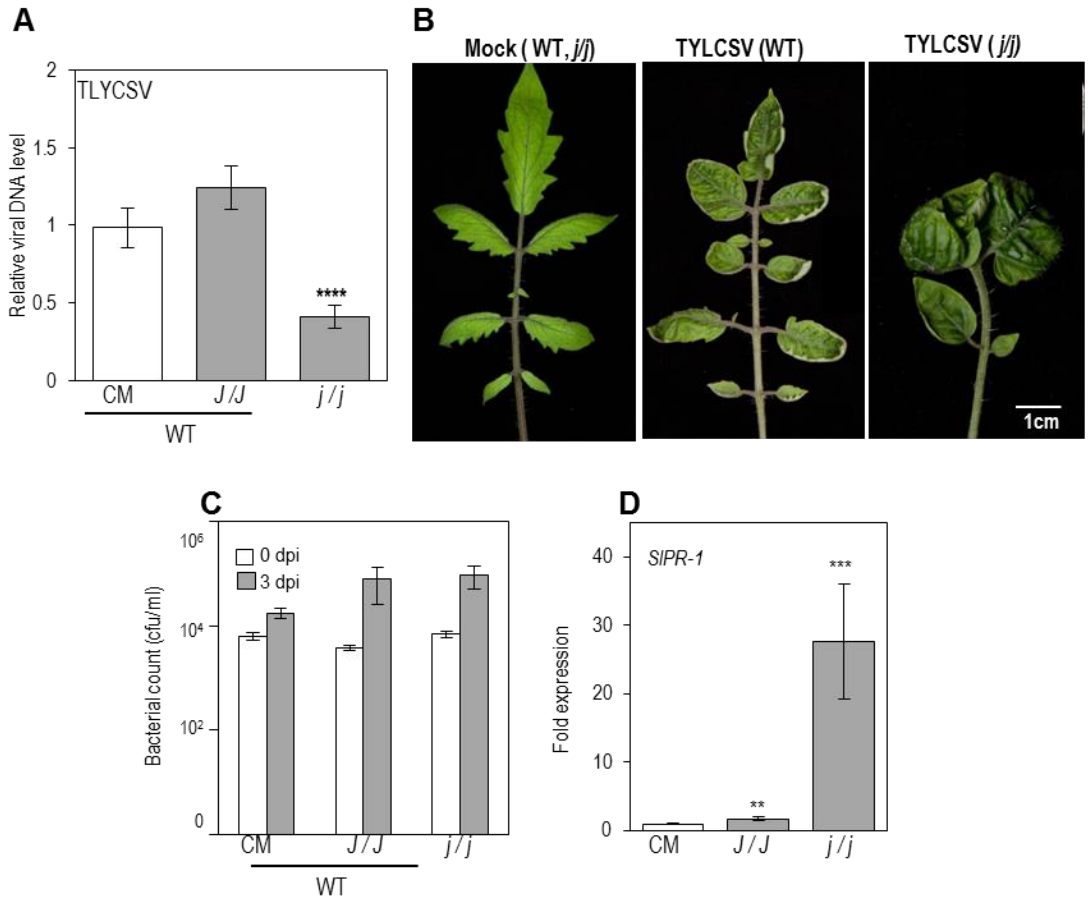


Figure 19. *jai1-1* tomato plants are more resistant to the *Agrobacterium*-mediated infection by TYLCSV. Six- to eight-week-old tomato (*L. esculentum*) plants were infected with TYLCSV. Wild-type (WT): Castlemart (CM) and homozygous JAI1 (J/J) and mutant homozygous *jai1-1* (j/j) were agroinoculated with TYLCSV. **(A)** Total DNA was extracted from apical leaves and viral DNA accumulation was measured by real-time PCR at 17 dpi. The values represent the average of ten infected plants. **(B)** Symptoms in WT: CM and JAI1 (J/J), and homozygous *jai1-1* (j/j) in mock-inoculated and TYLCSV-infected plants at 32 dpi. **(C)** Six- to eight-week-old tomato apical leaves from each tomato genotype were infiltrated with $5*10^5$ cfu/ml (DO_{600}=0.001) of *Agrobacterium* carrying a TYLCSV infectious clone. Bacterial count was obtained from the input (0dpi) and the growth (3dpi). Graph represents the mean value of five plants. This experiment was repeated at least twice with similar results. **(D)** Expression of the SA-responsive gene, *SlPR-1* in tomato genotypes. Values are the average of five plants. In A and D, bars represent standard error. Asterisks indicate samples that are statistically different from the control CM sample (****,p-value < 0.0001; ***,p-value < 0.005), according to a Student's t-test. Two independent experiments were performed with similar results; results from one representative replicate are shown.

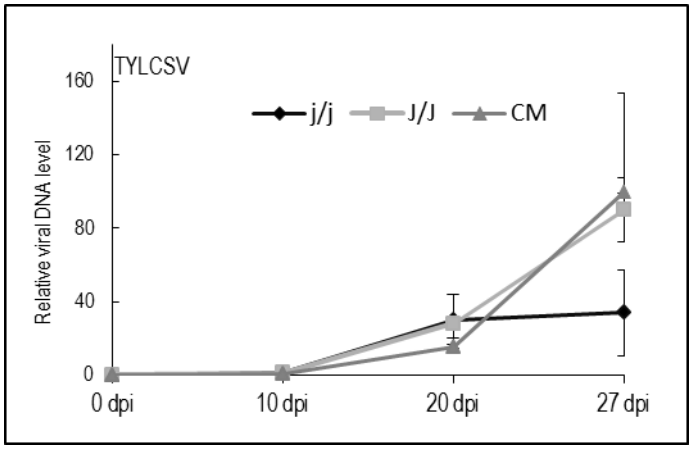


Figure 20. TYLCSV-infection mediated by whitefly in *jai1-1* tomato plants. Six- to eight-week-old tomato (*S. lycopersicum*) wild-type tomato cv. Castlemart (CM), homozygous JAI1 (J/J) and mutant homozygous *jai1-1* (j/j) plants were infected with TYLCSV using *Bemisia tabaci*-mediated inoculation. Total DNA was extracted from apical leaves and viral DNA accumulation measured by real-time PCR at 10, 20 and 27 dpi. Values are the mean of ten plants. Bars represent standard error. This experiment was performed only once (preliminary result).

C2 from TYLCSV does not interact with tomato JAZ proteins

Our previous results show that C2 from TYLCSV interacts with JAZ8 from *Arabidopsis* in yeast and *in planta*. To investigate whether this protein-protein interaction occurs between C2 and JAZ repressor from the virus' natural host, tomato, we tested the interaction between C2 and twelve members of the SlJAZ family in yeast, and could not find any positive result (Figure 21A). As controls we included a previously described C2 interactor, CSN5 (Lozano-Durán et al., 2011a) (Figure 21A). Because of the formation of homo/heterodimers reported in JAZ proteins from *Arabidopsis* (Pauwels and Goossens, 2011), we tested the expression of each prey SlJAZ protein using SlJAZ2 as bait; this experiment allowed detection of several positive interactions between SlJAZ2 and SlJAZ2, 3, 4, 5, 7, 8, 9, 10 and 11 (Figure 21B). Our controls support the idea that the negative interaction between C2 and SlJAZs is real, and not due to misexpression of the tested proteins.

The results shown in this section reveal that the activation of the JA response, through exogenous JA treatment, promotes TYLCSV infection in tomato plants. This effect is opposite to the one observed previously in *Arabidopsis*. This contradictory result might correlate with the observed differences in interaction between C2 and JAZ repressor proteins.

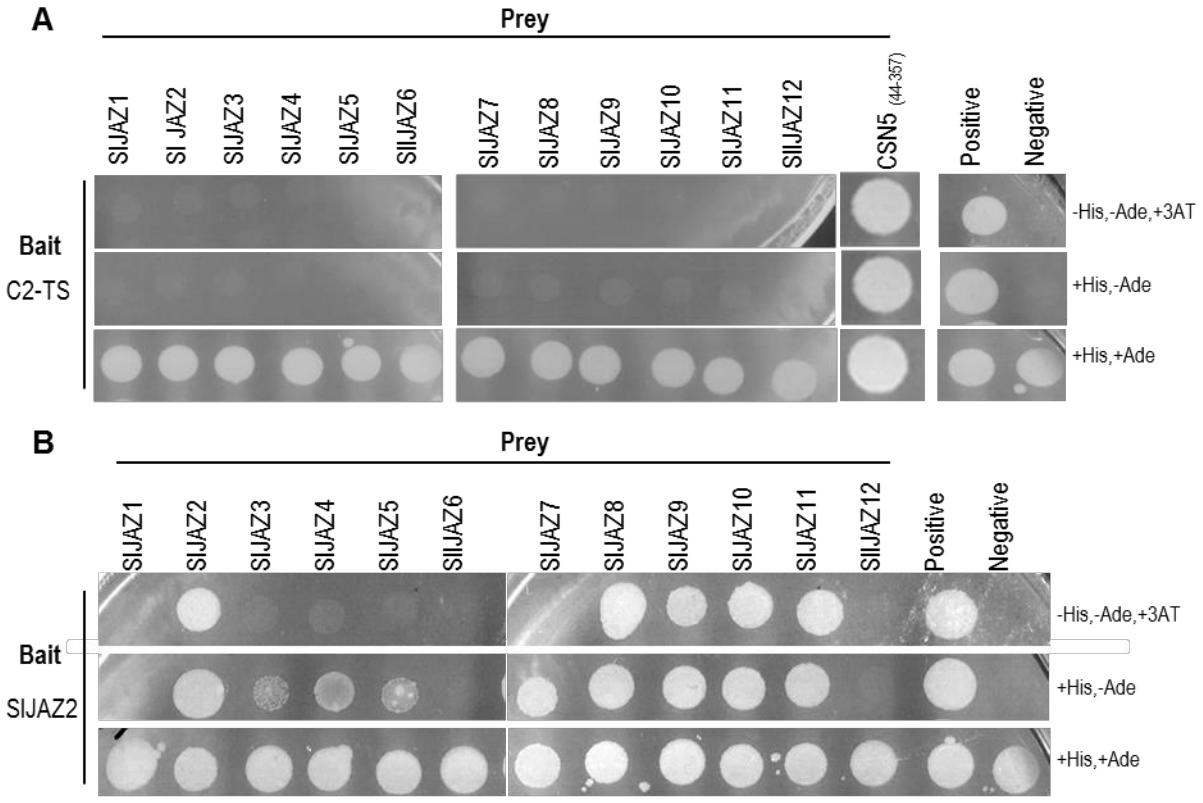


Figure 21. C2-TS does not interact with JAZ proteins from tomato *in yeast*. (A) Yeast cells co-transformed with pGBKT7-C2-TS $_{(1-78)}$ (bait) and pGADT7-SlJAZ (prey) or pGADT7-CSN5A $_{(44-357)}$ (prey, positive C2 interactor). **(B)** Yeast cells co-transformed with pGBKT7-SlJAZ2 (bait) and pGADT7-SlJAZ (prey). In A and B, yeast were selected and subsequently grown on medium containing histidine or lacking histidine and containing 2mM 3-amino-1,2,4-triazole (3-AT), to test for a weak or strong interaction, respectively; the same co-transformed clones were grown on medium containing histidine and adenine, as a co-transformation control. As a positive control, pGBTKT7-p53 was co-transformed with pGADT7-AgT; and as negative control, pGBTKT7-LamC was co-transformed with pGADT7-AgT. Two independent experiments were performed with similar results.

DISCUSSION

TYLCSV C2 suppresses JA responses affecting several layers of regulation

The results obtained here confirm that begomovirus C2 is inhibiting the response to JA in *Arabidopsis* since, besides the inhibition in the response to JA, the C2-transgenic plants are also less sensitive to a JA bacterial analogue, the bacterial toxin coronatine (COR). Besides, we observed that the number of differentially expressed genes after JA treatment is lower in C2 transgenic than in control plants, and that C2 strongly interacts with one of the JAZ proteins, JAZ8. These results imply that C2 might be interfering with the JA signalling pathway at several levels.

The transcriptomic analysis showed that C2 alters the response of only a subset of genes differentially expressed after JA-treatment, suggesting that C2 must be interfering with the JA-response pathway downstream of the receptor SCF^{COI1}. The interaction of C2 with JAZ8 points to this JAZ protein as the target that could explain the specific inhibitory effect on the JA pathway exerted by C2. JAZ8 is a special member of the JAZ family since it lacks LPIAR motif that seals JA in its binding pocket at the COI1-JAZ interface. As a consequence, JAZ8 is unable to associate strongly with COI1 in the presence of JA, and it is stabilized against JA-mediated degradation (Shyu et al., 2012). Although the mechanisms by which JAZ8 is removed from cells remain unknown, it has been postulated that stable JAZs are eliminated via COI1-independent proteolytic pathways, perhaps involving other F-box proteins. However, the ability of COR to destabilize JAZ8 in vivo, together with the inhibitory effect of MG132, indicates that JAZ8 can be degraded by a mechanism involving COI1 and the 26S proteasome (Shyu et al., 2012). The fact that the expression of C2 *in planta* reduces the accumulation of JAZ8 will be supporting a protein degradation mechanism of JAZ8 that is enhanced by the presence of the viral protein. In accordance, the reduction in TYLCV accumulation observed in *35S:JAZ8* plants suggests a negative impact of JAZ8 for the viral infection. Nevertheless, further work is required to fully understand the biological function of the C2-JAZ8 interaction.

When ectopically expressed in *Arabidopsis*, JAZ8 represses a subset of the JA-regulated responses involved in root growth and defence responses against the cartepillar *Spodoptera exigua* (Shyu et al., 2012). Although higher susceptibility of C2-transgenic plants to *P. syringe* or PVX suggests a partial C2-dependent suppression of the defence responses, whether the JA-responses controlled by JAZ8 are similar to those altered by C2 remains to be probed. A comparative analysis of the transcriptome of C2-transgenic plants with those from plants overexpressing or lacking JAZ8 could help to determine whether C2 alters the JAZ8-regulated pathways. Unfortunately, neither transcriptomic data nor JAZ8 knockout/down lines are available yet.

To sum up, we propose a tentative model according to which C2 would be affecting the JA signalling pathway at least at two levels: a) Inducing malfunction of the SCF^{COI1}; and b) Interacting with JAZ proteins. The effect of C2 at the first level would potentially have a generalized impact; the effect of the viral protein at the second level, however, would give rise to more specific changes. While the former would be detectable at basal conditions, it could be partially overcome after JA treatment; the latter, on the contrary, would be more clearly detectable after hormone treatment, and would imply the specific inhibition of certain processes. The feasibility of this hypothesis needs to be addressed in order to fully understand the impact of geminivirus C2 protein on JA signalling in their hosts.

Impact of JA signalling on TYLCV/TYLCSV infection

The results obtained by infection of hormone-treated *Arabidopsis* plants indicated that activation of the JA but not the SA signalling pathway impairs geminivirus infection and that this effect seems to be independent of the SA-JA crosstalk.

However, the reduction of the viral accumulation detected in the *aos/dde2* mutant plants do not seem to support this conclusion. The *aos/dde2* mutant is deficient in JA biosynthesis owing to disruption of the *ALLENE OXIDE SYNTHASE* (*AOS*) gene, which encodes the key enzyme of JA biosynthesis, resulting in male sterility and defective wound signal transduction (Park et al., 2002) . How the absence of JA biosynthesis and the external application of the hormone produce the same negative effect on the viral infection remains to be elucidated. The lack of effect in TYLCV accumulation of the *jin1* mutation is apparently also striking. The weak phenotype of the *jin1* mutant suggested the existence of other MYC-related genes. Those genes (MYC3 and MYC4) were identified in 2011 by Fernández-Calvo et al., showing that MYC3 and MYC4 are activators of JA-regulated programs that act additively with MYC2 to regulate specifically different subsets of the JA-dependent response, such as the defence against bacterial pathogens and insect herbivores. Interestingly, analyses of JA marker gene expression in single, double, and triple mutants revealed that all three MYC proteins are required for full responsiveness to JA. Thus, it is possible that the absence of effect in TYLCV infection of the *jin1* mutant could be due to functional redundancy. Infections of double and triple mutants will be required to clarify this question.

The results obtained in the infections of tomato plants are intriguing. The tomato *jai1* mutant seems to be more resistant to TYLCSV infection, but as we observed in the *Arabidopsis coi1-1* mutant, this could result in activated SA responses, which is detrimental to the T-DNA transfer. As in *Arabidopsis*, JA- but not SA-treatment alters the viral infection. However, in tomato exogenous application of the hormone enhances viral accumulation, as opposed to what happens in

Arabidopsis. This contradictory result could be due to differences in the role of JA between the two plants species, or differences between the two viral species used, TYLCVS and TYLCV. Further infections of tomato plants with TYLCV will clarify this point.

EXPERIMENTAL PROCEDURES

Microorganisms and general methods

Manipulations of *Escherichia coli* and *Saccharomyces cerevisiae* strains and nucleic acids were performed according to standard methods (Ausubel, 1998; Sambrook, 2001). *Agrobacterium tumefaciens* (*Agrobacterium*) GV3101 strain was used for the agroinfiltration assays in *Nicotiana benthamiana* and infections assays in tomato (*Solanum lycopersicum)* plants whereas LBA4404 was used for agroinfiltration and infections assays in *Arabidopsis thaliana. S. cerevisiae* strain pJ696 (*MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4*Δ*, gal80*Δ*, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*), a derivative of PJ69-4A (James et al., 1996), was used for the yeast two-hybrid experiments.

Plant DNA extraction using CTAB method was performed as described in (Lukowitz et al., 2000).

Plant materials and growth conditions

Unless otherwise stated, *A. thaliana* (*Arabidopsis*) plants accession Columbia (Col-0) wild-type (wt), and mutant derivatives were grown in growth chambers with 8 h light: 16 h dark cycles at 21°C. The following mutants or transgenics have been described elsewhere: *coi1-1* (Xie et al., 1998)*, jin1-1* (Berger et al., 1996), *aos/dde2-2* (von Malek et al., 2002), *35S:JAZ8* (Shyu et al., 2012). The mentioned lines were provided by the Nottingham *Arabidopsis* Stock Centre (NASC; http:// www.Arabidopsis.info) or by generous colleagues. Seeds from wt *Arabidopsis*, *jin1*, *aos/dde2* and *35S:JAZ8* were surface-sterilized and sown on MS (Duchefa, Haarlem, the Netherlands) agar plates (30g/L sucrose). *coi1-1* mutant seeds (F2) were grown in MS agar plates with 30g/L sucrose supply with 50μM methyl-jasmonate (MeJA; diluted in ethanol; Duchefa) for 15 d, and seedlings nonresponsive to MeJA (homozygous *coi1* mutants) were transferred to soil substrate for subsequent experiments. Corresponding wt control plants were grown in MS agar plates (30g/L sucrose) with 0.5% ethanol before transference to soil substrate. Plates were cold-treated for 2 to 6 days at 4 °C. Seedlings were grown at 20 °C with a 16 h light: 8 h dark photoperiod.

The transgenic *Arabidopsis* plants expressing C2 from *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (C2-TS), *Tomato yellow leaf curl virus* (TYLCV) (C2-TM) and the transgenic *N. benthamiana* plants expressing TYLCSV C2 are described elsewhere (Lozano-Durán et al., 2011a) . *N. benthamiana* plants were grown in soil at 22ºC in long day conditions 16 h light: 8 h dark photoperiod.

For root growth inhibition assays, MS plates were placed in a vertical orientation for 5 days, and seedlings were then transferred to MS plates containing 50μM MeJA. Root length was measured 5 days later using the ImageJ software (http://rsb.info.nih.gov/ij).

Sample preparation for the microarray analysis was done in a previous work (Rosa Lozano-Durán PhD thesis, 2010) (Lozano-Durán, 2010). For the transcriptomic analysis, T2 seedlings of C2-TS transgenic *Arabidopsis* plants were grown on MS with kanamycin for 7 days, and then treated with 50μM MeJA (JA) or mock solution for 10 hours. Three independent replicates were performed. For these analyses, T3 homozygous LUC2 (PRB1::LUC) transgenic plants (Santamaria et al., 2001) resistant to kanamycin were used as control. Previously, the hormonal response of LUC2 had been proven to be identical to that of the wild-type in the aforementioned assays.

Phytohormone treatments for the infection experiments were as follows: for MeJA (JA) treatments in *Arabidopsis* and tomato plants, a 50 μM MeJA solution or mock solution (0.5% ethanol in water) was used; for Salycilic acid (SA) treatments, a 0.5 mM SA solution or 100 μM SA solution (for *Arabidopsis* and tomato, respectively) and mock solution (water) were used. In both cases, Silwet L-77 was added to a final concentration of 0.1%, and the hormone or mock solution was applied to whole *Arabidopsis* or tomato plants by spraying every other day from two days after inoculation to 21 days post-inoculation (dpi) or 17 dpi for *Arabidopsis* or tomato, respectively.

Tomato plants wild-type (*S. lycopersicum*) cv. MoneyMaker, cv. Castlemart and *jai1-1* mutant (F2) (Li *et al.*, 2004) were grown in growth chambers with 16 h light: 8 h dark cycles at 22–28°C. The selection of homozygous *jai1-1* plants was done as previously described (Li et al., 2004).

For the agroinfiltration experiments, *N.benthamiana* plants were grown in soil at 22ºC in long day conditions (16 h light: 8 h dark photoperiod).

Bacterial inoculations

Bacterial inoculations were performed in collaboration with Alberto Macho at Carmen Beuzón's group, University of Málaga. *Pseudomonas syringae* pv *tomato* DC3000 (Cuppels, 1986), a mutant unable to secrete coronatine (*cfa- cma-*; (Brooks et al., 2004), a hrcC mutant (Mudgett and Staskawicz, 1999), or a strain expressing the avirulence factor AvrRpt2 (Macho et al., 2009) were grown at 28ºC in LB medium supplemented with rifampicin (15 μg/ml). Bacteria were suspended in 10 mM $MgCl_2$ before inoculations. Four to five-week old *Arabidopsis* plants were either inoculated by infiltrating with a $5x10^4$ cfu/ml bacterial suspension using a blunt syringe, or inoculated by dipping for 30 seconds in a $5x10^7$ cfu/ml bacterial suspension containing 0.02% silwet L-77 (Crompton Europe LTD). Symptoms were evaluated at 4 dpi. Samples were taken from inoculated

leaves at 4 dpi using a 10 mm-diameter cork borer. Three disks were taken per plant, placed into 1ml of 10 mM $MgCl_2$, and homogenized by mechanical disruption. Serial dilutions of the resulting bacterial suspensions were plated onto LB plates supplemented with of cycloheximide (2 μg/ml) and rifampicin (15 μg/ml).

Agrobacterium strain LB4404 carrying TYLCV-Mld (Navas-Castillo J., 1999) was grown at 28°C LB medium supplemented with rifampicin (50 μg/ml) and kanamycin (50 μg/ml). Replication was performed as described above for *P. syringae*, but after infection plated onto LB 50 μg/ml) and kanamycin (50 μg/ml).

PVX-GFP and TMV-GFP infections

Tobacco mosaic virus (TMV)-GFP and *Potato virus X* (PVX)-GFP were kindly provided by Dr. Peter Moffett and are described elsewhere (Peart et al., 2002). Infections in wild-type and transgenic C2-TS *N. benthamiana* were performed by agroinoculation as described in (Peart et al., 2002). GFP expression was monitored at 7 and 10 days post inoculation (dpi), and samples were taken at 10 dpi.

RNA extraction, cRNA preparation and Affymetrix GeneChip® hybridization

Preparation of samples was done in a previous work (Rosa Lozano-Durán PhD thesis, 2010). Seven-day-old wild-type and transgenic C2-TS *Arabidopsis* seedlings were treated with a 50 μM MeJA o mock solution for 10 hours as previously described. Total RNA was isolated from three replicates of MeJA- or mock-treated wild-type and transgenic C2-TS seedlings using TRIzol (Invitrogen) and subsequently cleaned using RNeasy MinElute Cleanup Kit (Invitrogen). RNA quantity and quality were assessed with a Nanodrop ND-1000 spectrophotometer (Labtech) and an Agilent 2100 bioanalyzer (Agilent Technologies), respectively.

Microarray hybridization was carried out at the Unité de Recherche en Génomique Végétale (Evry, France), using Affymetrix GeneChip® ATH1.

Microarray analysis

Analysis of the microarray data was performed using the following software: VirtualPlant 1.0 (www.virtualplant.org), Venny (www.bioinfogp.cnb.csic.es/tools/venny/), and TAIR GO annotation tool (http://www.*Arabidopsis*.org/tools/bulk/go/index.jsp).

Quantitative real-time PCR and semi-quantitative RT-PCR

Primer pairs for real-time PCR were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3/). Gene-specific primers were chosen so that the PCR products

were 100–300 bp. Total RNA was extracted from seedlings using RNAeasy Plant Mini Kit (Qiagen) and treated on column with DNase (Takara®). 1µg total RNA was used for first-strand cDNA synthesis using oligo(dT) primers and SuperScript II reverse transcriptase reagent (Invitrogen) following the manufacturer's instructions. For real-time PCR, the reaction mixture consisted of cDNA first-strand template, primer mix (10 µmol each) and SsoFast™EvaGreen®Supermix (BIO-RAD) in a total volume of 20 µl. The PCR conditions were: 10 minutes at 95°C, and 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. The reactions were performed using a MyiQ iCycler time cycler (BIO-RAD). A relative quantification real-time PCR method was used to compare expression of the genes in transgenic versus non-transgenic line (Panchuk et al., 2002).

Actin (*AtACT2*) was used as the internal control in *Arabidopsis* quantifications whereas *Internal transcript spacer 25SrDNA* (*ITS*) or *Elongation factor alpha (E1Fα)* were used as internal controls in tomato for viral DNA or transcripts levels quantifications, respectively. Relative quantification describes the change in expression of the target gene in a test sample relative to calibrator sample. For the validation of the microarray, the sample of LUC2 transgenic plants was used as the calibrator, with the expression level of the sample set to 1, data point is the mean value from three experimental replicate determinations. Three biological replicates were used. For viral quantification the sample of control (wt) virus-infected or untreated (-JA/-SA) virus-infected plants were used as the calibrator in *Arabidopsis* and tomato plants; with the values of the samples set to 1. Finally, for measuring the transcripts levels, the sample of pBIN-infected untreated (-JA/–SA) was used as calibrator in *Arabidopsis* and tomato plants, with the expression level of the sample set to 1. The list of primers used for real-time PCR assays is summarized in the table1.

For quantification of PXV-GFP and TMV-GFP, total RNA was extracted from the third leave of each infected *N. benthamiana* plant using RNAeasy Plant Mini Kit (Qiagen) and treated on column with DNase (Takara®). cDNA synthesis was performed as previously described. Virus-GFP accumulation was assessed by semi-quantitative PCR using primers for the GFP (Up-mGFP: AGTGGAGAGGGTGAAGGTGA; low-mGFP: AAAGGGCAGATTGTGTGGAC) and the following conditions: 94ºC, 30 seconds; 55ºC, 30 seconds; 72ºC, 40 seconds (22 cycles). Primers to amplify the *ITS* were used as control using the same PCR conditions, 16 cycles.

Yeast two hybrid assays

Yeast two hybrid assays using C2-TS and JAZ proteins were performed by Gemma Fernández Barbero at Roberto Solano's group, CNB, Madrid, Spain, as described in Chini et al., 2009. pGBKT7-C2-TS_{1-78}, pGADT7-$CSN5A_{(44-357)}$ constructs are described elsewhere (Lozano-Durán et al., 2011a). pGADT7-JAZ constructs are described in (Chini et al., 2009b). pGADT7-SlJAZ and

pGBKT7-SlJAZ constructs were kindly provide by Dr. Kirankuman S. Mysore from The Samuel Noble Roberts Foundation, OK, United States of America (Ishiga et al., 2013). Assays were performed as described (Castillo et al., 2004). Yeast were co-transformed and selected for bait and prey plasmids, as described (Yeast Protocols Handbook, Clontech Laboratories, Inc 2001; www.clontech.com).

Geminivirus infection assays

TYLCSV infections of tomato plants were performed by agroinoculation as previously described (Elmer et al., 1988). Plants were agroinoculated with pGreenTYA14 (binary vector containing a partial dimer of TYLCSV; accession number L27708) (Lozano-Durán et al., 2011a) in the axilary bud of the fourth/fifth leaf of 3-week-old wild-type tomato plants. For control, plants were mock inoculated with *A. tumefaciens* harbouring the empty binary vector pGreen-0229 (Hellens et al., 2000). Symptoms were evaluated every week until 32 dpi. Samples were taken at 17 dpi. DNA viral accumulation was performed as previously describe in the Chapter II.

TYLCV-Mld infections of *Arabidopsis* plants were performed by agroinoculating the infectious clone (pBIN19-TYLCV-Mild; accession number AF071228) (Navas-Castillo J., 1999). Samples were taken at 21 dpi. Viral DNA accumulation was quantified by real-time PCR.

Transient expression assays

Agrobacterium-mediated expression in *Arabidopsis* was performed as described in Zipfel et al (2006) with some modifications. *Agrobacterium* LBA4404 carrying a GUS-intron transgene cloned into pBIN19g vector (pBIN19-35S::GUS; kindly provided by Dr. Zipfel, The Sainsbury Laboratory, Norwich, UK). Bacteria were resuspended in infiltration solution (10 mM morpholineethanesulfonic acid pH 5.6, 10 mM $MgCl_2$, and 100 µM acetosyringone) at OD_{600} of 0.05 for injection into leaves of 4- to 5-week-old plants. At least 6 plants/genotype and 4 leaves/plant were used per experiment.

GUS assays

GUS staining was performed according to the protocol previously described by Ranjan et al., 2012 with minor modifications. Plant tissues were immersed in histochemical GUS staining buffer (100 mM $NaPO_4$ pH 7, 0.5 mM $K_3[Fe(CN)_6$, 0.5 mM $K_4[Fe(CN)_6]$, 20% Methanol, 0.3% Triton X-100 and 0.1% mg mL 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-gluc) (Duchefa Biochemie, Nederlands) on multi-well plates, vacuum-infiltrated (75 cm Hg) for 10 min three times, and then wrapped in aluminium foil and incubated at 37°C for 12 h. Samples were then washed several times with 95% ethanol until complete tissue clarification, stored in 50% glycerol and photographed.

Quantitative measurements for GUS activity were performed according to (Francis and Spiker, 2005).

Protein extraction and immunoprecipitation experiments

The co-Inmunoprecipitation assay was performed by Rosa Lozano-Durán and Alberto Macho at The Sainsbury Laboratory, Norwich, UK. Agroinfiltration of *N. benthamiana* plants was performed as described in Lozano-Durán et al., 2011. Protein extraction of *N. benthamiana* was performed as described in Schwessinger *et al.*,2011; immunoprecipitations were performed using the GFP-Trap® (Chromotek, Germany), following the manufacturer's instructions. The constructs to express GFP-C2 and JAZ8-HA have been previously described (Lozano-Durán et al., 2011a; Giménez-Ibánez et al., 2014).

Common name	Gene	Primers	Reference
Microarray validation and gene markers			
Glutamine-dependent asparagine synthase 1 (AtASN1)	At3g47340	CGCGCCTTTGAAAACGCTGTGA CGGCCAAGTGACGTGCAGTGAT	Castro *et al.*, in preparation
Allene oxide synthase (AtAOS1)	At5g42650	CCGACGGTGGGGAATAAACA GGACTACACAGGTGCGAACA	Castro *et al.*, in preparation
Galactinol synthase 1 (AtGOLS)	At2g47180	GGTTCACTACTGTGCAGCGGGTTC GACGGTGCGGTCACGTAGTT	Castro *et al.*, in preparation
Xyloglucan endotransglycosylase/hydrolase (AtXTH31)	At3g44990	TCCACTGGGAGTGGGTTCA	Castro *et al.*, in preparation
Basic pathogenesis-related protein 1 (AtPRB1)	At2g14580	GAATAAGGCTTCCCTGGCGT CTTGCAGCCTACGCGTCGGAA	This work
Ethylene-responsive transcription factor (AtTINY)	At5g25810	CTCCGTTCGACGTGTTTGTAT CCGAGGAGCTAGGGGAGATT	This work
Flagellin-sensitive 2 (AtFLS2)	At5g46330	CTTCTGACCATTGCGGTGGA ACCATTCACGGCTCTGCAGCA	Castro *et al.*, in preparation
Auxin-induced in root cultures 1 (AtAIR)	At4g12550	TCGCTTACGTGAGCAACGCG ATGGCTCCAAGAACCCCCCT	This work
Pathogenesis-related gene 1 (AtPR1)	At2g14610	CAGTGACACAAGTACCGATCTC CTTCCCTCGAAAGCTCAAGA	(Roberts et al., 2007)
Jasmonate-Zim-Domain protein 8 (AtJAZ8)	At1g30135	GTAAGGCCCACCAGAGTGTAT CGCAAGCAGAGAAATGAAAAC TGATGCTTTTGGATTTGGAAG	Zumaquero et al., in preparation

Common name	Gene	Primers	Reference
Jasmonate-Zim-Domain protein 10 (AtJAZ10)	At5g13220	AGCCTCCAGATCCCGATTTCT	Zumaquero et al., in preparation
Proteinase inhibitor 2 (SlPIN-2)		GCGACCTTCATAATTTCACCA AATTATCCATCATGGCTGTTCAC CCTTTTTGGATCAGATTCTCCTT	(Casteel et al., 2012)
Pathogenesis-related protein-P6 (SlPR-P6)		GTACTGCATCTTCTTGTTTCCA TAGATAAGTGCTTGATGTGCC	(Sarmento et al., 2011)
Pathogenesis-related protein 1(SlPR-1)		CCGTGCAATTGTGGGTGTC GAGTTGCGCCAGACTACTTGAGT	(Casteel et al., 2012)
Geminivirus quantification			
C4 TYLCV-Mld		AAGCAGGGCAGCACATTTCCATC CTGCGGCGTAAGCGTCATTG	(Rodriguez-Negrete et al., 2013)
CP TYLCSV		GGAGGCTGAACTTCGACAGC GGACTTTCAATGGGCCTTCAC	(Lozano-Durán et al., 2011b)
Housekeeping genes			
Actin (AtACT2)	At3g18780	ACTAAAACGCAAAACGAAAGCGGTT CTAAGCTCTCAAGATCAAAGGCTTA	(Love et al., 2005)
Internal transcript spacer 25SrDNA (ITS)		ATAACCGCATCAGGTCTCCA CCGAAGTTACGGATCCATTT	(Mason et al., 2008)
Elongation factor alpha (E1Fα)		ATTGGAAACGGATATGCTCCA TCCTTACCTGAACGCCTGTCA	(Nicot et al., 2005)

Table1. Oligonucleotides used in this study.

REFERENCES

- Agudelo-Romero, P., Carbonell, P., de la Iglesia, F., Carrera, J., Rodrigo, G., Jaramillo, A., Perez-Amador, M.A., Elena, S.F., 2008. Changes in the gene expression profile of Arabidopsis thaliana after infection with Tobacco etch virus. Virology journal 5, 92.

- Ascencio-Ibánez, J.T., Sozzani, R., Lee, T.J., Chu, T.M., Wolfinger, R.D., Cella, R., Hanley-Bowdoin, L., 2008. Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant physiology 148, 436-454.

- Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K., 1998. Current protocols in molecular biology.

- Berger, S., Bell, E., Mullet, J.E., 1996. Two Methyl Jasmonate-Insensitive Mutants Show Altered Expression of AtVsp in Response to Methyl Jasmonate and Wounding. Plant physiology 111, 525-531.

- Boter, M., Ruiz-Rivero, O., Abdeen, A., Prat, S., 2004. Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes & development 18, 1577-1591.

- Brooks, D.M., Hernandez-Guzman, G., Kloek, A.P., Alarcon-Chaidez, F., Sreedharan, A., Rangaswamy, V., Penaloza-Vazquez, A., Bender, C.L., Kunkel, B.N., 2004. Identification and characterization of a well-defined series of coronatine biosynthetic mutants of Pseudomonas syringae pv. tomato DC3000. Mol Plant Microbe Interact 17, 162-174.

- Brown JK, F.C., Briddon RW, Zerbini M, Moriones E, Navas-Castillo J, 2012. Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses. Academic press, London.

- Buchmann, R.C., Asad, S., Wolf, J.N., Mohannath, G., Bisaro, D.M., 2009. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. Journal of virology 83, 5005-5013.

- Cañizares M.C., Rosas-Díaz T. , Rodríguez-Negrete E., Hogenhou , S.A., Bedford I. D., Bejarano E. R., and, N.-C.J., Moriones, E., 2014. Arabidopsis thaliana, an experimental host for tomato yellow leaf curl disease-associated begomoviruses by agroinoculation and whitefly transmission. Plant Pathology.

- Casteel, C.L., Hansen, A.K., Walling, L.L., Paine, T.D., 2012. Manipulation of plant defense responses by the tomato psyllid (Bactericerca cockerelli) and its associated endosymbiont Candidatus Liberibacter psyllaurous. PLoS One 7, e35191.

- Castillo, A.G., Kong, L.J., Hanley-Bowdoin, L., Bejarano, E.R., 2004. Interaction between a geminivirus replication protein and the plant sumoylation system. Journal of virology 78, 2758-2769.

- Cuppels, D.A., 1986. Generation and Characterization of Tn5 Insertion Mutations in Pseudomonas syringae pv. tomato. Appl Environ Microbiol 51, 323-327.

- Cheng, Z., Sun, L., Qi, T., Zhang, B., Peng, W., Liu, Y., Xie, D., 2011. The bHLH transcription factor MYC3 interacts with the Jasmonate ZIM-domain proteins to mediate jasmonate response in Arabidopsis. Molecular plant 4, 279-288.

- Chini, A., Boter, M., Solano, R., 2009a. Plant oxylipins: COI1/JAZs/MYC2 as the core jasmonic acid-signalling module. The FEBS journal 276, 4682-4692.

- Chini, A., Fonseca, S., Chico, J.M., Fernandez-Calvo, P., Solano, R., 2009b. The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. Plant J 59, 77-87.

- Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., Solano, R., 2007. The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448, 666-671.

- Chung, H.S., Howe, G.A., 2009. A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in Arabidopsis. The Plant cell 21, 131-145.

- Chung, H.S., Koo, A.J., Gao, X., Jayanty, S., Thines, B., Jones, A.D., Howe, G.A., 2008. Regulation and function of Arabidopsis JASMONATE ZIM-domain genes in response to wounding and herbivory. Plant physiology 146, 952-964.

- Chung, H.S., Niu, Y., Browse, J., Howe, G.A., 2009. Top hits in contemporary JAZ: an update on jasmonate signaling. Phytochemistry 70, 1547-1559.

- Demianski, A.J., Chung, K.M., Kunkel, B.N., 2012. Analysis of Arabidopsis JAZ gene expression during Pseudomonas syringae pathogenesis. Molecular plant pathology 13, 46-57.

- Dong, X., van Wezel, R., Stanley, J., Hong, Y., 2003. Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. Journal of virology 77, 7026-7033.

- Durrant, W.E., Dong, X., 2004. Systemic acquired resistance. Annual review of phytopathology 42, 185-209.

- Elmer, J.S., Sunter, G., Gardiner, W.E., Brand, L., Browing, C.K., Bisaro, D., Rogers, S.G., 1988. Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNAs. Plant. Mol. Biol. 10, 225-234.

- Etessami, P., Callis, R., Ellwood, S., Stanley, J., 1988. Delimitation of essential genes of cassava latent virus DNA 2. Nucleic acids research 16, 4811-4829.

- Fernández-Calvo, P., Chini, A., Fernandez-Barbero, G., Chico, J.M., Gimenez-Ibanez, S., Geerinck, J., Eeckhout, D., Schweizer, F., Godoy, M., Franco-Zorrilla, J.M., Pauwels, L., Witters, E., Puga, M.I., Paz-Ares, J., Goossens, A., Reymond, P., De Jaeger, G., Solano, R., 2011. The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. The Plant cell 23, 701-715.

- Feys, B., Benedetti, C.E., Penfold, C.N., Turner, J.G., 1994. Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. The Plant cell 6, 751-759.

- Francis, K.E., Spiker, S., 2005. Identification of Arabidopsis thaliana transformants without selection reveals a high occurrence of silenced T-DNA integrations. The Plant journal : for cell and molecular biology 41, 464-477.

- García-Marcos, A., Pacheco, R., Manzano, A., Aguilar, E., Tenllado, F., 2013. Oxylipin biosynthesis genes positively regulate programmed cell death during compatible infections with the synergistic pair potato virus X-potato virus Y and Tomato spotted wilt virus. Journal of virology 87, 5769-5783.

- Geng, X., Jin, L., Shimada, M., Kim, M.G., Mackey, D., 2014. The phytotoxin coronatine is a multifunctional component of the virulence armament of Pseudomonas syringae. Planta 240, 1149-1165.

- Giménez-Ibánez, S., Boter, M., Fernandez-Barbero, G., Chini, A., Rathjen, J.P., Solano, R., 2014. The bacterial effector HopX1 targets JAZ transcriptional repressors to activate jasmonate signaling and promote infection in Arabidopsis. PLoS biology 12, e1001792.

- Grant, M.R., Jones, J.D., 2009. Hormone (dis)harmony moulds plant health and disease. Science 324, 750-752.

- Grunewald, W., Vanholme, B., Pauwels, L., Plovie, E., Inze, D., Gheysen, G., Goossens, A., 2009. Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. EMBO reports 10, 923-928.

- Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., Mullineaux, P.M., 2000. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant molecular biology 42, 819-832.

- Hou, X., Lee, L.Y., Xia, K., Yan, Y., Yu, H., 2010. DELLAs modulate jasmonate signaling via competitive binding to JAZs. Developmental cell 19, 884-894.

- Hu, P., Zhou, W., Cheng, Z., Fan, M., Wang, L., Xie, D., 2013. JAV1 controls jasmonate-regulated plant defense. Molecular cell 50, 504-515.

- Ishiga, Y., Ishiga, T., Uppalapati, S.R., Mysore, K.S., 2013. Jasmonate ZIM-domain (JAZ) protein regulates host and nonhost pathogen-induced cell death in tomato and Nicotiana benthamiana. PLoS One 8, e75728.

- James, P., Halladay, J., Craig, E.A., 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144, 1425-1436.

- Jeske, H., 2009. Geminiviruses. Current topics in microbiology and immunology 331, 185-226.

- Jung, C., Lyou, S.H., Yeu, S., Kim, M.A., Rhee, S., Kim, M., Lee, J.S., Choi, Y.D., Cheong, J.J., 2007. Microarray-based screening of jasmonate-responsive genes in Arabidopsis thaliana. Plant cell reports 26, 1053-1063.

- Katari, M.S., Nowicki, S.D., Aceituno, F.F., Nero, D., Kelfer, J., Thompson, L.P., Cabello, J.M., Davidson, R.S., Goldberg, A.P., Shasha, D.E., Coruzzi, G.M., Gutierrez, R.A., 2010. VirtualPlant: a software platform to support systems biology research. Plant physiology 152, 500-515.

- Kempema, L.A., Cui, X., Holzer, F.M., Walling, L.L., 2007. Arabidopsis transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs. Similarities and distinctions in responses to aphids. Plant physiology 143, 849-865.

- Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F., Kunkel, B.N., 2001. Resistance to Pseudomonas syringae conferred by an Arabidopsis thaliana coronatine-insensitive (coi1) mutation occurs through two distinct mechanisms. The Plant journal : for cell and molecular biology 26, 509-522.

- Kovak, M., Müller, A., Milanovic Jarh, D., Milavec, M., Düchting, P., and Ravnikar, M., 2009. Multiple hormone analysis indicates involvement of jasmonate signalling in the early defence of potato to potato virus YNTN. BIOLOGIA PLANTARUM 53, 195-199.

- Kunkel, B.N., Brooks, D.M., 2002. Cross talk between signaling pathways in pathogen defense. Current opinion in plant biology 5, 325-331.

- Laurie-Berry, N., Joardar, V., Street, I.H., Kunkel, B.N., 2006. The Arabidopsis thaliana JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during infection by Pseudomonas syringae. Molecular plant-microbe interactions : MPMI 19, 789-800.

- Lewsey, M.G., Gonzalez, I., Kalinina, N.O., Palukaitis, P., Canto, T., Carr, J.P., 2010. Symptom induction and RNA silencing suppression by the cucumber mosaic virus 2b protein. Plant signaling & behavior 5, 705-708.

- Li, L., Zhao, Y., McCaig, B.C., Wingerd, B.A., Wang, J., Whalon, M.E., Pichersky, E., Howe, G.A., 2004. The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. The Plant cell 16, 126-143.

- Liu, Y., Schiff, M., Dinesh-Kumar, S.P., 2004. Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to tobacco mosaic virus. The Plant journal : for cell and molecular biology 38, 800-809.

- Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., Solano, R., 2004. JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. The Plant cell 16, 1938-1950.

- Love, A.J., Geri, C., Laird, J., Carr, C., Yun, B.W., Loake, G.J., Tada, Y., Sadanandom, A., Milner, J.J., 2012. Cauliflower mosaic virus protein P6 inhibits signaling responses to salicylic acid and regulates innate immunity. PLoS One 7, e47535.

- Love, A.J., Yun, B.W., Laval, V., Loake, G.J., Milner, J.J., 2005. Cauliflower mosaic virus, a compatible pathogen of Arabidopsis, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. Plant physiology 139, 935-948.

- Lozano-Durán, 2010. Study of geminiviral subversion of the host cell: the case of C2 and the CSN complex. PhD Thesis Univesidad de Málaga.

- Lozano-Durán, R., Rosas-Diaz, T., Gusmaroli, G., Luna, A.P., Taconnat, L., Deng, X.W., Bejarano, E.R., 2011a. Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes and inhibit jasmonate signaling in Arabidopsis thaliana. The Plant cell 23, 1014-1032.

- Lozano-Durán, R., Rosas-Diaz, T., Luna, A.P., Bejarano, E.R., 2011b. Identification of host genes involved in geminivirus infection using a reverse genetics approach. PLoS One 6, e22383.

- Lukowitz, W., Gillmor, C.S., Scheible, W.R., 2000. Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. Plant physiology 123, 795-805.

- Luna, A.P., Morilla, G., Voinnet, O., Bejarano, E.R., 2012. Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. Molecular plant-microbe interactions : MPMI 25, 1294-1306.

- Macho, A.P., Guevara, C.M., Tornero, P., Ruiz-Albert, J., Beuzon, C.R., 2010. The Pseudomonas syringae effector protein HopZ1a suppresses effector-triggered immunity. The New phytologist 187, 1018-1033.

- Macho, A.P., Ruiz-Albert, J., Tornero, P., Beuzon, C.R., 2009. Identification of new type III effectors and analysis of the plant response by competitive index. Molecular plant pathology 10, 69-80.

- Mandadi, K.K., Scholthof, K.B., 2012. Characterization of a viral synergism in the monocot Brachypodium distachyon reveals distinctly altered host molecular processes associated with disease. Plant physiology 160, 1432-1452.

- Mansoor, S., Briddon, R.W., Zafar, Y., Stanley, J., 2003. Geminivirus disease complexes: an emerging threat. Trends in plant science 8, 128-134.

- Mason, G., Caciagli, P., Accotto, G.P., Noris, E., 2008. Real-time PCR for the quantitation of Tomato yellow leaf curl Sardinia virus in tomato plants and in Bemisia tabaci. Journal of virological methods 147, 282-289.

- Mauck, K.E., De Moraes, C.M., Mescher, M.C., 2010. Effects of Cucumber mosaic virus infection on vector and non-vector herbivores of squash. Communicative & integrative biology 3, 579-582.

- McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y.M., Buso, N., Cowley, A.P., Lopez, R., 2013. Analysis Tool Web Services from the EMBL-EBI. Nucleic acids research 41, W597-600.

- Melotto, M., Mecey, C., Niu, Y., Chung, H.S., Katsir, L., Yao, J., Zeng, W., Thines, B., Staswick, P., Browse, J., Howe, G.A., He, S.Y., 2008. A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. The Plant journal : for cell and molecular biology 55, 979-988.

- Melotto, M., Underwood, W., Koczan, J., Nomura, K., He, S.Y., 2006. Plant stomata function in innate immunity against bacterial invasion. Cell 126, 969-980.

- Morales, F.J., Anderson, P.K., 2001. The emergence and dissemination of whitefly-transmitted geminiviruses in Latin America. Archives of virology 146, 415-441.

- Mudgett, M.B., Staskawicz, B.J., 1999. Characterization of the Pseudomonas syringae pv. tomato AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. Molecular microbiology 32, 927-941.

- Navas-Castillo J., S.-C.S., and Díaz J.A., Sáez-Alonso E. and Moriones E., 1999. Tomato Yellow Leaf Curl Virus-Is Causes a Novel Disease of Common Bean and Severe Epidemics in Tomato in Spain. Plant Disease 83, 29-32.

- Nemhauser, J.L., Hong, F., Chory, J., 2006. Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell 126, 467-475.

- Nicaise, 2014. Crop immunity against viruses :out comes and future challenges. Front. Plant Sci.

- Nickstadt, A., Thomma, B.P., Feussner, I., Kangasjarvi, J., Zeier, J., Loeffler, C., Scheel, D., Berger, S., 2004. The jasmonate-insensitive mutant jin1 shows increased resistance to biotrophic as well as necrotrophic pathogens. Molecular plant pathology 5, 425-434.

- Nicot, N., Hausman, J.F., Hoffmann, L., Evers, D., 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Journal of experimental botany 56, 2907-2914.

- Niu, Y., Figueroa, P., Browse, J., 2011. Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. Journal of experimental botany 62, 2143-2154.

- Oka, K., Kobayashi, M., Mitsuhara, I., Seo, S., 2013. Jasmonic acid negatively regulates resistance to Tobacco mosaic virus in tobacco. Plant & cell physiology 54, 1999-2010.

- Pacheco, R., Garcia-Marcos, A., Manzano, A., de Lacoba, M.G., Camanes, G., Garcia-Agustin, P., Diaz-Ruiz, J.R., Tenllado, F., 2012. Comparative analysis of transcriptomic and hormonal responses to compatible and incompatible plant-virus interactions that lead to cell death. Molecular plant-microbe interactions : MPMI 25, 709-723.

- Panchuk, II, Volkov, R.A., Schoffl, F., 2002. Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in Arabidopsis. Plant physiology 129, 838-853.

- Park, J.H., Halitschke, R., Kim, H.B., Baldwin, I.T., Feldmann, K.A., Feyereisen, R., 2002. A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. The Plant journal : for cell and molecular biology 31, 1-12.

- Pauwels, L., Barbero, G.F., Geerinck, J., Tilleman, S., Grunewald, W., Perez, A.C., Chico, J.M., Bossche, R.V., Sewell, J., Gil, E., Garcia-Casado, G., Witters, E., Inze, D., Long, J.A., De Jaeger, G., Solano, R., Goossens, A., 2010. NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464, 788-791.

- Pauwels, L., Goossens, A., 2011. The JAZ proteins: a crucial interface in the jasmonate signaling cascade. The Plant cell 23, 3089-3100.

- Peart, J.R., Cook, G., Feys, B.J., Parker, J.E., Baulcombe, D.C., 2002. An EDS1 orthologue is required for N-mediated resistance against tobacco mosaic virus. The Plant journal : for cell and molecular biology 29, 569-579.

- Pieterse, C.M., Van Loon, L.C., 2004. NPR1: the spider in the web of induced resistance signaling pathways. Current opinion in plant biology 7, 456-464.

- Qi, T., Song, S., Ren, Q., Wu, D., Huang, H., Chen, Y., Fan, M., Peng, W., Ren, C., Xie, D., 2011. The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate Jasmonate-mediated anthocyanin accumulation and trichome initiation in Arabidopsis thaliana. The Plant cell 23, 1795-1814.

- Ranjan, R., Patro, S.. Pradhan, B., Kumar, A., Maiti, I. B., Dey, N. 2012. Development and functional analysis of novel genetic promoters using DNA shuffling, hybridization and a combination thereof. PLoS One 7 e31931

- Roberts, K., Love, A.J., Laval, V., Laird, J., Tomos, A.D., Hooks, M.A., Milner, J.J., 2007. Long-distance movement of Cauliflower mosaic virus and host defence responses in Arabidopsis follow a predictable pattern that is determined by the leaf orthostichy. The New phytologist 175, 707-717.

- Rodriguez-Negrete, E., Lozano-Duran, R., Piedra-Aguilera, A., Cruzado, L., Bejarano, E.R., Castillo, A.G., 2013. Geminivirus Rep protein interferes with the plant DNA methylation machinery and suppresses transcriptional gene silencing. The New phytologist 199, 464-475.

- Rojas, M.R., Hagen, C., Lucas, W.J., Gilbertson, R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annual review of phytopathology 43, 361-394.

- Sambrook, J., and Russell, D.W., 2001. Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.

- Santamaria, M., Thomson, C.J., Read, N.D., Loake, G.J., 2001. The promoter of a basic PR1-like gene, AtPRB1, from Arabidopsis establishes an organ-specific expression pattern and responsiveness to ethylene and methyl jasmonate. Plant Mol Biol 47, 641-652.

- Sarmento, R.A., Lemos, F., Dias, C.R., Kikuchi, W.T., Rodrigues, J.C., Pallini, A., Sabelis, M.W., Janssen, A., 2011. A herbivorous mite down-regulates plant defence and produces web to exclude competitors. PLoS One 6, e23757.

- Seal, S.E., Jeger, M.J., Van den Bosch, F., 2006. Begomovirus evolution and disease management. Adv Virus Res 67, 297-316.

- Shah, J., 2009. Plants under attack: systemic signals in defence. Current opinion in plant biology 12, 459-464.

- Shyu, C., Figueroa, P., Depew, C.L., Cooke, T.F., Sheard, L.B., Moreno, J.E., Katsir, L., Zheng, N., Browse, J., Howe, G.A., 2012. JAZ8 lacks a canonical degron and has an EAR motif that mediates transcriptional repression of jasmonate responses in Arabidopsis. The Plant cell 24, 536-550.

- Song, S., Qi, T., Huang, H., Ren, Q., Wu, D., Chang, C., Peng, W., Liu, Y., Peng, J., Xie, D., 2011. The Jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect Jasmonate-regulated stamen development in Arabidopsis. The Plant cell 23, 1000-1013.

- Spoel, S.H., Dong, X., 2008. Making sense of hormone crosstalk during plant immune responses. Cell host & microbe 3, 348-351.

- Sung, Y.K., Coutts, R.H., 1995. Mutational analysis of potato yellow mosaic geminivirus. The Journal of general virology 76 (Pt 7), 1773-1780.

- Sunter, G., Bisaro, D.M., 1991. Transactivation in a geminivirus: AL2 gene product is needed for coat protein expression. Virology 180, 416-419.

- Sunter, G., Bisaro, D.M., 1992. Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. The Plant cell 4, 1321-1331.

- Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., Browse, J., 2007. JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448, 661-665.

- Trinks, D., Rajeswaran, R., Shivaprasad, P.V., Akbergenov, R., Oakeley, E.J., Veluthambi, K., Hohn, T., Pooggin, M.M., 2005. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. Journal of virology 79, 2517-2527.

- Uppalapati, S.R., Ishiga, Y., Wangdi, T., Kunkel, B.N., Anand, A., Mysore, K.S., Bender, C.L., 2007. The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with Pseudomonas syringae pv. tomato DC3000. Molecular plant-microbe interactions : MPMI 20, 955-965.

- Valenzuela-Soto, J.H., Estrada-Hernandez, M.G., Ibarra-Laclette, E., Delano-Frier, J.P., 2010. Inoculation of tomato plants (Solanum lycopersicum) with growth-promoting Bacillus subtilis retards whitefly Bemisia tabaci development. Planta 231, 397-410.

- Vanholme, B., Grunewald, W., Bateman, A., Kohchi, T., Gheysen, G., 2007. The tify family previously known as ZIM. Trends in plant science 12, 239-244.

- Vanitharani, R., Chellappan, P., Pita, J.S., Fauquet, C.M., 2004. Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. Journal of virology 78, 9487-9498.

- Varsani, A., Navas-Castillo, J., Moriones, E., Hernandez-Zepeda, C., Idris, A., Brown, J.K., Murilo Zerbini, F., Martin, D.P., 2014. Establishment of three new genera in the family Geminiviridae: Becurtovirus, Eragrovirus and Turncurtovirus. Archives of virology 159, 2193-2203.

- Vicente, J., Cascon, T., Vicedo, B., Garcia-Agustin, P., Hamberg, M., Castresana, C., 2012. Role of 9-lipoxygenase and alpha-dioxygenase oxylipin pathways as modulators of local and systemic defense. Molecular plant 5, 914-928.

- Vigliocco, A., Bonamico, B., Alemano, S., Miersch, O., Abdala, G., 2002. Stimulation of jasmonic acid production in Zea mays L. infected by the maize rough dwarf virus-Rio Cuarto. Reversion of symptoms by salicylic acid. Biocell : official journal of the Sociedades Latinoamericanas de Microscopia Electronica ... et. al 26, 369-374.

- von Malek, B., van der Graaff, E., Schneitz, K., Keller, B., 2002. The Arabidopsis male-sterile mutant dde2-2 is defective in the ALLENE OXIDE SYNTHASE gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta 216, 187-192.

- Wager, A., Browse, J., 2012. Social Network: JAZ Protein Interactions Expand Our Knowledge of Jasmonate Signaling. Frontiers in plant science 3, 41.

- Wang, H., Buckley, K.J., Yang, X., Buchmann, R.C., Bisaro, D.M., 2005. Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. Journal of virology 79, 7410-7418.

- Wang, H., Hao, L., Shung, C.Y., Sunter, G., Bisaro, D.M., 2003. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. The Plant cell 15, 3020-3032.

- Wartig, L., Kheyr-Pour, A., Noris, E., De Kouchkovsky, F., Jouanneau, F., Gronenborn, B., Jupin, I., 1997. Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2, and C2 ORFs in viral pathogenesis. Virology 228, 132-140.

- Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., Turner, J.G., 1998. COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280, 1091-1094.

- Yang, J.Y., Iwasaki, M., Machida, C., Machida, Y., Zhou, X., Chua, N.H., 2008. betaC1, the pathogenicity factor of TYLCCNV, interacts with AS1 to alter leaf development and suppress selective jasmonic acid responses. Genes & development 22, 2564-2577.

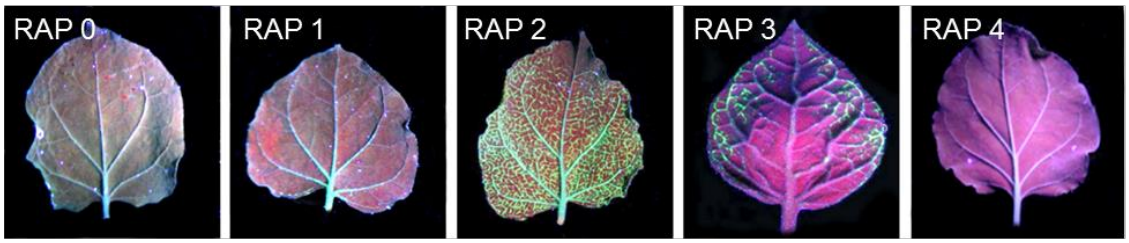
- Zhang, P.J., Broekgaarden, C., Zheng, S.J., Snoeren, T.A., van Loon, J.J., Gols, R., Dicke, M., 2013. Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in Arabidopsis thaliana. The New phytologist 197, 1291-1299.

- Zhu, F., Xi, D.H., Yuan, S., Xu, F., Zhang, D.W., Lin, H.H., 2014. Salicylic acid and jasmonic acid are essential for systemic resistance against tobacco mosaic virus in Nicotiana benthamiana. Molecular plant-microbe interactions : MPMI 27, 567-577.

- Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., Felix, G., 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 125, 749-760.

Chapter II

IDENTIFICATION OF HOST GENES INVOLVED IN GEMINIVIRUS INFECTION USING A REVERSE GENETICS APPROACH



Chapter published in the following articles:

Rosa Lozano-Durán*, Tábata Rosas-Díaz*, Ana P. Luna and Eduardo R. Bejarano. 2011. Identification of plant genes required for *Tomato yellow leaf curl Sardinia virus* infection using a reverse genetics approach. PLoS ONE 2011; 6(7):e22383.*These authors contributed equally to this work.

Czosnek H., Eybishtz A., Sade D., Gorovits R., Bejarano E., Rosas-Díaz T. and Lozano-Duran R. 2012.Tobacco Rattle virus-based Post Transcriptional Gene Silencing (TRV-PTGS) to discover host genes involved in the infection by the Tomato yellow leaf curl virus family and in the establishment of resistance to the virus. Viruses. DOI:10.3390/v5030998.

ABSTRACT

Geminiviruses, like all viruses, rely on the host cell machinery to establish a successful infection, but the identity and function of these required host proteins remain largely unknown. *Tomato yellow leaf curl Sardinia virus* (TYLCSV), a monopartite geminivirus, is one of the causal agents of the devastating Tomato yellow leaf curl disease (TYLCD). The transgenic 2IRGFP *N. benthamiana* plants, used in combination with virus-induced gene silencing (VIGS), entail an important potential as a tool in reverse genetics studies to identify host factors involved in TYLCSV infection. Using these transgenic plants, we have made an accurate description of the evolution of TYLCSV replication in the host in both space and time. Moreover, we have determined that TYLCSV and *Tobacco rattle virus* (TRV) do not dramatically influence each other when co-infected in *N. benthamiana*, what makes the use of TRV-induced gene silencing in combination with TYLCSV for reverse genetic studies feasible. Finally, we have tested the effect of silencing candidate host genes on TYLCSV infection, identifying eighteen genes potentially involved in this process, fifteen of which had never been implicated in geminiviral infections before. Seven of the analyzed genes have a potential anti-viral effect, whereas the expression of the other eleven is required for a full infection. Interestingly, almost half of the genes altering TYLCSV infection play a role in postranslational modifications. Therefore, our results provide new insights into the molecular mechanisms underlying geminivirus infections, and at the same time reveal the 2IRGFP/VIGS system as a powerful tool for functional reverse genetics studies.

INTRODUCTION

Geminiviruses are a large family of plant viruses with circular, single stranded DNA genomes packaged within geminate particles (Rojas et al., 2005). The *Geminiviridae* family (Stanley et al., 2005) is divided into seven genera according to their genome organization and biological properties. The genus *Begomovirus* includes members that are transmitted by whiteflies, infect dicotyledonous plants, and may have either bipartite or monopartite genomes. *Tomato yellow leaf curl Sardinia virus* (TYLCSV) is a member of the *Begomovirus* genus, and is one of the causal agents of the Tomato yellow leaf curl disease (TYLCD), which can cause up to 100% yield losses in tomato fields (Moriones and Navas-Castillo, 2000; Czosnek, 2007; Diaz-Pendon et al., 2010). TYLCSV has a monopartite genome of 2.8 kb in size, which encodes six proteins and contains an intergenic region (IR) comprising the origin of replication and viral promoters. The open reading frames (ORFs) in the complementary sense orientation encode a replication-associated protein (Rep, also known as C1), a transcriptional activator protein (TrAP, also known as C2), and a replication enhancer protein (REn, also known as C3); a small ORF, C4, is located within the Rep ORF but in a different reading frame. The virion strand contains two ORFs encoding the coat protein (CP) and a small protein named V2 (Czosnek, 2007; Diaz-Pendon et al., 2010).

To establish a successful infection, viruses must create a proper environment for viral propagation, which involves hijacking the cellular machinery for viral functions and, at the same time, preventing or counteracting the plant defence mechanisms. To fulfil these requirements, viral proteins trigger changes in the cell at all levels: transcriptional, translational and posttranslational. Identifying the host genes involved in viral replication, movement, and generally all those processes that lead to the establishment of a successful infection, could provide valuable new targets to ultimately generate viral resistance.

The advances in high-throughput technologies and bioinformatics have made possible to assess gene expression massively, providing an insight into the host's transcriptional responses to viral infections in a genome-wide fashion. These transcriptomic studies, together with proteomic studies, are providing an unprecedented vision of the "host-side" of the plant-virus interaction, leading to the identification of host genes whose function or expression is altered as a consequence of the infection. Geminiviruses have also been recently the subject of this kind of study, unveiling host genes differentially expressed either during the infection (Ascencio-Ibanez et al., 2008; Andleeb et al., 2010; Sahu et al., 2010) or upon expression of a viral protein (Trinks et al., 2005; Andleeb et al., 2010; Lozano-Durán et al., 2011). However, despite all this information being available, it is still a daunting task to determine the exact role of these host

genes in the infection process. Notably, this is particularly challenging in the case of monopartite geminiviruses, in which gene replacement with marker genes is not feasible, and thus are more tedious to monitor. In a previous work, we described the generation of *Nicotiana benthamiana* transgenic plants containing a GFP (Green fluorescence protein) expression cassette flanked by two repeats of TYLCSV IR as a tool to monitor TYLCSV replication (Morilla et al., 2006). These plants, named 2IRGFP, entail an important potential as a tool in reverse genetics studies to identify host factors involved in the viral infection, when used in combination with VIGS (virus-induced gene silencing) technology. Although the feasibility of this approach was previously confirmed by silencing the Proliferating cellular nuclear antigen (*PCNA*) and SUMO conjugating enzyme (*SCE1*) genes (Castillo et al., 2007), its use in a larger screening required an optimization of the conditions.

In this work, we explore further the potential of 2IRGFP *N. benthamiana* plants in combination with VIGS to identify host genes with a role in geminivirus infection. We have achieved an accurate description of the dynamics of viral replication by monitoring GFP expression in both space and time, explored the limitations of the strategy to be used in a reverse-genetics screening, and unveiled the effect of silencing selected *N. benthamiana* genes, most of them previously identified in transcriptomic or protein-protein interaction studies, in geminivirus infection. Using this strategy, we have identified eighteen genes involved in several cellular processes whose silencing alters TYLCSV infection. Notably, for fifteen of these genes this is the first description of a role in viral infections. Hence, our results provide new insights into the molecular mechanisms underlying geminivirus infections, and at the same time reveal the 2IRGFP/VIGS system as a powerful tool for functional reverse genetics studies.

RESULTS

Dynamics of *Tomato yellow leaf curl Sardinia virus* infection in transgenic 2IRGFP *N. benthamiana* plants is not altered by co-infection with *Tobacco rattle virus*

Traditionally, the development of geminivirus infections has been monitored by symptom evaluation and quantification of viral DNA by nucleic acid hybridization or PCR (Czosnek et al., 1988; Ber et al., 1990). These methods, however, have important limitations to monitor the infection in both space and time. Symptom evaluation is semi-quantitative at best, and does not necessarily correlate with viral accumulation. Hybridization or PCR studies, on the other hand, are destructive methods that are not able to discriminate if the viral molecules accumulated in a certain plant organ or tissue have been produced *in situ* or, on the contrary, have been originated elsewhere and subsequently transported. Due to these restrictions, a comprehensive study of the dynamics of the geminivirus infection, considering active replication and not merely virus accumulation, is still lacking.

In a previous work (Morilla et al., 2006), we developed *N. benthamiana* transgenic plants that overexpress GFP in those cells where the virus is replicating. During TYLCSV infection, these plants, named 2IRGFP, display a Rep-dependent GFP overexpression driven by the generation of mGFP replicons. Since overproduction of GFP correlates with TYLCSV active replication, these plants provide an unprecedented opportunity to monitor TYLCSV infection. For this purpose, 2IRGFP plants were infected with TYLCSV (three independent experiments, 20 plants each), GFP expression was exhaustively monitored and samples were collected at different times post-infection. For each time point, three plants were sampled (one per independent experiment); for each of the sampled plants, the three most apical leaves were taken, and tissue printing was performed with the main root. Total DNA was extracted from the harvested leaves, and both mGFP replicons and viral DNA were detected by DNA hybridization.

According to the extension and intensity of GFP expression in leaves, we visually distinguished five phenotypes, which we named RAP (for Replication-Associated Phenotype) 0, 1, 2, 3 and 4, as depicted in Figure1. Leaves from uninfected plants show a low expression of GFP extended through the whole leaf surface (RAP0). In RAP1, which corresponds to the first stage of the virus infection, GFP overexpression appears in some of the vascular bundles and the background GFP expression is not extensively silenced. RAP2 represents the stage of maximum GFP accumulation, in which an intense green fluorescence is observed as a continuous pattern through the leaf vascular bundles, and the GFP expression background in the leaf lamina has faded. RAP3 is the last stage in GFP expression, where GFP can only be detected in distinct areas of the leaf vascular bundles, before it completely disappears (RAP4).

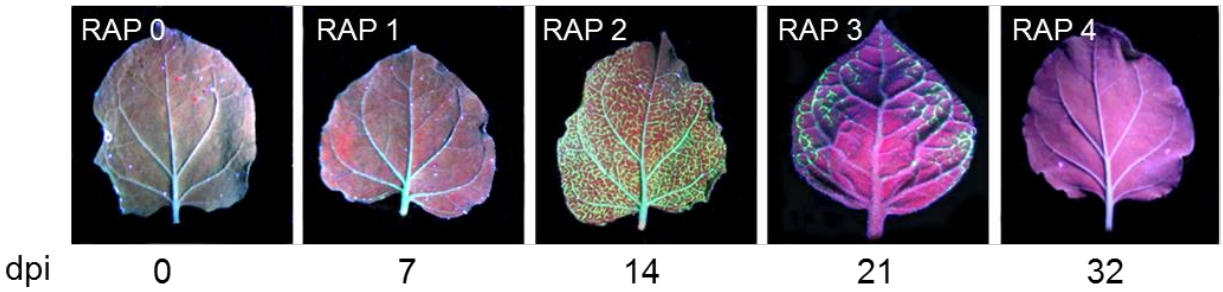


Figure 1. **Phenotypes of TYLCSV-infected 2IRGFP *N. benthamiana* leaves.** Extension and intensity of GFP expression in the leaves of TYLCSV-infected plants corresponding to RAP phenotypes (for Replication-Associated Phenotype) 0, 1, 2, 3 and 4 at different days post-inoculation (dpi).

The average evolution of GFP expression in the leaves of TYLCSV-infected plants is depicted in Figure 2A. At 7 days post-infection (dpi), GFP over-expression associated to the RAP1 phenotype can already be observed in some, but not most, plants, and accumulation of mGFP replicons and viral DNA is already detectable (Figure 2B). One week later, at 14 dpi, the maximum levels of viral replication, monitored as GFP overexpression (RAP2), are reached in the most apical leaves. As expected, this increase in GFP correlates with a higher accumulation of mGFP replicons and viral DNA. This viral DNA is most likely the result of previous viral replication in the root, or even in the aerial parts of the plant. It is noteworthy that viral DNA could be detected in roots as early as 7 dpi, before GFP expression is clearly noticeable; bearing in mind that the root is a sink organ, this is probably the result of transport from leaves where the virus is actively replicating (Figure 2B).

Once an extensive description of the dynamics of TYLCSV infection has been achieved, detecting changes in the timing or pattern of GFP over-expression due to silencing of a given host gene should be easy and reliable. *Tobacco rattle virus* (TRV)-based silencing vectors have been widely used and offer several advantages over other viral vectors, such as their abilities to mediate VIGS in the absence of TRV-derived symptoms and to target host RNAs in the growing points of plants. To accurately evaluate the impact of TRV infection on the evolution of the RAP phenotype, we monitored the GFP expression in 2IRGFP plants co-infected with TRV and TYLCSV (three independent experiments, 20 plants each). TRV/TYLCSV co-infected plants showed the same pattern of RAP phenotypes described for TYLCSV infected plants; the only detectable difference between single and double infected plants is a slight delay of approximately two days in the appearance of RAP phenotypes.

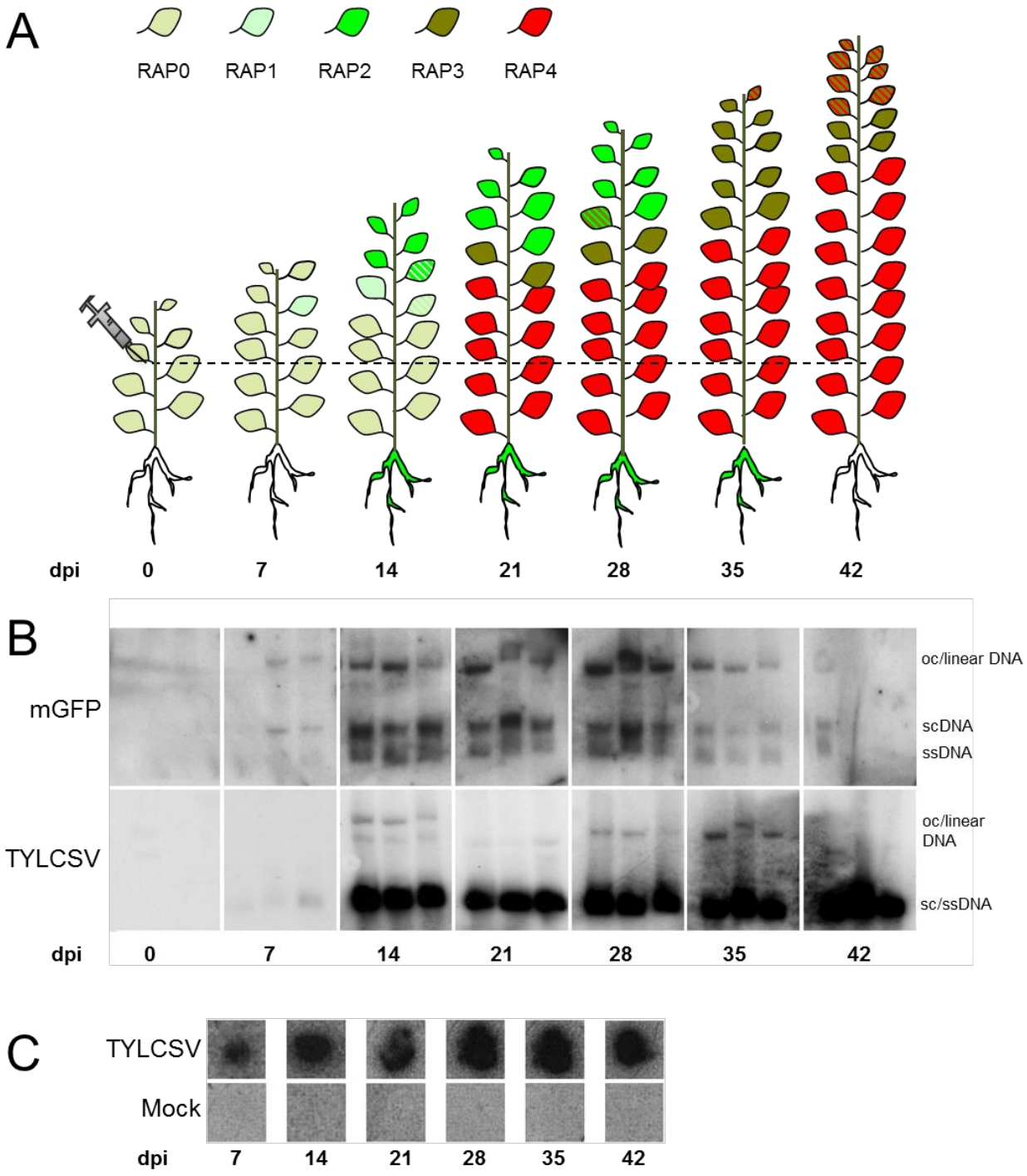


Figure 2. Phenotypic and molecular analysis of TYLCSV-infected 2IRGFP *N. benthamiana* plants. **(A)** Evolution of RAP phenotypes in TYLCSV-infected transgenic *N. benthamiana* 2IRGFP plants. The diagram displays the average RAP phenotypes of leaves and the induction of GFP in roots at different

(***Figure 2 legend continued***) days post-infection (dpi). Leaves containing areas of two different colours indicate an equivalent coexistence of RAP phenotypes in the population. In roots, green colour indicates GFP overexpression. The depicted results are the average of 60 infected plants. The dashed line marks the inoculation point. **(B)** Detection of episomal replicons (mGFP) and virus (TYLCSV) in leaves of infected plants. DNA was extracted from the three most apical leaves of three independent plants infected with TYLCSV. Undigested DNA was blotted and hybridized with probes specific for mGFP or TYLCSV. Bands representing open circle (oc), supercoiled (sc) or single-stranded (ss) forms of DNA are indicated. **(C)** Detection of virus (TYLCSV) in roots of infected plants in tissue printing.The RAP2 phenotype is maintained in the apical leaves up to 28 dpi, while GFP silencing is extensively detected from 21 dpi in the rest of the leaves. The decrease in GFP over-expression observed from 35 dpi onwards (Figure 2A) correlates with the reduction of mGFP replicons (Figure 2B); the viral accumulation, however, is high, most likely due to previous rounds of replication. As seen in this figure, TYLCSV is also replicating in the roots between 14 and 35 dpi, as indicated by GFP overexpression. The appearance of GFP in roots correlated with presence of viral DNA in the tissue printing (Figure 2C) until 42 dpi, when no GFP can be observed but accumulation of viral DNA is detected.

TYLCSV infection does not revert TRV-induced gene silencing in *N. benthamiana*

Since several proteins encoded by TYLCSV can function as suppressors of gene silencing (Luna et al., 2012) TYLCSV infection might interfere with the TRV-induced silencing. To test this possibility, we evaluated the effect of TYLCSV infection on the silencing of either a GFP transgene or the endogenous *Sulfur (Sul)* gene. To determine the impact of TYLCSV infection on the silencing of the GFP transgene*, N. benthamiana* plants constitutively expressing GFP (line 16c) (Voinnet and Baulcombe, 1997; Ruiz et al., 1998) were co-infected with TRV:*GFP* and TYLCSV or infected with TRV:GFP alone as a control. Infection with TRV:*GFP* triggered the silencing of the transgene, and this silencing was fully extended by 15 dpi (Figure 3A). Co-infection with TYLCSV did not alter this silencing phenotype, indicating that TYLCSV does not interfere with the TRV-induced GFP silencing (Figure 3A).

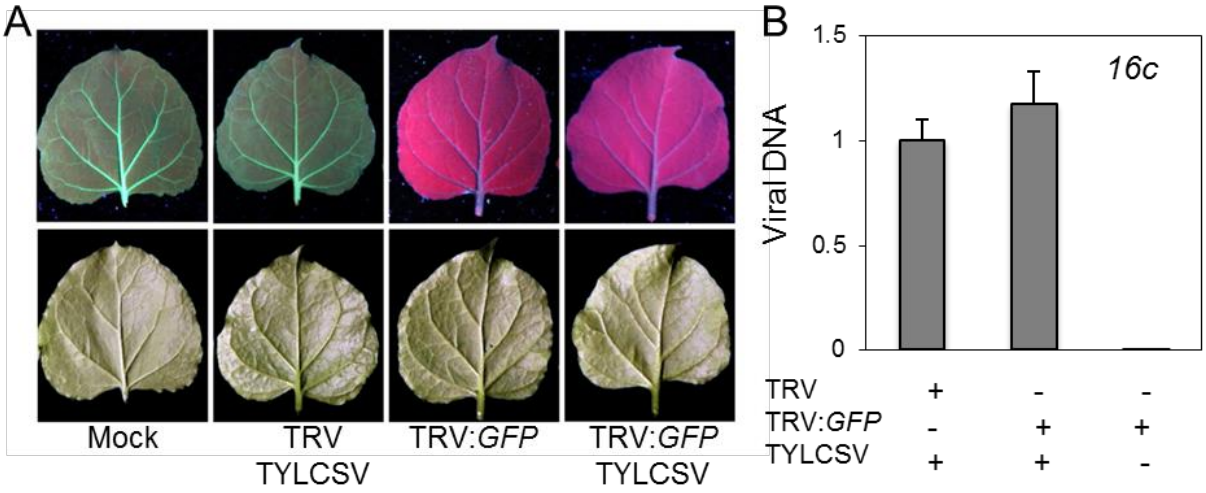


Figure 3. Effect of TYLCSV infection on TRV-induced silencing of *GFP*. (A) Leaves from 16c transgenic *N. benthamiana* plants 15 days after inoculation with TRV:*GFP*, or co-inoculation with TRV or TRV:*GFP*/TYLCSV. **(B)** Relative amount of TYLCSV DNA determined by quantitative real-time PCR. Values are the mean of five replicates. Bars represent standard error.

The *Sul* gene was chosen to evaluate the effect of TYLCSV infection on the silencing of an endogenous gene, for it produces a readily visible phenotype when silenced, derived from its involvement in chlorophyll synthesis (Kjemtrup et al., 1998). 2IRGFP *N. benthamiana* plants were co-infected with TRV:*Sul* and TYLCSV or infected with TRV:*Sul* alone as a control. Once again, co-infection with TYLCSV did not affect the silencing phenotype of TRV:*Sul* infected plants (Figure 4A), indicating that TYLCSV does not alter the TRV-induced silencing of this endogenous gene.

Quantification of TYLCSV accumulation using quantitative real-time PCR shows that TRV-induced silencing of either *GFP* or *Sul* does not affect TYLCSV accumulation (Figured 3B, 4B).

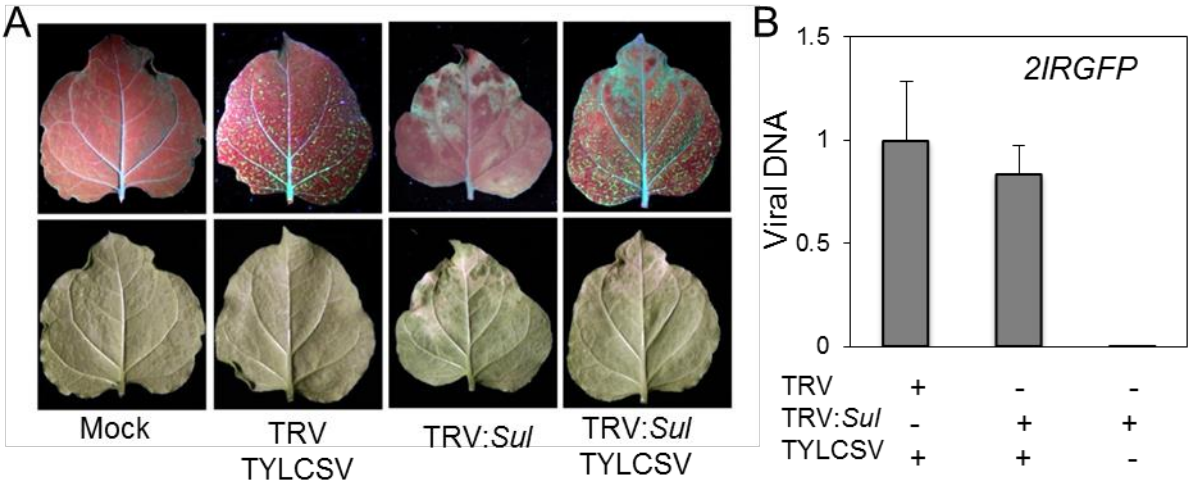


Figure 4. Effect of TYLCSV infection on TRV-induced silencing of *Sul*. (A) Leaves from 2IRGFP transgenic *N. benthamiana* plants 15 days after inoculation with TRV:*Sul*, or co-inoculation with TRV or TRV:*Sul*/TYLCSV. **(B)** Relative amount of TYLCSV DNA determined by quantitative real-time PCR. Values are the mean of five replicates. Bars represent standard error.

Simultaneous TRV-induced silencing of multiple genes in *N. benthamiana* plants

One drawback to VIGS is that it very often does not produce a uniform silencing throughout the plant. If the silencing of the gene does not generate a readily visible phenotype, it will be very difficult to distinguish silenced from non-silenced tissues, what would dramatically complicate the interpretation of results. A strategy to compensate for the lack of uniformity of VIGS would incorporate an internal reference to monitor the level of silencing. This system would act as a control for the VIGS vector, marking the silenced areas with a visible phenotype. The use of internal markers for VIGS based in visual phenotypes has been implemented in several plant species and has proven very successful for empowering the method as a tool in reverse genetics. Some works have demonstrated that the simultaneous silencing of several genes is

possible by including multiple gene sequences in the same silencing vector (Chen et al., 2004; Spitzer et al., 2007; Orzaez et al., 2009). With the aim of developing a visual reporter system to mark silenced areas in *N. benthamiana* leaves, we decided to follow two different approaches: (*i*) Test if the silencing triggered by two distinct TRV constructs co-localize, and (*ii*) Test if the silencing triggered by two different gene sequences cloned in tandem in the same TRV vector co-localize. For these assays we used two gene sequences whose silencing produces a readily visible phenotype: the *Sul* gene and *PCNA* (Peele et al., 2001; Morilla et al., 2006).

The results obtained are presented in Figure 5. In our system, silencing of the two marker genes does not significantly co-localize when the two TRV clones are co-inoculated in the plant. Only 13.6% of the new leaves in co-inoculated plants displayed both phenotypes, and the percentages of leaves showing each phenotype considered separately are lower than in single inoculations, indicating that co-inoculation apparently leads to a decreased silencing efficiency. A similar effect is observed when both genes are cloned in tandem in the same TRV vector, although the percentage of leaves showing simultaneous *Sul*- and *PCNA*-silenced phenotypes is slightly higher (20%) (Figure 5). Segregation of the silencing phenotypes warns against the use of this strategy as a marker system for gene silencing in *N. benthamiana* leaves.

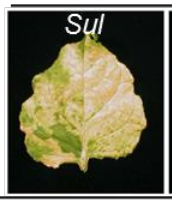
	Silencing phenotype (%)			
	 *Sul*	 *PCNA*	 *Sul+PCNA*	 WT
Silencing construct				
TRV:*Sul*	97.7	-	-	2.3
TRV:*PCNA*	-	45.5	-	54.6
TRV:*Sul-PCNA*	29.1	13.9	20	37
TRV:*Sul* + TRV:*PCNA*	40.9	20.4	13.6	25.1

Figure 5. **Simultaneous TRV-induced silencing of *PCNA* and *Sul.*** Percentage of leaves located above the infiltration point displaying the silencing phenotype of either *PCNA, Sul* or both in *N. benthamiana* plants inoculated with TRV:*Sul*, TRV:*PCNA* or TRV:*SulPCNA*, or co-inoculated with TRV:*Sul* and TRV:*PCNA.* For each inoculation, n=10 plants. The data correspond to leaves collected approximately 28 days after the infection.

Selection and cloning of candidate genes

As a first step in the identification of host genes required for TYLCSV infection, we made a selection of candidate genes following several criteria: (*i*) Genes encoding proteins known to physically interact with geminivirus proteins; (*ii*) Genes exclusively or preferentially expressed in phloem tissues; (*iii*) Genes transactivated by the C2 homologue from the geminiviruses *Mungbean yellow mosaic virus* and *African cassava mosaic geminivirus*; (*iv*) Genes involved in cellular processes potentially required for geminivirus infection (Table 1). A total of 114 genes were initially included as candidate genes.

Although silencing could be reached by expressing a DNA fragment of 21 to 23 nucleotides bearing 100% identity to the target gene (Thomas et al., 2001), this is often not efficient at triggering silencing and longer sequences must be used (Thomas et al., 2001; Ekengren et al., 2003). The highest efficiency of VIGS appears to be achieved using fragments in the range of 300-500 nucleotides with multiple stretches of more than 23 nucleotides identity (Burch-Smith et al., 2004; Liu and Page, 2008). Because of their different sources, our candidate genes belong to different species. Cloning 300-500 bp fragments of the *N. benthamiana* homologous gene would be the strategy of choice; unfortunately, the *N. benthamiana* genome had not been sequenced when this work was done and thus the gene sequences were in most cases not available. To circumvent this difficulty, we carried out homology analyses in all selected genes to identify sequences of 300-500 bp conserved in different plant species, including *Arabidopsis* and tomato. The use of heterologous gene sequences to silence their respective orthologs in *N. benthamiana* has been previously reported (Senthil-Kumar et al., 2007). Chosen sequences were further analysed with Invitrogen Block-iT^{TM} RNAi designer (https://rnaidesigner.invitrogen.com/rnaiexpress/) to localize potential efficient siRNAs within the sequence: the fragment of choice was that containing the largest number of predicted siRNA molecules. The selection process is depicted in the flow diagram in Figure 6.

After this analysis, 54 out of the initial 114 genes were maintained as candidate genes (Table 1). Since the sequence of these selected genes was highly conserved, we decided to use the *Arabidopsis* cDNAs to generate the VIGS constructs, with the aim of rendering this strategy faster and more homogeneous. We ordered the 37 *Arabidopsis* cDNA clones that were available at NASC (European *Arabidopsis* Stock Centre) (Table S1) and the selected 300-500 bp fragment for each cDNA was PCR-amplified and cloned in the TRV RNA2-based VIGS vector pTV00 (Ratcliff et al., 2001). The primers used to amplify each fragment are included in Table 2.

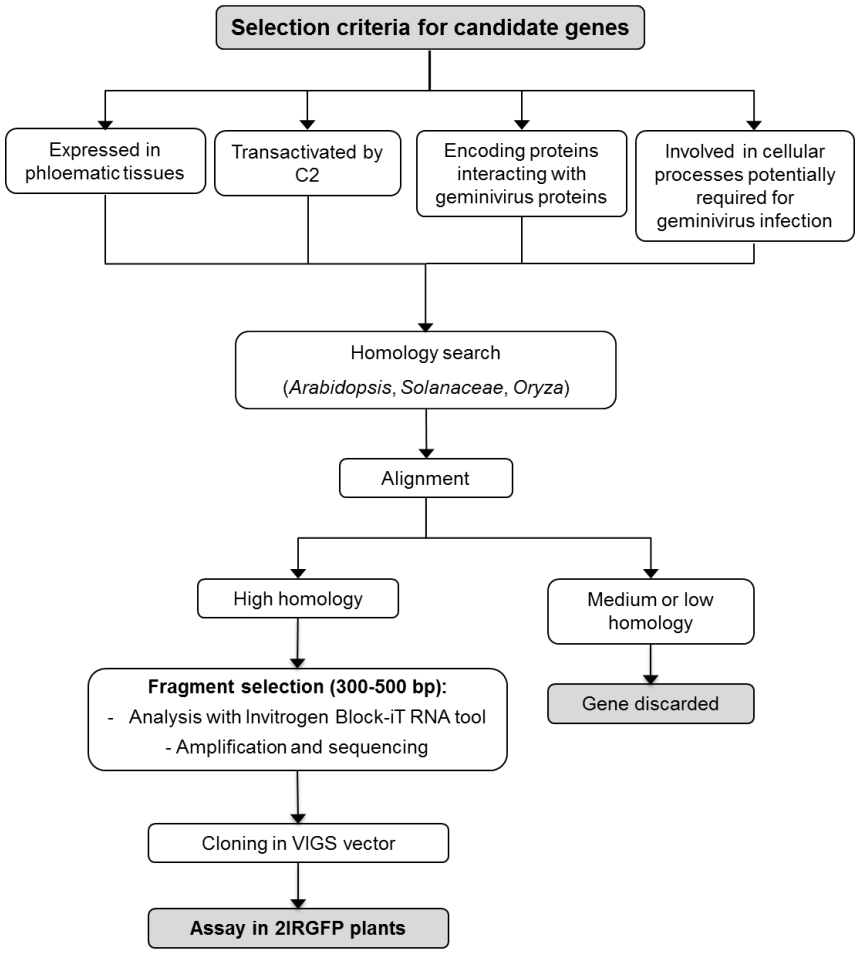


Figure 6. Gene selection strategy. Flow diagram depicting the strategy used for selecting the candidate genes to be tested using the 2IRGFP plants/TRV-based system.

Screening of candidate genes in *N. benthamiana* 2IRGFP plants

Once the time course of TYLCSV infection in 2IRGFP plants had been established, we followed the strategy depicted in Figure 7 to test the potential effect of candidate gene silencing on TYLCSV infection (Table 1). Summing up, we induced gene silencing for each candidate host gene in 2IRGFP plants using TRV constructs, and subsequently infected these plants with TYLCSV. Plants infiltrated with the empty TRV vector and infected with TYLCSV were used as a control; plants infiltrated with the *Sul*-containing TRV vector were used as a control of VIGS efficiency. GFP overexpression was monitored daily from 9 to 15 dpi under UV light.

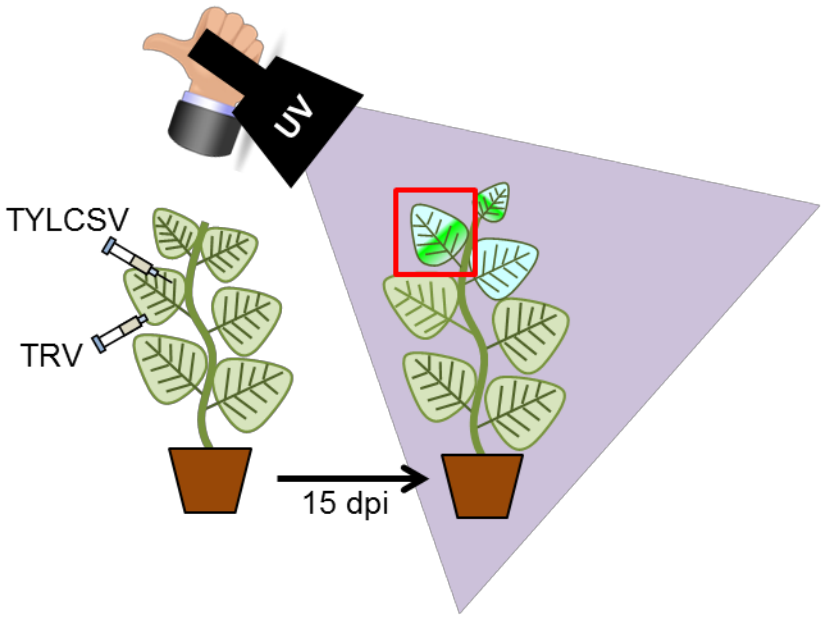


Figure 7. Screening strategy to assess the effect of candidate gene silencing in 2IRGFP transgenic *N. benthamiana* plants. (A) Plants were co-inoculated with a TRV:*Gene* construct and TYLCSV. GFP expression was monitored daily from 9 to 15 dpi. Five plants were used per construct; experiments were repeated at least twice.

According to the effect of their silencing on TYLCSV infection, measured as time of appearance and intensity of GFP expression, we grouped the tested host genes into three classes: those whose silencing did not cause changes in GFP expression (group A), or those whose silencing promoted earlier (group B) or later/lower/null (group C) GFP expression (Table 1; examples of each class are shown in Figure 8).

Representative genes belonging to groups A (*SKL2*, *ECR1*), B (*UBA1*, *GLO1* and *RPA32*) and C (*HSC70*, *ASK2*, and *δ-COP*) were chosen to evaluate the impact of their silencing on TYLCSV infection, measured as viral DNA accumulation. For this purpose, 2IRGFP *N. benthamiana* plants were co-inoculated with the TRV derivative clones and TYLCSV. At 15 dpi, total DNA was extracted from the pooled three most apical leaves of each plant and the relative amount of viral DNA was determined using quantitative real-time PCR (two independent experiments, 5 plants each**)**. The mean values of TYLCSV accumulation are represented in Figure 8B. As expected from the GFP overexpression data, silencing of *UBA1* or *GLO1* and silencing of *RPA32* tripled and doubled TYLCSV accumulation, respectively. On the other hand, silencing of *HSC70* and *ASK2* reduced TYLCSV accumulation by 70 and 30%, respectively. Strikingly, silencing of the *δ-COP* subunit completely abolished TYLCSV accumulation.

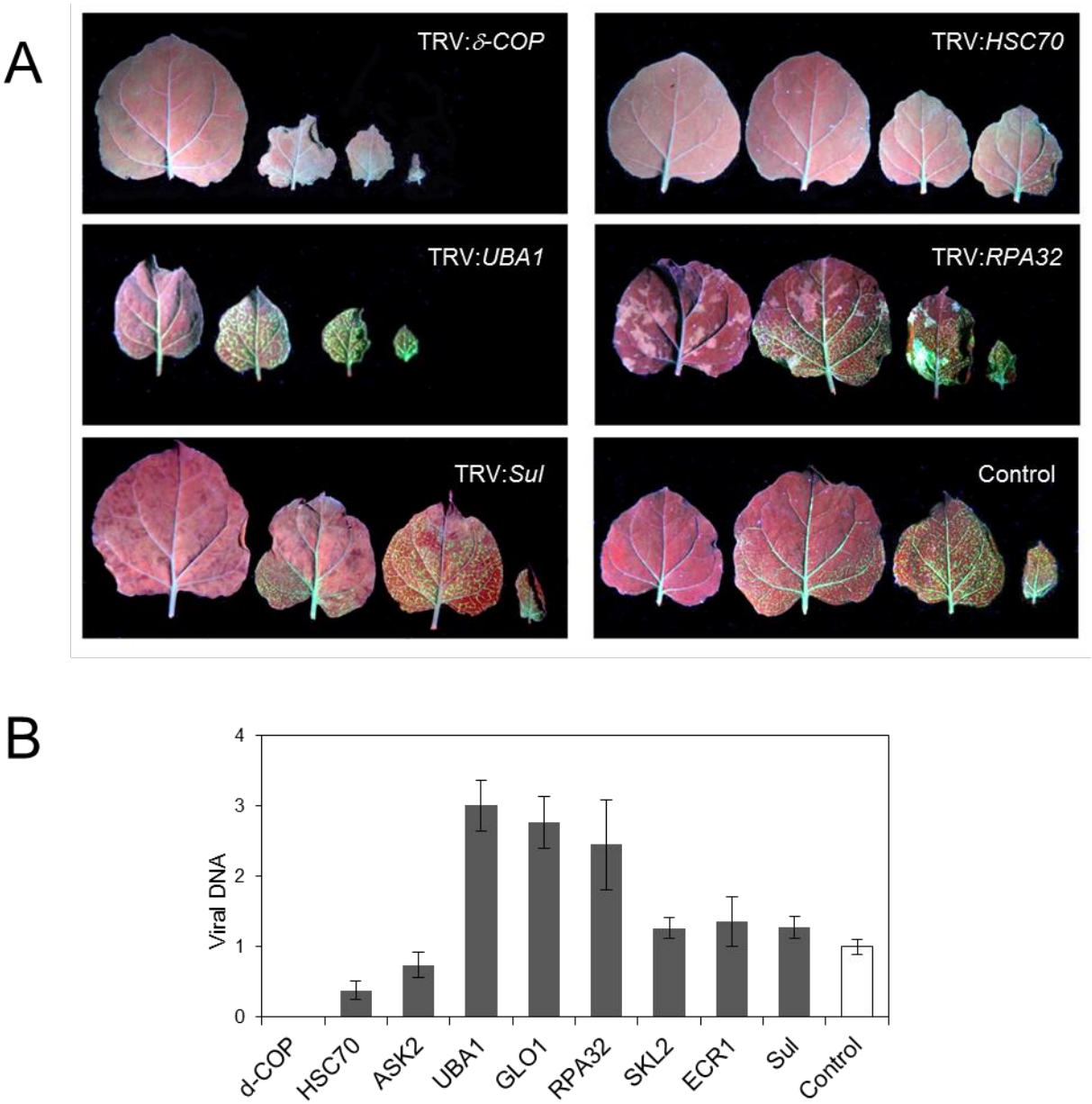


Figure 8. Screening of candidate genes in 2IRGFP transgenic *N. benthamiana* plants. (A) GFP expression in the four most apical leaves of 2IRGFP transgenic plants co-infected with TYLCSV and representative TRV constructs. **(B)** Relative amount of TYLCSV DNA in leaves of plants co-infected with TYLCSV and TRV constructs to induced the silencing of either *COATOMER DELTA SUBUNIT* (*δ-COP*), *HEAT SHOCK COGNATE 70* (*HSC70*), *SKP1-LIKE 2* (*ASK2*), *UBIQUITIN ACTIVATING ENZYME 1* (*UBA1*), *LACTOYLGLUTATHIONE LYASE* (*GLO1*), *PUTATIVE SHIKIMATE KINASE* (*SKL2*), *RUB-ACTIVATING ENZYME SUBUNIT (ECR1*), *REPLICATION ASSOCIATED PROTEIN A* (*RPA32*), *SULFUR* (*Sul*) or no gene (empty vector, as control). Viral DNA was quantified by quantitative real-time PCR. Values are the mean of five replicates. Bars represent standard error. The sample of TYLCSV and pTV00 co-infected plants was used as the calibrator, with the accumulation of the TYLCSV capsid protein gene set to 1.

DISCUSSION

Replication dynamics of TYLCSV

Transgenic 2IRGFP *N. benthamiana* plants have proven to be an accurate and sensitive tool that allows monitoring TYLCSV infection real-time and in a non-destructive manner. Using these transgenic plants, we have been able to describe the dynamics of TYLCSV infection in great detail, determining in which tissues the virus is replicating on an average infection at a certain time. To our knowledge, this is the first description of the replication dynamics of a geminivirus infection in both space and time, as most of the previous studies reflect viral DNA accumulation but not active replication.

According to our results, TYLCSV replication can be detected in leaves placed above the inoculation point at 7 dpi. One week later (14 dpi), viral replication is taking place in the apical leaves of all inoculated plants, where it is maintained at a high level until 28 dpi. From that moment onwards, the rate of viral replication decreases, and eight weeks after the inoculation it is only detectable in limited areas of apical leaves. These observations suggest that the virus is able to maintain the replication of its genome, in the aerial parts of the host plant, only in certain leaves and during a limited period of time. Additionally, the virus is also able to replicate in roots between 14 and 35 dpi. Interestingly, while we observe a direct correlation between the changes in GFP expression and the accumulation of episomal replicons (mGFP), the amount of viral DNA seems to be maintained even when viral replication can no longer be detected. These data suggest that, although both DNA molecules are produced by the same mechanism, mGFP replicons must be degraded whereas the viral DNA is not, maybe as a result of its encapsidation.

Double infection with TYLCSV and TRV does not significantly affect TYLCSV infection or TRV-induced silencing

We have demonstrated that co-infection with TRV does not dramatically affect TYLCSV infection in *N. benthamiana*. This fact makes it feasible to use TYLCSV in combination with TRV-mediated VIGS as a tool in reverse genetics studies to identify host factors involved in the geminivirus infection. We observed, however, a slight delay in the development of TYLCSV infection when in combination with TRV. This delay makes the use of appropriate controls (co-infection with the empty TRV vector) of special importance for this type of analysis. Although TYLCSV, like all geminiviruses, encodes suppressors of gene silencing (Luna et al., 2012), it does not noticeably affect TRV-induced gene silencing in *N. benthamiana* plants. In agreement with these results, TRV-mediated VIGS has been successfully used in combination with geminiviral infections in tomato in a recent work (Eybishtz et al., 2010).

Despite our efforts, the attempt to establish a visual reporter system based on the silencing of the *Sulfur* gene has been fruitless. Although simultaneous silencing of two genes is achieved by both co-infiltration of independent TRV-based constructs or by infiltration with a TRV construct harbouring multiple gene sequences (Figure 5), silencing does not significantly co-localize in any case, and the extension of the silencing of each gene considered independently diminishes (Figure 5). Even though the reasons for this outcome remain obscure, the absence of significant co-localization makes it impractical to use this co-silencing approach as a marker for VIGS. A similar effect of simultaneous silencing in *N. benthamiana* had been previously described (Peele et al., 2001).

Identification of host genes involved in TYLCSV infection

Using our reverse genetics approach, based on the use of transgenic 2IRGFP *N. benthamiana* plants, we have been able to demonstrate that silencing of 18 out of 37 analysed host genes alters TYLCSV infection.

Bearing in mind the limitations of VIGS, and since we have not tested the silencing of those candidate genes in which no effect on TYLCSV infection could be detected (group A), we cannot rule out the possibility that we may have false negatives: some of the tested genes might not have been efficiently silenced, and thus their potential impact on the viral infection would go unnoticed. For this reason, we cannot assess that those tested candidate genes without an obvious effect on TYLCSV infection do not play a role in the viral infection. False positive results, on the other hand, would be more difficult to obtain in our experimental system, and as long as the proper controls are being used we consider the positive results as reliable. In this context, a reasonable concern would be the possibility of silencing unwanted host genes as a consequence of sequence homology with the target host gene. In order to evaluate this undesired effect, we performed a BLAST homology search with every sequence used for VIGS, confirming that the only hit in each case was the selected target gene. However, and since the *N. benthamiana* genome has been released after the publication of this work, these analyses should be repeated.

Additionally, it is noteworthy that this screening method tests the candidate gene in the context of the infection, and consequently those genes identified should be biologically relevant.

Out of the eighteen genes whose silencing alters TYLCSV infection, seven have a potential anti-viral effect, since TYLCSV replication is enhanced when they are silenced (group B), whereas the expression of the other eleven is required for a full infection, for their silencing negatively impacts this process (group C).

Among the genes affecting TYLCSV infection, there are three (*NSI*, *GRAB2* and *RPA32*) whose deregulation was previously shown to modify the geminivirus infection or replication (Xie et al., 1999; McGarry et al., 2003; Carvalho et al., 2006; Singh et al., 2006).

An earlier work showed that overexpression of the nuclear acetyltransferase NSI, a protein that interacts with the Nuclear shuttle protein (NSP) of the geminivirus *Cabbage leaf curl virus* (CaLCuV), enhances the efficiency of infection (McGarry et al., 2003), suggesting a role of protein acetylation in coordinating replication of the viral genome with its export from the nucleus. This positive effect of NSI in the geminivirus infection is supported by the data obtained with TYLCSV, which demonstrate that silencing of NSI negatively affects viral infection. On the other hand, silencing of the geminivirus RepA binding gene (*GRAB2*) during TYLCSV infection has an opposite effect on viral propagation to that previously reported for a different geminivirus species (Xie et al., 1999). This gene encodes a NAC-containing protein isolated in wheat for its interaction with *Wheat dwarf virus* (WDV) RepA (Xie et al., 1999). Even though GRAB2 overexpression inhibits WDV replication in wheat cells, the reason for this remains unclear, and could be ascribed to different roles of GRAB2 on the viral DNA cycle (Xie et al., 1999). Our results show that reduction in gene expression of GRAB2 has a deleterious effect on TYLCSV infection, suggesting that correct GRAB2 expression is required for full infectivity. Replication Protein A (RPA32) has been shown to interact with *Mungbean yellow mosaic India virus* (MYMIV) Rep (Singh et al., 2007) and modulate the functions of Rep by enhancing its ATPase, but down-regulating its nicking and closing activities. Strikingly, even though RPA32 seems to promote the transient replication of a plasmid bearing MYMIV origin of replication *in planta* (Singh et al., 2007), in our system its silencing seems to enhance the viral infection. We do not have a feasible explanation for this contradictory phenotype at the moment, and further work will be needed to decipher it.

The roles of other host genes whose silencing affects TYLCSV infection might be deduced from their known cellular functions. Therefore, we will briefly discuss below the potential roles of a group of identified host factors with known cellular functions in postranslational modifications, stress responses, metabolism or intracellular transport.

It is worth noting that 8 out of these 18 genes are involved in processes related to protein modifications or protein metabolism, such as ubiquitination, rubylation, phosphorylation, acetylation or protein folding.

Four of these genes encode components or regulators of the ubiquitin or ubiquitin-like pathways: Ubiquitin activating enzyme (*UBA1)*, RING-type E3 ubiquitin ligase (*RHF2A*), SKP1-like 2 (*ASK2*) and a subunit of the de-rubylating CSN complex (*CSN3*).

Ubiquitination has been shown to contribute to multiple levels of plant defence, including resistance to viruses (reviewed in (Dreher and Callis, 2007) and (Citovsky et al., 2009)). Specifically, several recent works have suggested the existence of links between ubiquitination and geminivirus infection [6, 10, 36, 37]. Since the tomato UBA1 interacts with TYLCSV C2 (F. Hèricourt et al., in preparation), the finding that silencing of this host gene leads to an earlier

TYLCSV infection suggests that the interaction with the viral C2 protein might lead to the inhibition of the enzyme, which would be consistent with the previously described general negative impact of C2 on the ubiquitination in the host (Lozano-Durán et al., 2011). On the other hand, the expression of the RING-type E3 ubiquitin ligase *RFH2A* silenced in this work is up-regulated following CaLCuV infection (Ascencio-Ibanez et al., 2008) or infiltration with virulent *Pseudomonas syringae* (*Arabidopsis* eFP browser: http://esc4037-shemp.csb.utoronto.ca/efp/cgi-bin/efpWeb.cgi), which may indicate an involvement in plant defence. Such a hypothetical role would explain why the silencing of this gene promotes the viral infection.

The SCF complex seems to be an important target during geminivirus infection, since several geminiviral proteins interfere with or hijack the SCF function (Lozano-Durán and Bejarano, 2011; Lozano-Durán et al., 2011) . The fact that three of the genes whose silencing alters TYLSCV infection are components or regulators of these complexes supports this idea. *ASK2* is a member of a gene family encoding SKP1-like proteins that can be assembled into distinct SCF complexes, and plays a role in a large number of cellular processes such as cell division, development, osmotic stress or drought tolerance (Boudsocq et al., 2004; Liu et al., 2004; Umezawa et al., 2004). *ASK2* expression is down-regulated by challenge with bacteria, fungi or elicitors (*Arabidopsis* eFP browser) but transactivated by geminivirus C2 in *Arabidopsis* protoplasts (Trinks et al., 2005), suggesting a possible involvement in plant defence acting as a negative regulator. If this is the case, it could explain the adverse effect of its silencing on TYLCSV infection.

CSN3 is one of the eight subunits of the CSN complex, which derubylates cullins and thus regulates the activity of ubiquitin Cullin RING Ligases (CRLs). Recently, geminivirus C2 protein was shown to interfere with the activity of this complex over CULLIN1, most likely through the interaction with CSN5, the catalytic subunit, therefore altering ubiquitination in the host cell (Lozano-Durán et al., 2011). Given that geminivirus infection on *Arabidopsis csn5a* mutant plants takes place less efficiently that in wild-type plants (Lozano-Durán and Bejarano, 2011, Journal of Plant Pathology and Microbiology), it might be feasible that geminiviruses could be redirecting the activity of the CSN complex, rather that generally impairing it. Since depletion of any of the CSN subunits results in the loss of the complex (reviewed in (Serino and Deng, 2003)), it would not be surprising that silencing of CSN3 results in a hindered infection.

Among the host genes that seem to be required for the viral infection, since their silencing delay or suppress TYLCSV replication, we identified two encoding protein kinases that interact with TYLCSV C4 (Héricourt et al., in preparation): *BAM1* (Barely any meristem 1) and *SK4-1/SKK* (Shaggy-related kinase kappa). *BAM1* encodes a CLAVATA1-related receptor kinase-like protein required for both shoot and flower meristem function, which is also involved in leaf and

gametophyte development (DeYoung et al., 2006; Deyoung and Clark, 2008; Guo et al., 2010). Interestingly, *BAM1* expression is down-regulated after challenge with fungi, bacteria or elicitors (*Arabidopsis* eFP browser). In such a scenario, silencing of this gene might lead to an activation of defence responses in the plant. Alternatively, since this protein interacts with TYLCSV C4 (Héricourt et al., in preparation), this gene product might be required for some viral function.

Shaggy-like protein kinases like *SK4-1/SKK* have been shown to interact with other geminiviral C4 proteins, and this interaction is required to trigger disease symptoms (Piroux et al., 2007; Dogra et al., 2009) and for C4 function to suppress gene silencing (Dogra et al., 2009). Our results confirm the previous idea that these kinases might be required for geminivirus infection, since silencing of *SK41/SKK* negatively impacts TYLCSV infection.

Five of the identified genes potentially involved in TYLCSV infection have a role in stress responses: *HSC70-1* (Heat shock protein cognate 70), *RD21* (responsive to dehydration 21), *PLP2* (patatin-like protein), *GLO1* (lactoylglutatione lyase) and *AOC1* (allene oxide cyclase 1). HSC70-1 is one of the five cytosolic members of the heat shock protein 70 family in *Arabidopsis* (Sung et al., 2001). Infection with several plant viruses, such as the geminivirus *Beet curly top virus*, induces the expression of members of this gene family in systemically infected tissues (Escaler et al., 2000; Aparicio et al., 2005). HSC70 is a major interactor of SGT1 (Noel et al., 2007), which has proven required for resistance to viruses (Dielen et al., 2010; Komatsu et al., 2010). A chloroplastic HSC70 from *Arabidopsis*, CPHSC70-1 (At4g24280), has been recently shown to interact with *Abutilon mosaic virus* movement protein, and this interaction seems to be important for viral transport and symptom induction (Krenz et al., 2010). Although the role of HSC70 induction in plant-virus interactions is uncertain, it might be expected to fulfil a requirement for rapid protein maturation and turnover during a short virus multiplication cycle. Alternatively, there is evidence that HSC70 may play a role in virus cell-to-cell movement. Our results show that silencing of HSC70-1 results in an impaired TYLCSV infection, supporting that over-production of this protein is required for a full viral infection.

RD21 is a cysteine protease whose homologue in tomato is able to interact with TYLCSV V2 (F. Héricourt et al., in preparation). RD21 has been recently shown to be the target protease of the serpin AtSerpin1 (Lampl et al., 2010). In animals, serpins are protease inhibitors involved in several physiological processes, including innate immunity. The expression of *RD21* is up-regulated following inoculation with *Botrytis cinerea* or *Pseudomonas syringae (Arabidopsis* eFP browser*)*, or upon CaLCuV infection (Ascencio-Ibanez et al., 2008), suggesting a possible role of RD21 in plant defence, which would in turn explain why the silencing of this gene promotes the viral infection.

PLP2 encodes a lipid acyl hydrolase that accumulates upon infection with CaLCuV (Ascencio-Ibanez et al., 2008), fungi and bacteria and negatively affects resistance to the last two types of

pathogens (La Camera et al., 2005). On the contrary, it has been shown to contribute to resistance to *Cucumber mosaic virus* by inducing HR (La Camera et al., 2009). Since this gene product is proposed to positively regulate the biosynthesis of oxylipins providing fatty acid precursors (La Camera et al., 2009), silencing of this gene might result in increased salicylic acid signalling, which could explain the impairment of TYLCSV infection.

GLO1 is part of the glyoxalase system, involved in detoxification of methylglioxal (MG), a cytotoxic byproduct of glycolysis (reviewed in (Yadav et al., 2008)). Overexpression of the glyoxalase pathway in transgenic tobacco and rice plants has been found to keep in check the increase of ROS and MG under stress conditions by maintaining glutathione homeostasis and antioxidant enzyme levels (reviewed in (Yadav et al., 2008)), and overexpression of *GLO1* has been related to enhanced tolerance to abiotic stresses (Mustafiz et al., 2010; Sun et al., 2010). A possible role for reactive oxygen species as a requirement for virus replication (Clarke et al., 2002) and for antioxidative mechanisms as antagonizing viral infection (Sun et al., 2010) has been proposed. Moreover, viral infections have been shown to induce oxidative stress in plants (Diaz-Vivancos et al., 2006; Rimmer et al., 2006; Amari et al., 2007; Diaz-Vivancos et al., 2008; Garcia-Marcos et al., 2009; Song et al., 2009) and geminivirus infection alters the expression of oxidative stress-related genes (Ascencio-Ibanez et al., 2008) . Given that silencing of *GLO1* triggers an earlier TYLCSV infection, it would be feasible that its interaction with C3 (F. Héricourt et al., in preparation) might be interfering with this enzyme to promote pathogenicity.

AOC1 is one of four genes that encode this enzyme in *Arabidopsis*, which catalyzes an essential step in jasmonic acid biosynthesis. This gene is repressed upon CaLCuV infection (Ascencio-Ibanez et al., 2008), maybe as a consequence of the opposite regulation between jasmonate and salicylic acid signalling pathways, since the latter is activated in this geminivirus-host interaction. Due to this counter-regulation, silencing of this gene might result in activation of the salicylic acid pathway in response to TYLCSV, explaining its negative effect on the viral infection.

Viruses heavily rely on cytoplasmic transport systems for their propagation. Among the host factors involved in TYLCSV infection, we have identified one gene required for vesicular trafficking (Coatomer delta subunit, *δ-COP*) and another one involved in transport between the cytoplasm and the nucleus (Importin alpha isoform 4, *IMPAA-4*).

δ-COP encodes a component of the polymeric coatomer coat complexes COPI. The precise role of the COPI remains unclear, although it has been associated with vesicular transport within the Golgi apparatus and from the Golgi apparatus to the ER (Lee et al., 2004). Vesicular trafficking has been previously shown to play a role in geminivirus infection, since interaction with synaptotagmin SYTA has proven required for CaLCuV cell-to-cell movement and systemic spread (Lewis and Lazarowitz, 2010). Interestingly, silencing of this gene completely abolishes

TYLCSV infection in our system, suggesting that vesicular trafficking is essential for viral infection. The effect of this gene on TYLCSV infection is further studied in Chapter III. IMPAA-4 is one of the members of the importin α gene family in eukaryotes. Importin α is a component of the nuclear pore-targeting complex (PTAC) that acts as an adaptor by recognizing the nuclear localization signal (NLS) sequences and binding to importin β. Importin β is the carrier component of PTAC, and targets the complex to the nuclear pore by binding to nuclear pore proteins (Cook et al., 2007; Terry et al., 2007). Importin α has been shown to interact with the CP from the geminivirus MYMV (Guerra-Peraza et al., 2005), and this interaction might serve for docking of viruses to the nucleus and facilitating nuclear localization of the CP during encapsidation. In this context, the finding that silencing of *IMPA-4* favours the viral infection seems counterintuitive; however, the fact that this gene is overexpressed in response to several pathogens and elicitors (*Arabidopsis* eFP browser) suggests that this host factor might also play a role in plant defence, providing a possible explanation for the observed phenotype. Additionally, TYLCSV CP could rely on the interaction with a different host protein for its nuclear import.

Besides the aforementioned cellular processes, others seem to be involved in TYLCSV infection. Silencing of genes selected because of their specific expression or overexpression in phloem tissue and required for phenylpropanoid metabolism (4-coumarate:CoA ligase1, *4CL1*) or secondary cell wall synthesis (Bearskin2B, *BRN2*) delay or promote TYLCSV infection, respectively. BRN2 is a member of the Class IIB NAC transcription factor family. In *Arabidopsis*, this protein has been suggested to regulate cell maturation in cells that undergo terminal differentiation with strong cell wall modifications (Bennett et al., 2010). *4CL1* is involved in the last step of the general phenylpropanoid pathway, channeling carbon flow into branch pathways of the phenylpropanoid metabolism. Interestingly, silencing of this gene leads to increased cellulose content and reduced amounts of total lignin (Yang et al., 2010).

As illustrated in the examples above, the use of this approach has allowed the identification of novel plant genes with a role in the geminivirus infection, which sheds light on the underlying biological processes, therefore paving the way for the development of strategies to counteract these devastating diseases. Given the previously mentioned advantages of this 2IRGFP/VIGS system, it can be considered an easy, fast and effective tool to determine the role of host genes in geminivirus infections, and might be of great assistance to speed up this kind of functional studies. However, using VIGS to target a specific gene requires information about its nucleotide sequence. This used to be a limitation when working with *N. benthamiana,* before the release of the genome draft (www.solgenomics.net). We tried to circumvent this difficulty by using nucleotide sequence information from *Arabidopsis* and closely related species. Nowadays, the

availability of the *N. benthamiana* genome provides the opportunity to develop the full potential of the VIGS/2IRGFP strategy to identify host factors involved in geminivirus infection.

Identity	Function	Selection criteria	Reference	ACC *A. thaliana*
Group A (no detected effect on infection)				
A-type cyclin-dependent kinase (*CDK2*)	Cell cycle control	Cellular process	(Ascencio-Ibanez et al., 2008)	AT3G48750
Cullin-associated and neddylation-dissociated (*CAND1*)	Protein metabolism	TrAP/C2 interaction	Hericourt *et al.* (in preparation)	AT2G02560
DNA polymerase alpha 2 (*POLA2*)	DNA metabolism	Cellular process	(Shultz et al., 2007)	AT1G67630
DNA polymerase delta small subunit (*POLD2*)	DNA metabolism	Cellular process	(Shultz et al., 2007)	AT2G42120
E2F transcription factor 1 (*E2FB*)	Transcription	Cellular process	(Ascencio-Ibanez et al., 2008)	AT5G22220
Geminivirus Rep-interacting kinase (*GRIK1*)	Signal transduction	Rep interaction	(Kong and Hanley-Bowdoin, 2002)	AT3G45240
Histone 3 K4-specific methyltransferase SET7/9	Unknown	TrAP/C2 interaction	Hericourt *et al.* (in preparation)	AT1G21920
Homologue to co-chaperone DNAJ-like protein (*ATJ3*)	Protein folding	C3 interaction	Hericourt *et al.* (in preparation)	AT3G44110
NSP interacting kinase 2 (*NIK2*)	Signal transduction	Phloem over-expression	(Vilaine et al., 2003)	AT3G25560
Putative nucleic acid binding/transcription factor (*JDK*)	Unknown	TrAP/C2 interaction	Hericourt *et al.* (in preparation)	AT5G03150
Putative transcriptional activators with NAC domain (*ATAF1*)	Transcription	C3 interaction	(Selth et al., 2005)	AT1G01720
Putative shikimate kinase (*SKL2*)	Unknown	CP interaction	Hericourt *et al.* (in preparation)	AT2G35500
Retinoblastoma-related protein (*RBR*)	Cell cycle control	Rep interaction	(Ach et al., 1997; Kong et al., 2000)	AT3G12280
RUB-activating enzyme subunit (*ECR1*)	Protein modification	Cellular process	(Woodward et al., 2007; Ascencio-Ibanez et al., 2008)	AT5G19180
Scarecrow-like protein (*SCL13*)	Transcription	Phloem over-expression	(Vilaine et al., 2003)	AT4G17230
SNF1-related protein kinase (*AKIN11*)	Signal transduction	TrAP/C2 interaction	(Wang et al., 2003)	AT3G29160
SUMO activating enzyme (*SAE1B*)	Protein metabolism	Cellular process	(Lois, 2010)	AT5G50580
Transcription factor IIA gamma chain (*TFIIA-S*)	Transcription	Phloem over-expression	(Asano et al., 2002)	AT4G24440
Wound inducive gene (*F14P1.1*)	Stress	C4 interaction	Hericourt *et al.* (in preparation)	AT1G19660
Group B (promote earlier infection)				
Bearskin 2 (*BRN2*)	Transcription	Phloem over-expression	(Asano et al., 2002)	AT4G10350
Importin alpha isoform 4 (*IMPA-4*)	Transport	CP interaction	(Guerra-Peraza et al., 2005)	AT1G09270
Lactoylglutathione lyase (*GLO1*)	Stress	C3 Interaction	Hericourt *et al.* (in preparation)	AT1G15380
Replication protein A32 (*RPA32/RPA2*)	DNA metabolism	Rep interaction	(Singh et al., 2006)	AT3G02920
Dehydration responsive 21 (*RD21*)	Stress	V2 interaction	Hericourt *et al.* (in preparation)	AT1G47128
RING-type E3 ubiquitin ligase (*RHF2A*)	Protein modification	Transactived by TrAP/C2	(Vilaine et al., 2003)	AT5G22000
Ubiquitin activating enzyme (*UBA1*)	Protein modification	TrAP/C2 Interaction	Hericourt *et al.* (in preparation)	AT2G30110
Group C (delay, reduce or prevent the infection)				
4-coumarate:CoA ligase (*AT4CL1*)	Metabolism	Phloem over-expression	(Asano et al., 2002)	AT1G51680
Allene oxide cyclase (*AOC1*)	Metabolism	Phloem over-expression	(Vilaine et al., 2003)	AT3G25760
Barely any meristem 1 *(BAM1)*	Protein modification	C4 interaction	Hericourt *et al.* (in preparation)	AT5G65700
Coatomer delta subunit (δ-*COP*)	Protein transport	C3 interaction	Hericourt *et al.* (in preparation)	AT5G05010
COP9 signalosome subunit 3 (*CSN3*)	Protein modification	Cellular process	(Schwechheimer and Isono, 2010)	AT5G14250
Geminivirus Rep A-binding (*GRAB2*)	Transcription	Rep interaction	(Xie et al., 1999)	AT5G61430
Heat shock protein cognate 70 (*HSC70*)	Protein modification	Phloem over-expression	(Vilaine et al., 2003)	AT5G02500

Identity	Function	Selection criteria	Reference	ACC *A. thaliana*
Nuclear acetyltransferase (*NSI*)	Signal transduction	NSP Interaction	(Carvalho et al., 2006)	AT1G32070
Patatin-like protein 2 (*PLP2*)	Stress	Phloem over-expression	(Vilaine et al., 2003)	AT2G26560
Shaggy-related kinase kappa (*SK4-1/SKK)*	Protein modification	C4 interaction	Hericourt *et al.* (in preparation)	AT1G09840
SKP1-like 2 (*ASK2*)	Protein modification	Transactived by TrAP/C2	(Trinks et al., 2005)	AT5G08590

Table 1. List of candidate genes. The criterion for selection is indicated in each case. The accession numbers (ACC) of the homologous Arabidopsis gene used in the VIGS experiments are indicated in this case.

EXPERIMENTAL PROCEDURES

Microorganisms, plants and general methods

Manipulations of *Escherichia coli* and nucleic acids were performed according to standard methods (Ausubel et al., 1998; Sambrook and Russell, 2001). *E. coli* strain DH5-α was used for subcloning. All PCR-amplified fragments cloned in this work were fully sequenced. *Agrobacterium tumefaciens* GV3101 strain was used for the delivery of *Tobacco rattle virus* (TRV) RNA2-based vectors and TYLCSV infective clone; *A. tumefaciens* C58c1 was used for the delivery of the TRV RNA1-based construct pBINTRA6 (Ratcliff et al., 2001).

2IRGFP *N. benthamiana* plants were grown in soil at 22ºC in short day conditions (8 h light/ 16 h dark photoperiod).

Plasmids and cloning

cDNA clones of the selected candidate genes were obtained from NASC (Table 2). Fragments (300-500 bp) from the selected genes were generated by PCR with specific primers (Table 2) and cloned in pGEMT-easy (Promega). *Spe*I/*Apa*I fragments from the pGEMT clones containing the selected sequenced were subcloned into *Spe*I/*Apa*I sites of TRV RNA2-based vector pTV00 (Ratcliff et al., 2001) to yield the correspondent TRV used to silencing the plants genes.

To yield the TRV:*GFP* construct, a 383 bp *BamH*I-*Cla*I fragment from pSMGFP (Davis and Vierstra, 1998) was cloned into *BamH*I-*Cla*I of pTV00. To yield the TRV:*SuI* construct, a 450 bp fragment of the *Sulfur* gene amplified from *Arabidopsis* cDNA using At*Sulfur* primers (Table 2) was digested with *Kpn*I and cloned into the *Kpn*I site of pTV00. To yield the TRV:*SulPCNA* construct a 450 bp *Kpn*I fragment from TRV:*SuI* was subcloned into *KpnI* site of TRV:*PCNA* (Morilla et al., 2006).

Geminivirus infection assays and detection of viral and mGFP DNA

Viral infections of 2IRGFP *N. benthamiana* plants were performed by the agro-inoculation technique as previously described (Elmer et al., 1988). Plants were agro-inoculated with plasmid pGreenTYA14 (binary vector containing a partial dimer of TYLCSV-ES[2] (Lozano-Durán et al., 2011)) in the axilary bud of the fourth/fifth leaf of 3-week-old wild-type or transgenic 2IRGFP *N. benthamiana* plants. For control, plants were mock inoculated with *A. tumefaciens* culture harbouring the empty binary vector pGreen-0229 (Hellens et al., 2000).

Viral and mGFP DNAs were detected by gel blot hybridization. Total plant DNA was extracted from *N. benthamiana* leaves at different days post-infection. Two micrograms of undigested total DNA per sample were used. As probe for TYLCSV detection, we used a *BamH*I DNA fragment from pGreenTYA14 (Lozano-Durán et al., 2011) containing a full-length genome of TYLCSV-ES.

For mGFP detection we used a *BamH*I-*Sac*I DNA fragment from pSMGFP comprising the complete GFP open reading frame (Davis and Vierstra, 1998).

For quantitative real-time PCR, total plant DNA was extracted from *N. benthamiana* leaves at 15 dpi. The reaction mixture consisted of approximately 10 ng total DNA, primer mix (3 µM each) and SYBR Green Master Mix (TaKaRa, Kyoto, Japan) in a total volume of 25 µl. The PCR conditions were: 10 minutes at 95°C, and 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. The reactions were performed using a Rotor-Gene real time cycler (QIAGEN, Hamburg Germany). A relative quantification real-time PCR method using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to compare the amount of the TYLCSV capsid protein gene (amplified using primers GGAGGCTGAACTTCGACAGC and GGACTTTCAATGGGCCTTCAC) between different infections/experiments. The 25S ribosomal DNA interspacer (ITS) (amplified using primers ATAACCGCATCAGGTCTCCA and CCGAAGTTACGGATCCATTT) was used as the internal control.

Virus Induced Gene Silencing assay

Virus induced gene silencing with TRV in *N. benthamiana* plants was performed according the method described by (Ratcliff et al., 2001) . Briefly, independent cultures of *A. tumefaciens* GV3101 carrying pTV00 or pTV00-based constructs and *A. tumefaciens* C58c1 carrying pBINTRA6 were grown overnight in LBroth medium plus appropriate antibiotics. Cultures were resuspended in VIGS buffer (10 mM morpholineethanesulfonic acid pH 5.6, 10 mM $MgCl_2$, and 100 µM acetosyringone) adjusting optical density to OD_{600}=1, and incubated overnight at room temperature in the dark. Cultures containing pBINTRA6 plasmid and pTV00 or pTV00-derived plasmid were mixed at a 1:1 ratio. Approximately 1 mL of this mixed culture was used to infiltrate the underside of two leaves of each 3-week-old 2IRGFP *N. benthamiana* plant.

Gene	TAIR clone	Primers
AtSulfur	-	AACCGAGGTACCGTTTGCCC CAGAAGGTACCAAAGCCTTTGAGC
A-type cyclin-dependent kinase (CDK2)	U83549	ATATGATCAAAACATATC CCCATGATTCTGAAAATC
Cullin-associated and neddylation-dissociated (CAND1)	U25698	TATTGGTTCACTTGTTCC GATAGATTAATGTGAAGC
DNA polymerase alpha 2 (POLA2)	U61004	CAGATTTCATCAATCCCG TCTGAATATTACATTTGC

Gene	TAIR clone	Primers
DNA polymerase delta small subunit (POLD2)	U24549	TCTTCACCTTATAACACC
		TAGGAATGCAGATCAACC
E2F transcription factor 1 (E2F1)	U25624	CTTAAGAACAGGATTCAG
		GTCTATTGGTCCCATTG
Geminivirus Rep-interacting kinase (GRIK1)	U09417	GCTAAGTCGTTCCAAACG
		ACCTCAATGAGATTAACG
Homologue to co-chaperone DNAJ-like protein (ATJ)	C104790	CTCTTGAGGATGTGTACC
		TCCAGTGACAGTGTCAGG
Histone 3 K4-specific methyltransferase SET7/9 (T26F17.15)	U09782	GAGAGTTGGGCTAGAGG
		CTTGCTTACGACCTTCATGC
Putative nucleic acid binding/transcription factor/zinc ion binding (JDK)	PYAT5G03150	GGCCACAACCTTCCATGG
		TAATGCGTCGCAGAACGC
Retinoblastoma-related protein (RB)	C104790	GGTTCCATAGATGCATGC
		GAGATGGCATTGGTTCAGC
NSP interacting kinase 2 (NIK2)	U21612	TGACGATTACTTTGAAGC
		CTATTCTATCGTAGTTGC
Putative transcriptional activators with NAC domain (ATAF1)	U82383	TCAGAATTATTACAGTTGC
		CGTGCATGATCCAATTGG
SNF1-related protein kinase (AKIN11)	U21346	CATCCTCATATTATTCGG
		AATTTACCTGATATAACC
SUMO activating enzyme (SAE1B)	U18656	GTGTTGATTTTTTCGAGAAG
		ACGTCCTTCAGTTTCCTC
Transcription factor IIA gamma chain	U20073	GGGGAAGTAGAGAATGGC
		GCATCTCACTGTGTGAGC
Scarecrow-like protein (SCL13)	U14082	AGATCTTGGAAGCAATAG
		ATATGGGAAGTTCACAAC
Wound inducive gene (F14P1.1)	U10100	TTAACTCTAGTGTAGTGG
		AGGCATTACCCACCTTTG
Putative shikimate kinase (SKL2)	U13498	TATCCACCAGCACTATCG
		GATTTCAGTTGAATCTCC
RUB-activating enzyme (ECR1)	U13340	AAGATTGTGTCTGCATGC
		GCTCTTTGTCCAAACACG
Responsive to dehydratation 21 (RD21A)	U11707	AACTTACAGCGAGGAATCG
		AGGCTTGATGGGAGATGG

Gene	TAIR clone	Primers
Lactoylglutathione lyase (GLO1)	U17691	ATCTCACGTCACTGAACC
		CCTTCTTCTACTAATGCC
Bearskin 2 (BRN2)	C103095	ACCAAAAGTTTGAGATGG
		TCTACATACCACCCATCC
Nuclear import factor importin alpha	U18321	TTTATTCTGATGATCCTC
		TGAGAACAAGATTCCTGC
Replication protein 32 (RPA32/RPA2)	U82360	ACGGCAACGCTGCTTTCG
		CACAGATCGTTTCCCTTG
RING-type E3 ubiquitin ligase	C105154	AGGGGCATTTGACTTCGG
		TTCGTTCTTCAATCTCAGC
Ubiquitin activating enzyme (UBA1)	U21814	GCAAGAACTCAACAATGC
		ACTTCAGAGAAAACAACC
Allene oxide cyclase (AOC1)	U82314	AAGTTCAAGAACTGAGCG
		AACCTGTCCGTAGGCACC
COP9 signalosome subunit 3 (CSN3)	U14097	CAGGTGTTAATTTCTCTAC
		CAAACGCTTGGAGGAGACCTG
Geminivirus Rep A-binding (GRAB2)	U15787	AGAGCAGATGGATTTACC
		AGAATTTTCCTTCAAGCC
Heat shock protein cognate 70 (HSC70)	U09493	ATTGACTCTCTATACGAG
		ACCTTAGGGATACGGGTAG
Isoform of 4-coumarate:CoA ligase	U21582	CGAATTCGCCACTAAGCC
		GTCGACGTAACGAGCTTC
Nuclear acetyltransferase (NSI)	U82318	GCTTATTGGTATGGCACG
		GAAAGAACAGTTTTATCC
Patatin-like protein 2 (PLP2)	U16519	CTACTTTGATGTAATAGC
		ATAGTAGGTTGAAGATGC
Receptor protein kinase–like proteína (BAM1)	U25612	TCGGTGAGGTGCTTCACG
		TCCGACAGGTTTTCTTCC
Shaggy-related kinase kappa (ASK-KAPPA)	U16839	ATCAGAACATGTTGTTGG
		ATCACGGTGACAAAGACC
SKP1-like gene (ASK2)	U82510	ATCCGGTTCAAGGAGGTG
		CGTGCTTGCCATCATATTC
Coatomer delta subunit (δ-COP)	U16159	TCTTGTTACAACTAAGC
		TTCAAGTCTTCCAGATCC

Table 2. Oligonucleotides and TAIR clones used for amplifying and cloning fragments of the selected genes.

REFERENCES

- Ach, R.A., Durfee, T., Miller, A.B., Taranto, P., Hanley-Bowdoin, L., Zambryski, P.C., Gruissem, W., 1997. RRB1 and RRB2 encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. Molecular and cellular biology 17, 5077-5086.

- Amari, K., Diaz-Vivancos, P., Pallas, V., Sanchez-Pina, M.A., Hernandez, J.A., 2007. Oxidative stress induction by Prunus necrotic ringspot virus infection in apricot seeds. Physiol Plant 131, 302-310.

- Andleeb, S., Amin, I., Bashir, A., Briddon, R.W., Mansoor, S., 2010. Transient expression of betaC1 protein differentially regulates host genes related to stress response, chloroplast and mitochondrial functions. Virology journal 7, 373.

- Aparicio, F., Thomas, C.L., Lederer, C., Niu, Y., Wang, D., Maule, A.J., 2005. Virus Induction of Heat Shock Protein 70 Reflects a General Response to Protein Accumulation in the Plant Cytosol. Plant Physiol 138, 529-536.

- Asano, T., Masumura, T., Kusano, H., Kikuchi, S., Kurita, A., Shimada, H., Kadowaki, K., 2002. Construction of a specialized cDNA library from plant cells isolated by laser capture microdissection: toward comprehensive analysis of the genes expressed in the rice phloem. Plant J 32, 401-408.

- Ascencio-Ibanez, J.T., Sozzani, R., Lee, T.J., Chu, T.M., Wolfinger, R.D., Cella, R., Hanley-Bowdoin, L., 2008. Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant physiology 148, 436-454.

- Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., Struhl, K., 1998. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.

- Bennett, T., van den Toorn, A., Sanchez-Perez, G.F., Campilho, A., Willemsen, V., Snel, B., Scheres, B., 2010. SOMBRERO, BEARSKIN1, and BEARSKIN2 regulate root cap maturation in Arabidopsis. Plant Cell 22, 640-654.

- Ber, R., Navot, N., Zamir, D., Antignus, Y., Cohen, S., Czosnek, H., 1990. Infection of tomato by tomato yellow leaf curl virus: suceptibility to infection, symptom development and accumulation of viral DNA. Arch. Virology.

- Boudsocq, M., Barbier-Brygoo, H., Lauriere, C., 2004. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. J Biol Chem 279, 41758-41766.

- Burch-Smith, T.M., Anderson, J.C., Martin, G.B., Dinesh-Kumar, S.P., 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J 39, 734-746.

- Carvalho, M.F., Turgeon, R., Lazarowitz, S.G., 2006. The geminivirus nuclear shuttle protein NSP inhibits the activity of AtNSI, a vascular-expressed Arabidopsis acetyltransferase regulated with the sink-to-source transition. Plant Physiol 140, 1317-1330.

- Castillo, Morilla G., Lozano R., Collinet D., Perez-Luna A., and, K.A., E.R., B., 2007. Identification of Plant Genes Involved in TYLCV Replication, Tomato Yellow Leaf Curl Virus Disease.

- Citovsky, V., Zaltsman, A., Kozlovsky, S.V., Gafni, Y., Krichevsky, A., 2009. Proteasomal degradation in plant-pathogen interactions. Semin Cell Dev Biol 20, 1048-1054.

- Clarke, S.F., Guy, P.L., Burritt, D.J., Jameson, P.E., 2002. Changes in the activities of antioxidant enzymes in response to virus infection and hormone treatment. Physiol Plant 114, 157-164.

- Cook, A., Bono, F., Jinek, M., Conti, E., 2007. Structural biology of nucleocytoplasmic transport. Annu Rev Biochem 76, 647-671.

- Czosnek, H., 2007. Tomato Yellow Leaf Curl Disease. Management, molecular biologya, breeding for resistance. Spinger, Dordretch.

- Czosnek, H., Ber, R., Navot, N., Zamir, D., 1988. Detection of tomato yellow leaf curl virus in lysates of plants and insects by hybridization with a viral DNA probe. Plant disease 72, 949-951.

- Chen, J., Jiang, C., Gookin, T., Hunter, D., Clark, D., Reid, M., 2004. Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence. Plant Molecular Biology 55, 521-530.

- Davis, S.J., Vierstra, R.D., 1998. Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol 36, 521-528.

- DeYoung, B.J., Bickle, K.L., Schrage, K.J., Muskett, P., Patel, K., Clark, S.E., 2006. The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in Arabidopsis. Plant J 45, 1-16.

- Deyoung, B.J., Clark, S.E., 2008. BAM receptors regulate stem cell specification and organ development through complex interactions with CLAVATA signaling. Genetics 180, 895-904.

- Diaz-Pendon, J.A., Canizares, M.C., Moriones, E., Bejarano, E.R., Czosnek, H., Navas-Castillo, J., 2010. Tomato yellow leaf curl viruses: menage a trois between the virus complex, the plant and the whitefly vector. Mol Plant Pathol 11, 441-450.

- Diaz-Vivancos, P., Clemente-Moreno, M.J., Rubio, M., Olmos, E., Garcia, J.A., Martinez-Gomez, P., Hernandez, J.A., 2008. Alteration in the chloroplastic metabolism leads to ROS accumulation in pea plants in response to plum pox virus. J Exp Bot 59, 2147-2160.

- Diaz-Vivancos, P., Rubio, M., Mesonero, V., Periago, P.M., Barcelo, A.R., Martinez-Gomez, P., Hernandez, J.A., 2006. The apoplastic antioxidant system in Prunus: response to long-term plum pox virus infection. J Exp Bot 57, 3813-3824.

- Dielen, A.S., Badaoui, S., Candresse, T., German-Retana, S., 2010. The ubiquitin/26S proteasome system in plant-pathogen interactions: a never-ending hide-and-seek game. Mol Plant Pathol 11, 293-308.

- Dogra, S.C., Eini, O., Rezaian, M.A., Randles, J.W., 2009. A novel shaggy-like kinase interacts with the Tomato leaf curl virus pathogenicity determinant C4 protein. Plant molecular biology 71, 25-38.

- Dreher, K., Callis, J., 2007. Ubiquitin, hormones and biotic stress in plants. Ann Bot 99, 787-822.

- Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P., Martin, G.B., 2003. Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. The Plant journal : for cell and molecular biology 36, 905-917.

- Elmer, J.S., Sunter, G., Gardiner, W.E., Brand, L., Browing, C.K., Bisaro, D., Rogers, S.G., 1988. Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNAs. Plant. Mol. Biol. 10, 225-234.

- Escaler, M., Aranda, M.A., Thomas, C.L., Maule, A.J., 2000. Pea embryonic tissues show common responses to the replication of a wide range of viruses. Virology 267, 318-325.

- Eybishtz, A., Peretz, Y., Sade, D., Gorovits, R., Czosnek, H., 2010. Tomato yellow leaf curl virus infection of a resistant tomato line with a silenced sucrose transporter gene LeHT1 results in inhibition of growth, enhanced virus spread, and necrosis. Planta 231, 537-548.

- Garcia-Marcos, A., Pacheco, R., Martianez, J., Gonzalez-Jara, P., Diaz-Ruiz, J.R., Tenllado, F., 2009. Transcriptional changes and oxidative stress associated with the synergistic interaction between Potato virus X and Potato virus Y and their relationship with symptom expression. Mol Plant Microbe Interact 22, 1431-1444.

- Guerra-Peraza, O., Kirk, D., Seltzer, V., Veluthambi, K., Schmit, A.C., Hohn, T., Herzog, E., 2005. Coat proteins of Rice tungro bacilliform virus and Mungbean yellow mosaic virus contain multiple nuclear-localization signals and interact with importin alpha. J Gen Virol 86, 1815-1826.

- Guo, Y., Han, L., Hymes, M., Denver, R., Clark, S.E., 2010. CLAVATA2 forms a distinct CLE-binding receptor complex regulating Arabidopsis stem cell specification. Plant J 63, 889-900.

- Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., Mullineaux, P.M., 2000. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant molecular biology 42, 819-832.

- Kjemtrup, S., Sampson, K.S., Peele, C.G., Nguyen, L.V., 1998. Gene silencing from plant DNA carried by a Geminivirus. Plant Journal 14, 91-100.

- Komatsu, K., Hashimoto, M., Ozeki, J., Yamaji, Y., Maejima, K., Senshu, H., Himeno, M., Okano, Y., Kagiwada, S., Namba, S., 2010. Viral-induced systemic necrosis in plants involves both programmed cell death and the inhibition of viral multiplication, which are regulated by independent pathways. Mol Plant Microbe Interact 23, 283-293.

- Kong, L.J., Hanley-Bowdoin, L., 2002. A geminivirus replication protein interacts with a protein kinase and a motor protein that display different expression patterns during plant development and infection. The Plant cell 14, 1817-1832.

- Kong, L.J., Orozco, B.M., Roe, J.L., Nagar, S., Ou, S., Feiler, H.S., Durfee, T., Miller, A.B., Gruissem, W., Robertson, D., Hanley-Bowdoin, L., 2000. A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. The EMBO journal 19, 3485-3495.

- Krenz, B., Windeisen, V., Wege, C., Jeske, H., Kleinow, T., 2010. A plastid-targeted heat shock cognate 70kDa protein interacts with the Abutilon mosaic virus movement protein. Virology 401, 6-17.

- La Camera, S., Balague, C., Gobel, C., Geoffroy, P., Legrand, M., Feussner, I., Roby, D., Heitz, T., 2009. The Arabidopsis patatin-like protein 2 (PLP2) plays an essential role in cell death execution and differentially affects biosynthesis of oxylipins and resistance to pathogens. Mol Plant Microbe Interact 22, 469-481.

- La Camera, S., Geoffroy, P., Samaha, H., Ndiaye, A., Rahim, G., Legrand, M., Heitz, T., 2005. A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in Arabidopsis. Plant J 44, 810-825.

- Lampl, N., Budai-Hadrian, O., Davydov, O., Joss, T.V., Harrop, S.J., Curmi, P.M., Roberts, T.H., Fluhr, R., 2010. Arabidopsis AtSerpin1, crystal structure and in vivo interaction with its target protease RESPONSIVE TO DESICCATION-21 (RD21). J Biol Chem 285, 13550-13560.

- Lee, M.C., Miller, E.A., Goldberg, J., Orci, L., Schekman, R., 2004. Bi-directional protein transport between the ER and Golgi. Annu Rev Cell Dev Biol 20, 87-123.

- Lewis, J.D., Lazarowitz, S.G., 2010. Arabidopsis synaptotagmin SYTA regulates endocytosis and virus movement protein cell-to-cell transport. Proceedings of the National Academy of Sciences of the United States of America 107, 2491-2496.

- Liu, E., Page, J.E., 2008. Optimized cDNA libraries for virus-induced gene silencing (VIGS) using tobacco rattle virus. Plant Methods 4, 5.

- Liu, F., Ni, W., Griffith, M.E., Huang, Z., Chang, C., Peng, W., Ma, H., Xie, D., 2004. The ASK1 and ASK2 genes are essential for Arabidopsis early development. Plant Cell 16, 5-20.

- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

- Lois, L.M., 2010. Diversity of the SUMOylation machinery in plants. Biochem Soc Trans 38, 60-64.

- Lozano-Durán, R., Bejarano, E.R., 2011. Geminivirus C2 protein might be the key player for geminiviral co- option of SCF-mediated ubiquitination. Plant signaling & behavior 6, 999-1001.

- Lozano-Durán, R., Rosas-Diaz, T., Gusmaroli, G., Luna, A.P., Taconnat, L., Deng, X.W., Bejarano, E.R., 2011. Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes and inhibit jasmonate signaling in Arabidopsis thaliana. The Plant cell 23, 1014-1032.

- Luna, A.P., Morilla, G., Voinnet, O., Bejarano, E.R., 2012. Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. Molecular plant-microbe interactions : MPMI 25, 1294-1306.

- McGarry, R.C., Barron, Y.D., Carvalho, M.F., Hill, J.E., Gold, D., Cheung, E., Kraus, W.L., Lazarowitz, S.G., 2003. A novel Arabidopsis acetyltransferase interacts with the geminivirus movement protein NSP. Plant Cell 15, 1605-1618.

- Morilla, G., Castillo, A.G., Preiss, W., Jeske, H., Bejarano, E.R., 2006. A versatile transreplication-based system to identify cellular proteins involved in geminivirus replication. J Virol 80, 3624-3633.

- Moriones, E., Navas-Castillo, J., 2000. Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. Virus Res 71, 123-134.

- Mustafiz, A., Sahoo, K.K., Singla-Pareek, S.L., Sopory, S.K., 2010. Metabolic engineering of glyoxalase pathway for enhancing stress tolerance in plants. Methods Mol Biol 639, 95-118.

- Noel, L.D., Cagna, G., Stuttmann, J., Wirthmuller, L., Betsuyaku, S., Witte, C.P., Bhat, R., Pochon, N., Colby, T., Parker, J.E., 2007. Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates Arabidopsis immune responses. Plant Cell 19, 4061-4076.

- Orzaez, D., Medina, A., Torre, S., Fernandez-Moreno, J.P., Rambla, J.L., Fernandez-Del-Carmen, A., Butelli, E., Martin, C., Granell, A., 2009. A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation. Plant Physiol 150, 1122-1134.

- Peele, C., Jordan, C.V., Muangsan, N., Turnage, M., Egelkrout, E., Eagle, P., Hanley-Bowdoin, L., Robertson, D., 2001. Silencing of a meristematic gene using geminivirus-derived vectors. The Plant journal : for cell and molecular biology 27, 357-366.

- Piroux, N., Saunders, K., Page, A., Stanley, J., 2007. Geminivirus pathogenicity protein C4 interacts with Arabidopsis thaliana shaggy-related protein kinase AtSKeta, a component of the brassinosteroid signalling pathway. Virology 362, 428-440.

- Ratcliff, F., Montserrat, A., Martin-Hernandez, Baulcombe, D., 2001. Tobacco rattle virus as a vector for analysis of gene function by silencing. The Plant Journal 25, 237-245.

- Rimmer, J., Singh, A., Banwell, P., Clarke, P.M., Rhys Evans, P., 2006. The use of octyl-2-cyanoacrylate (Dermabond) tissue adhesive for skin closure in head and neck surgery. Ann R Coll Surg Engl 88, 412-413.

- Rojas, M.R., Hagen, C., Lucas, W.J., Gilbertson, R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annual review of phytopathology 43, 361-394.

- Ruiz, M.T., Voinnet, O., Baulcombe, D.C., 1998. Initiation and maintenance of virus-induced gene silencing. Plant Cell 10, 937-946.

- Sahu, P.P., Rai, N.K., Chakraborty, S., Singh, M., Chandrappa, P.H., Ramesh, B., Chattopadhyay, D., Prasad, M., 2010. Tomato cultivar tolerant to Tomato leaf curl New Delhi virus infection induces virus-specific short interfering RNA accumulation and defence-associated host gene expression. Mol Plant Pathol 11, 531-544.

- Sambrook, J., Russell, D.W., 2001. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

- Schwechheimer, C., Isono, E., 2010. The COP9 signalosome and its role in plant development. Eur J Cell Biol 89, 157-162.

- Selth, L.A., Dogra, S.C., Rasheed, M.S., Healy, H., Randles, J.W., Rezaian, M.A., 2005. A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. The Plant cell 17, 311-325.

- Senthil-Kumar, M., Hema, R., Anand, A., Kang, L., Udayakumar, M., Mysore, K.S., 2007. A systematic study to determine the extent of gene silencing in Nicotiana benthamiana and other Solanaceae species when heterologous gene sequences are used for virus-induced gene silencing. New Phytol 176, 782-791.

- Serino, G., Deng, X.W., 2003. The COP9 signalosome: regulating plant development through the control of proteolysis. Annu Rev Plant Biol 54, 165-182.

- Shultz, R.W., Tatineni, V.M., Hanley-Bowdoin, L., Thompson, W.F., 2007. Genome-wide analysis of the core DNA replication machinery in the higher plants Arabidopsis and rice. Plant Physiol 144, 1697-1714.

- Singh, D.K., Islam, M.N., Choudhury, N.R., Karjee, S., Mukherjee, S.K., 2006. The 32 kDa subunit of replication protein A (RPA) participates in the DNA replication of Mung bean yellow mosaic India virus (MYMIV) by interacting with the viral Rep protein. Nucleic Acids Res 35, 755-770.

- Singh, D.K., Islam, M.N., Choudhury, N.R., Karjee, S., Mukherjee, S.K., 2007. The 32 kDa subunit of replication protein A (RPA) participates in the DNA replication of Mung bean yellow mosaic India virus (MYMIV) by interacting with the viral Rep protein. Nucleic Acids Res 35, 755-770.

- Song, X.S., Wang, Y.J., Mao, W.H., Shi, K., Zhou, Y.H., Nogues, S., Yu, J.Q., 2009. Effects of cucumber mosaic virus infection on electron transport and antioxidant system in chloroplasts and mitochondria of cucumber and tomato leaves. Physiol Plant 135, 246-257.

- Spitzer, B., Zvi, M.M., Ovadis, M., Marhevka, E., Barkai, O., Edelbaum, O., Marton, I., Masci, T., Alon, M., Morin, S., Rogachev, I., Aharoni, A., Vainstein, A., 2007. Reverse genetics of floral scent: application of tobacco rattle virus-based gene silencing in Petunia. Plant Physiol 145, 1241-1250.

- Stanley, J., Bisaro, D.M., Briddon, R.W., Brown, J.K., Fauquet, C.M., Harrison, B.D., Rybicki, E.P., Stenger, D.C., 2005. Geminiviridae, in: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), In Virus Taxonomy. VIIIth Report of the International Committee on Taxonomy of Viruses, Elsevier/Academic Press, London, pp. 301-326.

- Sun, W., Xu, X., Zhu, H., Liu, A., Liu, L., Li, J., Hua, X., 2010. Comparative transcriptomic profiling of a salt-tolerant wild tomato species and a salt-sensitive tomato cultivar. Plant Cell Physiol 51, 997-1006.

- Sung, D.Y., Vierling, E., Guy, C.L., 2001. Comprehensive expression profile analysis of the Arabidopsis Hsp70 gene family. Plant Physiol 126, 789-800.

- Terry, L.J., Shows, E.B., Wente, S.R., 2007. Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. Science 318, 1412-1416.

- Thomas, C.L., Jones, L., Baulcombe, D.C., Maule, A.J., 2001. Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in Nicotiana benthamiana using a potato virus X vector. Plant J 25, 417-425.

- Trinks, D., Rajeswaran, R., Shivaprasad, P.V., Akbergenov, R., Oakeley, E.J., Veluthambi, K., Hohn, T., Pooggin, M.M., 2005. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. Journal of virology 79, 2517-2527.

- Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K., Shinozaki, K., 2004. SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in Arabidopsis thaliana. Proc Natl Acad Sci U S A 101, 17306-17311.

- Vilaine, F., Palauqui, J.C., Amselem, J., Kusiak, C., Lemoine, R., Dinant, S., 2003. Towards deciphering phloem: a transcriptome analysis of the phloem of Apium graveolens. Plant J 36, 67-81.

- Voinnet, O., Baulcombe, D.C., 1997. Systemic signalling in gene silencing. Nature 389, 553.

- Wang, H., Hao, L., Shung, C.Y., Sunter, G., Bisaro, D.M., 2003. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. The Plant cell 15, 3020-3032.

- Woodward, A.W., Ratzel, S.E., Woodward, E.E., Shamoo, Y., Bartel, B., 2007. Mutation of E1-CONJUGATING ENZYME-RELATED1 decreases RELATED TO UBIQUITIN conjugation and alters auxin response and development. Plant Physiol 144, 976-987.

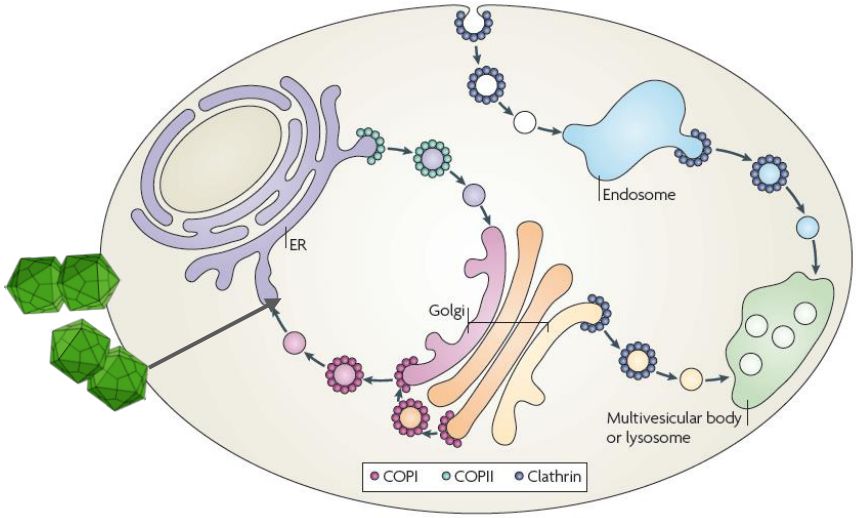
- Xie, Q., Sanz-Burgos, A.P., Guo, H., Garcia, J.A., Gutierrez, C., 1999. GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Mol Biol 39, 647-656.

- Yadav, S.K., Singla-Pareek, S.L., Sopory, S.K., 2008. An overview on the role of methylglyoxal and glyoxalases in plants. Drug Metabol Drug Interact 23, 51-68.

- Yang, J., Chen, F., Yu, O., Beachy, R.N., 2010. Controlled silencing of 4-coumarate:CoA ligase alters lignocellulose composition without affecting stem growth. Plant Physiol Biochem 49, 103-109.

Chapter III

ACTIVE RETROGRADE TRANSPORT IS REQUIRED FOR INFECTION BY GEMINIVIRUSES



ABSTRACT

In recent years, it has become evident that vesicle trafficking is engaged in the plant's immune system to actively defend against potential pathogens. The coat protein complex COPI, which mediates the vesicle transport from the Golgi apparatus to the endoplasmic reticulum in the so-called "retrograde" pathway, consists of several coatomer subunits, which function together with the GTPase ADP-RIBOSYLATION FACTOR (ARF1). Several viruses cause a remodelling of COPI structures and biogenesis within their host plants. For plant RNA viruses, these interactions were demonstrated to be relevant to the infections process. An involvement of the retrograde pathway has been shown for animal DNA viruses. Recently, our findings have shown that the delta subunit (δ-COP) protein of the COPI complex is required for a full viral infection of a plant DNA virus (the geminivirus *Tomato yellow leaf curl Sardinia virus,* TYLCSV). Moreover, the δ-COP protein was shown to interact with the C3 protein from TYLCSV in yeast. The main aim of this work is determine the role of retrograde transport in geminivirus infection and in plant-pathogen interactions. In this work, we demonstrate that an intact retrograde trafficking pathway is crucial for geminivirus infection. Silencing of either *δ-COPI* or *ARF1* completely abolishes infection by TYLCSV and the unrelated geminivirus *Beet curly top virus* (BCTV), but does not affect infectivity of the RNA viruses *Potato virus X* (PVX) or *Tobacco mosaic virus* (TMV) or the plant pathogenic bacterial strain *Pseudomonas syringae* pv *tomato* DC3000 Δ*hopQ1-1*. Taken together, these results suggest that an intact retrograde pathway is specifically required by geminivirus infection, but does not have a general impact over others pathogens in *Nicotiana benthamiana*.

INTRODUCTION

Geminiviruses are insect-transmitted plant viruses that cause some of the most economically important diseases in vegetable and field crops worldwide (Mansoor et al., 2003; Rojas et al., 2005). The incidence and severity of geminivirus diseases has greatly increased in the past 20 years (Navas-Castillo et al., 2011); a comprehensive understanding of the molecular and cellular events underlying geminiviral infection will be a cornerstone in the prevention of these devastating diseases.

Geminivirus are divided into seven genera (*Begomovirus, Mastrevirus, Curtovirus, Becurtovirus, Eragrovirus, Topocuvirus* and *Turncurtovirus*), based on genome organization, nucleotide sequence similarities and biological properties (Brown JK, 2012; Varsani et al., 2014). Geminiviral genomes consist of one (monopartite) or two (bipartite) circular single-stranded DNA (ssDNA) molecules, with replicative double-stranded DNA (dsDNA) that is transcribed in the nucleus of infected plant cells and packaged in icosahedral twinned particles (Zhang et al., 2001; Bottcher et al., 2004; Rojas et al., 2005; Krupovic et al., 2009). Their genomes encode 6–8 proteins involved in viral replication, movement, transmission and pathogenesis (Hanley-Bowdoin et al., 2013). All geminivirus genome components possess an intergenic region (IR), which contains divergent promoters responsible for the expression of viral genes. The genome of monopartite begomoviruses comprises, in the complementary sense orientation, an open reading frame (ORF) encoding the replication-associated protein (Rep/C1), a transcription activator protein (TrAP/C2), and a replication enhancer protein (REn/C3), partially overlapping with the latter; a small ORF, C4, is located within the Rep ORF but in a different reading frame (Hanley-Bowdoin et al., 2000; Jeske, 2009). In the virion sense orientation, monopartite begomoviral genomes contain genes encoding the coat protein (CP) and a pathogenicity determinant (V2). The CP forms the viral capsid and mediates vector transmission (Briddon et al., 1990). The V2 protein functions to inhibit post-transcriptional gene silencing (PTGS) (Luna et al., 2012; Zhang et al., 2012). V2 has also been shown to provide the movement function for monopartite viruses.

Most of our knowledge of geminivirus movement comes from the bipartite geminiviruses, which encode two well characterized proteins a nuclear shuttle protein (NSP) and movement protein (MP) that mediate viral DNA movement into and out of the nucleus and between cells (reviewed in (Hanley-Bowdoin et al., 2013)). Little is known about the movement proteins of monopartite geminiviruses, in which CP acts as the NSP (Poornima Priyadarshini CG, 2011), whereas MP function is mediated by V2 alone or in a complex with C4 (Rojas et al., 2001). NSP from *Bean dwarf mosaic virus* interacts with histone H3, raising the possibility that viral DNA moves as a

minichromosome (Zhou et al., 2011). The begomovirus *Squash leaf curl virus* forms ER tubules in sink tissue, and these tubules might accommodate a compacted minichromosome (Ward et al., 1997). *Abutilon mosaic virus* MP interacts with a chloroplast heat shock cognate 70 protein (HSC70), MP from *Cabbage leaf curl virus* interacts with a synaptotagmin protein (SYTA), and the C3 protein from *Tomato yellow mosaic Sardinia virus* (TYLCSV) interacts with the delta subunit of Coatomer (COPI) complex, (δ-COP) protein (Lewis and Lazarowitz, 2010; Krenz et al., 2012). Remarkably, downregulation of these three proteins restricts or delays viral infection, suggesting that geminiviruses recruit host transport systems for their movement as an essential part of their infection cycle. However, the role of host transport and vesicle trafficking proteins during the geminivirus infection still remains unclear.

The COPI complex is composed of seven subunits (α, β, β′, γ, δ, ε, and ζ) that form a cage-like structure expected to be similar to the clathrin coat (Boehm et al., 2001; Lee and Goldberg, 2010). Cytoplasmic vesicles containing a COPI coat are best known for their involvement in retrograde transport of cargo from the Golgi apparatus to the endoplasmic reticulum (ER) (Thompson and Brown, 2012). The coat GTPase that drives COPI coat formation is ADP-RIBOSYLATION FACTOR 1 (ARF1), which is typically represented by a gene family (e.g. six members in *Arabidopsis* named *ARFA1*). ARF1 specifically recruits COPI proteins to transport vesicles, thereby mediating retrograde vesicle transport from the Golgi to the endoplasmic reticulum (Kirchhausen, 2000). In plants, ARF1 appears to have multiple roles. It has been shown that ARF1 is distributed to the Golgi and post-Golgi compartments that bud from the Golgi apparatus, and may be also involved in endocytosis (Xu and Scheres, 2005; Stefano et al., 2006; Matheson et al., 2007).

Further research has described that the COPI complex and ARF1 protein, as components of trafficking vesicles, play an important role in the response to a wide range of pathogens. It has been demonstrated that ARF1 also has a critical function in the pathogenesis of bacteria as *Salmonella* and *Escherichia coli* (Moss and Vaughan, 1991; Nagai et al., 2002); on the other hand, COPI is involved in entry and infection capacity of virus SV40 and in the formation of a complex protein necessary for influenza virus and Enterovirus 71 replication (Richards et al., 2002; Konig et al., 2010; Wang et al., 2012) in vertebrates. Moreover, ARF1 and/or COPI are important for the formation or maintenance of Hepatitis C virus (HCV) replication complexes (Tai et al., 2009).

To date, there are fewer examples of the role of vesicle trafficking in plants in comparison to vertebrate's pathogenesis. Nomura et al (2006) reported that the *Arabidopsis* ARF-GEF AtMIN7 is specifically targeted by HopM1, a virulence factor from *Pseudomonas syringae* (AtMIN7 encodes one of the eight members of the *Arabidopsis* ARF guanine nucleotide exchange factor –GEF-

protein family). Coemans et al. (2008) provided evidence for a role of ARF1 in disease response: loss-of-function of ARF1 affects non-host resistance to *Pseudomonas cichorii* and partially compromises *N* gene-mediated resistance towards *Tobacco mosaic virus* (TMV) in *Nicotiana benthamiana*. Additionally, it has been shown that inhibition of ARF1 activity disrupts the replication of *Red clover necrotic mosaic virus* (RCNMV) and *Tobacco etch virus* (TEV), two plant RNA viruses (Wei and Wang, 2008; Hyodo et al., 2013), suggesting importance of an active retrograde transport for infectivity of RNA viruses in plants. Finally, silencing of *N. benthamiana δ-COPI* subunit or yeast *COP1* impairs, respectively, TYLCSV infection or *Tomato bushy stunt virus* (TBSV) replication (Jiang et al., 2006; Lozano-Durán et al., 2011b).

In this work, we demonstrate that an intact retrograde pathway, a process involving δ-COP and ARF1, is crucial for geminivirus infection. Silencing of either *δ-COPI* or *ARF1* completely abolishes infection by the begomovirus TYLCSV or the curtovirus *Beet curly top virus* (BCTV), but does not affect infectivity of the RNA viruses *Potato virus X* (PVX) or *Tobacco mosaic virus* (TMV) or the plant pathogenic bacterial strain *Pseudomonas syringae* pv *tomato* (*Pto*) DC3000 Δ*hopQ1-1*. Taken together, these results suggest that an intact retrograde transport pathway is specifically required for geminivirus infection, but does not have a general impact over other plant pathogens.

RESULTS

Characterization of *δ-COP* and *ARF1* silencing in *Nicotiana benthamiana* plants

In Chapter II we identified δ-COP as an essential host factor for TYLCSV infection in *N. benthamiana*. δ-COP is a component of the COPI complex, which functions in retrograde trafficking of vesicles. With the objective to analyze in detail the role of the retrograde pathway during geminivirus infection, we first decided to confirm whether the resistance phenotype to TYLCSV infection observed in *δ-COP*-silenced *N. benthamiana* 2IR plants (Chapter II), is also produced in *δ-COP*-silenced wild-type plants. Besides, we tested if silencing of *ARF1* (another component involved in the same functional pathway) has a similar impact on the viral infection.

For silencing of *δ-COP*, we used the TRV-*δ-COP* construct described in Chapter II; for silencing of *ARF1*, we cloned a fragment of 399bp of the *NbARF1* gene into the TRV vector as described previously by Coemans et al. (2008). Fully expanded leaves of 10 plants were agroinoculated with TRV-*δ-COP*, TRV-*ARF1* or TRV as control for silencing.

The silencing phenotype was evaluated in the mock plants at 7-15 days post inoculation (dpi) (two independent experiments, 5 plants each). Remarkably, the silencing phenotypes of *δ-COP* and *ARF1* in *N. benthamiana* plants were very similar, causing curling and yellowing in leaves and stunting of the plants (Figure 1A). In both cases, this phenotype could be observed from 8 dpi, gradually becoming more severe after this point. To determine the silencing efficiency of the TRV-*δ-COP* and the TRV-*ARF1* constructs, we quantified the mRNA transcript levels of *Nbδ-COP* and *NbARF1* genes in the TRV-infected plants at 10 and 15 dpi. For each time point, the two youngest leaves of five plants were taken, pooled, and total RNA was extracted and used in real-time PCR with gene-specific primers (See Experimental procedures). For both genes, a reduction of 65% at 10 dpi and 75% at 15 dpi, when compared with TRV control plants, could be observed (Figure 1B).

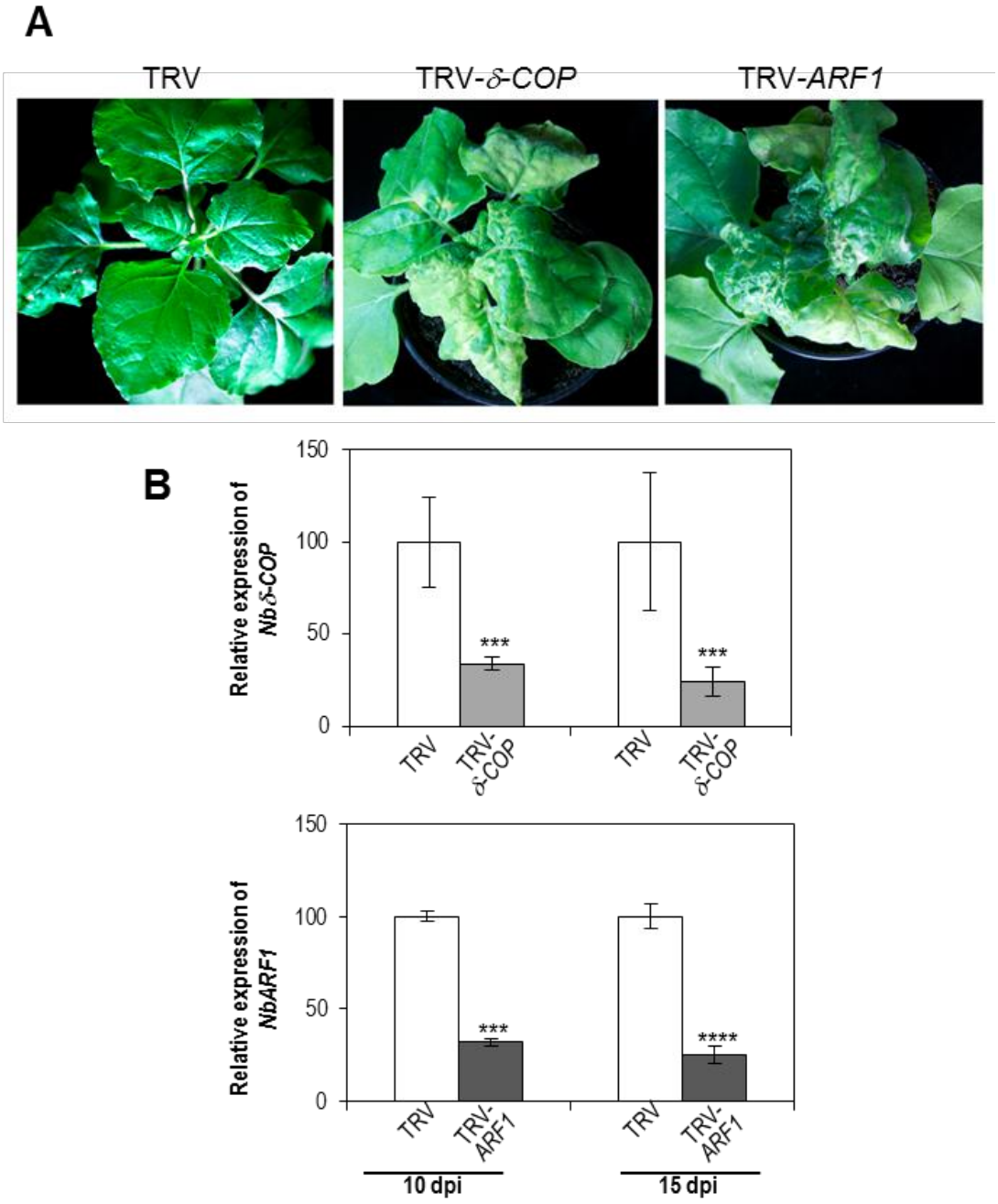


Figure 1. Virus-induced gene silencing of *δ-COP* and *NbARF1* in *Nicotiana benthamiana*. The two youngest leaves of five-week-old *N. benthamiana* plants were infiltrated with TRV-*δ-COP*, TRV-*ARF1* or with TRV as a control. (A) Phenotype of *δ-COP*- or *ARF1*-silenced plants as compared to control plants (TRV). Pictures were taken at 15 dpi. (B) *Nbδ-COP P* and *NbARF1* transcript levels measured by real-time PCR at 10 and 15 dpi. Total RNA was purified using three apical leaves of each plant. Bars represent the average of five plants. Asterisks indicate samples that are statistically significant different from the control silenced plants (****,p-value < 0.0001; ***, p-value < 0.005), according to a Student's. This experiment was repeated twice with similar results; results from one representative experiment are shown.

Silencing of *δ-COP* or *ARF1* abolishes geminivirus infection

The amount of viral DNA accumulated was determined at 15dpi. The results confirmed that *δ-COP* silencing caused a complete impairment of TYLCSV accumulation in *N. benthamiana* wild-type plants. When *NbARF1* was silenced, we observed a similar impairment of TYLCSV accumulation. Five plants per construct were co-agroinoculated with an infective TYLCSV clone and five with empty binary plasmids as mock for geminivirus infection (Figure 2). In order to evaluate the anti-viral effects of *δ-COP* and *ARF1* silencing over the infection of a different geminivirus species, the curtovirus *Beet curly top virus* (BCTV), we followed the same strategy described previously. Interestingly, silencing of either gene significantly diminished or abolished BCTV accumulation as well, which reached non-detectable levels in *δ-COP* -silenced plants and was reduced by 85% in *ARF1*-silenced plants (Figure 2). These results suggest that an intact retrograde trafficking pathway is required for geminivirus infection.

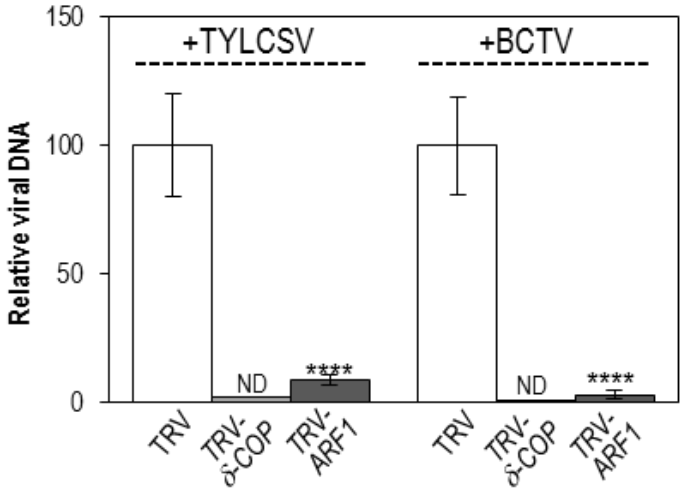


Figure 2. Virus-Induced gene silencing of *δ-COP* and *ARF1* abolishes geminivirus accumulation. Relative amount of TYLCSV and BCTV DNA in leaves of plants co-infected with TYLCSV or BCTV and TRV-*δ-COP*, TRV-*ARF1* or TRV as control. Viral DNA was measured by real time PCR at 15 dpi. Values represent the average of five plants. Bars represent standard error. Asterisks indicate samples that are statistically significant different from the control silenced plants (****p-value < 0.0001), according to a Student's. This experiment was repeated three times with similar results; results from one representative experiment are shown. ND: non-detected.

Silencing of *δ-COP* or *ARF1* does not affect the infection with RNA viruses or with the plant pathogenic bacterial strain *Pseudomonas syringae* pv *tomato* DC3000 Δ*hopQ1-1*

Because we have shown that the retrograde pathway is required for geminivirus infection, we decided to examine the role of this pathway in other plant-pathogen interactions. In order to do this, we decided to test the impact of *δ-COP and ARF1* genes on the infection by two RNA plant viruses, *Tobacco mosaic virus* (TMV) and *Potato virus X* (PVX), as well as the plant pathogenic bacterial strain *Pto* DC3000 Δ*hopQ1-1*.

For this purpose, we inoculated *N. benthamiana* plants with the TRV-*ARF1* and TRV-*δ-COP* constructs and TRV as control and, at same time, inoculated them with green fluorescent protein (GFP)-expressing TMV/PVX (TMV-GFP and PVX-GFP, respectively). Total RNA was purified using three apical leaves of each plant at 15 dpi and *GFP* transcripts were quantified by real-time PCR (two independent experiments, 5 plants each).

We observed that neither *δ-COP* nor *ARF1* silencing had any effect on TMV-GFP or PVX-GFP infection, since it did not cause changes in GFP expression at 15 dpi (Figure 3).

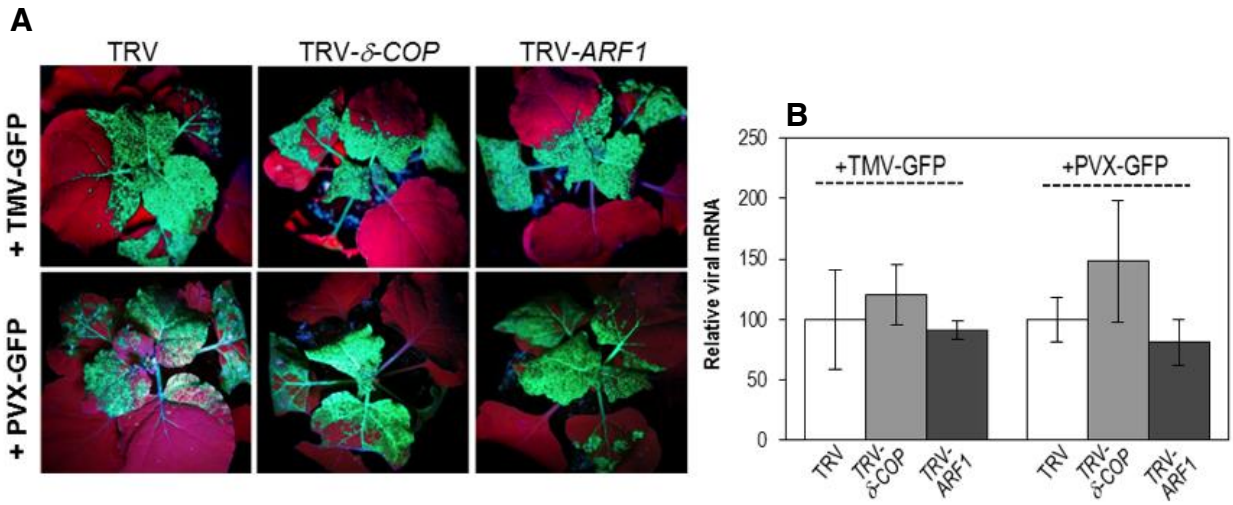


Figure 3. Virus-Induced gene silencing of *δ-COP* and *ARF1* does not affect the infection by RNA viruses. (A) TMV-GFP and PVX-GFP infection in *δ-COP* - or *ARF1*- silenced *N. benthamiana* plants under UV light. **(B)** Relative viral accumulation in apical leaves of TMV-GFP or PVX-GFP-inoculated *δ-COP*- or *ARF1*-silenced plants. *GFP* mRNA levels was determined by real time PCR at 15 dpi using *ITS* gene as endogenous control. Values represent the average of five plants. Bars represent standard error. This experiment was repeated two times with similar results; results from one representative experiment are shown.

In order to test whether δ-COP and ARF1 are required for the infection by *Pto* DC3000 Δ*hopQ1-1*, described previously as pathogenic in *N. benthamiana* (Wei and Wang, 2008), we inoculated *N. benthamiana* plants with the *TRV-δ-COP* and *TRV-ARF1* constructs and TRV as control and at 10 dpi we inoculated the bacteria. Bacterial growth was assessed at 0 and 3 days after inoculation (two independent experiments, 3 plants each). The results show no significant difference between the bacterial growth in the *δ-COP* - or *ARF1*- silenced plants and the TRV control plants (Figure 4).

Taken together, these results indicate that lack of *δ-COP* and *ARF1* affects geminivirus infection specifically, but not other plant-pathogen interactions.

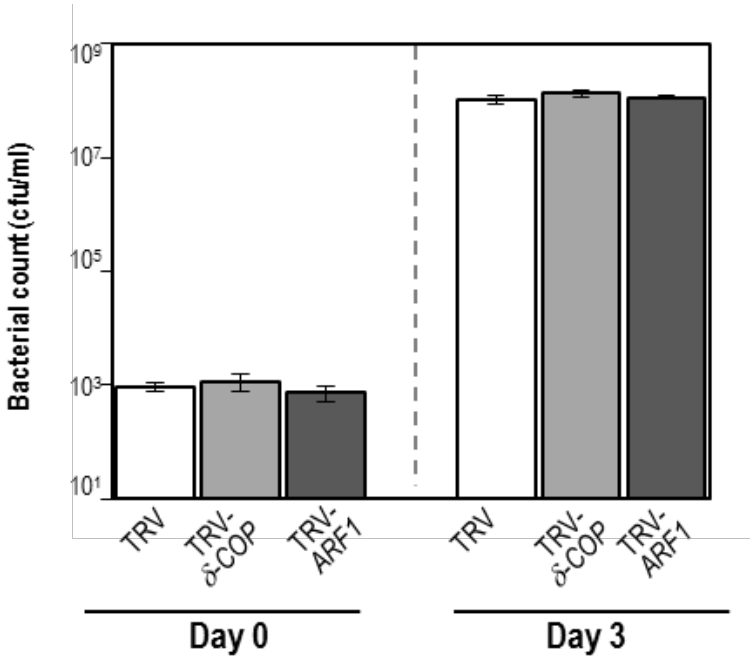


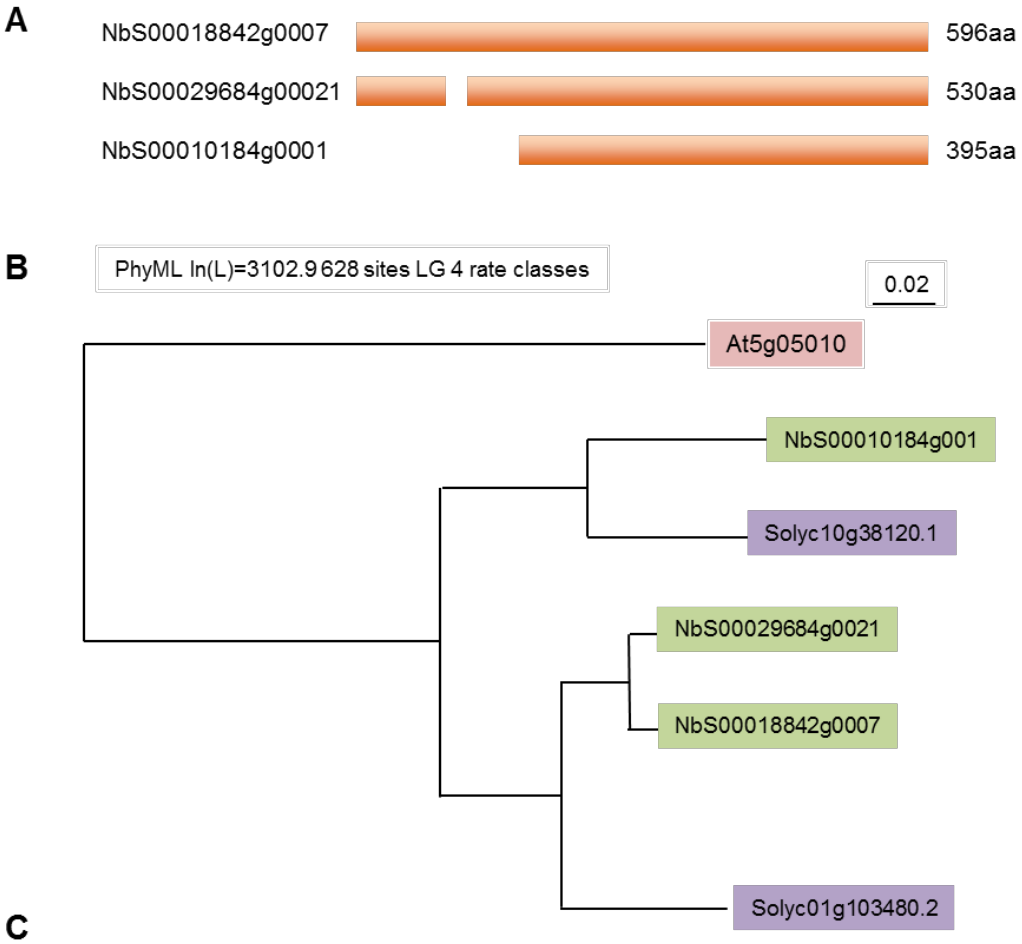
Figure 4. ***Pto*** **DC3000** **Δ*hopQ1-1*** **growth in** ***δ-COP*** **- and** ***ARF1*****-silenced or contro**l **plants**. Ten days after inoculation with the TRV-derived constructs, *N. benthamiana* plants were infiltrated with *Pto* DC3000 Δ*hopQ1-1* 5x10^4 cfu/ml. Bacterial growth was determined at 0 and 3 dpi. Two independent experiments were performed with similar results; the graph represents one of two biological replicates. Values represent the average of three plants. Bars represent standard error.

DISCUSSION

Analysis of *δ-COP* and *ARF1* silencing in *N. benthamiana* plants

Although virus-induced gene silencing (VIGS) has been successfully used for functional genetic studies of plant-geminivirus interactions (Czosnek et al., 2013), one of its limitations lies on the difficulty to predict the specificity of the silencing construct, especially when working with genomes which are not accurately annotated. To address the specificity of the *δ-COP* and *ARF1* silencing constructs in *N. benthamiana,* we carried out a sequence analysis of the fragments used to induce silencing.

In the case of δ-*COP,* we used the *Arabidopsis* gene to generate the VIGS clone, since the *N. benthamiana* gene sequence was not available. To determine if the *Arabidopsis* sequence was capable of silencing the *N. benthamiana* δ-*COP,* we carried out a blast analysis using the *N. benthamiana* draft genome available in the Solgenomics database (Niben.genome.v0.4.4.transcripts; www.solgenomics.net). This analysis reveals three complete cDNAs annotated as Coatomer subunit δ that encompass a sequence highly homologue to the *Arabidopsis* δ-*COP* subunit (*At*δ-*COP*) fragment used (from 84 to 87%). Two of those clones (NbS00018842g0007 and NbS00029684g00021) encoded almost identical proteins (98.3% identity), which show similar length (596 aa and 530 aa) and high identity (73 and 74%) to *At*δ-*COP* (Figure 5 and Supplemental Figure 1). The other clone (NbS00010184g0001) encodes a smaller protein (395aa) that lacks the N-terminus sequence of the protein, since the start codon used by the other two clones is not present in this sequence, and showed 68% identity to the *Arabidopsis* homologue (At5g05010). Interestingly, the *Solanum lycopersicum* genome contains two genes annotated as δ−*COP*, which, as in *N. benthamiana*, encode a large (528 aa) and a short version (328 aa, lacking the N-terminus) of the protein that are highly homologous to the *N. benthamiana* counterparts (92% and 90% respectively). Taking into account this homology and the amphidiploid nature of *N. benthamiana*, it seems that its genome contains two genes encoding a full δ −COP and at least one encoding a truncated version, absent in most plants species but present in other *Solanaceae* species such as tomato (Figure 5 and supplemental Figure 1). Given that all *N. benthamiana* cDNAs contain a sequence showing high identity to the *Arabidopsis* fragment used to silence Nbδ-*COP*, it is expected that the expression of all of them will be impaired in the VIGS experiments. The fact that we detected a reduction in the mRNA accumulation by real-time PCR using primers that match perfectly with sequences identical in all three cDNAs (Supplemental Figure 2) confirms the inhibition of the expression all the putative Nbδ−*COP*.



Sequence	1	2	3	4	5	6
1: Arabidopsis_At5g05010	100.0	74.1	73.7	72.4	68.5	67.9
2: NbS00029684g0021.1	74.1	100.0	98.3	92.5	85.3	85.4
3: NbS00018842g0007.1	73.7	98.3	100.0	92.7	85.1	84.9
4: Solyc01g103480.2.1	72.4	92.5	92.7	100.0	82.7	83.9
5: NbS00010184g0001.1	68.5	85.3	85.1	82.7	100.0	90.3
6: Solyc10g038120.1.1	67.9	85.4	84.9	83.9	90.3	100.0

Figure 5. Sequence analysis of *Coatomer subunit δ* from *N. benthamiana* (*Nbδ-COP*). (A) Three complete cDNAs annotated as *Nbδ-COP*, two clones (NbS00018842g0007 and NbS00029684g00021) encoded almost identical proteins, which show similar length (596 aa and 530 aa), and one cDNA clone (NbS00010184g0001) encodes a smaller protein (395aa) that lacks the N-terminus sequence. **(B)** Phylogenetic tree and **(C)** Percentage of identity among δ-COP protein sequences from the Solanaceae *N. benthamiana* and *S. lycopersicum* and the homologue from *Arabidopsis* (At5g05010). ClustalW alignment was used to generate the table. Sea View (Gouy et al., 2010) was used to perform the phylogenetic analysis and generate the figure.

A similar analysis between the *NbARF1* fragment used for silencing and the *N. benthamiana* draft genome reveals homology with five cDNA clones annotated as *ADP-RIBOSYLATION FACTOR*. Only one of those clones correspond to a complete cDNA (NbS00009707g0011) that encompass a sequence highly similar to the *NbARF1* fragment used (97%). A blast search with the *N. benthamiana* ARF1 protein from the GeneBank database (Acc: ABF74733) and the *N. benthamiana* genome reveals 38 proteins that showed significant homology. Among them, the protein NbS00009707g0011 seems to correspond to the GeneBank sequence (Acc: ABF74733.1) used to design the VIGS vector, since it has the same length (181aa) and showed the highest percentage of identity (96%). Phylogenetic analysis with all *Arabidopsis* ARF1 proteins showed that NbS00009707g0011 (*NbARF1)* is highly homologue to a group ARF1 named ARFA1 than include *AtARFA1A*, previously identify as *AtARF1* (Figure 6), although this is the only member of the ARFA1 group identified to date in *N. benthamiana*. However, taking into account that the ARFA1 group has six gene members in *A. thaliana* (*AtARFA1*a to *AtARFA1f)* and in *S. lycopersium* (Figure 6), it is expected that the *N. benthamiana* genome will contain at least a similar number of genes encoding ARFA1 proteins.

Considering the high identity among all ARFA1 members in the *Arabidopsis* genome, we carried out an analysis using the nucleotide zone with the highest identity (>79%) and length (>185) nt of each *AtARFA1* genes and the *NbARF1* sequence used in the VIGS experiments. As we expected, the *NbARF1* fragment used for silencing has a high homology with all the members of the *AtARFA1* group, suggesting that we have likely silenced all the orthologous *ARFA1* family members in *N. benthamiana* (Supplemental Figure 3). The fact that in a previous report (Gebbie et al., 2005), the expression of antisense of *AtARFA1c* in *Arabidopsis* inhibited all other five members of the group supports the idea that RNA silencing constructs for one of the genes could inhibit the expression of all *ARFA1* genes.

Disruption of the plant retrograde pathway impairs infection by geminiviruses

δ-COP and ARF1 proteins are involved in the COPI-dependent retrograde trafficking pathway. Consistently, the phenotypes observed in *N. benthamiana* upon silencing the genes encoding these proteins were very similar, and resembled those previously described for *ARF1*-silenced *N. benthamiana* and *Arabidopsis* plants (Gebbie et al., 2005; Coemans et al., 2008). The stunted phenotype of these plants can probably be attributed to reduced cell expansion. In addition, reduced cell division was observed in ARF1-suppressed *Arabidopsis* plants (Gebbie et al., 2005), as well as in plants containing a mutation in GNOM, a GEF of ARF1 (Geldner et al., 2003). Although we did not detect any significant differences in the phenotype generated by silencing

ARF1 or *δ-COP*, genetic data suggests that ARF1 could also play an additional role in the biogenesis of the COPII vesicles at ER exit sites (ERES) (Lee et al., 2002). Taking into account that the VIGS *ARF1* construct used in this work is likely to silence the entire *ARFA1* gene family, we cannot rule out the possibility that the *ARF1* silencing could have an additional effect in vesicle transport, beyond that derived from the impairment of the COPI-dependent retrograde pathway.

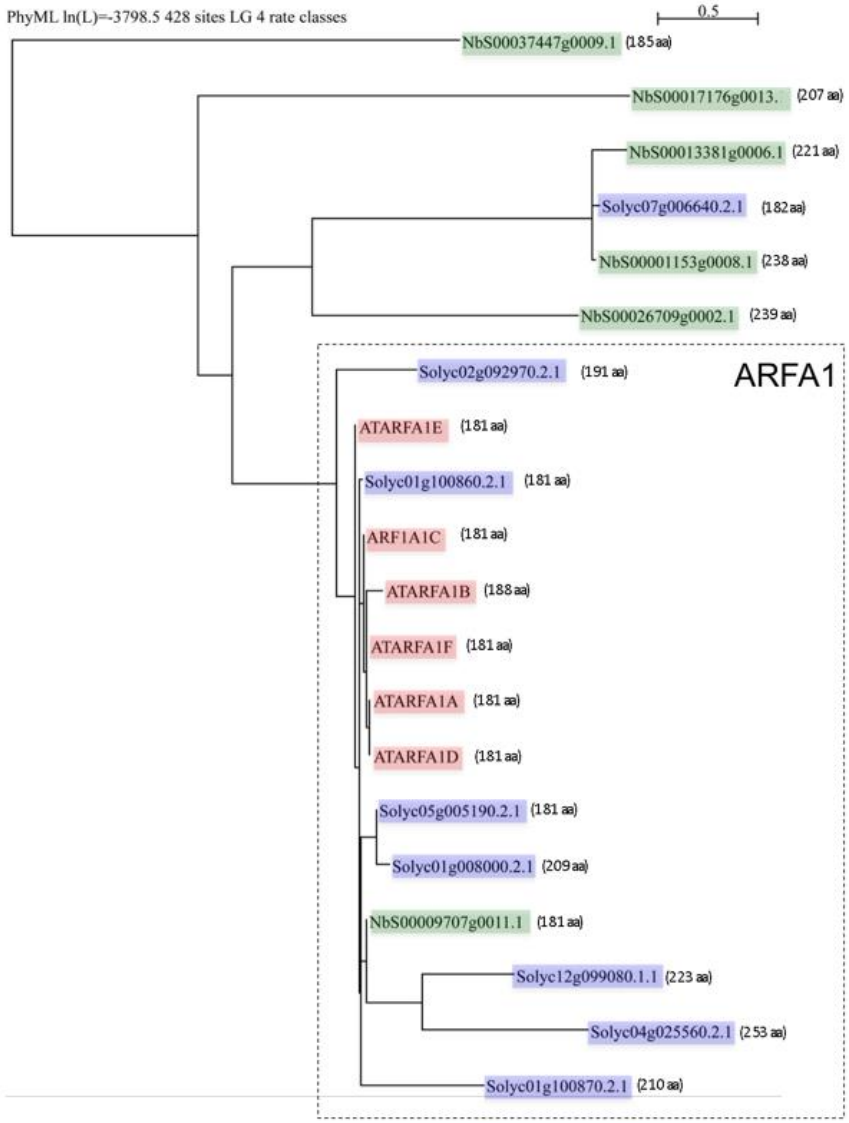


Figure 6. Phylogenetic tree of the ARF1 proteins from *Solanaceae (N. benthamiana and S. lycopersicum)* and the members of group ARFA1 from *Arabidopsis*. Three major clusters are depicted: the first and the second clusters represent the most divergent *N. benthamiana* and *S. lycopersicum* proteins that showed significant homology to ARF1; the third cluster includes proteins from the group ARFA1 from *Arabidopsis* (ARFA1A to ARFA1F), the ARF1 *N. benthamiana* protein (NbS00009707g0011) and seven ARF1 proteins from *S. lycopersicum*. The tree shows that the *N. benthamiana* protein NbS00009707g0011 is the only member which is closely related to the ARF1 proteins. Sea View (Gouy et al., 2010) was used to perform the phylogenetic analysis and generate the figure.

Plant vesicle trafficking pathways are engaged in the plant's immune system to actively defend against potential pathogens (Frei dit Frey and Robatzek, 2009). By contrast, some invasive plant pathogens have evolved means to utilize these trafficking pathways to their benefit (Jiang et al., 2006; Wei and Wang, 2008; Hyodo et al., 2013) or block them in order to suppress immunity and cause disease (Nomura et al., 2006). Our results show that geminiviruses need an intact retrograde transport to complete their infection process, as it was reported previously for some RNA viruses, as *Tomato bushy stunt virus* (TBSV), *Red clover necrotic mosaic virus* (RCNMV) or *Tobacco etch virus* (TEV) (Jiang et al., 2006; Wei and Wang, 2008; Hyodo et al., 2013). Notably, plant RNA viruses replicate in the cytoplasm and promote the formation of virus-induced membrane structures to increase the local concentration of components required for replication and provide a scaffold for tethering the replicase complexes. However, geminiviruses are DNA viruses that replicate in the nucleus. The viral DNA then traffics out of the nucleus bound to the viral NSP (bipartite geminiviruses) or the CP (monopartite geminiviruses) and shuttles to the cytoplasm, where a second viral protein (MP or V2/C4) traps these complexes to direct them to and across the cell wall (Hanley-Bowdoin et al., 2013). We suggest two potential models for the involvement of the retrograde trafficking pathway in the geminivirus cycle: (i) vesicle trafficking may play an important role in the viral movement and therefore in cell-to-cell dispersion of the virus; or (ii) retrograde transport is needed to make functional host or viral proteins required for the infection available.

Two groups reported that ER-tubules and exo/endocytosis are crucial elements for movement of bipartite begomoviruses (Lewis and Lazarowitz, 2010; Krenz et al., 2012). From genetic data, obtained with a dominant-negative Synaptotagmin (SYTA) *Arabidopsis* mutant that causes depletion of plasma membrane-derived endosomes and inhibits cell-to-cell trafficking of *Cabagge leaf curl virus* (CaLCuV), the authors propose a model based on animal viruses, according to which geminiviruses would acquire an envelope from an internal membrane and transit through the remainder of the secretory pathway for release. Although δ-COP and ARF1 are not involved directly in exo/endocytosis, impairment of the retrograde pathway could indirectly alter these processes, since it would block the recycling of proteins essential for the vesicle transport from the ER to Golgi.

In a second possible scenario, virus infection would require host proteins that enter the secretory pathway in the ER and are subsequently transported to the Golgi. Indirect impairment of the anterograde pathway produced by *δ-COP* and *ARF1* silencing would interfere with the protein modifications that are performed in the Golgi. In addition, this negative effect on the viral infection could be due to the inhibition of glycosylation of viral proteins. Although glycosylation of plant viral proteins has been not reported yet, there is large evidence that proteins from animal viruses are glycosylated during the infection and that these modifications are important for viral virulence and

immune evasion (Vigerust and Shepherd, 2007; Rogers and Heise, 2009). VIGS of components of the anterograde transport such as *Sar1, Sec13, Sec23, Sec24* or *Sec31*, will allow determining whether the anterograde transport is, as we hypothesize, also required for the geminivirus infections.

Since we only carried out systemic infections, we cannot determine if the function of retrograde transport is required for viral replication and/or movement. Replication assays in silenced *N. benthamiana* leaves will be useful to determine what infection process is impaired, shedding light on the feasibility of the proposed scenarios.

Interestingly, we detected that the C3 protein from TYLCSV interacts with the δ-COP subunit from *Arabidopsis* in yeast (Hericourt et al., in preparation). Although C3 does not contain any of the described cargo motifs sequences that are located in the C-terminal region of the protein such as KK, KKXX, KXKXX or KDEL (Zerangue et al., 2001; Popoff et al., 2011), it contains a sequence (KYK/NXX) that resemblance those motives, located at the end of the C-terminus and well conserved among begomoviruses. Besides, there are reports of cargo protein transported within COPI vesicles that do not have coatomer interacting motifs (Cosson et al., 1998). Experiments to confirm the interaction between C3 and δ-COP *in planta* and to determine if, as other cargo putative proteins, C3 also interacts with ARF1 are currently in progress. Whether the interaction between C3 and δ-COP has a biological function is still an open question that requires further experiments.

Surprisingly, in the case of the RNA viruses TMV or PVX we did not observe any impact on the expression or accumulation of virus-derived GFP when *δ-COP* and *ARF1* were silenced. These results are in agreement with those previously reported by (Coemans et al., 2008) describing that ARF1 loss-of-function by VIGS did not affect the susceptibility to TMV in *N. benthamiana* plants. However, as mentioned above, there are other works showing that the inhibition of ARF1 or COPI functions reduced the accumulation of plants RNA viruses (TBSV, RCNMV or TEV). In two of those works, the authors used a dominant-negative mutant of ARF1 or Brefeldin A (BFA), a well-known fungal metabolite, to inhibit COPI function (Wei and Wang, 2008; Hyodo et al., 2013). Interestingly, both BFA treatment and expression of the mutant version of *ARF1* in plant cells not only inhibits the COPI pathway but also compromises COPII vesicle trafficking (Lee et al., 2002; Stefano et al., 2006). Therefore, although we cannot rule out the possibility that the results represent differences in the viral requirements for different transport vesicles, the inhibition of viral infection detected in those experiments could be due to direct impairment of COPII-dependent transport from the ER to the Golgi and not of COPI-dependent retrograde transport. In the case of TBSV, Jiang and colleagues (2006) used a yeast replication assay to screen for essential genes and found that down

regulation of COPI suppresses the replication of the tombusviruses. Whether impairment of the COPI complex has the same effect in plants remains to be determined.

Previous data obtained by VIGS of *ARF1* in *N. benthamiana* strongly implicate this gene in the non-host resistance to bacteria, since it severely hampered non-host resistance towards *P. cichorii* but did not alter the susceptibility towards the pathogen *Pto* pv *tabaci*. Our results obtained with other pathogenic pathovar, *Pto* Δ*hopQ1-1*, is in agreement with the previous results, since we did not observe any significant difference between bacterial multiplication in *ARF1*- and *δ-COP*-silenced and control plants.

In conclusion, our data unveil a role of the COPI-dependent retrograde transport, through δ-COP and ARF1, as an essential pathway for geminivirus infection. However, this transport is not involved in a general susceptibility mechanism to pathogens, because there is no change in the susceptibility to plant RNA viruses or pathogenic bacteria when the pathway is disrupted.

EXPERIMENTAL PROCEDURES

Virus Induced Gene Silencing

Virus-induced gene silencing was performed as described in Chapter II. The TRV-*δ-COP* clone was described in Chapter II. The TRV-*ARF1* (pTV:ARF1) construct was generated as described previously by Coemans *et al*., 2008. - In order to evaluate silencing efficiency, total RNA was extracted from silenced *N. benthamiana* leaves at 10 and 15 dpi, and mRNA accumulation was quantified by real-time PCR employing specific primers for *Nbδ-COP* (CCCAAATTGGTTGGTACAGG and GACAGCAGCCTCAGTGTCTC) and *NbARF1* (AATGACAGAGACCGTGTTGTTGA and ACAGCATCCCGAAGCTCATC).

Geminivirus infection assays and detection of viral DNA

Viral infections of *N. benthamiana* plants were performed by the agroinoculation technique as previously described (Elmer et al., 1988). Plants were agroinoculated with plasmid pGreenTYA14 (binary vector containing a partial dimer of TYLCSV-ES[2] (Lozano-Durán et al., 2011a)) or pBIN-BCTV (Briddon et al., 1989) in the axilary bud of the fourth/fifth leaf of 3-week-old wild-type *N. benthamiana* plants. For control, plants were inoculated with an *A. tumefaciens* culture harbouring the empty binary vector pGreen-0229 (Hellens et al., 2000).

For quantification of geminiviral accumulation by quantitative real-time PCR, total plant DNA was extracted from *N. benthamiana* leaves at 15 dpi. TYLCSV CP or the BCTV C4 genes were amplified using primers GGAGGCTGAACTTCGACAGC and GGACTTTCAATGGGCCTTCAC and CTACACGAAGATGGGCAACCT and TGACGTCGGAGCTGGTTTAG, respectively. Quantitative real-time PCR was performed as described below.

RNA virus infection assays

Tobacco mosaic virus (TMV)-GFP and *Potato virus X* (PVX)-GFP were kindly provided by Dr. Peter Moffett and are described elsewhere (Peart et al., 2002). Infections in *N. benthamiana* wild-type plants were performed by agroinoculation adjusting optical density to OD_{600}=0.01. GFP expression was monitored up to 15 dpi and samples were taken. Total RNA was extracted from the three apical leaves of each infected *N. benthamiana* plant. For quantification of PXV-GFP and TMV-GFP, virus-derived GFP expression was assessed by real-time PCR as described below using primers for the GFP (GAGGGATACGTGCAGGAGAG and GATCCTGTTGACGAGGGTGT).

cDNA synthesis and quantitative real-time PCR

Total RNA was extracted using TRIzure (Bioline) and treated with DNase (TaKaRa, Kyoto, Japan). 1µg total RNA was used for first-strand cDNA synthesis using oligo dT-17 primers and SuperScript II reverse transcriptase reagent (Invitrogen) following the manufacturer's instructions.

For quantitative real-time PCR, the reaction mixture consisted of approximately 10 ng total DNA or cDNA, primer mix (10 µM each) and SsoFast™ EvaGreen® Supermix (BIO-RAD) in a total volume of 20 µl. The PCR conditions were: 10 minutes at 95°C, and 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. The reactions were performed using a MyiQ icycler (BIO-RAD). For the quantification of viral amount, specific primers were used as described above; in all cases, the endogenous gene *25S ribosomal DNA interspacer* (*ITS*) was used as the normalizer, with primers ATAACCGCATCAGGTCTCCA and CCGAAGTTACGGATCCATTT. A relative quantification real-time PCR method using the $2^{-\Delta\Delta CT}$ method was used to compare the amount of each viral and endogenous gene (Livak and Schmittgen, 2001).

Bacterial inoculation

The silenced plants were infected with the bacterial strain *Pto* DC3000 Δ*hopQ1-1*, a mutant unable to produce the HopQ1-1 protein and previously described as pathogenic in *N. benthamiana* (Wei et al., 2007). Bacteria were grown at 28ºC in solid LB medium supplemented with cycloheximide (Cx) (2 µg/ml) for 48h, then suspended in 10 mM $MgCl_2$ (OD_{600}= 0.0001, $5x10^4$ cfu/ml) before inoculation. The bacterial cell suspension was thereafter infiltrated into two leaves of *N. benthamiana* plants using a needle-less syringe. Three days after the infection, three small leaf discs (10 mm in diameter) were punched out of the infiltrated areas of six plants. The leaf discs were subsequently ground in 1000 µL of 10 mM $MgCl_2$, and serial dilutions were plated onto LB plates supplemented with Cx (2 µg/ml). Following incubation at 28 °C for 48 h, colonies were counted to determine bacterial growth.

REFERENCES

- Boehm, M., Aguilar, R.C., Bonifacino, J.S., 2001. Functional and physical interactions of the adaptor protein complex AP-4 with ADP-ribosylation factors (ARFs). The EMBO journal 20, 6265-6276.

- Bottcher, B., Unseld, S., Ceulemans, H., Russell, R.B., Jeske, H., 2004. Geminate structures of African cassava mosaic virus. Journal of virology 78, 6758-6765.

- Briddon, R.W., Pinner, M.S., Stanley, J., Markham, P.G., 1990. Geminivirus coat protein gene replacement alters insect specificity. Virology 177, 85-94.

- Briddon, R.W., Watts, J., Markham, P.G., Stanley, J., 1989. The coat protein of beet curly top virus is essential for infectivity. Virology 172, 628-633.

- Brown JK, F.C., Briddon RW, Zerbini M, Moriones E, Navas-Castillo J, 2012. Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses. Academic press, London.

- Coemans, B., Takahashi, Y., Berberich, T., Ito, A., Kanzaki, H., Matsumura, H., Saitoh, H., Tsuda, S., Kamoun, S., Sagi, L., Swennen, R., Terauchi, R., 2008. High-throughput in planta expression screening identifies an ADP-ribosylation factor (ARF1) involved in non-host resistance and R gene-mediated resistance. Molecular plant pathology 9, 25-36.

- Cosson, P., Lefkir, Y., Demolliere, C., Letourneur, F., 1998. New COP1-binding motifs involved in ER retrieval. The EMBO journal 17, 6863-6870.

- Czosnek, H., Eybishtz, A., Sade, D., Gorovits, R., Sobol, I., Bejarano, E., Rosas-Diaz, T., Lozano-Duran, R., 2013. Discovering host genes involved in the infection by the Tomato Yellow Leaf Curl Virus complex and in the establishment of resistance to the virus using Tobacco Rattle Virus-based post transcriptional gene silencing. Viruses 5, 998-1022.

- Elmer, J.S., Sunter, G., Gardiner, W.E., Brand, L., Browning, C.K., Bisaro, D.M., Rogers, S.G., 1988. Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNAs. Plant molecular biology 10, 225-234.

- Frei dit Frey, N., Robatzek, S., 2009. Trafficking vesicles: pro or contra pathogens? Current opinion in plant biology 12, 437-443.

- Gebbie, L.K., Burn, J.E., Hocart, C.H., Williamson, R.E., 2005. Genes encoding ADP-ribosylation factors in Arabidopsis thaliana L. Heyn.; genome analysis and antisense suppression. Journal of experimental botany 56, 1079-1091.

- Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., Jurgens, G., 2003. The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 112, 219-230.

- Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular biology and evolution 27, 221-224.

- Hanley-Bowdoin, L., Bejarano, E.R., Robertson, D., Mansoor, S., 2013. Geminiviruses: masters at redirecting and reprogramming plant processes. Nature reviews. Microbiology 11, 777-788.

- Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S., Robertson, D., 2000. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Critical reviews in biochemistry and molecular biology 35, 105-140.

- Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., Mullineaux, P.M., 2000. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant molecular biology 42, 819-832.

- Hyodo, K., Mine, A., Taniguchi, T., Kaido, M., Mise, K., Taniguchi, H., Okuno, T., 2013. ADP ribosylation factor 1 plays an essential role in the replication of a plant RNA virus. Journal of virology 87, 163-176.

- Jeske, H., 2009. Geminiviruses. Current topics in microbiology and immunology 331, 185-226.

- Jiang, Y., Serviene, E., Gal, J., Panavas, T., Nagy, P.D., 2006. Identification of essential host factors affecting tombusvirus RNA replication based on the yeast Tet promoters Hughes Collection. Journal of virology 80, 7394-7404.

- Kirchhausen, 2000. Three ways to make a vesicle. Nature Reviews Molecular Cell Biology 1, 187-198.

- Konig, R., Stertz, S., Zhou, Y., Inoue, A., Hoffmann, H.H., Bhattacharyya, S., Alamares, J.G., Tscherne, D.M., Ortigoza, M.B., Liang, Y., Gao, Q., Andrews, S.E., Bandyopadhyay, S., De Jesus, P., Tu, B.P., Pache, L., Shih, C., Orth, A., Bonamy, G., Miraglia, L., Ideker, T., Garcia-Sastre, A., Young, J.A., Palese, P., Shaw, M.L., Chanda, S.K., 2010. Human host factors required for influenza virus replication. Nature 463, 813-817.

- Krenz, B., Jeske, H., Kleinow, T., 2012. The induction of stromule formation by a plant DNA-virus in epidermal leaf tissues suggests a novel intra- and intercellular macromolecular trafficking route. Frontiers in plant science 3, 291.

- Krupovic, M., Ravantti, J.J., Bamford, D.H., 2009. Geminiviruses: a tale of a plasmid becoming a virus. BMC evolutionary biology 9, 112.

- Lee, C., Goldberg, J., 2010. Structure of coatomer cage proteins and the relationship among COPI, COPII, and clathrin vesicle coats. Cell 142, 123-132.

- Lee, M.H., Min, M.K., Lee, Y.J., Jin, J.B., Shin, D.H., Kim, D.H., Lee, K.H., Hwang, I., 2002. ADP-ribosylation factor 1 of Arabidopsis plays a critical role in intracellular trafficking and maintenance of endoplasmic reticulum morphology in Arabidopsis. Plant physiology 129, 1507-1520.

- Lewis, J.D., Lazarowitz, S.G., 2010. Arabidopsis synaptotagmin SYTA regulates endocytosis and virus movement protein cell-to-cell transport. Proceedings of the National Academy of Sciences of the United States of America 107, 2491-2496.

- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

- Lozano-Durán, R., Rosas-Diaz, T., Gusmaroli, G., Luna, A.P., Taconnat, L., Deng, X.W., Bejarano, E.R., 2011a. Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes and inhibit jasmonate signaling in Arabidopsis thaliana. The Plant cell 23, 1014-1032.

- Lozano-Durán, R., Rosas-Diaz, T., Luna, A.P., Bejarano, E.R., 2011b. Identification of host genes involved in geminivirus infection using a reverse genetics approach. PLoS One 6, e22383.

- Luna, A.P., Morilla, G., Voinnet, O., Bejarano, E.R., 2012. Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. Molecular plant-microbe interactions : MPMI 25, 1294-1306.

- Mansoor, S., Briddon, R.W., Zafar, Y., Stanley, J., 2003. Geminivirus disease complexes: an emerging threat. Trends in plant science 8, 128-134.

- Matheson, L.A., Hanton, S.L., Rossi, M., Latijnhouwers, M., Stefano, G., Renna, L., Brandizzi, F., 2007. Multiple roles of ADP-ribosylation factor 1 in plant cells include spatially regulated recruitment of coatomer and elements of the Golgi matrix. Plant physiology 143, 1615-1627.

- Moss, J., Vaughan, M., 1991. Activation of cholera toxin and Escherichia coli heat-labile enterotoxins by ADP-ribosylation factors, a family of 20 kDa guanine nucleotide-binding proteins. Molecular microbiology 5, 2621-2627.

- Nagai, H., Kagan, J.C., Zhu, X., Kahn, R.A., Roy, C.R., 2002. A bacterial guanine nucleotide exchange factor activates ARF on Legionella phagosomes. Science 295, 679-682.

- Navas-Castillo, J., Fiallo-Olive, E., Sanchez-Campos, S., 2011. Emerging virus diseases transmitted by whiteflies. Annual review of phytopathology 49, 219-248.

- Nomura, K., Debroy, S., Lee, Y.H., Pumplin, N., Jones, J., He, S.Y., 2006. A bacterial virulence protein suppresses host innate immunity to cause plant disease. Science 313, 220-223.

- Peart, J.R., Cook, G., Feys, B.J., Parker, J.E., Baulcombe, D.C., 2002. An EDS1 orthologue is required for N-mediated resistance against tobacco mosaic virus. The Plant journal : for cell and molecular biology 29, 569-579.

- Poornima Priyadarshini CG, A.M., Tippeswamy R, Savithri HS., 2011. Functional characterization of coat protein and V2 involved in cell to cell movement of Cotton leaf curl Kokhran virus-Dabawali. PLoS One 6 (11) e26929.

- Popoff, V., Adolf, F., Brugger, B., Wieland, F., 2011. COPI budding within the Golgi stack. Cold Spring Harbor perspectives in biology 3, a005231.

- Richards, A.A., Stang, E., Pepperkok, R., Parton, R.G., 2002. Inhibitors of COP-mediated transport and cholera toxin action inhibit simian virus 40 infection. Molecular biology of the cell 13, 1750-1764.

- Rogers, K.M., Heise, M., 2009. Modulation of cellular tropism and innate antiviral response by viral glycans. Journal of innate immunity 1, 405-412.

- Rojas, M.R., Hagen, C., Lucas, W.J., Gilbertson, R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annual review of phytopathology 43, 361-394.

- Rojas, M.R., Jiang, H., Salati, R., Xoconostle-Cazares, B., Sudarshana, M.R., Lucas, W.J., Gilbertson, R.L., 2001. Functional analysis of proteins involved in movement of the monopartite begomovirus, Tomato yellow leaf curl virus. Virology 291, 110-125.

- Stefano, G., Renna, L., Chatre, L., Hanton, S.L., Moreau, P., Hawes, C., Brandizzi, F., 2006. In tobacco leaf epidermal cells, the integrity of protein export from the endoplasmic reticulum and of ER export sites depends on active COPI machinery. The Plant journal : for cell and molecular biology 46, 95-110.

- Tai, A.W., Benita, Y., Peng, L.F., Kim, S.S., Sakamoto, N., Xavier, R.J., Chung, R.T., 2009. A functional genomic screen identifies cellular cofactors of hepatitis C virus replication. Cell host & microbe 5, 298-307.

- Thompson, J.A., Brown, J.C., 2012. Role of Coatomer Protein I in Virus Replication. Journal of virology & antiviral research 1.

- Varsani, A., Navas-Castillo, J., Moriones, E., Hernandez-Zepeda, C., Idris, A., Brown, J.K., Murilo Zerbini, F., Martin, D.P., 2014. Establishment of three new genera in the family Geminiviridae: Becurtovirus, Eragrovirus and Turncurtovirus. Archives of virology 159, 2193-2203.

- Vigerust, D.J., Shepherd, V.L., 2007. Virus glycosylation: role in virulence and immune interactions. Trends in microbiology 15, 211-218.

- Wang, J., Wu, Z., Jin, Q., 2012. COPI is required for enterovirus 71 replication. PLoS One 7, e38035.

- Ward, B.M., Medville, R., Lazarowitz, S.G., Turgeon, R., 1997. The geminivirus BL1 movement protein is associated with endoplasmic reticulum-derived tubules in developing phloem cells. Journal of virology 71, 3726-3733.

- Wei, C.F., Kvitko, B.H., Shimizu, R., Crabill, E., Alfano, J.R., Lin, N.C., Martin, G.B., Huang, H.C., Collmer, A., 2007. A Pseudomonas syringae pv. tomato DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant Nicotiana benthamiana. The Plant journal : for cell and molecular biology 51, 32-46.

- Wei, T., Wang, A., 2008. Biogenesis of cytoplasmic membranous vesicles for plant potyvirus replication occurs at endoplasmic reticulum exit sites in a COPI- and COPII-dependent manner. Journal of virology 82, 12252-12264.

- Xu, J., Scheres, B., 2005. Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. The Plant cell 17, 525-536.

- Zerangue, N., Malan, M.J., Fried, S.R., Dazin, P.F., Jan, Y.N., Jan, L.Y., Schwappach, B., 2001. Analysis of endoplasmic reticulum trafficking signals by combinatorial screening in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 98, 2431-2436.

- Zhang, J., Dong, J., Xu, Y., Wu, J., 2012. V2 protein encoded by Tomato yellow leaf curl China virus is an RNA silencing suppressor. Virus research 163, 51-58.

- Zhang, S.C., Wege, C., Jeske, H., 2001. Movement proteins (BC1 and BV1) of Abutilon mosaic geminivirus are cotransported in and between cells of sink but not of source leaves as detected by green fluorescent protein tagging. Virology 290, 249-260.

- Zhou, Y., Rojas, M.R., Park, M.R., Seo, Y.S., Lucas, W.J., Gilbertson, R.L., 2011. Histone H3 interacts and colocalizes with the nuclear shuttle protein and the movement protein of a geminivirus. Journal of virology 85, 11821-11832.

Supplemental Figure 1

ClustalW alignment among the δ-COP protein from *Arabidopsis*, *N. benthamiana* and S. *lycopersicum.*

```
A.thaliana_At5g05010          ------------------------------------MVVLAAAIVVKSGKVIVSRHYVDM
NbS00029684g0021.1            ------------------------------------MVVLAASIISKSGKALVSRQFVDM
NbS00018842g0007.1            ------------------MFFFSFLKALNLRFKIQVVVLAASIISKSGKALVSRQFVDM
Solyc01g103480.2.1            MVVL--------------------------------AASIISKSGKALVSRQFVDM
NbS00010184g0001.1            ------------------------------------------------------------
Solyc10g038120.1.1            ------------------------------------------------------------

A.thaliana_At5g05010          SRIRIEGLLAAFPKLVGMGKQHTYIETENVRYVYQPIEALFLLLVTTKQSNILEDLATLT
NbS00029684g0021.1            SRIRIEGYLAAFPKLVGTGKQHTYIETDNVRYVYQPIESLYLLLVTNKQSNILEDLETLR
NbS00018842g0007.1            SRIRIEGYLAAFPKLVGTGKQHTYIETDNVRYVYQPIESLYLLLVTNKQSNILEDLETLR
Solyc01g103480.2.1            SRIRIEGYLAAFPKLVGIGKQHTYIETENVRYVYQPIESLYLLLVTNKQSNILEDLETLR
NbS00010184g0001.1            ------------------------------------------------------------
Solyc10g038120.1.1            ------------------------------------------------------------

A.thaliana_At5g05010          LLSKL-----------------------------------------------VPEYSMS
NbS00029684g0021.1            LLSKL-----------------------------------------------VPEYCHS
NbS00018842g0007.1            LLSKLVSFSPLLIMKLNYPLFLALFISAHRILLLFKRNAFPLLVFSSFFNLQNVPEYSYS
Solyc01g103480.2.1            LLSKL-----------------------------------------------VPEYSYS
NbS00010184g0001.1            ------------------------------------------------------------
Solyc10g038120.1.1            ------------------------------------------------------------

A.thaliana_At5g05010          LDEEGISRASFELIFAFDEVISLGHKESVTVAQVKQYCEMESHEEKLHKLVMQSKINDTK
NbS00029684g0021.1            LDEEGIGSTSFELIFAFDEVISLGHKENVTVTQVKQYCEMESHEERLHKLVLQNKINETK
NbS00018842g0007.1            LDEEGIGSTAFELIFAFDEVISLGHKENVTVTQVKQYCEMESHEERLHKLVLQNKINETK
Solyc01g103480.2.1            LDEEGIGRTAFELIFAFDEVISLGHKENVTVTQVKQYCEMESHEERLHKLVLQNKINETK
NbS00010184g0001.1            ---------------------------------------MESHEEKLHKLVLQSKINETK
Solyc10g038120.1.1            ---------------------------------------MESHEEKLHKLVLQSKINETK
                                                                        ******:*****:*.***:**

A.thaliana_At5g05010          DVMKRKANEIDKSKIEKNK--PGGFSSMGSMGSGRLESGFNE-LSISSGGGGGYGSGSGF
NbS00029684g0021.1            DVMKRKASEIGKSKIEKNRGEKGGFMSLQSMGSGRIDTGFGSDSGISSGGTGGFGSGSGF
NbS00018842g0007.1            DVMKRKASEIGKSKIEKNRGEKGGFMSLQSMGSGRIDTGFGSDSGISSGGTGGFGSGSGF
Solyc01g103480.2.1            DVMKRKASEIDKSKIERNRGDKGGFMSLQSMSSGRIDTGFGSDSGISNIGGNGS---GGF
NbS00010184g0001.1            DVMKRKANEIDKSKIEKNRGEKGGFMSLQSMGSGRIHTGFGSDTNLSSLGGGGFGSDSGL
Solyc10g038120.1.1            DVMKRKASEIDKSKIERNRGEKGGFMSLQSMGSGRMDTGFGSDTNLSSLGGSG----SGF
                              *******.** *****:*:   *** *: **.***:.:** .   :*. *  *    .*:

A.thaliana_At5g05010          GMISDVDPINTKPKDRSRSSVTAPPKSSGMKLGKSGK-NQLMESLKA-EGEDVIEDVKP-
NbS00029684g0021.1            GLSPDVDTFSTKSKGRPAASATAPPKGLGMQLGKNQKTNQFLESLKA-EGEVIVEDVRPS
NbS00018842g0007.1            GLSPDVDTFSTKSKGRPAASATAPPKGLGMQLGKNQKTNQFLESLKAEEGEVIVEDVRPS
Solyc01g103480.2.1            ALPPDVDTFSTKSKGRPAASATAPPKGLGMQLGKTQKTNQFLESLKA-EGEVIVEDVRPS
NbS00010184g0001.1            GPSTDLDSFSTKSKGRPAASATAPPKGLGMQLGKTQRANQFLESLKA-EGEVIVEDVRPS
Solyc10g038120.1.1            GPSTDVDSFSTKSKGRPVASATGPPKGLGMQLGKTQRTNQFLQSLKA-EGEVIVEDVRPS
                              .   ::* :.** * *  :*.*.***. **:***. : **:::**** *** ::***:*

A.thaliana_At5g05010          TGQSKAAAPPPTDPFTLTVEEKLNVALRRDGGLSSFDMQGTLSLQILNQEDGFVQVQIAT
NbS00029684g0021.1            IGQAKPAAAPLTDPVTLTVEEKINVTLKRDGGVSNFDVQGTLSLQILNQEDAFIQVQIET
NbS00018842g0007.1            IGQAKPAAAPLTDPVTLTVEEKINVTLKRDGGVSNFDVQGTLSLQILNQEDAFIQVQIET
Solyc01g103480.2.1            VGQAKPAAAPLTDPVTLTVEEKINVTLKRDGGLSNFVVQGTLSLQILNQEDAFIQVQIET
NbS00010184g0001.1            IGQSKPAAPPPTDPVTLTVEEKINVTLKRDGGIGNFDVQGTLSLQILNQEDGHIQVQVET
Solyc10g038120.1.1            IGPSKPPAPPPTDPVTLTIEEKINVTLKRDGGISNFNVQGTLSLQILNQEDGLIQVQIET
                               * :*  * * ***.***:***:**:*:****:..* :*************. :***: *
```

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A.thaliana_At5g05010            GENPEILFKTHPNINRDMFNNENILGLKRPDQPFPTGQGGDGVGLLRWRMQRADESMVPL
NbS00029684g0021.1              SGNPTILFKTHPNMNKELFSNENILGLKDPNRPFPTGQGGDGVSLLRWRMQTADESILPL
NbS00018842g0007.1              SGNPAILFKTHPNMNKELFSNENILGLKDPNRPFPTGQGGDGVCLLRWRMQTADESILPL
Solyc01g103480.2.1              SGNPAILFKAHPNMNKELFANENILGLKDPNRSFPTGQGGDGVSLLRWRMQSTDESILPL
NbS00010184g0001.1              SGNPAILFKTHPNINKELFSKENILGLKDPNRPFPANQSGDGVNLLKWRMQSADESILPL
Solyc10g038120.1.1              SGNPAIHFNTHPNINKELFSNENILGLKEPSRPFPANQSGDGVSLLRWRMQSADESILPL
                                . ** * *::***:*:::* :******* *.: **: *.**** **:**** :***::**

A.thaliana_At5g05010            TINCWPSVSGNETYVSLEYEASSMFDLTNVIISVPLPALREAPSVRQCDGEWRYDPRNSV
NbS00029684g0021.1              TINCWPSVSGSETYVNIEYETPAQIDLQNVVISVPLPALREAPNVQQIDGEWRYDPRNSV
NbS00018842g0007.1              TINCWPSVSGSETYVNIEYETPAQIDLQNVVISVPLPALREAPNVQQIDGEWRYDPRNSV
Solyc01g103480.2.1              TINCWPSVSGSETYVNIEYETPAQIDLQNVVISVPLPALREAPNVQQIDGEWRYDSRNSV
NbS00010184g0001.1              TINCWPSVSGNETYVNIEYETPAQIDLQNAVISVPLPALREVPRIQQIDGEWRYDSRNAV
Solyc10g038120.1.1              TINCWPSVSGNETYVNIEYETPAQTDLQNVVIFVPLPALRDAPRIQQIDGEWRYDSRNSV
                                **********.****.:***: :  ** *.:* *******:.* ::* ******* **:*

A.thaliana_At5g05010            LEWSILLIDNSNRSGSMEFVVPPVDSSVFFPISVQFAATSTYSGLKVTGMIPLRGGGGAT
NbS00029684g0021.1              LEWSVLLIDNSNRSGALEFVVPAADPSVFFPISARFTASRTFSDLKVVNILPIKG--GPT
NbS00018842g0007.1              LEWSVLLIDNSNCSGVLEFVVPAADPSVFFPISARFTASRTFSDLKVVNILPIKG--GPT
Solyc01g103480.2.1              LEWSVLLIDNSNRSGSLEFVVPAADPSAFFPISAQFTSSRTFSDVKVVNVLPLKG--GAT
NbS00010184g0001.1              LEWSIVLIDNSNRSGSLEFVVPAADPDVFFPISTRFTASRTFSDLKVANILPLKG--GSA
Solyc10g038120.1.1              LEWSVVLIDNSNRSGSLEFVVPAADPDVFFPISARFTASRTFSDLKVANILPLKG--GSP
                                ****::****** ** :***** .* ..*****.:*::: *:* :**. ::*::*  *

A.thaliana_At5g05010            PRFVQRTQLIAQNYQVI
NbS00029684g0021.1              PKHSQRTQLATETYQVV
NbS00018842g0007.1              PKHSQRTQLSTETYQVV
Solyc01g103480.2.1              PKHSQRTLLATESYQVV
NbS00010184g0001.1              PKFFQRMLLATENYHVV
Solyc10g038120.1.1              PKFSQRTLLASENYQVV
                                *:. **  * ::.*:*:
```

Supplemental Figure 2

Alignment showing the position of primers used to quantify transcripts level of *Nbδ-COP* by real-time PCR

```
                                →
                    Nbδ-COP Fw  CCCCAAATTGGTTGTTACAGG
NbS00018842g0007.1  ATCTTGCAGCTTTCCCCAAATTGGTTGGTACAGGAAAGCAGCATACATAT 195
NbS00029684g0021.1  ATCTTGCAGCTTTCCCCAAATTGGTTGGTACAGGAAAGCAGCATACATAT 380
NbS00010184g0001.1  ATCTTGCAGCCTTCCCCAAATTGGTTGGAACAGGAAAGCAACATACATAT 307


NbS00018842g0007.1  ATTGAGACTGACAATGTGCGATATGTTTATCAACCGATAGAATCTTTGTA 245
NbS00029684g0021.1  ATTGAGACTGACAATGTGCGATATGTTTATCAACCGATAGAGTCTTTGTA 430
NbS00010184g0001.1  GTTGAGACTGAAAACGTGCGTTATGTTTATCAGCCGATTGAATCTCTATA 357

                                                                GAGA
NbS00018842g0007.1  CTTGCTACTTGTGACCAACAAACAGAGCAACATTCTTGAAGATCTGGAGA 295
NbS00029684g0021.1  CTTGCTACTTGTGACCAACAAACAGAGCAACATTCTTGAAGATCTTGAGA 480
NbS00010184g0001.1  CTTGCTGCTTGTGACCAACAAACAGAGCAACATTCTTGAAGATTTAGAGA 407
                                ←
                    CACTGAGGCTGCTGTC Nbδ-COP Rv
NbS00018842g0007.1  CACTGAGGCTGCTGTCTAAA 315
NbS00029684g0021.1  CACTGAGGCTGCTGTCTAAA 500
NbS00010184g0001.1  CACTGAGGCTGCTGTCTAAA 427
```

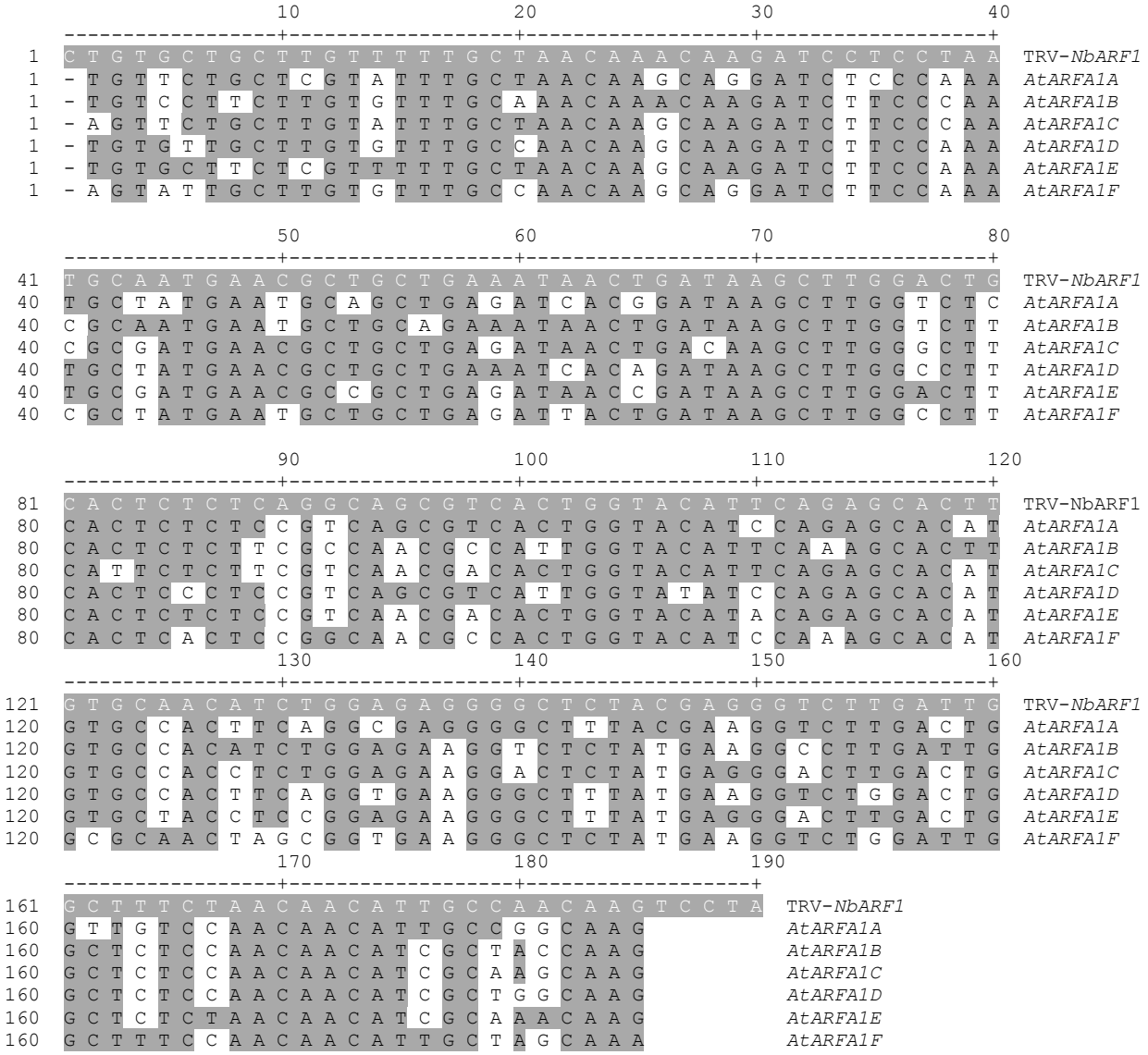
Supplemental Figure 3

(A) Dot-Plot among *NbARF1* used to silence and all members of AtARF1A group. **(B)** Alignment report of *AtARFA1* members and the target used for TRV-*NbARF1* construct. Grey boxes indicate the 100% identity nucleotides between both sequences. (Analysis was performed using DNAstar MegAling Sowfware).

A)

***AtARFA1* (nt)**	***NbARF1* (nt)**	**Identity (%)**	**Length (nt)**
A.356>545	1>190	82.1	190
B.356>540	1>185	84.3	185
C.356>545	1>190	82.6	190
D.356>540	1>185	81.6	185
E.356>545	1>190	84.7	190
F.356>546	1>191	79.1	191

B)



CONCLUSIONS

1. Transcriptional analysis of transgenic *Arabidopsis* plants expressing TYLCSV C2 unveils several cellular processes altered by this viral protein. Among them, the response to jasmonates, which is repressed by C2, is the most affected functional category.

2. Transcriptional analysis of transgenic *Arabidopsis* plants expressing TYLCSV C2 upon exogenous jasmonate treatment points at a C2-mediated repression of specifically certain JA-induced responses; therefore, it is doubtful that this suppression can be through the inhibition of ubiquitin E3-ligase SCF^{COI1}.

3. Exogenous JA treatment has a negative effect over geminivirus infection in *Arabidopsis*, and this effect is independent of the SA-JA crosstalk.

4. C2 interacts specifically with AtJAZ8 in yeast and in *planta*. AtJAZ8 seems to be destabilized by C2 *in planta*.

5. Overexpression of AtJAZ8 exerts a negative effect on the geminivirus infection in *Arabidopsis*.

6. Activation of JA signalling promotes TYLCSV infection in tomato. This effect is opposite to the one observed in *Arabidopsis*, and correlates with a lack of interaction between C2 and the AtJAZ8 orthologues in tomato SlJAZ9, 10 and 11 .

7. C2 from TYLCSV may specifically interfere with the JA response at multiple levels in a host-dependent manner.

8. The transgenic 2IRGFP *N. benthamiana* plants, used in combination with virus-induced gene silencing (VIGS), entail an important potential as a tool in reverse genetics studies to identify host factors involved in TYLCSV infection.

9. Silencing of candidate host genes followed by TYLCSV infection in 2IRGFP *N. benthamiana* plants identified eighteen genes potentially involved in this process, fifteen of which had never been implicated in geminiviral infections before.

10. An intact retrograde trafficking is required for a full geminivirus infection. Gene silencing of two of the main components of this pathway, δ-COP and ARF1, severely impairs TYLCSV and BCTV infection in *N. benthamiana,* but does not affect other plant-pathogen interactions.

CONCLUSIONES

1. El análisis transcriptómico de las plantas transgénicas de *Arabidopsis* que expresan C2 de TYLCSV revelan múltiples alteraciones en procesos celulares causados por ésta proteína viral. Entre los procesos más destacados están la represión de la respuesta a jasmonatos a metabolismo secundario.

2. El análisis transcriptómico de las plantas transgénicas de *Arabidopsis* que expresan C2 de TYLCSV tratadas con jasmonato exógeno, puntualiza que la represión causada por C2 es a través la respuesta específica de genes inducidos por JA; por lo tanto es dudoso que ésta inhibición sea a través de la inhibición de la E3-ligasa SCF^{COI1}.

3. El tratamiento exógeno con JA tiene un efecto negativo sobre la infección por geminivirus en *Arabidopsis*, y éste efecto es independiente del antagonismo SA-JA.

4. C2 interacciona específicamente con AtJAZ8 *in planta* y en levaduras. Además, C2 parece que desestabiliza a AtJAZ8 *in planta*.

5. La sobre-expresión de JAZ8 tiene un efecto negativo sobre la infección de los geminivirus en *Arabidopsis*.

6. El tratamiento exógeno con JA promueve la infección de TYLCSV en tomate. El efecto es opuesto al observado en *Arabidopsis,* y correlaciona con el resultado de la no interacción entre C2 y los ortólogos de AtJA8 en tomate SlJAZ9, 10 y 11.

7. C2 de TYLCSV puede interferir específicamente con la respuesta de JA a diferentes niveles de forma dependiente del hospedador.

8. Las plantas transgénicas de *N. benthamiana* 2IRGFP usadas en combinación con el silenciamiento inducido por virus (VIGS), son una herramienta de gran utilidad en estudios de genética reversa para poder identificar factores del hospedador involucrados con la infección de TYLCSV.

9. El silenciamiento de genes candidatos del hospedador que alteran la infección de TYLCSV, permitió la identificación de dieciocho genes que están involucrados en el proceso de infección, donde quince de ellos no se habían descrito hasta la fecha para la infección por geminivirus.

10. Los geminivirus requiere un sistema de transporte retrógrado activo. El silenciamiento génico de dos de los principales componentes de ésta ruta, δ-COP y ARF1, afecta negativamente la infección de TYLCSV y BCTV, pero no altera la interacción con otros patógenos de planta.

RESUMEN

INTRODUCCIÓN Y OBJETIVOS

La gran mayoría de los virus que infectan plantas poseen un genoma de ARN de cadena positiva, si bien existen algunas familias que presentan un genoma de ADN. Lo que contrasta con los virus que infectan procariotas, vertebrados e invertebrados, que en su mayoría tienen un genoma de ADN. Hasta el momento se han descrito tres familias de virus de plantas de ADN: (i) *Caulimoviridae*, con un genoma de ADN de doble cadena, (ii) *Nanoviridae* y (iii) *Geminiviridae*, ambos con un genoma de ADN de cadena simple que forma una pequeña molécula circular. Si se midiese el éxito de un virus desde una perspectiva humana, en términos del impacto económico que producen, los miembros de estas tres familias de virus de plantas con genomas de ADN podrían considerarse como muy exitosos, particularmente los geminivirus.

Las enfermedades de plantas asociadas a geminivirus eran ya bien conocidas a principios del siglo XX debido a su impacto económico en los cultivos, y ya por entonces se asoció su dispersión a una serie de insectos vectores. La etiología de muchas de estas enfermedades sigue siendo desconocida, debido sobre todo al pequeño tamaño de estos virus y a su asociación específica a floema. El incremento en el número y distribución de insectos vectores, la creciente dependencia de los monocultivos, y el mayor movimiento global de plantas, convirtieron a las enfermedades producidas por geminivirus en un problema de primer orden para las cosechas de las regiones tropicales y subtropicales. Este problema creciente conllevó la caracterización de los geminivirus como agentes causantes de la enfermedad, y los convirtió en los primeros virus de ADN de cadena simple identificados en plantas.

Los geminivirus son además de ser los mejor caracterizados, porque presentan ciertos atributos que han facilitado su estudio: (i) poseen un genoma de pequeño tamaño (entre 2.5 y 5.0 kb), (ii) su forma replicativa es una molécula circular de ADN de doble cadena, lo cual los hace fácilmente manipulables aplicando métodos estándar de Biología Molecular, y además permite su inoculación en plantas mediante protocolos que prescinden de su insecto vector natural. Estas características han permitido que los geminivirus se hayan empleado como herramienta para estudiar la replicación del ADN y la regulación de la expresión génica, tanto en plantas monocotiledóneas como en dicotiledóneas. Además, como los geminivirus generan replicones extracromosómicos de alto número de copias, su potencial para la expresión de transgenes in planta, así como para el silenciamiento de genes endógenos, ha generado un considerable interés.

Debido a la relevancia que tiene el estudio de los geminivirus, cada vez es más necesario el desarrollo de herramientas que permitan conocer los mecanismos que conllevan al desarrollo de una enfermedad, sea con finalidades básicas y aplicadas; así como también tener un mayor conocimiento de aquéllos factores de la planta hospedadora necesarios para la respuesta y/o defensa contra los geminivirus. Por ello, el objetivo de ésta tesis es: Explorar las interacciones funcionales entre los geminivirus y sus hospedadores. Para abarcar éste objetivo la tesis se dividirá en tres capítulos:

1. Revelar la importancia de la proteína C2 de los geminivirus durante la señalización de jasmonatos y la respuesta de defensa en planta.
2. Llevar a cabo la identificación de genes del hospedador involucrados en la infección por geminivirus usando una aproximación de genética reversa.
3. Analizar el papel que cumple el tráfico vesícula retrogrado durante la infección por geminivirus.

Capítulo I

Los geminivirus son virus de plantas con genomas circulares de DNA de cadena sencilla que infectan numerosas especies de interés agronómico en todo el mundo, provocando cuantiosas pérdidas que pueden llegar hasta el 100% de la cosecha. Los genomas de estos virus están muy reducidos, y codifican sólo 6 u 8 proteínas, dependiendo de la especie. Esta reducción genómica hace que el virus dependa de factores celulares para el desarrollo de la infección y la compleción de su ciclo vital, incluyendo las fases de replicación y tráfico dentro de la célula o en la planta. Dado que los geminivirus precisan proteínas de la planta hospedadora, la identificación de dichas proteínas supondría un importante paso hacia la comprensión del proceso de infección, lo que en último término podría suponer un importante aporte en la lucha contra la enfermedad. La proteína C2 (también conocida como L2, AC2, AL2 o TrAP) es una proteína multifuncional codificada por los geminivirus. En los geminivirus del género *Begomovirus*, C2 actúa como un factor de transcripción necesario para la expresión de genes virales tardíos, y también dispara la transactivación de genes del hospedador. Ambos efectos se producen a través de un mecanismo indirecto, ya que C2 no es capaz de unir DNA de manera específica de secuencia. A la proteína C2 de geminivirus también se le han atribuido una serie de funciones adicionales, como supresión de respuestas de defensa de la planta o inhibición del sileciamiento, tanto post-transcripcional como transcripcional. Las plantas son organismos sésiles que se ven obligados a afrontar constantemente variaciones ambientales y ataques de patógenos. Para poder montar una

respuesta rápida y efectiva ante los cambios ambientales, incluyendo el ataque de patógenos, las plantas dependen extensamente de la plasticidad proteómica. La ubiquitinación es una modificación posttraduccional muy dinámica que controla la mayoría de los eventos de degradación proteica en eucariotas. Este proceso consiste en la conjugación de un pequeño péptido, llamado ubiquitina, a una proteína diana que, en la mayoría de los casos, es degradada por el proteasoma 26S como consecuencia. Según análisis proteómicos y genómicos, en plantas la ubiquitinación compite con la transcripción como el mecanismo regulador principal. La ubiquitinación tiene lugar a través de una cascada enzimática que comprende tres enzimas: una enzima E1 o activadora, una enzima E2 o conjugadora, y una enzima E3 o ligasa de ubiquitina. Esta última enzima, E3 ligasa, es la encargada de unir el sustrato y, por tanto, la que confiere especificidad al proceso. Hay distintas clases de E3 ligasas de ubiquitina en plantas, pero la familia más abundante es la que comprende los complejos Cullin RING Ligasas (CRLs). Entre éstos, el complejo basado en CULINA 1, también denominado SCF (por Skp1/Cullin1/F-box), es el mayor y mejor caracterizado por su papel en numerosos procesos celulares, tales como la respuesta a hormonas, la señalización por luz, o el desarrollo floral. Estos complejos SCF están compuestos por cuatro subunidades: CULINA 1, una proteína homóloga a Skp1 (proteína *Skp-phase kinase-associate*) (llamadas ASKs en *Arabidopsis*), la subunidad RBX1, que contiene el dominio RING, y una proteína con dominio F-box que une el sustrato y, por tanto, determina la proteína que será ubiquitinada. El genoma de *Arabidopsis* codifica más de 700 proteínas F-box, y el genoma de arroz casi el mismo número, lo que sugiere que estos complejos SCF podrían ubiquitinar, potencialmente, un elevado número de sustratos diferentes. Resulta llamativa la expansión de la familia de proteínas F-box en plantas, considerablemente mayor que en otros eucariotas, aunque las razones de este fenómeno aún no están claras.

Se ha demostrado que la ubiquitinación contribuye a la defensa en plantas a múltiples niveles. Numerosos resultados indican que la ubiquitinación tiene un importante papel, específicamente, en la defensa de la planta frente a virus: componentes de la ruta de ubiquitinación modifican su expresión en respuesta a la infección viral; se han descrito interacciones entre proteínas virales y proteínas implicadas en este proceso; en algunos casos concretos, se ha detectado ubiquitinación de proteínas virales; el silenciamiento del complejo CSN compromete la resistencia a *Tobacco mosaic virus* (TMV) mediada por gen N en *N. benthamiana*; SGT1 es necesario para que se produzca necrosis y limitación del crecimiento viral frente a *Plantago asiatica mosaic virus* (PlAMV) y *Potato virus X* (PVX); la proteína F-box ACIF se necesita para la resistencia y la HR disparada por TMV. Además, se han descrito casos de usurpación viral de la maquinaria de ubiquitinación, y más específicamente de complejos SCF: la proteína P0 de polerovirus contiene un dominio F-box que permite su incorporación a un complejo SCF para mediar la degradación de AGO1,

modulando el silenciamiento génico; la proteína Clink de nanovirus también contiene un dominio F-box, y puede unir SKP1 y la proteína de ciclo celular pRBR (homóloga a retinoblastoma de animales), afectando a la regulación del ciclo celular en la planta, lo que favorecería su propia replicación. Los jasmonatos son un grupo de hormonas vegetales que desempeñan un papel crucial en la defensa de la planta frente a diversos patógenos. La señalización por jasmonatos depende del complejo SCF^{COI1}, que actúa a modo de receptor hormonal. Actualmente no existe demasiada información disponible acerca del papel de los jasmonatos en interacciones planta-virus, pero algunos trabajos recientes indican que dichas interacciones son un tema emergente en el estudio de virus vegetales: la estimulación de la producción de ácido jasmónico en maiz tolerante al virus to *Maize rough dwarf virus-Río Cuarto* sugiere que el alto contenido foliar y en raíz de jasmonatos podría estar ligado a la tolerancia a la enfermedad; la señalización por jasmonatos está implicada en la defensa temprana frente a *potato virus* Y^{NTN}; el silenciamiento de *COI1* compromete la resistencia a TMV mediada por gen N; la infección por el geminivirus *Cabbage leaf curl virus* (CaLCuV) dispara una represión de la ruta de señalización de jasmonatos en plantas de *Arabidopsis*; un factor de patogenicidad del geminivirus *Tomato yellow leaf curl China virus* (TYLCCNV) provoca la represión de determinados genes de respuesta a jasmonatos; la infección con *Tobacco etch virus* (TEV) causa una represión de la respuesta a esta hormona.

Las plantas C2 muestran múltiples fenotipos derivados de la interferencia con el funcionamiento de complejos SCF. En trabajos previos de nuestro de trabajo se demostró, por primera vez, que la proteína C2 interfiere con la ruta de ubiquitinación y con la señalización de jasmonatos y que el tratamiento exógeno con metil-jasmonato interfiere con la infección por geminivirus, por lo que la supresión de la respuesta a jasmonatos por C2 podría suponer un beneficio para el virus, lo que explicaría su conservación en la familia *Geminiviridae*.

El análisis transcripcional de las plantas transgénicas de *Arabidopsis* que expresan la C2 de TYLCSV apoya esta hipótesis, ya que un enriquecimiento funcional de los genes reprimidos en estas plantas muestra una clara represión de las respuestas a jasmonatos y de defensa, entre otros procesos. Además, el 70% de los procesos reprimidos en las plantas C2 aparecen activados en plantas silvestres de *Arabidopsis* tratadas con metiljasmonato, lo que sugiere que la represión de la respuesta a jasmonatos es responsable de la mayoría de efectos causados por C2 en la planta.

Con objeto de estudiar específicamente la interferencia de C2 con la respuesta a jasmonatos en la planta, se llevó a cabo un estudio más detallado de la respuesta de las plantas transgénicas que expresan C2 a esta hormona. Por un lado, se demostró la menor sensibilidad de estas plantas, dependiente de los niveles de expresión de C2, a metil-jasmonato, para las distintas proteínas C2 y para todas las concentraciones de hormona ensayada. Además, y dado que el complejo SCF^{COI1}

no es sólo el receptor para jasmonatos, sino también para la toxina bacteriana coronatina, se determinó la menor sensibilidad de las plantas C2 a esta toxina.

Por otro lado, se realizaron nuevos análisis transcriptómicos comparando plantas transgénicas C2 y plantas silvestres en condiciones basales o tratadas con metil jasmonato. Aún en condiciones basales, las plantas C2 presentan una supresión de la respuesta a jasmonatos y de diversos procesos relacionados con defensa frente a patógenos. Consecuentemente, estas plantas son más susceptibles a las infecciones por el patógeno bacteriano *Pseudomonas syringae pv tomato* DC3000 y por el virus de RNA *Potato virus X* (PVX). Cuando se comparan las plantas C2 y las plantas control tratadas con metiljasmonato a nivel transcriptómico, dos procesos aparecen claramente reprimidos en presencia de C2: procesos relacionados con defensa frente a patógenos y metabolismo secundario disparado por la señalización por jasmonatos. Estos dos son procesos fundamentales activados por la hormona, y ambos son dependientes de COI1. En este trabajo se ha descrito que esta interferencia de C2 con la respuesta a jasmonatos tiene lugar a distintos niveles. Por un lado, tal y como se ha descrito, C2 interfiere parcialmente con el funcionamiento del complejo SCF^{COI1}. Por otro lado, C2 interacciona con proteínas JAZ en levaduras y *en planta*. Una hipótesis interesante sería que los geminivirus no sólo están interfiriendo con la ubiquitinación en la planta hospedadora, sino redirigiéndola. C2 u otras proteínas virales podrían, por tanto, estar promoviendo la expresión o la sobreexpresión de determinadas proteínas F-box de la planta, llevando a la degradación de ciertos sustratos potencialmente perjudiciales para la infección por el geminivirus, como podrían ser reguladores positivos de la respuesta de defensa o reguladores negativos del ciclo celular. En última instancia, proponemos que geminivirus C2 podrían estar interfiriendo con la respuesta jasmonato en varios niveles.

Capítulo II

El género *Begomovirus* de la familia *Geminiviridae* incluye a miembros que son transmitidos por la mosca blanca, infectan plantas dicotiledóneas, y puede tener cualquiera de los dos genomas bipartitos o monopartitos. *Tomato yellow leaf curl Sardinia virus* (TYLCSV) es un miembro del género *Begomovirus*, y es uno de los agentes causales de la enfermedad del rizado amarillo del tomate (TYLCD). TYLCSV tiene un genoma monopartito de 2,8 kb de tamaño, que codifica seis proteínas y contiene una región intergénica (IR) que comprende el origen de replicación y los promotores virales. Los marcos de lectura abierta (ORFs) en la orientación de sentido complementaria codifican una proteína de replicación asociado (Rep, también conocido como C1), una proteína activador transcripcional (TRAP, también conocida como C2), y una proteína de potenciador de la replicación (REn, también conocida como C3); una pequeña ORF, C4, se

encuentra dentro de la Rep ORF pero en un marco de lectura diferente. La cadena virión contiene dos ORFs que codifican la proteína de cubierta (CP) y una pequeña proteína denominada V2.

Para establecer una infección exitosa, los virus deben crear un entorno adecuado para la propagación viral, que implica el secuestro de la maquinaria celular para las funciones virales y, al mismo tiempo, evitar o contrarrestar los mecanismos de defensa de la planta. Para cumplir estos requisitos, las proteínas virales provocan cambios en la célula en todos los niveles: transcripcional, traduccional y post-raduccional. La identificación de los genes de acogida que participan en la replicación viral, el movimiento, y en general todos aquellos procesos que conducen a la creación de una infección exitosa, podría proporcionar nuevos objetivos valiosos para generar en última instancia, la resistencia viral.

Los avances en tecnologías de alto rendimiento y la bioinformática han hecho posible evaluar la expresión génica de forma masiva, proporcionando una visión de las respuestas de la transcripción del huésped a las infecciones virales en una moda en todo el genoma. Estos estudios de transcriptómica, junto con los estudios proteómicos, están proporcionando una visión sin precedentes "del lado del hospedardor" de la interacción planta-virus, lo que lleva a la identificación de genes de la planta hospedadora cuya función o expresión se ve alterada como consecuencia de la infección. Los geminivirus también han sido recientemente objeto de este tipo de estudio, los genes expresados diferencialmente del hospedador revelando ya sea durante la infección o después de la expresión de una proteína viral. Sin embargo, a pesar de que toda esta información esté disponible, sigue siendo una tarea de grandes proporciones para determinar el papel exacto de estos genes del huésped en el proceso de infección. Particularmente es difícil en el caso de los geminivirus monopartitas, en el que la sustitución de genes con genes marcadores no es factible, y por lo tanto son más tediosos de monitorear.

En nuestro laboratorio se han generado plantas transgénicas de *Nicotiana benthamiana* denominadas 2IRGFP. Estas plantas contienen un cassette de expresión de la GFP bajo el control del promotor 35S flanqueado por dos repeticiones directas de la región intergénica del genoma de TYLCSV. Cuando estas plantas son infectadas con TYLCSV presentan una sobreexpresión de la GFP dependiente de la actividad de la proteína viral REP, que dispara la formación de replicones mGFP. La acumulación de GFP actúa como marcador de la replicación de TYLCSV, permitiendo la detección y seguimiento de este proceso de manera rápida, sencilla, semi-cuantitativa y a tiempo real. En consecuencia, las plantas 2IRGFP son una herramienta inestimable para el estudio de la dinámica de infección de TYLCSV. Además, en combinación con una técnica de silenciamiento como el silenciamiento génico inducido por virus (VIGS), las plantas 2IRGFP son una poderosa herramienta en estudios de genética reversa dirigidos a la identificación de genes de la planta necesarios para la infección viral. Aunque la viabilidad de este enfoque se confirmó previamente

por silenciar el antígeno nuclear celular proliferante (PCNA) y SUMO conjugación enzima (SCE1) genes, su uso en una proyección más grande requiere una optimización de las condiciones.

En la presente tesis doctoral se ha realizado una descripción detallada de la dinámica de infección de TYLCSV en plantas 2IRGFP, siguiendo la sobreexpresión de GFP en la planta a lo largo del tiempo, y confirmando estos datos con hibridaciones moleculares para determinar la acumulación de replicones mGFP así como la acumulación viral. Los máximos niveles de replicación viral se detectaron entre los 14 y los 35 días post-inoculación (dpi); dentro de este intervalo, el virus también se está replicando activamente en las raíces. El silenciamiento génico inducido con vectores virales basados en TRV puede utilizarse con éxito en plantas 2IRGFP, y no modifica la infección por TYLCSV sustancialmente. Por tanto, es factible utilizar esta herramienta para realizar una búsqueda de genes de la planta requeridos para la infección viral mediante genética reversa, siguiendo una aproximación de genes candidatos. Con esta idea, se realizó una lista de genes candidatos siguiendo tres diferentes criterios:

(i) Genes que codifican proteínas para las que se ha descrito una interacción con proteínas virales.

(ii) Genes expresados exclusiva o preferencialmente en floema, tejido al que están limitado TYLCSV y otros geminivirus. (iii) Genes descritos como transactivados por la proteína viral AC2.

(iv) Genes implicados en procesos celulares potencialmente necesarios en la infección por geminivirus (como por ejemplo replicación de DNA).

Los genes candidatos se sometieron a un subsiguiente paso de selección, basado en el grado de conservación de los mismos en distintas especies. Aquellos genes que se consideraron como conservados, 54 en total, se analizaron en el programa *Invitrogen Block-iT™ RNAi designer*, y para cada uno se seleccionó una región entre 300 y 500 pares de bases que potencialmente reuniese las condiciones teóricas óptimas para disparar silenciamiento. Estos fragmentos se clonaron en el vector de silenciamiento basado en TRV.

De acuerdo con el efecto de su silenciamiento sobre la infección TYLCSV, medida como tiempo de aparición y la intensidad de la expresión de GFP, se agruparon los genes del huésped en tres clases: aquellos cuyo silenciamiento no causó cambios en la expresión (grupo A), o aquellos cuya silenciamiento adelantó (grupo B) o por el contrario retrasó o llegó a ser nula (grupo C) la expresión de GFP (Tabla 1; ejemplos de cada clase se muestran en la Figura 8).

Genes representativos pertenecientes a los grupos A (*SKL2, ECR1*), B (*UBA1, GLO1 y RPA32*) y C (*HSC70, ASK2, y deltaCOP*) fueron escogidos para evaluar el impacto de su silenciamiento sobre la infección de TYLCSV, medida como la acumulación de ADN viral. Para este fin, las plantas de *N. benthamiana* 2IRGFP fueron co-inoculadas con los clones derivados TRV y TYLCSV. Los valores medios de acumulación TYLCSV se corroboran lo observado previamente con los datos de la sobreexpresión de GFP, el silenciamiento de *UBA1* o *GLO1* y silenciamiento de

RPA32 triplicó y duplicó acumulación TYLCSV, respectivamente. Por otro lado, el silenciamiento de *HSC70* y *ASK2* redujo la acumulación TYLCSV por 70 y 30%, respectivamente. Sorprendentemente, el silenciamiento de la subunidad *deltaCOP* del complejo COPI (δ-COP) anuló por completo la acumulación TYLCSV.

En resumen, hemos identificado 18 genes implicados en varios procesos celulares cuyos silenciamiento altera infección TYLCSV. En particular, 15 de estos genes son descritos por primera vez como factores implicados en infecciones virales. Por lo tanto, nuestros resultados proporcionan nuevos conocimientos sobre los mecanismos moleculares que subyacen a las infecciones por geminivirus, y al mismo tiempo revelan el sistema 2IRGFP/ VIGS como una poderosa herramienta para los estudios de genética funcional reversa.

Capítulo III

Éste capítulo se inició por el sorprendente hallazgo que se obtuvo en el capítulo II, donde observamos que silenciamiento del gen que codifica para subunidad delta del complejo de coatomero COPI δ-COP imposibilita por completo la infección de TYLCSV en plantas de *N. benthamiana*. Además, sumado a que trabajos previos de nuestro grupo identificaron en sistemas de doble híbrido en levaduras que que la proteína C3 (REn) de TYLCSV interacciona con δ-COP. Cabe destacar que COPI es un componente importante para el tráfico retrógrado que va dirigido desde el aparato de Golgi hacia el retículo endoplasmático (RE), y que el movimiento retrógrado es parte crucial del tráfico de vesículas tanto en plantas como animales. Cada vez existen más evidencias que los virus de RNA como de DNA usurparían/bloquearían el movimiento retrógrado para facilitar su replicación y/o movimiento. Sumando los datos del impacto biológico y molecular de δ-COP sobre TYLCSV con los que contábamos en ése momento, nos planteamos el objetivo de éste capítulo que era determinar la el papel que cumple el tráfico retrógrado en las infecciones por geminivirus.

La mayoría de nuestro conocimiento actual sobre el movimiento de los geminivirus proviene de los *Begomovirus* bipartitos, poco se sabe acerca de las proteínas de movimiento de los geminivirus monopartitos, donde la proteína CP actúa como el NSP, mientras que la función de MP está mediada por V2 solo o en un complejo con C4. Se sabe que NSP de *Bean dwarf mosaic virus* interaccciona con la histona H3, aumentando la posibilidad de que el ADN viral se mueve como un mini-cromosoma. El begomovirus *Squash leaf curl virus* forma túbulos en el RE del tejido floemático y estos túbulos podrían encajar en un mini-cromosoma compactado. La proteína MP de *Abutilon mosaic virus* interacciona con una proteína de choque térmico de cloroplasto (HSC70),

mientras que la MP de *Cabbage leaf curl virus* interacciona con una proteína denominada sinaptotagmina (SYTA). Sorprendentemente, la represión de estas proteínas del hospedador, incluyendo δ-COP que interacciona con C3 de TYLCSV, restringe o retrasa la infección viral, lo que podría sugierir que los geminivirus reclutan a los sistemas de transporte de acogida para su movimiento como una parte esencial de su ciclo de infección. Sin embargo, el papel de las proteínas de transporte del hospedador y el tráfico de vesículas durante la infección por geminivirus queda por determinar.

El complejo COPI está compuesto de siete subunidades (α, β, β ', γ, δ, ε, y ζ) que forman una estructura en forma de jaula. Las vesículas citoplasmáticas que contienen una cubierta formada por COPI son más conocidos por su implicación en el transporte retrógrado de la carga desde el aparato de Golgi a RE. Las cubiertas de COPI también pueden estar implicadas en la formación de vesículas recubiertas a lo largo del propio RE y compartimientos del endosoma.

Toda formación de vesículas está dirigida por una GTP-asa específica. ADP-RIBOSILACIÓN 1 (ARF1) es la GTPasa que impulsa la formación de las vesículas de COPI. ARF1 está formado por una gran familia de genes que también funciona en otros procesos de recubrimiento. Se ha demostrado que ARF1 recluta específicamente proteínas COPI para el transporte de vesículas, mediando de ese modo el tráfico de vesículas retrógrado.

Trabajo de investigación han descrito que las proteínas COPI y ARF1, como miembros de vesículas tráfico, juegan un papel importante en la respuesta a una amplia gama de patógenos. Se ha demostrado que ARF1 también tiene una función crítica en la patogénesis de las bacterias como *Salmonella* y *Escherichia coli.* Por otro lado, COPI está implicada en la capacidad de entrada y la infección del virus SV40 y en la formación de un complejo de proteínas necesario para el virus de la influenza y replicación del Enterovirus 71 de replicación en vertebrados. Por otra parte, ARF1 y/o COPI son importantes para la formación o mantenimiento de los complejos de replicación del virus de la hepatitis C.

Actualmente, hay menos ejemplos de la función de tráfico de vesículas en las plantas en comparación con la patogénesis de vertebrados. Se ha reportado que la *Arabidopsis* ARF-FMAM (AtMIN7) es degradada mediante la interacción específica con HopM1, un factor de virulencia de *Pseudomonas syringae*. Además existe evidencia del papel de ARF1 en respuesta de defensa en planta: la pérdida de la función de ARF1 afecta la resistencia de no huésped para *Pseudomonas cichorii* y compromete parcialmente la resistencia mediada por el gen *N* en *N. benthamiana* contra *Tobacco mosaic virus* (TMV). Además, se ha demostrado que la inhibición de la actividad ARF1 afecta negativamente la replicación de *Red clover necrotic mosaic virus* (RCNMV) y de *Tobacco etch virus* (TEV), dos virus de ARN de plantas, lo que sugiere importancia de un transporte retrógrado activo para la infectividad de los virus ARN en las plantas. Como se mostró en el

capítulo II, el silenciamiento de gen de la subunidad *δ-COP* impide completamente la infección de TYLCSV en plantas de *N. benthamiana*, lo que sugiere una función crucial del complejo COPI en la replicación TYLCSV, el movimiento o la transcripción.

Por ello, el objetivo de éste capítulo consistió en probar cuál es el papel que cumple el tráfico retrógrado durante la infección por geminivirus. Para ello se silenció mediante VIGS otro miembro importante de la formación de las vesículas, como lo es ARF1. Además, con la finalidad evaluar si la alteración del tráfico retrógrado tiene un papel durante la defensa frente a otro patógenos, se infectaron las plantas con dos virus de RNA como TMV y *Potato virus X* (PVX), así como con una bacteria *Pseudomonas syringae* pv tomate DC3000 ΔhopQ1-1 (Pto).

Con los resultados obtenidos en éste capítulo, demostramos que el silenciamiento de tanto de *δ-COP* o *ARF1* suprime completamente la infección por el TYLCSV o de otro miembro del género *Curtovirus* como *Beet curly top virus* (BCTV), pero no afecta a la infectividad del virus de ARN como PVX o TMV. También observamos que el silenciamiento de ambos componentes del transporte retrógrado no afecta a la capacidad replicativa de la bacteria patogénica *Pto*.

En resumen, podríamos concluir que un transporte retrógrado, a través de δ-COP *y* ARF1*,* intacto es importante para una infección exitosa de los geminivirus. Esto no se debería principalmente a un mecanismo de defensa general porque la alteración de la transporte retrógrado no afecta la infectividad de otros patógenos en plantas de *N. benthamiana*.

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Geminiviruses Subvert Ubiquitination by Altering CSN-Mediated Derubylation of SCF E3 Ligase Complexes and Inhibit Jasmonate Signaling in *Arabidopsis thaliana* [C][W]

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Viruses must create a suitable cell environment and elude defense mechanisms, which likely involves interactions with host proteins and subsequent interference with or usurpation of cellular machinery. Here, we describe a novel strategy used by plant DNA viruses (Geminiviruses) to redirect ubiquitination by interfering with the activity of the CSN (COP9 signalosome) complex. We show that geminiviral C2 protein interacts with CSN5, and its expression in transgenic plants compromises CSN activity on CUL1. Several responses regulated by the CUL1-based SCF ubiquitin E3 ligases (including responses to jasmonates, auxins, gibberellins, ethylene, and abscisic acid) are altered in these plants. Impairment of SCF function is confirmed by stabilization of yellow fluorescent protein–GAI, a substrate of the SCF^{SLY1}. Transcriptomic analysis of these transgenic plants highlights the response to jasmonates as the main SCF-dependent process affected by C2. Exogenous jasmonate treatment of *Arabidopsis thaliana* plants disrupts geminivirus infection, suggesting that the suppression of the jasmonate response might be crucial for infection. Our findings suggest that C2 affects the activity of SCFs, most likely through interference with the CSN. As SCFs are key regulators of many cellular processes, the capability of viruses to selectively interfere with or hijack the activity of these complexes might define a novel and powerful strategy in viral infections.

INTRODUCTION

Members of the Geminivirus family are plant viruses with circular, single-stranded DNA genomes (Rojas et al., 2005) that infect a wide range of plant species and cause extensive losses in food and fiber crops. Geminiviruses have highly reduced genomes, encoding only six to eight proteins. Due to limiting coding capacity, to successfully accomplish infection, these viruses must rely on both their own multifunctional proteins and the host cell machinery to replicate, move within and between cells, and avoid plant defense mechanisms (Hanley-Bowdoin et al., 2004).

C2 (also known as L2, AC2, AL2, or TrAP, for transcriptional activator protein) is a multifunctional protein encoded by geminiviruses. In viruses belonging to the genus *Begomovirus*, C2 acts as a transcription factor required for the expression of viral genes needed late in infection (Sunter and Bisaro, 1992) and triggers transactivation of host genes (Trinks et al., 2005) through an indirect mechanism. C2 is also a pathogenicity factor that suppresses host defenses: constitutive expression of truncated C2 from the begomovirus *Tomato golden mosaic virus* or the related L2 protein from the curtovirus *Beet curly top virus* (BCTV) in transgenic plants conditions an enhanced susceptibility phenotype (Sunter et al., 2001) that correlates with their ability to interact with and inactivate SNF1-related kinase (Sunter et al., 2001; Hao et al., 2003). C2 and L2 are also gene silencing suppressors of both posttranscriptional gene silencing and transcriptional gene silencing (reviewed in Raja et al., 2010).

Plants are sessile organisms forced to face environmental variations and continuously challenged by potential pathogens. To mount a rapid response, plants extensively rely on proteomic plasticity, which is partially driven by ubiquitination, a highly dynamic posttranslational modification that controls most of the protein degradation events in eukaryotes. According to proteomic and genetic analyses, ubiquitination rivals transcription as the dominant regulatory mechanism in plants (Vierstra, 2009). Ubiquitination occurs through an enzymatic cascade comprising an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase that binds the substrate and thus confers specificity. In plants, the most abundant family of E3 ligases comprises the multisubunit Cullin RING Ligases (CRLs). Among these, the Cullin1-based group, also named SCF (for Skp1/Cullin1/F-box), is the largest and best characterized because of its

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unveiled roles in many cellular processes, such as hormonal responses (reviewed in Dreher and Callis, 2007; Santner and Estelle, 2009), light signaling (Dieterle et al., 2001; Harmon and Kay, 2003; Marrocco et al., 2006), or floral meristem and organ identity (Kuroda et al., 2002; Wang et al., 2003). SCF complexes are composed of four subunits: Cullin1 (CUL1), SKP1/ASK (S-phase kinase-associated protein), the RING subunit RBX1 (RING box 1), and an F-box substrate binding protein. The *Arabidopsis thaliana* genome encodes more than 700 predicted F-box proteins, which suggests a high targeting potential (Hua et al., 2011).

The activity of Cullin RING ligases is regulated by a cycle of covalent attachment and removal of a ubiquitin-like protein named RUB (for Related to Ubiquitin; known as Nedd8 in fission yeast and animals) (del Pozo and Estelle, 1999; reviewed in Hotton and Callis, 2008), which is needed for robust CRL activity (Lyapina et al., 2001). One of the regulators of this activity is a conserved protein complex named CSN (COP9 signalosome; reviewed in Wei et al., 2008). The CSN complex is comprised of eight subunits, named CSN1 to CSN8, where CSN5 is the only catalytic subunit described to date. The best-characterized biochemical activity assigned to the CSN is the isopeptidase activity that removes the RUB moiety from the cullin component of the CRL, which is essential for the function of CRLs in vivo. In addition to the CSN holocomplex, several other subcomplexes are formed by a subset of CSN subunits or by CSN5 and other proteins, but the composition and number of these small complexes still remain unclear (Mundt et al., 2002; Oron et al., 2002; Gusmaroli et al., 2004; Fukumoto et al., 2005; Tomoda et al., 2005). Ubiquitination has been shown to contribute to multiple levels of plant defense (reviewed in Dreher and Callis, 2007). Specifically, several lines of evidence suggest that SCF complexes function in plant–virus interactions: (1) SGT1, an essential SKP1-interacting eukaryotic protein, is required for host and nonhost resistance, virus-induced necrosis, and restrain of viral growth of *Plantago asiatica mosaic virus* and *Potato virus X* (Komatsu et al., 2010); (2) virus-induced gene silencing of SKP1, SGT1, or the CSN complex compromised *N* gene–mediated resistance to *Tobacco mosaic virus* (TMV) in *Nicotiana benthamiana* (Liu et al., 2002); (3) the F-box protein ACIF is needed for TMV-triggered hypersensitive response in *Nicotiana tabacum* and affects *N* gene–mediated responses to TMV (van den Burg et al., 2008).

A large number of both animal and plant viruses have been described to interfere, inhibit, or usurp the ubiquitination machinery in the cell (reviewed in Isaacson and Ploegh, 2009) by encoding their own ubiquitination components (ubiquitin-like proteins, E3 ligases, adaptors, or deubiquitinating enzymes) or redirecting host ubiquitination. In plants, the Polerovirus P0 protein carries an F-box domain that allows its incorporation into an SCF complex to mediate degradation of AGO1, modulating gene silencing (Baumberger et al., 2007; Bortolamiol et al., 2007). The nanovirus Clink protein is also an F-box protein and can bind to both SKP1 and the cell cycle protein pRBR, affecting cell cycle regulation (Aronson et al., 2000).

Ubiquitination controls most of the hormonal responses in plants (reviewed in Dreher and Callis, 2007; Santner and Estelle, 2009). Among them, the jasmonate response, dependent on the SCF^{COI1} complex, plays a crucial role in pathogen defenses. Not much information about the role of jasmonates on viral infection is currently available, but recent works revealed jasmonate signaling as an emerging topic in plant–virus interaction research (Vigliocco et al., 2002; Liu et al., 2004; Agudelo-Romero et al., 2008; Ascencio-Ibáñez et al., 2008; Yang et al., 2008; Kovac et al., 2009).

In this article, we demonstrate that geminiviruses, through their C2 protein, interact and interfere with the derubylation activity of the CSN complex. The activity of the CSN over CUL1 seems to be compromised when C2 is present; consequently, processes regulated by SCF complexes are altered. Since SCFs are key regulators of many cellular processes, the capability of geminiviruses to selectively interfere with or hijack the activity of these complexes might represent a powerful strategy in the viral infection. According to our results, one of the main targets of geminiviral inhibition of SCFs might be the suppression of the jasmonate response. This work demonstrates that geminiviruses are capable of interfering with the ubiquitination pathway and jasmonate signaling through a novel mechanism.

RESULTS

TYLCSV C2 Is Required for Full Infection

Several geminiviral proteins are required to accomplish full infection. Among them, C2 from several begomoviruses has been shown to be needed for viral propagation (Etessami et al., 1988; Wartig et al., 1997). To confirm if this is also applicable to *Tomato yellow leaf curl Sardinia virus* Spain isolate (TYLCSV; accession number L27708), one of the begomoviruses responsible for the Tomato yellow leaf curl disease (TYLCD) in Spain, we constructed a null mutant virus for the C2 gene with a T-C transition in the start codon, hereafter called TYLCSV $C2_{T2C}$. This mutation also affects the nucleotide sequence of *Rep* (for Replication-associated protein) viral gene but does not result in an amino acid change. TYLCSV $C2_{T2C}$ was unable to infect tomato, while *N. benthamiana* plants infected with the mutant developed very mild or no symptoms and the level of viral DNA was severely reduced (see Supplemental Figure 1A online). To confirm that the viral DNA accumulated in plants infected with the mutant does not result from replication of revertants, we extracted DNA from young leaves collected at 28 d after inoculation (DAI) from plants inoculated with the mutant virus (three infected plants). A 625-bp fragment containing the mutation site was PCR amplified and fully sequenced. All analyzed fragments contained the mutation, confirming that the T-C transition is stable in infected plants.

To determine if the mutant is affected in replication, we evaluated the level of viral DNA accumulated in agroinfiltrated leaf patches of *N. benthamiana*. Total DNA was extracted 7 d after infiltration and hybridized with a TYLCSV probe. TYLCSV $C2_{T2C}$ DNA accumulates to levels comparable to those of the wild-type virus, indicating that the mutant is not impaired in replication (see Supplemental Figure 1B online). These results indicate that C2 from the Spanish isolate of TYLCSV is required for the establishment of a systemic infection but not for viral replication.

C2 Interacts with the Plant CSN5

Protein–protein interactions between viral and host proteins are one of the main mechanisms used by viruses to create a proper environment for the infection. To identify plant proteins interacting with C2, we performed a wide yeast two-hybrid screen using an *Arabidopsis* cDNA library (F. Hericourt et al., unpublished data). For the screening, a partial clone of C2, named C2-TS_{1-78}, lacking 59 amino acids of the C terminus, was expressed fused to GAL4 DBD. This truncated C2 protein lacks the transcriptional activation domain, since this domain has been previously shown to activate the expression of yeast two-hybrid GAL4 system reporter genes by itself (Hartitz et al., 1999). One of five clones identified in the screening (from 2×10^7 transformants) corresponds to a truncated version of the *Arabidopsis* CSN5A lacking the 44 N-terminal amino acids ($CSN5A_{44-357}$).

CSN5A is the only catalytic subunit of the conserved eukaryotic multiprotein complex named CSN described to date. CSN, originally identified through genetic screening as a negative regulator of photomorphogenesis in *Arabidopsis*, has been subsequently involved in the regulation of a wide variety of signaling and developmental processes in multiple organisms across all eukaryotic kingdoms, and its activity has proven to be essential. In *Arabidopsis*, transgenic lines expressing dominant-negative versions of CSN5A (Gusmaroli et al., 2004) or mutants partially defective in CSN5 activity (Gusmaroli et al., 2007) display severe pleiotropic developmental defects. On the other hand, complete loss of function of any of the eight CSN subunits results in a lethal phenotype characterized by postembryonic arrest at seedling stage (Gusmaroli et al., 2007). Despite its involvement in the regulation of a plethora of developmental and environmental responses, the major biochemical activity ascribed to date to the CSN is the removal of RUB1 from cullins.

To analyze if the interaction between C2 and CSN5 is conserved throughout the geminivirus family, we assayed the interaction of *Arabidopsis* CSN5A with C2 homologs from two other geminivirus species: C2 from the begomovirus *Tomato yellow leaf curl virus* (TYLCV), another causal agent of TYLCD, and L2 from the curtovirus BCTV, which is able to infect *Arabidopsis.* Hereafter, C2-TS stands for TYLCSV C2, C2-TM stands for TYLCV C2, and L2-BC stands for BCTV L2. Although C2 and L2 share similar roles, since both inhibit RNA silencing and act as pathogenicity factors, they show some functional divergence: C2 also functions as a transcription factor, while apparently L2 does not. Like for TYLCSV C2, partial clones encoding C-terminal truncated TYLCV C2 or BCTV L2, named C2-TM_{1-78} and L2-BC_{1-108}, respectively, were used for the yeast two-hybrid assays. The C2 protein of both geminivirus species was shown to interact with $CSN5A_{44-357}$ in a yeast two-hybrid assay (Table 1), indicating that this interaction is conserved among geminiviruses.

Phylogenetic analysis shows that CSN5 is highly conserved among plants (see Supplemental Figure 2 online). In *Arabidopsis*, unlike any other plant species described so far, there are two different CSN5 subunits, named CSN5A and CSN5B. These subunits display very different abundance and incorporate into distinct CSN complexes (CSN^{CSN5A} and CSN^{CSN5B}) that play unequal roles in the regulation of plant development (Gusmaroli et al., 2004).

Table 1. Interaction between Geminivirus C2 and the Plant CSN5 in Yeast

Bait	Prey	Interaction
$C2_{1-78}$-TS	$CSN5A_{44-357}$	Yes
$C2_{1-78}$-TM	$CSN5A_{44-357}$	Yes
$L2_{1-108}$-BC	$CSN5A_{44-357}$	Yes
P53	$CSN5A_{44-357}$	No
$C2_{1-78}$-TS	$CSN5B_{44-358}$	Yes
$C2_{1-78}$-TM	$CSN5B_{44-358}$	Yes
$L2_{1-108}$-BC	$CSN5B_{44-358}$	Yes
P53	$CSN5B_{44-358}$	No
$C2_{1-78}$-TS	$SlCSN5_{57-367}$	Yes
$C2_{1-78}$-TM	$SlCSN5_{57-367}$	Yes
$L2_{1-108}$-BC	$SlCSN5_{57-367}$	Yes
P53	$SlCSN5_{57-367}$	No
$C2_{1-78}$-TS	AgT	No
$C2_{1-78}$-TM	AgT	No
$L2_{1-108}$-BC	AgT	No

C2-TS stands for TYLCSV C2; C2-TM stands for TYLCV C2; L2-BC stands for BCTV L2. CSN5A and CSN5B are from *Arabidopsis*; SlCSN5 is CSN5 from tomato cultivar Moneymaker. Interaction was indicated by the ability of cells to grow on medium lacking His and Ade and containing 50 mM 3-aminotriazole. P53 stands for the murine p53 protein fused to the GAL4 DBD (pGBKT7-53; Clontech), and AgT stands for the SV40 large T antigen fused to the GAL4 AD fused to the GAL4 (pGADT7-T; Clontech); both are used as negative controls.

To determine if C2 also interacts with CSN5B, we cloned a partial *CSN5B* clone, equivalent to the *CSN5A* partial clone isolated in the screening, lacking the 44 N-terminal residues ($CSN5B_{44-358}$), and found that all three tested C2/L2 proteins are able to interact with *Arabidopsis* $CSN5B_{44-358}$ (Table 1). Given that TYLCSV and TYLCV are important pathogens for tomato (*Solanum lycopersicum*) crops and tomato is a host for BCTV, we also tested the interaction between C2/L2 and tomato CSN5. We cloned the CSN5 cDNA from *S. lycopersicum* cultivar Moneymaker (AC:FN820438) and generated a partial clone to express a truncated protein similar to the *Arabidopsis* CSN5A and CSN5B used in the binding assays (residues 57 to 367, $SlCSN5_{57-367}$). There are three amino acid differences between the cloned CSN5, obtained from Moneymaker, and a previously identified CSN5 obtained from the VFNT cultivar (AC:AF175964). Yeast two-hybrid assays demonstrate that all three tested C2 proteins are also able to interact with $SlCSN5_{57-367}$ (Table 1), suggesting that C2/L2-CSN5 interaction is also conserved in other plant species.

It has been previously reported that CSN5 interacts with the GAL4 DNA binding domain (Nordgård et al., 2001); thus, the isolation of CSN5 in a GAL4-based yeast two-hybrid screening should be considered cautiously. However, CSN5 homologs from different organisms have been isolated in this kind of screening in several independent studies (Kameda et al., 2006; Cho et al., 2008; Tanguy et al., 2008), and the interactions have been confirmed by other methods. Curiously, in two out of the three cited works, the isolated clone was partial, lacking at least the 44 N-terminal amino acids. We found that the complete CSN5 proteins from both *Arabidopsis* (CSN5A and CSN5B) and tomato, fused to the GAL4 AD, strongly interact with any protein

fused to the GAL4 DBD or with the empty GAL4 DBD-containing vector. However, this unspecific interaction does not occur when the truncated version of CSN5 is used instead.

To confirm the C2/L2-CSN5 interaction in planta, we used a bimolecular fluorescence complementation (BiFC) assay. *N. benthamiana* leaves were coinfiltrated with *Agrobacterium tumefaciens* cells to express N-terminal fusions of C2 or L2 with N- or C-yellow fluorescent protein (YFP) and N-terminal fusions of *Arabidopsis* CSN5A or tomato CSN5 with C- or N-YFP. The infiltrated leaves were analyzed under the confocal microscope 3 d after infiltration. YFP fluorescence was observed in cells coinfiltrated with constructs corresponding to NYFP-CSN5A and any of the CYFP-C2/L2 constructs or vice versa. Similar results were obtained when leaves were coinfiltrated with tomato CSN5 and C2 constructs (Figure 1A). By contrast, expression of CSN5 or any of C2/L2 constructs alone (data not shown) or coexpression of any of those constructs with the β-glucuronidase protein (Kertbundit et al., 1991) fused to NYFP or CYFP did not restore the YFP fluorescence (Figure 1). Interactions between C2/L2 and CSN5 seem to occur mainly in the nuclei, as they colocalize with 4',6-diamidino-2-phenylindole (DAPI) staining (Figure 1A). The nuclear localization of C2 from some begomoviruses has been previously reported (van Wezel et al., 2001; Sharma et al., 2010). To confirm that this is also the case for C2-TS, subcellular localization was examined using a green fluorescent protein (GFP)-C2-TS fusion. Three days after agroinfiltration of the construct in *N. benthamiana* leaves, agroinfiltrated patches were observed under the confocal microscope. GFP-C2-TS localized mainly in the nucleus, as demonstrated by the colocalization with DAPI staining (Figure 1B).

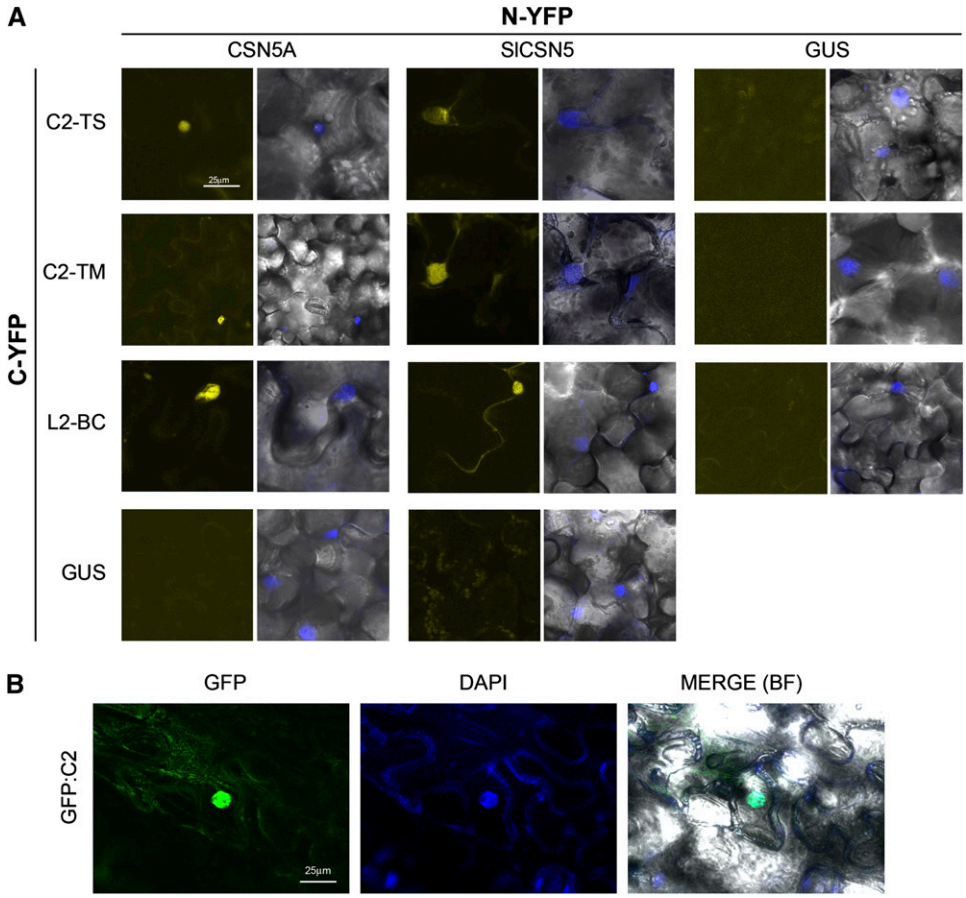

Figure 1. In Vivo Interaction between Geminivirus C2 and the Plant CSN5.

(A) BiFC analyses showing interaction between geminivirus C2/L2 (C2-TS stands for TYLCSV C2; C2-TM stands for TYLCV C2; L2-BC stands for BCTV L2) and the plant CSN5 (CSN5 stands for *Arabidopsis* CSN5A; SlCSN5 stands for *S. lycopersicum* cultivar Moneymaker CSN5). GUS stands for *A. thaliana* β-glucuronidase, used as a negative control. *N. benthamiana* leaves coinfiltrated with constructs expressing C2/L2, CSN5, or β-glucuronidase fused to the YFP C terminus (CYFP) or N terminus (NYFP) were observed under the confocal microscope 3 d after infiltration. Leaves were infiltrated with a 4 μg/mL DAPI solution 3 d after infiltration and observed under the confocal microscope 0.5 to 5 h later. No differences were observed between the two pair-wise combinations; only one of the combinations is shown.

(B) Subcellular localization of GFP-C2-TS fusion protein in epidermal cells of *N. benthamiana*. *N. benthamiana* leaves infiltrated with a construct expressing a GFP-C2-TS fusion protein were infiltrated with a 4 μg/mL DAPI solution 3 d after infiltration and observed under the confocal microscope 0.5 to 5 h later. GFP-C2-TS is mainly localized into the nucleus. GFP fluorescence, DAPI staining, and merge, including bright-field channel, are shown.

Taken together, these results demonstrate that C2/L2 from TYLCSV, TYLCV, and BCTV associate with CSN5 mainly in the nucleus of plant cells.

Expression of Viral C2/L2 Protein in Transgenic *Arabidopsis* Lines Specifically Interferes with CUL1 Derubylation without Affecting the Proper Assembly of CSN and SCF Complexes

To determine if the interaction of C2/L2 with CSN5 might be affecting the derubylation activity of the CSN complex, we compared the relative levels of rubylated and derubylated cullins between the wild-type and transgenic *Arabidopsis* plants expressing C2/L2 from TYLCSV, TYLCV, and BCTV (details of these transgenic lines are shown in Supplemental Figure 3 online). None of these C2/L2-expressing transgenic lines displayed noticeable defects in development or morphology. Protein extracts from wild-type, transgenic C2/L2 lines and the *csn5a-1* mutant (as a control) were subjected to immunoblot analysis using antibodies against *Arabidopsis* CUL1, CUL3, and CUL4, the three *Arabidopsis* cullins known to form CRLs. As shown in Figure 2A, the relative level of rubylated CUL1 observed in all transgenic lines expressing C2/L2 is higher than that of the wild-type plants, whereas we did not observe clear changes in the relative rubylation levels of CUL3 or CUL4 (see Supplemental Figure 4 online). This result suggests that C2/L2 may be hindering the derubylating activity of the CSN complex specifically over CUL1. It is noteworthy that the total cellular levels of CUL3 and CUL1 are slightly increased in the C2/L2 transgenic plants; however, no changes in CUL4 accumulation are detected.

Viral proteins have often been shown to sequester host proteins to co-opt or redirect pivotal cellular machineries to viral function. In this context, it is possible to speculate that C2/L2 might sequester CSN5, preventing its assembly into the complex, or that C2/L2-CSN5 interaction might affect the distribution of CSN5 between the CSN holocomplex and the CSN5-containing subcomplex forms. Based on this idea, a gel filtration experiment was performed in which the fractionation pattern of CSN5 was analyzed (Figure 2B). The comparison of the gel filtration profiles of wild-type plants and transgenic lines expressing C2/L2 demonstrates that CSN5 is normally assembled into both the CSN holocomplex, where it exercises its derubylation activity, and into the subcomplex forms. Keeping in mind that the expression of C2/L2 results in the accumulation of preferentially rubylated CUL1, it is also possible that these viral proteins could alter CUL1 assembly into the SCF complex. However, the analyses of CUL1 fractionation patterns did not reveal any significant changes in the presence of C2/L2 (Figure 2C). These results imply that C2/L2 does not interfere with the proper assembly of CUL1 into the SCF complex. This is in agreement with the observation that RBX1 and SKP1, two other components of the SCF complex, accumulate in the same fractions in one representative C2 transgenic line (Figure 2D). Taken together, these data indicate that C2/L2 does not interfere with the assembly of either the CSN or the SCF complexes and that the observed accumulation of rubylated CUL1 is therefore consistent with a specific interference of C2/L2 with the CSN-mediated CUL1 derubylation.

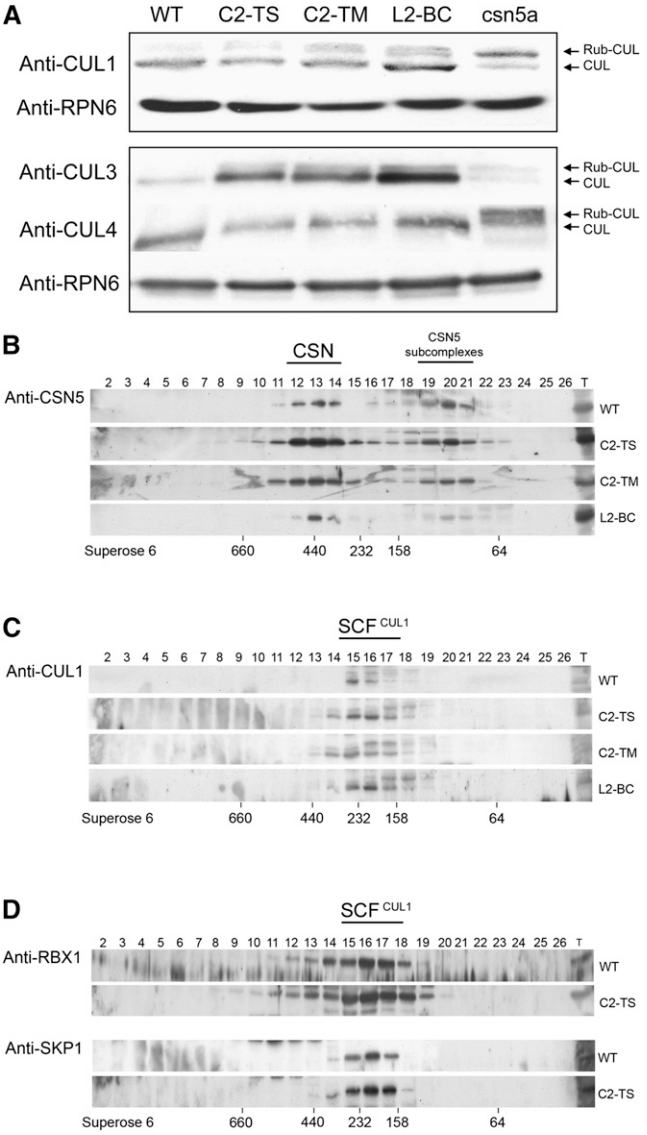

Figure 2. Inmmunoblot Analysis of 2-Week-Old Wild-Type, *csn5a*, and Kanamycin-Resistant Transgenic C2-TS, C2-TM, and L2-BC *Arabidopsis* Seedlings.

(A) Detection of *Arabidopsis* CUL1, CUL3, and CUL4. Total proteins were subjected to SDS-PAGE and immunoblot analysis with α-CUL1, α-CUL3, and α-CUL4. Equal protein loads were confirmed using α-RPN6 (RPN6 is a non-ATPase regulatory subunit of the 26S proteasome). Rubylated (Rub-CUL) and derubylated Cullins (CUL) are indicated by arrows. WT, wild type. **(B)** to **(D)** Immunoblot analyses of Superose 6 gel filtration fractions. Column fractions were subjected to SDS-PAGE and immunobloted with α-CSN5 **(B)**, α-CUL1 **(C)**, α-RBX1, or α-ASK1 **(D)**. Fraction numbers are indicated. Lane T contains the total unfractionated extracts.

C2/L2 *Arabidopsis* Transgenic Plants Share Phenotypes with *cul1* Mutants, Including Altered SCF-Dependent Hormonal Responses

Given that C2/L2 transgenic plants display an altered CUL1 rubylated/derubylated ratio, CUL1 function could be impaired in

these plants. *cul1* mutants are altered in a plethora of developmental processes, such as root growth, skotomorphogenesis, and hormonal responses (Moon et al., 2007; Gilkerson et al., 2009); consequently, it is conceivable that the C2/L2 transgenic plants could also display these defects.

To evaluate root growth rate in the C2/L2 transgenic plants, the root length of 4-d-old seedlings was measured every 24 h for 4 d. Data show that C2/L2 transgenic roots are smaller and grow more slowly than wild-type roots (Figure 3A).

Skotomorphogenesis is also altered in transgenic C2/L2 plants: etiolated transgenic seedlings differ from the wild type in hypocotyl length. All three transgenic lines display significantly shorter hypocotyls than the wild type as determined by Mann-Whitney rank sum test (Figure 3B). The reduction in hypocotyl size correlates with the RNA expression level of the transgenes (see Supplemental Figure 5 online).

Because SCF complexes play a role in the signaling pathways of several hormones, and most of these responses have been shown to be altered in *cul1* mutants (Moon et al., 2007; Gilkerson et al., 2009), we investigated how C2/L2 transgenic lines respond to ethylene, auxins, gibberellins, and jasmonates. In all cases, we measured inhibition of primary root elongation caused by treatment with the exogenous compound (1-aminocyclopropane-1-carboxylic acid [ACC], 2,4-D, gibberellin A3 [GA3], or methyl jasmonate [MeJA]) as a measure of the response to the hormone. The results show that *Arabidopsis* transgenic plants expressing C2 or L2 were less sensitive to 2,4-D, GA_3, and MeJA and more sensitive to ACC (Figure 4). The differential sensitivity of C2/L2 transgenic plants is thus consistent with a malfunction of the corresponding SCF complex in all cases. We tested the differential sensitivity to MeJA in independent transgenic lines expressing different levels of C2/L2 mRNA and found a correlation between lower sensitivity and higher mRNA expression (see Supplemental Figure 6A online). To confirm these results, quantitative real-time PCR was used to quantify the mRNA expression level of marker genes for each of the assayed hormones. We selected ERF1 and ERS1 as marker genes for the ethylene response, PIN1 and IAA19 for the auxin response, MFC19.13 and MHJ24.10 for the gibberellin response, and OPR3 and JR1 for the jasmonate response. In all cases, the expression level of the marker genes correlated with the observed differential sensitivity phenotype (Figure 5). We also tested if the expression of C2-TS alters the sensitivity to hormones in a different plant species: transgenic *N. benthamiana* plants containing a TYLCSV C2 expression cassette were tested for their sensitivity to auxins and jasmonates. As shown in Supplemental Figure 6B online, transgenic *N. benthamiana* plants expressing C2-TS are also

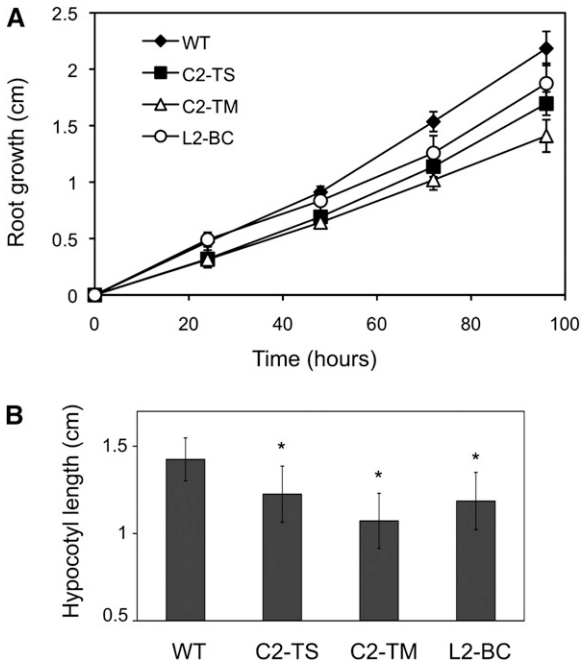

Figure 3. Root and Hypocotyl Length Analysis of C2/L2 Transgenic Plants.

(A) Total root length of transgenic C2-TS, C2-TM, and L2-BC or wild-type *Arabidopsis* seedlings (WT) was measured every 24 h beginning 4 d after germination ($n \geq 14$). Bars represent SE.

(B) Hypocotyl length of 9-d-old dark-grown transgenic C2-TS, C2-TM, and L2-BC or wild-type *Arabidopsis* seedlings. Bars represent SD. Asterisks indicate a statistically significant difference when compared with the wild-type value according to Mann-Whitney rank sum test. $n \geq 30$; the experiment was repeated three times.

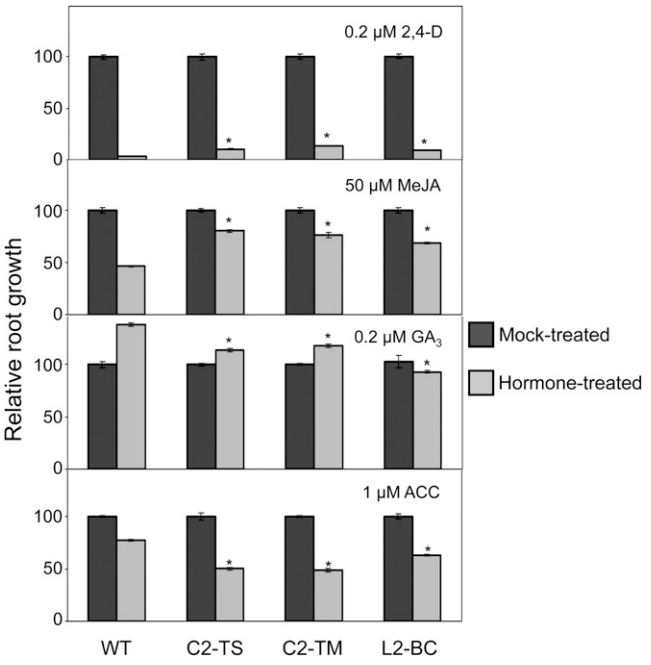

Figure 4. Reduced Auxin, Jasmonate, and Gibberellin Response and Enhanced Ethylene Response in Transgenic C2/L2 *Arabidopsis* Lines.

Hormone sensitivity was measured as root growth inhibition. Experiments were repeated at least three times independently; results from one of the replicates are represented ($n \geq 15$). Five-day-old seedlings were grown on exogenous hormone for an additional 5 d. Bars represent SE. Asterisks indicate a statistically significant difference when compared with the wild-type value according to Mann-Whitney rank sum test. WT, wild type.

less sensitive to 2,4-D and MeJA, which demonstrates that C2-mediated lower sensitivity to these hormones is not host specific.

Besides the previously described phenotypes, *Arabidopsis* C2/L2 transgenic plants are more resistant to drought (Figure 6A). This enhanced tolerance correlated with a slower weight loss in detached leaves (Figure 6B), suggesting that the stomata are more efficiently closed in these plants. Recently, an F-box protein named DOR was described to function as an inhibitor for abscisic acid (ABA)-induced stomatal closure under drought stress, most probably through its activity in a SCF^{DOR} complex (Zhang et al., 2008). The *DOR* gene is preferentially expressed in the guard cells and affects the stomatal response to ABA: guard cells of the *dor* mutant are hypersensitive to this hormone. However, other well-characterized responses to ABA, such as the inhibition of seed germination or the reduction of vegetative growth, are not altered in this mutant (Zhang et al., 2008), consistently with the specific expression pattern. Based on our previous findings that C2/L2 seems to be interfering with the SCF complexes, it would be feasible to speculate that a defective SCF^{DOR} activity could result in increased ABA sensitivity in the guard cells, which would in turn explain the observed drought tolerance phenotype. In line with this idea, when we tested the stomatal response to exogenously applied ABA, we found that the stomata in the C2/L2 transgenic plants are indeed more responsive to ABA (Figures 6C and 6D), even though the sensitivity to this hormone is not higher when measured as inhibition of either seed germination or root growth.

C2/L2 Hinders the Degradation of GAI, Target of the SCF^{SLY1} E3

If the differential sensitivity to hormones observed in the C2/L2-expressing lines is due to the inhibition of the SCF complexes, the substrates of these complexes must be accumulating in the presence of C2/L2. To check this possibility, we took advantage of a YFP-GAI expression construct (kindly provided by David Alabadí, Instituto de Biología Molecular y Celular de Plantas, Spain). GAI is a DELLA protein that is degraded by the SCF^{SLY1} in the presence of gibberellins. When the construct expressing YFP-GAI is agroinfiltrated in *N. benthamiana* leaves, yellow fluorescence can be observed in the nuclei 3 DAI (Figure 7A), indicating the expression and accumulation of the fusion protein. This fluorescence diminishes and eventually disappears when the leaves are treated with 100 μM GA_3, since the hormone treatment triggers the ubiquitination of the fusion protein by the SCF^{SLY1} and its subsequent degradation by the 26S proteasome. As shown in Figure 7A, when we coinfiltrate YFP-GAI and C2/L2 expression construct, the decrease in fluorescence after GA_3 treatment is less dramatic, indicating a stabilization of the DELLA protein caused by C2/L2 protein. These results were confirmed by immunoblot analyses using an anti-GFP antibody (Figure 7B). GA_3 treatment clearly reduced the amount of YFP-GAI when this fusion protein is agroinfiltrated alone, but no significant differences were observed when it is coinfiltrated with any of the C2/L2 expression constructs. As an internal

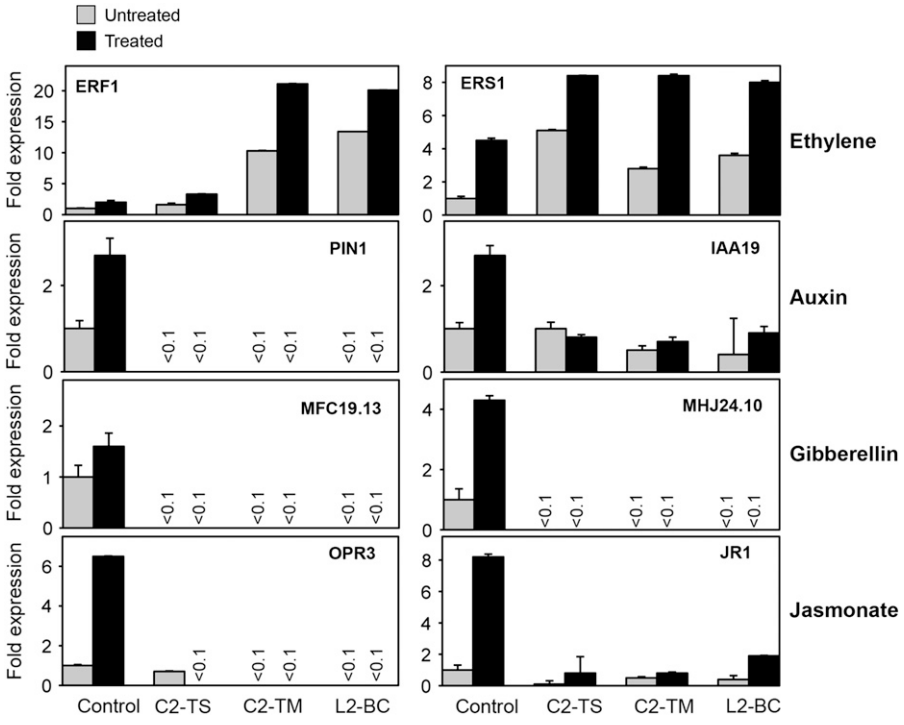

Figure 5. Expression Level of Hormone-Responsive Genes in C2/L2 Transgenic Plants.

Relative expression level of marker genes of the ethylene (*ERF1* and *ERS2*), auxin (*PIN1* and *IAA19*), gibberellin (*MFC19.13* and *MHJ24.10*), and jasmonate (*OPR3* and *JR1*) response in mock- or hormone-treated transgenic C2-TS, C2-TM, and L2-BC and control *Arabidopsis* seedlings determined by quantitative real-time PCR. C2/L2-expressing lines are compared with the control in each condition. Actin was used as the internal control.

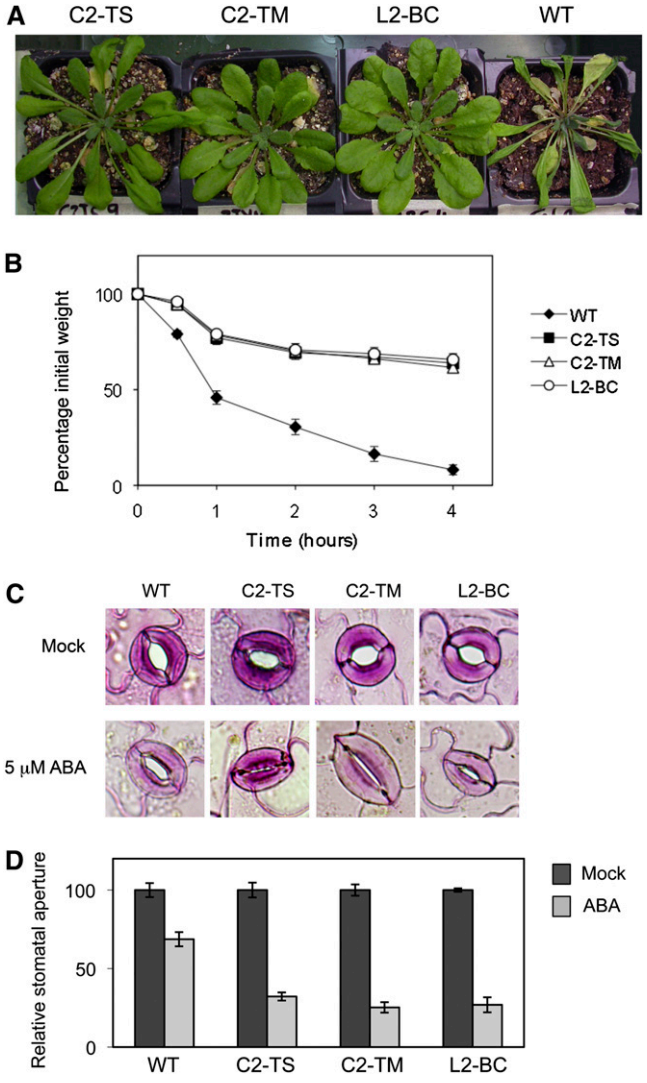

Figure 6. Drought-Related Phenotypes of Transgenic C2-TS, C2-TM, and L2-BC and Wild-Type *Arabidopsis* Plants.

(A) Phenotype of 7-week-old plants after 10 d without water supply. WT, wild type.

(B) Weight loss in detached leaves. Rosette leaves from 4-week-old plants were detached, placed on weighing dishes, and allowed to dry at room conditions. Weight of the samples was recorded at 0.5, 1, 2, 3, and 4 h. $n = 5$; bars represent SE.

(C) Stomata in epidermal peels after inducing stomatal aperture and treating with 5 μM ABA or mock solution for 1 h.

(D) Stomatal aperture in epidermal peels after treatment with 5 μM ABA or mock solution. The experiments were repeated three times independently; at least 30 stomatal apertures were measured in each condition. Bars represent SD.

[See online article for color version of this figure.]

control, a GFP expression construct was agroinfiltrated alone or coinfiltrated with the C2/L2 expression construct. No differences in fluorescence (data not shown) or GFP protein accumulation were detected between treated and untreated plants (Figure 7B).

Transcriptomic Analysis Reveals a Clear Suppression of Jasmonate Responses in C2 Plants

To further characterize the global effects on gene expression induced by C2, we performed a transcriptomic analysis of the transgenic *Arabidopsis* plants expressing TYLCSV C2. Microarray examination reveals 606 genes that were upregulated and 644 that were downregulated in the transgenic plants with a P value below 0.05 compared with control plants. These microarray results were validated by quantitative real-time PCR (see Supplemental Figure 7 online). When we subjected the two groups of genes with altered expression to functional enrichment, we found several biological processes affected by C2-TS, including response to hormone stimulus and defense response (Table 2). As expected, the expression of genes involved in the

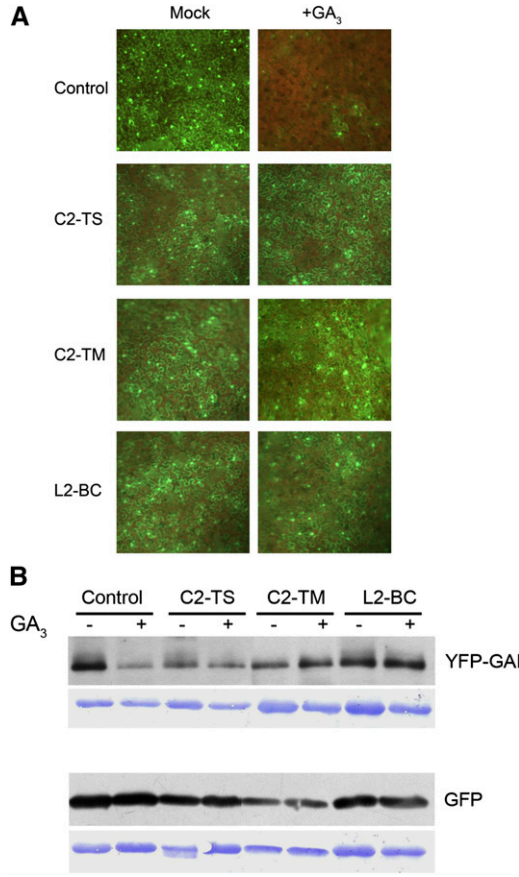

Figure 7. In Vivo Degradation Assay of YFP-GAI Fusion Protein.

(A) The construct expressing YFP-GAI was agroinfiltrated in *N. benthamiana* leaves alone (control) or coinfiltrated with constructs expressing C2-TS, C2-TM, or L2-BC. Three days after infiltration, agroinfiltrated leaves were sprayed with 100 μM GA3 or mock solution and visualized under the epifluorescence microscope 1 to 2 h later.

(B) Detection of YFP-GAI or GFP (as a control) in *N. benthamiana* leaves agroinfiltrated with the construct expressing YFP-GAI alone or together with constructs expressing C2-TS, C2-TM, and L2- BC and treated with 100 μM GA3 or mock solution. Total proteins were subjected to SDS-PAGE and immunoblot analysis with α-GFP, which also recognizes YFP. Coomassie blue staining of the protein blot is shown as loading control.

hormonal responses previously tested is also altered: for example, the gibberellin-responsive GASA4 and GASA5 are downregulated, whereas the ethylene-responsive ATERF4, PDX1L4, and HRE1 are upregulated.

Although the number of up- and downregulated genes in the transgenic C2-TS plants is similar, the nonredundant analysis of GO categories did not reveal any specific hormonal response affected among the upregulated genes. Instead, in the subset of repressed genes, it is especially noticeable the presence of processes related to plant defense and response to jasmonates. Among these downregulated genes, some hallmarks of the jasmonates biosynthetic and perception pathways can be found (see Supplemental Table 3 online). When the list of downregulated genes is compared with that of the upregulated genes in a Columbia (Col-0) plant after MeJA treatment (jasmonate-responsive genes) (Nemhauser et al., 2006), the intersection contains 114 common genes (Figure 8). Gene ontology (GO) analysis of these 114 genes reveals that 32 out of 45 (71%) of the GO categories reported as overrepresented for the whole set of downregulated genes in C2-expressing plants are also overrepresented in this intersectional subset (Table 3). On the other hand, intersections between the set of upregulated genes in C2-expressing plants with either up- or downregulated genes in MeJA-treated plants or between the downregulated genes in C2-expressing plants and upregulated genes in MeJA-treated plants give no significant functional terms exceeding the P value cutoff of 0.01. Given that processes related to jasmonates biosynthesis and perception appear to be clearly repressed by C2, we can infer that interference with the jasmonate pathway might to some degree account for the suppression of the defense response, maybe linking this phenotype to the ability of C2 to hinder the activity of the SCF^{COI1}. Taking these results together, we conclude that the inhibition of the jasmonates response is the main process affecting downregulation of transcription in the C2-TS–expressing plants.

Jasmonate Treatment Reduces the Susceptibility to Geminivirus Infection

Previous results demonstrated that expression of *Tomato golden mosaic virus* C2 or BCTV L2 in *N. benthamiana* plants produced an enhanced susceptibility to DNA and RNA viruses (Sunter et al., 2001), suggesting that C2/L2 proteins have the ability to suppress host stress or defense responses. Since jasmonate signaling has been extensively implicated in defense responses (reviewed in Bari and Jones, 2009), the changes in hormonal sensitivity observed in the C2/L2 transgenic plants could be responsible for this enhanced susceptibility phenotype, suggesting that repression of the jasmonate response could favor viral infection. To determine whether jasmonate response affects geminivirus infection, we inoculated MeJA and mock-treated *Arabidopsis* plants with BCTV. Total DNA was extracted from these samples and subjected to nucleic acid hybridization with a viral probe. Results from symptom evaluation and viral DNA accumulation are presented in Figure 9. The application of exogenous MeJA results in milder symptoms and lower viral DNA accumulation, indicating a disruption of the geminivirus infection by this compound.

DISCUSSION

C2, a Protein Required for Virus Infectivity, Interacts with CSN5 and Exerts a Specific Effect on CUL1 Rubylation State

Using TYLCSV C2 as bait protein in a yeast two-hybrid screening, we isolated the *Arabidopsis* protein CSN5A. Binding assays in yeast and plant confirm C2-CSN5A interaction and demonstrate that this viral protein also binds the tomato ortholog and the *Arabidopsis* paralog (CSN5B). Interaction experiments using these three CSN5 proteins further demonstrate that they interact with the C2 protein from another begomovirus, TYLCV, and with the homologous protein in the curtovirus BCTV. Taken together, these results suggest that binding to CSN5 is a conserved function of geminivirus C2/L2 protein.

Although interactions with components of the CRLs have been described previously for DNA and RNA viruses, only few examples of interactions with the CSN complex have been reported, all limited to animal viruses (Mahalingam et al., 1998; Oh et al., 2006; Tanaka et al., 2006; Hsieh et al., 2007). Even though, as for C2-CSN5, those interactions seem to play a role during virus infection (Oh et al., 2006; Tanaka et al., 2006; Hsieh et al., 2007), the mechanisms proposed point to a redirection of proteosomal degradation rather than to an effect on the derubylating activity of the CSN complex itself.

Given that the main biochemical activity of the CSN complex is the derubylation of cullins, we checked the rubylated/derubylated ratio of CUL1, CUL3, and CUL4 in transgenic *Arabidopsis* lines expressing geminivirus C2/L2 and found that these plants contain a higher proportion of rubylated CUL1; nevertheless, CUL3 and CUL4 rubylation ratio is not altered. Although we cannot rule out the possibility that subtle changes in CUL3 to CUL4 ratio cannot be detected by immunoblots, this result suggests that C2/L2-CSN5 interaction specifically inhibits the derubylation activity of CSN over CUL1. In spite of the fact that the precise mechanism conferring specificity to the action of C2 remains elusive, the interaction between several CSN subunits and SCF components in plants (Schwechheimer et al., 2001) raises the possibility that the reduction in CUL1 derubylation could be the result of the specific interference of C2 with the CSN-SCF binding.

Strikingly, even though the CUL3 rubylated/derubylated ratio is not affected by the presence of C2/L2, the transgenic plants expressing these viral genes show a slight increase in the accumulation of CUL3, which seems to be the result of post-translational regulation, since the level of CUL3 mRNA is reduced in these plants (see Supplemental Figure 8 online). Interestingly, a genetic interaction has been described for *CSN5* and *CUL3*, and CSN5 and CUL3 have been proposed to regulate each other's abundance in an opposite manner (Gusmaroli et al., 2007). In this context, one would expect that C2/L2 interference with CSN function would trigger the same reduction in CUL3 accumulation. However, C2/L2 expression is instead accompanied by an increase in CUL3 abundance. The fact that CUL1 derubylation is affected in the same transgenic lines allows the intriguing possibility that a defective SCF activity could be responsible for CUL3 accumulation. Alternatively, a tantalizing, nonexclusive hypothesis could be that C2-mediated blocking of

Table 2. Gene Ontology Analysis of Differentially Expressed Genes

Nonredundant GO Categories	Level	Differentially Expressed (%)	Expected Frequency	P Value
Downregulated Genes				
Response to stress	3	16.7% (107)	5.5%	8.98e-20
Secondary metabolic process	3	6.7% (43)	1.3%	1.84e-15
Immune response	3	3.3% (21)	0.6%	5.27e-07
Catabolic process	3	6.2% (40)	2.2%	9.73e-06
Defense response	3	5.5% (35)	1.9%	8.21e-05
Nitrogen compound metabolic process	3	4.7% (30)	1.6%	0.00041
Cellular biosynthetic process	4	13.2% (85)	4.8%	9.45e-13
Organic acid metabolic process	4	7.3% (47)	2.2%	7.80e-09
Aromatic compound metabolic process	4	4.5% (29)	1.0%	1.32e-08
Response to wounding	4	2.8% (18)	0.4%	2.05e-07
Innate immune response	4	3.1% (20)	0.6%	6.74e-07
Heterocycle metabolic process	4	3.1% (20)	0.7%	1.89e-05
Pigment metabolic process	4	2.3% (15)	0.4%	2.04e-05
Cellular catabolic process	4	5.9% (38)	2.1%	2.59e-05
Amine metabolic process	4	4.7% (30)	1.4%	3.01e-05
Response to water	4	2.5% (16)	0.5%	0.00010
Response to jasmonic acid stimulus	4	2.5% (16)	0.5%	0.00031
Sulfur metabolic process	4	1.9% (12)	0.3%	0.00167
Carboxylic acid metabolic process	5	7.3% (47)	2.2%	7.27e-09
Aromatic compound biosynthetic process	5	3.3% (21)	0.6%	1.92e-07
Response to heat	5	2.5% (16)	0.4%	7.85e-06
Nitrogen compound biosynthetic process	5	3.0% (19)	0.6%	2.71e-05
Response to water deprivation	5	2.5% (16)	0.5%	4.14e-05
Response to oxidative stress	5	3.3% (21)	0.8%	5.57e-05
Response to cold	5	2.8% (18)	0.7%	0.00129
Amino acid derivative biosynthetic process	6	3.6% (23)	0.6%	5.91e-09
Defense response, incompatible interaction	6	2.6% (17)	0.3%	5.64e-08
Cellular carbohydrate metabolic process	6	4.2% (27)	1.2%	4.32e-05
Response to desiccation	6	1.1% (7)	0.1%	9.12e-05
Amino acid metabolic process	6	4.0% (26)	1.2%	9.23e-05
Biogenic amine metabolic process	6	1.4% (9)	0.2%	0.00025
Toxin catabolic process	6	1.1% (7)	0.1%	0.00398
Porphyrin catabolic process	6	0.8% (5)	0.0%	0.00412
Jasmonic acid and ethylene-dependent systemic resistance	7	1.2% (8)	0.1%	0.00010
Indole derivative biosynthetic process	7	1.2% (8)	0.1%	0.00010
Phenylpropanoid biosynthetic process	7	2.2% (14)	0.4%	0.00015
Amino acid biosynthetic process	7	2.5% (16)	0.5%	0.00017
Biogenic amine biosynthetic process	7	1.2% (8)	0.1%	0.00076
Indolalkylamine metabolic process	7	0.9% (6)	0.1%	0.00439
Flavonoid biosynthetic process	8	1.6% (10)	0.2%	0.00029
Indoleacetic acid biosynthetic process	8	0.8% (5)	0.0%	0.00054
Tryptophan metabolic process	8	0.9% (6)	0.1%	0.00439
Glycosinolate biosynthetic process	9	1.1% (7)	0.1%	2.89e-05
Jasmonic acid metabolic process	9	1.1% (7)	0.1%	5.27e-05
Oxylipin biosynthetic process	9	1.1% (7)	0.1%	5.27e-05
Upregulated genes				
Response to hormone stimulus	3	5.6% (34)	2.4%	0.00738

Nonredundant GO categories identified as enriched among down- or upregulated genes in C2-TS expressing *Arabidopsis* plants versus control plants. GO category levels are indicated. The percentages of genes belonging to each category are reported for the differentially expressed genes and for the genes present in the microarray. The absolute number of differentially expressed genes belonging to each category is reported in parentheses.

the derubylation of CUL1 could increase the number of CSN complexes available to remove RUB from other cullins, thus reducing CUL3 autodegradation. It has been previously proposed that CSN5A reduced activity could trigger the autoubiquitination and degradation of rubylated CUL1 (Stuttmann et al., 2009). However, in spite of a clearly higher rubylated/derubylated CUL1 ratio, we did not observe any destabilization of rubylated CUL1 in either transgenic lines expressing C2/L2 or in *csn5a* mutants, in agreement with previous data indicating that CUL1 is not destabilized in the same *csn5a* background (Gusmaroli et al., 2007).

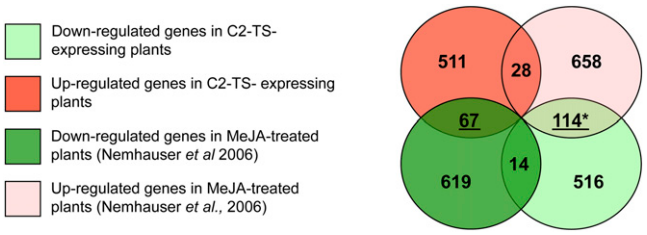

Figure 8. Venn Diagrams Depicting the Intersection between Upregulated or Downregulated Genes in C2-TS Transgenic Plants or MeJA-Treated Col-0 Plants (Nemhauser et al., 2006).

The number of genes in each category is indicated. Numbers of genes higher than those expected in a random distribution, with $\alpha = 0.05$, are underlined. The significance of matches being higher than explained by random sampling was tested by assuming a Poisson distribution from a mean value calculated as $\mu = G1x\ (G2/Gt)$, since any gene in G1 has a G2/Gt possibility to belong to G2, and being G1 and G2 the subsets of genes to be compared and Gt the total number of genes represented in the microarray. An asterisk by the number of genes in an intersection indicates that there are overrepresented GO terms in that subset of genes (Table 2).
[See online article for color version of this figure.]

C2 Affects the Activity of Several SCF Complexes

Since C2/L2 expression increases the CUL1 rubylated/derubylated ratio, it is reasonable to expect that CUL1-based SCF functions would be compromised in the presence of C2/L2. Transgenic C2/L2 plants share phenotypes with *csn* and *cul1* mutants, including decreased sensitivity to auxins, gibberellins, and jasmonates and enhanced sensitivity to ethylene and ABA in the guard cells, results that are consistent with a general impairment in the activity of several known SCF complexes (SCF^{TIR1}, SCF^{SLY1}, SCF^{COI1}, $SCF^{EBF1/2}$, and SCF^{DOR}) and the subsequent accumulation of their substrates. The stabilization of YFP-GAI, target of the SCF^{SLY1} complex, produced when C2/L2 is expressed supports this hypothesis.

The observation that the pleiotropic defects of transgenic C2/L2-expressing plants are not as severe as the multifaceted developmental phenotype of *cul1* or *csn5* mutants could be explained by the fact that C2/L2 does not completely impair derubylation but rather hinders it, so that the downstream changes are more subtle. On the other hand, and given that the CSN and the SCF complexes are essential for cell viability, it might also be feasible that the expression of C2/L2 could be counterselected, and consequently the expression level of the selected transgenic lines would be low. The fact that overexpression of some C2 proteins from a potato virus X–based vector induces severe developmental changes and the subsequent collapse of the plant (A.P. Luna and E.R. Bejarano, unpublished data) is in agreement with this idea. Another possibility would be that the effect of C2 on the SCF complexes could be specific rather than generalized, as suggested by the transcriptomic data. Curiously, Stuttmann et al. (2009) propose that defects in cullin derubylation may be tolerated without causing obvious physiological defects.

C2 Expression Modulates Jasmonate Responses

Although transgenic C2/L2 *Arabidopsis* plants display multiple phenotypes derived from the interference with the function of the SCF complexes, microarray analysis of C2-TS transgenic plants highlighted the jasmonate response as the main SCF-dependent hormone signaling pathway impaired in these plants. Reduction in responsiveness to jasmonates has been reported for transgenic plants expressing antisense RNA of *CSN5* (Schwechheimer et al., 2002), as well as for *csn* (Feng et al., 2003) and *cul1* mutants (Ren et al., 2005; Moon et al., 2007). Jasmonates are important plant signaling molecules that mediate biotic and abiotic stress responses as well as several aspects of growth and development. Plants respond to jasmonates by degrading the JAZ family of transcriptional regulators in a SCF^{COI1}complex- and a proteasome-dependent manner (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). Therefore, it is likely that C2/L2 may alter the jasmonate response through its effect on CUL1 rubylation. However, and given that C2/L2 is a multifunctional protein, we cannot rule out that the observed phenotype might be driven, partially or completely, by a different mechanism, such as the transcriptional activation activity of this viral protein.

Several lines of evidence indicate that the suppression of the jasmonate response is required for geminivirus infectivity: (1) MeJA treatment of *Arabidopsis* plants reduces BCTV infection; (2) infection of *Arabidopsis* with CaLCuV induces repression of the jasmonate response (Ascencio-Ibáñez et al., 2008); (3) the pathogenesis factor βC1 from DNAβ of TYLCCNV can suppress expression of several jasmonate-responsive genes (Yang et al., 2008). Moreover, the fact that both localization of a large number of geminiviruses (e.g., TYLCSV, TYLCV, and BCTV) and jasmonate synthesis occur preferentially in the phloem cells (Stenzel et al., 2003) makes the suppression of the jasmonate response a feasible target during infection. This suppression could have a direct effect in virus movement or replication by leading to several changes in the plant advantageous for the virus, such as the inhibition of the synthesis of secondary metabolites deleterious for viral replication or movement (e.g., phenylpropanoids; (Kandan et al., 2002; Matros and Mock, 2004), or might be aimed at circumventing phloem cell wall in growth development (Amiard et al., 2007).

Additionally, the interference with the jasmonate signaling may have an impact on the viral insect vector. Jasmonates are the hormones mediating plant defense against insects and could therefore be indirectly affecting geminivirus spread. Both TYLCSV and TYLCV are transmitted by the whitefly *Bemisia tabaci*, and it has been described that whitefly nymphs trigger the expression of jasmonate-responsive genes, which are important in slowing nymphal development (Kempema et al., 2007; Valenzuela-Soto et al., 2010). Through the suppression of the jasmonate response, the virus might be accelerating its vector's cycle, thus enhancing its own spread. On the other hand, this suppression could also prevent the synthesis of secondary metabolites that could interfere with the interaction between plant and insect (Bleeker et al., 2009).

Table 3. GO Analysis of the Intersection between C2 Downregulated or Upregulated Genes and the Upregulated Genes in the MeJA Microarray from Nemhauser et al. (2006)

Downregulated Genes	Level	Differentially Expressed (%)	Expected Frequency	P Value
Nonredundant GO Categories				
Response to stress	3	22.8% (26)	5.5%	5.04e-08
Secondary metabolic process	3	8.8% (10)	1.3%	0.00024
Immune response	3	6.1% (7)	0.7%	0.00140
Catabolic process	3	3.5% (4)	0.2%	0.00383
Defense response	3	5.3% (6)	0.5%	0.00160
Nitrogen compound metabolic process	3	9.6% (11)	1.5%	0.00011
Cellular biosynthetic process	4	19.3% (22)	7.3%	0.00263
Organic acid metabolic process	4	18.4% (21)	2.1%	4.07e-12
Aromatic compound metabolic process	4	9.6% (11)	1.0%	1.36e-06
Response to wounding	4	9.6% (11)	0.4%	6.46e-11
Innate immune response	4	6.1% (7)	0.7%	0.00140
Heterocycle metabolic process	4	7.9% (9)	0.6%	4.53e-06
Amine metabolic process	4	5.3% (6)	0.1%	1.64e-06
Response to jasmonic acid stimulus	4	10.5% (12)	0.5%	3.64e-11
Carboxylic acid metabolic process	5	18.4% (21)	2.1%	3.94e-12
Aromatic compound biosynthetic process	5	5.3% (6)	0.6%	0.00577
Nitrogen compound Biosynthetic process	5	7.9% (9)	0.6%	2.49e-06
Amino acid derivative biosynthetic process	6	5.3% (6)	0.6%	0.00702
Defense response, incompatible interaction	6	5.3% (6)	0.5%	0.00160
Amino acid metabolic process	6	8.8% (10)	1.1%	5.29e-05
Biogenic amine metabolic process	6	5.3% (6)	0.1%	1.64e-06
Toxin catabolic process	6	3.5% (4)	0.2%	0.00383
Jasmonic acid and ethylene-dependent systemic resistance	7	5.3% (6)	0.1%	2.38e-07
Indole derivative biosynthetic process	7	5.3% (6)	0.1%	3.56e-07
Amino acid biosynthetic process	7	7.0% (8)	0.5%	7.10e-06
Biogenic amine biosynthetic process	7	4.4% (5)	0.1%	3.67e-05
Indolalkylamine metabolic process	7	4.4% (5)	0.1%	3.72e-06
Indoleacetic acid biosynthetic process	8	2.6% (3)	0.0%	0.00035
Trp metabolic process	8	4.4% (5)	0.1%	3.72e-06
Glycosinolate biosynthetic process	9	2.6% (3)	0.1%	0.00930
Jasmonic acid metabolic process	9	5.6% (3)	0.1%	2.48e-08
Oxylipin biosynthetic process	9	5.6% (3)	0.1%	3.35e-08

GO category levels are indicated. The percentages of genes belonging to each category are reported for the differentially expressed genes and for the genes present in the microarray. The absolute number of differentially expressed genes belonging to each category is reported in parentheses.

C2/L2 Might Facilitate Co-Option of the SCF-Mediated Ubiquitination

According to our results, it seems that C2/L2 would be capable of hindering the activity of several SCF complexes in the plant cell, presumably conferring some biological advantage for the viral infection, such as suppression of hormone-mediated plant defense responses. It is an appealing hypothesis, however, that the virus might be not only impairing the function of the SCF complexes, but rather also redirecting them toward certain target proteins whose degradation would be advantageous for the viral infection. The fact that the overexpression of a given F-box protein can circumvent the general malfunction of the SCF complexes (Denti et al., 2006; Stuttmann et al., 2009) raises the idea that geminiviruses could be co-opting the SCF-mediated ubiquitination pathway for their own advantage through the promotion of the expression of selected adaptor subunits. It would be interesting to look for F-box proteins upregulated during geminivirus infection or in heterologous expression of geminiviral proteins to localize possible targets of this theoretical mechanism.

Some plant viruses have been shown to co-opt the SCF machinery by encoding their own viral F-box proteins, which are assembled into plant SCF complexes (Baumberger et al., 2007; Bortolamiol et al., 2007; Lageix et al., 2007), triggering the ubiquitination of plant proteins that interfere with viral infection. Thus, it would be a tempting hypothesis that, to maximize the effect of their encoded F-box proteins, promoting their efficient incorporation in the maximum possible number of SCF complexes, these plant viruses might have developed means to interfere with the assembly/disassembly cycle of SCF complexes, which is based in rubylation and derubylation.

In summary, viruses typically encode a few multifunctional proteins that enable them to redirect the host replication and transcriptional machineries to viral templates, reprogram host cells to provide an environment favorable for the viral infection, and counteract host defenses. The results obtained in this work

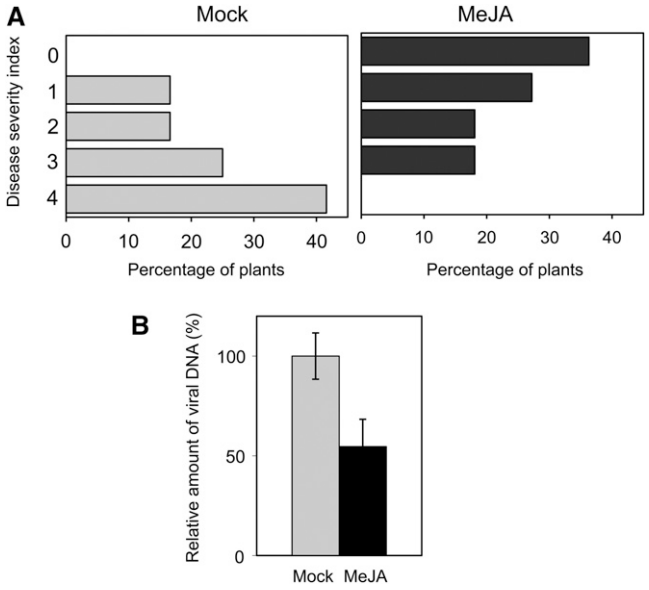

Figure 9. BCTV Infection of *Arabidopsis* Plants Treated with MeJA or Mock Solution.

Four- to five-week-old *Arabidopsis* plants were agroinoculated with BCTV, treated every other day with MeJA or mock solution (12 plants per treatment), and scored for the appearance of symptoms at 28 DAI. Total DNA was extracted from each plant independently and subjected to DNA gel blot to quantify viral DNA accumulation. The experiment was repeated twice; no differences in symptom development or viral DNA accumulation were observed between the replicates. The results of one of the experiments are shown.

(A) Symptom severity at 28 DAI according to the severity index described in Baliji et al. (2007), where 0 represent symptomless plants and 1 to 4 represent plants showing increasing symptom severity.

(B) Relative viral DNA accumulation. Bars represent SE.

unveil a powerful strategy used by geminiviruses, which involves the interaction with a hub regulator of protein ubiquitination, a mechanism that could allow the virus to trigger wide changes in the cellular homeostasis. Additional studies will be required to further dissect the molecular mechanisms underlying this strategy and to determine whether this is a generalized tactic for viruses.

METHODS

Microorganisms and General Methods

Manipulations of *Escherichia coli* and *Saccharomyces cerevisiae* strains and nucleic acids were performed according to standard methods (Ausubel et al., 1998; Sambrook and Russell, 2001). *E. coli* strain DH5-α was used for subcloning. All PCR-amplified fragments cloned in this work were fully sequenced. *Agrobacterium tumefaciens* GV3101 strain was used for the agroinfiltration assays, and LBA4404 was used for plant transformation. *S. cerevisiae* strain pJ696 (*MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*), a derivative of PJ69-4A (James et al., 1996), was used for the two-hybrid experiments. Plant DNA gel blots were performed as described by Castillo et al. (2004).

Plant Materials and Growth Conditions

Wild-type *Arabidopsis thaliana* used in this study is the Col ecotype. Seeds were surface sterilized and sown on Murashige and Skoog (MS) agar plates with 30 g/liter sucrose. Plates were cold treated for 2 to 6 d at 4°C. Seedlings were grown at 20°C under fluorescent white light (fluence rate of 40 to 60 μmol m^{-2} s^{-1}) with a 16-h-light/8-h-dark photoperiod. For far-red light treatments, seedlings were grown under continuous far-red light (fluence rate of 110 μmol m^{-2} s^{-1}). For dark-grown seedlings, plates were wrapped in several layers of aluminum foil.

For root growth inhibition assays, MS plates were placed in a vertical orientation for 5 d, and seedlings were then transferred to MS plates containing the tested hormone. Root length was scanned 5 d later using ImageJ software (http://rsb.info.nih.gov/ij). The hormones and concentrations used in the root growth inhibition assays were the following: 2,4-D (Duchefa Biochemie; 0.1 μM), MeJA (Duchefa Biochemie; 50 or 100 μM), GA_3 (Duchefa Biochemie; 0.2 μM), and ACC (Sigma-Aldrich A3903; 1 μM).

MeJA treatments for the infection experiments were as follows: a 50 μM MeJA solution or mock solution (containing 50 μM ethanol) were applied to 4-week-old *Arabidopsis* plants by spray every other day from 1 d before the inoculation to 28 DAI.

For the agroinfiltration experiments, *N.benthamiana* plants were grown in soil at 22°C in long-day conditions (16-h-light/8-h-dark photoperiod). For the root growth inhibition assays, wild-type and transgenic C2 *N. benthamiana* seeds were surface sterilized and sown on MS agar plates, and the seedlings were subjected to the corresponding treatments described for *Arabidopsis*.

The *csn5a-1* mutant (SALK_063436 line) was previously described (Gusmaroli et al., 2007).

For the transcriptomic analysis, T2 seedlings were grown on MS with kanamycin for 7 d and then were treated with hormone-containing or mock solutions at the indicated concentrations for the indicated time: 1 μM 2,4D, 1 h; 50 μM MeJA, 10 h; 1 μM GA_3, 1 h; 10 μM ACC, 1 h. Three independent replicates were performed. For these analyses, transgenic kanamycin-resistant plants containing with an expression cassette to express the firefly luciferase (*LUC*) reporter gene (Murray et al., 2002) were used as the control, and all seedlings were selected in kanamycin. Previously, the hormonal responses of the LUC plants were proved to be identical to those of the wild-type in the aforementioned assays.

Drought Tolerance Test

For the drought tolerance test, plants were initially grown on soil under a normal watering regime for 6 to 7 weeks. Watering was then halted and observations were taken after a further 10 d without water supply.

Weight Loss Measurements

For weight loss measurements, rosette leaves from 4-week-old plants were detached, placed on weighing dishes, and allowed to dry at room conditions. Weight of the samples was recorded at 0, 0.5, 1, 2, 3, and 4 h, and the percentage of initial weight was calculated for each point.

Stomatal Aperture Measurements

Rosette leaves from 4- to 5-week-old plants were exposed to white light for 2 h (fluence rate of 40 to 60 μmol m^{-2} s^{-1}) while submerged in a solution containing 50 mM KCl, 10 μM $CaCl_2$, 0.01% Tween 20, and 10 mM MES-KOH, pH 6.15, to induce stomatal aperture. Subsequently, 5 μM ABA (Sigma-Aldrich A4906) or mock solution was added to the buffer, and the samples were incubated under the same conditions for 1 h. Epidermal peels were stained with toluidine blue and observed under the microscope (TCS NT; Leica). Stomatal aperture was measured using ImageJ software.

Plasmids and Cloning

TYLCSV $C2_{T2C}$ mutant virus was generated by two-sided splicing by overlap extension (Ho et al., 1989). Primers pairs $C2_{T2C}$-F/Fragment2$C2_{T2C}$ –R and $C2_{T2C}$-R/ Fragment1$C2_{T2C}$ –F were used in the two initial PCR reactions; subsequent amplification used primers Fragment1$C2_{T2C}$ –F/ Fragment2$C2_{T2C}$ –R. The PCR product was cloned into the *Eco*RV site of pBluescript SKII+ (Stratagene) to yield pBSSK-TYA14NdeI/NcoI. An *Nde*I/*Nco*I fragment containing the wild-type C2 start codon in pGreen-TYA14 was replaced by the *Nde*I/*Nco*I fragment in pBSSK-TYA14Nde/NcoI to generate pGreenTYA14$C2_{T2C}$.

For the yeast two-hybrid constructs, cDNA from *Arabidopsis* and tomato (*Solanum lycopersicum*) were generated from total RNA extracted from seedlings and leaves respectively. One microgram of total RNA was used for first-strand cDNA synthesis using oligo(dT) primers and SuperScript II reverse transcriptase reagent (Invitrogen) following the manufacturer's instructions. *Arabidopsis CSN5A*$_{44\text{-}357}$ and *CSN5B*$_{44\text{-}358}$ and tomato *JAB* $(CSN5)_{57\text{-}357}$ were PCR amplified and cloned into pGADT7 vector (Clontech). Full *C2*/*L2* open reading frame (ORF) from TYLCSV (accession number L27708), TYLCV (accession number AF071228), and BCTV (accession number AF379637) were amplified by PCR and cloned into pGBKT7 vector (Clontech).

For the BiFC experiments, cDNA from *Arabidopsis* and tomato were generated as indicated for the yeast two-hybrid constructs. *Arabidopsis CSN5* and tomato *JAB* ORFs were PCR amplified and cloned into the *pENTR*/*D-TOPO* vector. Full *C2*/*L2* ORF from TYLCSV, TYLCV, and BCTV was amplified by PCR and cloned into the *pENTR*/*D-TOPO* vector. Cloned ORFs were inserted by LR reaction (Invitrogen) into the binary pBiFP vectors pBiFP2 and pBiFP3 (de Lucas et al., 2008) containing the N- or C-terminal fragments of the eYFP fluorescent protein (NYFP and CYFP). The resulting constructs were used to transform *A. tumefaciens* GV3101.

For plant transformation and transient expression, PCR fragments containing the *C2*/*L2* full ORF of TYLCSV, TYLCV, and BCTV were blunt-cloned in pBluescript SKII+ (Stratagene) (TYLCV *C2* and BCTV *L2*) or cloned into the *Hpa*I site of pSXSN1 (M.A. Sanchez-Durán et al., unpublished data) (TYLCSV *C2*) to yield pC2TM, pL2BC, and pSXC2TS, respectively. Fragments containing TYLCV *C2* and BCTV *L2* full ORFs were obtained from pC2TM and pL2BC by *Hpa*I/*Kpn*I digestion and subcloned in the *Hpa*I/*Kpn*I sites of pBINX (M.A. Sanchez-Durán et al., unpublished data) to yield pBINX-C2-TM and pBINX-L2-BC. A fragment comprising an expression cassette containing the TYLCSV *C2* full ORF was obtained from pSXC2TS by *Xba*I digestion and subcloned into the *Xba*I site of the binary vector pBIN+ (van Engelen et al., 1995) to yield pBIN-C2-TS.

For the subcellular localization study, TYLCSV *C2* ORF was fused to GFP at its N terminus, and the fusion protein or GFP alone was cloned under the control of the 35S promoter in a pBINX1 (M.A. Sánchez-Durán et al., unpublished data) to yield pBINX1-GFP-C2 and pBINX1-GFP. *GFP* ORF was PCR amplified from pSMGFP (Davis and Vierstra, 1998).

Supplemental Table 1 online contains all the oligonucleotides used in this study. Supplemental Table 2 online summarizes the engineering of the plasmids used in this work.

Phylogenetic Analysis

Amino acid plant CSN5 homolog proteins were aligned with ClustalW (http://www.ebi.ac.uk/clustalw/index.html). Afterwards, the alignment was checked and manually adjusted. The resultant aligned sequences (see Supplemental Data Set 1 online) were used to construct a phylogenetic tree (unrooted) using neighbor-joining clustering based on pairwise mean character differences conducted in SEAVIEW (SEAVIEW 4,2,12; http://pbil.univ-lyon1.fr/software/seaview.html; Gouy et al., 2010). Statistical support of branches was assessed by bootstrap analyses using 1000 resamples of the data matrix.

Yeast Two-Hybrid Assay

The yeast strain PJ696, which contains the reporter genes *lacZ*, *HIS3*, and *ADE2*, was used in the two-hybrid assays (Fields and Song, 1989). Assays were performed as described (Castillo et al., 2004). Yeast were cotransformed and selected for bait and prey plasmids, as described Yeast Protocols Handbook (Clontech Laboratories).

BiFC Assays

Different combination of the *A. tumefaciens* clones expressing the fusion proteins (NYFP-CSN5/CYFP-C2/L2 or NYFP-C2/L2/CYFP-CSN5) were coinfiltrated into the abaxial surface of 2- to 3-week-old *N. benthamiana* plants as described (Voinnet et al., 2003). The p19 protein of *Tomato bushy stunt virus* (pBIN61-p19, kindly provided by Olivier Voinnet, Strasbourg, France) was used to suppress gene silencing. *A. tumefaciens* strains containing the pBiFP constructs or the p19 plasmid were at a D_{600} ratio of 1:1:1 for infiltration. Fluorescence was visualized in epidermal cell layers of the leaves after 3 d of infiltration using a Leica DMR confocal microscope.

Subcellular Localization

A. tumefaciens GV3101 was transformed with pBIN1-GFP-C2 or pBIN1-GFP plasmids and coinfiltrated with p19 as described above. *A. tumefaciens* strains containing pBINX1-GFP-C2 or pBIN1-GFP (as a control) and the p19 silencing plasmid were at a D_{600} ratio of 1:1 for infiltration. Fluorescence was visualized in epidermal cell layers of the leaves after 3 d of infiltration using a Leica DMR fluorescence microscope.

Plant Transformation

Arabidopsis transformation was performed by floral dip (Clough and Bent, 1998) using *A. tumefaciens* GV3101 containing pBIN-C2-TS, pBINX-C2-TM, or pBINX-L2-BC. Transformants were selected with kanamycin (50 μg/mL). *N. benthamiana* plants were transformed with pBIN-C2-TS as described by Morilla et al. (2006). Ten independent lines per construct were selected and subjected to expression analysis by RNA gel blot. Unless otherwise indicated, T2 seeds from the lines C2-TS 9, C2-TM 1, and L2-BC 4 were used in this work. Further information about the transgenic lines is provided in Supplemental Figure 3 online.

Transient Expression Assays

For the in vivo degradation assay, the YFP-GAI construct (kindly provided by David Alabadí, Instituto de Biología Molecular y Celular de Plantas, Valencia, Spain) and the C2/L2 expression constructs were used to transform *A. tumefaciens* GV3101. Three days after infiltration, the agroinfiltrated leaves were sprayed with a 100 μM GA_3 solution or with mock solution containing the GA_3 solvent (ethanol). Fluorescence was visualized 1 to 2 h later using an epifluorescence Leica microscope MZ FLIII.

Immunoblot and Gel Filtration Assays

Immunoblot and gel filtration analyses of plant extracts were performed as described by Gusmaroli et al. (2004). In all cases in which equal loading was required, the same samples were probed with α-RPN6 to confirm equal loading. For quantitative experiments, multiple exposures were obtained to assure that the film was not saturated. The antibodies used in this study are as follows: α-CUL1 (Wang et al., 2002), α-CUL3 (Figueroa et al., 2005), α-CUL4 (Chen et al., 2006), α-CSN5 (Kwok et al., 1998), α-RBX1 (Schwechheimer et al. 2002), α-SKP1 (Gray et al., 1999), and α-GFP (kindly provided by Olivier Voinnet, Strasbourg, France).

Quantitative Real-Time PCR

Primer pairs for real-time PCR were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/; see Supplemental Table 1 online). Gene-specific primers were chosen so that the PCR products were 100 to 300 bp. Total RNA was extracted using RNAeasy Plant Mini Kit (Qiagen) and treated on column with Dnase (Qiagen). One microgram of total RNA was used for first-strand cDNA synthesis using oligo(dT) primers and Super-Script II reverse transcriptase reagent (Invitrogen) following the manufacturer's instructions. For real-time PCR, the reaction mixture consisted of cDNA first-strand template, primer mix (5 μmol each) and SYBR Green Master Mix (Quanta Biosciences) in a total volume of 25 μL. The PCR conditions were as follows: 10 min at 95°C and 40 cycles of 30 s at 95°C and 30 s at 60°C. The reactions were performed using a Rotor-Gene real time cycler (Qiagen). A relative quantification real-time PCR method was used to compare expression of the genes in transgenic versus non-transgenic line (Panchuk et al., 2002). Relative quantification describes the change in expression of the target gene in a test sample relative to calibrator sample. Actin was used as the internal control. The sample of LUC transgenic plants was used as the calibrator, with the expression level of the sample set to 1. Each data point is the mean value from three experimental replicate determinations. Each cDNA sample used is a mixture from three biological replicates at a ratio of 1:1:1.

Transcriptomic Studies

Microarray analysis was performed at the Unité de Recherche en Génomique Végétale (Evry, France) using the CATMA arrays, containing 24,576 gene-specific tags corresponding to 22,089 genes from *Arabidopsis* (Crowe et al., 2003; Hilson et al., 2004). For each point, three independent biological replicates were produced. For each biological repetition, RNA samples were obtained by pooling RNAs from 8 to 10 plants. Samples were collected on plants at 1.10 to 1.12 developmental growth stages (Boyes et al., 2001), cultivated in MS plus kanamycin. Total RNA was isolated from three replicates of the 35S:LUC and transgenic C2-TS seedlings using TRIzol (Invitrogen) and subsequently cleaned using the RNeasy MinElute cleanup kit (Invitrogen). RNA quantity and quality were assessed with a Nanodrop ND-1000 spectrophotometer (Labtech) and an Agilent 2100 bioanalyzer (Agilent Technologies), respectively. For each comparison and each biological replicate, a dye-swap was performed (i.e., six hybridizations per comparison). The labeling of cRNAs with Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products), the hybridization to the slides, and the scanning were performed as described by Lurin et al. (2004).

For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green), and no background was subtracted. An array-by-array normalization was performed to remove systematic biases. First, spots considered badly formed features were excluded. Then, a global intensity-dependent normalization using the loess procedure (see Yang and Speed, 2002) was performed to correct the dye bias. Finally, for each block, the log-ratio median calculated over the values for the entire block was subtracted from each individual log-ratio value to correct effects on each block, as well as print tip, washing, and/or drying effects.

Differential analysis was based on the log ratios averaged on the dye-swap: the technical replicates were averaged to get one log-ratio per biological replicate, and these values were used to perform a paired *t* test. For each spot, the empirical variance was calculated and then a trimmed variance was calculated from spots, which did not display extreme variance. The spots that were excluded were those with a specific variance/common variance ratio smaller than the α-quantile of a χ^2 distribution of two degree of liberty or greater than the 1-α-quantile of a χ^2 distribution of two degree of liberty with α equal to 0.0001 (the same order of magnitude as the probe number). The raw P values were adjusted by the Bonferroni method, which controls the family-wise error rate to keep a strong control of the false positives in a multiple-comparison context. We considered as being differentially expressed the spots with a Bonferroni P value ≤ 0.05. A detailed description of the normalization step and of the variance modeling used in the differential analysis is available in Gagnot et al. (2008).

Geminivirus Infection Assays

Twenty plants were agroinoculated with pGreenTYA14 (binary vector containing a partial dimer of TYLCSV; see Supplemental Table 2 online) or pGreenTYA14C2$_{T2C}$ (the same construct carrying a T-C transition in the start codon of the C2 ORF). For control, five plants were mock inoculated with *A. tumefaciens* harboring the empty binary vector pGreen-0229 (Hellens et al., 2000). Symptoms were evaluated every week until 42 DAI. Samples were taken at 21 DAI.

BCTV viral infections of *Arabidopsis* were performed by agroinoculation using wild-type virus (Briddon et al., 1989). Symptoms were evaluated every week until 28 DAI. Samples were taken at 28 DAI.

Viral DNA accumulation was quantified by DNA gel blot hybridization of total plant DNA. Two micrograms of total DNA were used. Membranes were hybridized with TYLCSV or BCTV radiolabeled probes. Viral DNA accumulation was quantified by phosphorimager analyses of DNA gel blots and normalized to genomic DNA.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes used in this article are as follows: AT1G22920 (CSN5A), AT1G71230 (CSN5B), AT4G02570 (CUL1), AT1G26830 (CUL3A), AT1G69670 (CUL3B), AT5G46210 (CUL4), AT3G23240 (ERF1), AT2G40940 (ERS1), AT1G73590 (PIN1), At3g15540 (IAA19), At5g45460 (MFC19.13), At5g64120 (MHJ24.10), At2G06050 (OPR3), and At3G16470 (JR1). Microarray data from this article were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE24475 and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project AU07-12_GeminiSelSup) according to the Minimum Information About a Microarray Experiment standards.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Infection of *N. benthamiana* Plants with TYLCSV Wild Type or C2$_{T2C}$ Mutant.

Supplemental Figure 2. Comparison of CSN5 from Several Plant Species.

Supplemental Figure 3. Characterization of the Transgenic *Arabidopsis* Lines Expressing C2/L2.

Supplemental Figure 4. CUL1 and CUL3 Protein Accumulation in Transgenic *Arabidopsis* Lines Expressing C2/L2.

Supplemental Figure 5. Hypocotyl Length of 9-d-Old Dark-Grown Transgenic C2-TS, C2-TM, and L2-BC or Wild-Type *Arabidopsis* Seedlings.

Supplemental Figure 6. Reduced Response to Hormones in Transgenic *Arabidopsis* and *N. benthamiana* Plants Expressing C2.

Supplemental Figure 7. Evaluation of the Expression of Two Differentially Expressed Genes in Transgenic C2-TS *Arabidopsis* Plants for Microarray Validation.

Supplemental Figure 8. Relative Expression of CUL3 in C2-TS, C2-TM, and L2-BC or Control *Arabidopsis* Seedlings Determined by Quantitative Real-Time PCR.

Supplemental Table 1. Oligonucleotides Used in This Study.

Supplemental Table 2. Plasmids Generated in This Work.

Supplemental Table 3. Hallmark Genes of the Jasmonate Response Downregulated in the Microarray of C2-TS Transgenic Plants.

Supplemental Data Set 1. Text File of Alignment Corresponding to Phylogenetic Analysis in Supplemental Figure 2.

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REFERENCES

Agudelo-Romero, P., Carbonell, P., de la Iglesia, F., Carrera, J., Rodrigo, G., Jaramillo, A., Pérez-Amador, M.A., and Elena, S.F. (2008). Changes in the gene expression profile of *Arabidopsis thaliana* after infection with Tobacco etch virus. Virol. J. **5:** 92.

Amiard, V., Demmig-Adams, B., Mueh, K.E., Turgeon, R., Combs, A.F., and Adams III, W.W. (2007). Role of light and jasmonic acid signaling in regulating foliar phloem cell wall ingrowth development. New Phytol. **173:** 722–731.

Aronson, M.N., Meyer, A.D., Györgyey, J., Katul, L., Vetten, H.J., Gronenborn, B., and Timchenko, T. (2000). Clink, a nanovirus-encoded protein, binds both pRB and SKP1. J. Virol. **74:** 2967–2972.

Ascencio-Ibáñez, J.T., Sozzani, R., Lee, T.J., Chu, T.M., Wolfinger, R.D., Cella, R., and Hanley-Bowdoin, L. (2008). Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant Physiol. **148:** 436–454.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1998). Current Protocols in Molecular Biology. (New York: John Wiley & Sons).

Baliji, S., Sunter, J., and Sunter, G. (2007). Transcriptional analysis of complementary sense genes in spinach curly top virus and functional role of C2 in pathogenesis. Mol. Plant Microbe Interact. **20:** 194–206.

Bari, R., and Jones, J.D. (2009). Role of plant hormones in plant defence responses. Plant Mol. Biol. **69:** 473–488.

Baumberger, N., Tsai, C.H., Lie, M., Havecker, E., and Baulcombe, D.C. (2007). The Polerovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. Curr. Biol. **17:** 1609–1614.

Bleeker, P.M., Diergaarde, P.J., Ament, K., Guerra, J., Weidner, M., Schütz, S., de Both, M.T., Haring, M.A., and Schuurink, R.C. (2009). The role of specific tomato volatiles in tomato-whitefly interaction. Plant Physiol. **151:** 925–935.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., and Ziegler-Graff, V. (2007). The Polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Curr. Biol. **17:** 1615–1621.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., and Görlach, J. (2001). Growth stage-based phenotypic analysis of *Arabidopsis*: A model for high throughput functional genomics in plants. Plant Cell **13:** 1499–1510.

Briddon, R.W., Watts, J., Markham, P.G., and Stanley, J. (1989). The coat protein of beet curly top virus is essential for infectivity. Virology **172:** 628–633.

Castillo, A.G., Kong, L.J., Hanley-Bowdoin, L., and Bejarano, E.R. (2004). Interaction between a geminivirus replication protein and the plant sumoylation system. J. Virol. **78:** 2758–2769.

Chen, H., Shen, Y., Tang, X., Yu, L., Wang, J., Guo, L., Zhang, Y., Zhang, H., Feng, S., Strickland, E., Zheng, N., and Deng, X.W. (2006). *Arabidopsis* CULLIN4 forms an E3 ubiquitin ligase with RBX1 and the CDD complex in mediating light control of development. Plant Cell **18:** 1991–2004.

Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J.M., Lorenzo, O., García-Casado, G., López-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666–671.

Cho, J.H., Kim, H.B., Kim, H.S., and Choi, S.B. (2008). Identification and characterization of a rice MCM2 homologue required for DNA replication. BMB Rep. **41:** 581–586.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Crowe, M.L., et al. (2003). CATMA: A complete Arabidopsis GST database. Nucleic Acids Res. **31:** 156–158.

Davis, S.J., and Vierstra, R.D. (1998). Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol. Biol. **36:** 521–528.

del Pozo, J.C., and Estelle, M. (1999). The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. Proc. Natl. Acad. Sci. USA **96:** 15342–15347.

de Lucas, M., Davière, J.M., Rodríguez-Falcón, M., Pontin, M., Iglesias-Pedraz, J.M., Lorrain, S., Fankhauser, C., Blázquez, M.A., Titarenko, E., and Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. Nature **451:** 480–484.

Denti, S., Fernandez-Sanchez, M.E., Rogge, L., and Bianchi, E. (2006). The COP9 signalosome regulates Skp2 levels and proliferation of human cells. J. Biol. Chem. **281:** 32188–32196.

Dieterle, M., Zhou, Y.C., Schäfer, E., Funk, M., and Kretsch, T. (2001). EID1, an F-box protein involved in phytochrome A-specific light signaling. Genes Dev. **15:** 939–944.

Dreher, K., and Callis, J. (2007). Ubiquitin, hormones and biotic stress in plants. Ann. Bot. (Lond.) **99:** 787–822.

Etessami, P., Callis, R., Ellwood, S., and Stanley, J. (1988). Delimitation of essential genes of cassava latent virus DNA 2. Nucleic Acids Res. **16:** 4811–4829.

Feng, S., Ma, L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N., and Deng, X.W. (2003). The COP9 signalosome interacts physically with SCF COI1 and modulates jasmonate responses. Plant Cell **15:** 1083–1094.

Fields, S., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. Nature **340:** 245–246.

Figueroa, P., Gusmaroli, G., Serino, G., Habashi, J., Ma, L., Shen, Y., Feng, S., Bostick, M., Callis, J., Hellmann, H., and Deng, X.W. (2005). *Arabidopsis* has two redundant Cullin3 proteins that are essential for embryo development and that interact with RBX1 and BTB proteins to form multisubunit E3 ubiquitin ligase complexes in vivo. Plant Cell **17:** 1180–1195.

Fukumoto, A., Tomoda, K., Kubota, M., Kato, J.Y., and Yoneda-Kato,

N. (2005). Small Jab1-containing subcomplex is regulated in an anchorage- and cell cycle-dependent manner, which is abrogated by ras transformation. FEBS Lett. **579:** 1047–1054.

Gagnot, S., Tamby, J.P., Martin-Magniette, M.L., Bitton, F., Taconnat, L., Balzergue, S., Aubourg, S., Renou, J.P., Lecharny, A., and Brunaud, V. (2008). CATdb: A public access to Arabidopsis transcriptome data from the URGV-CATMA platform. Nucleic Acids Res. **36** (Database issue): D986–D990.

Gilkerson, J., Hu, J., Brown, J., Jones, A., Sun, T.P., and Callis, J. (2009). Isolation and characterization of cul1-7, a recessive allele of CULLIN1 that disrupts SCF function at the C terminus of CUL1 in *Arabidopsis thaliana*. Genetics **181:** 945–963.

Gouy, M., Guindon, S., and Gascuel, O. (2010). SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. **27:** 221–224.

Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. Genes Dev. **13:** 1678–1691.

Gusmaroli, G., Feng, S., and Deng, X.W. (2004). The *Arabidopsis* CSN5A and CSN5B subunits are present in distinct COP9 signalosome complexes, and mutations in their JAMM domains exhibit differential dominant negative effects on development. Plant Cell **16:** 2984–3001.

Gusmaroli, G., Figueroa, P., Serino, G., and Deng, X.W. (2007). Role of the MPN subunits in COP9 signalosome assembly and activity, and their regulatory interaction with *Arabidopsis* Cullin3-based E3 ligases. Plant Cell **19:** 564–581.

Hanley-Bowdoin, L., Settlage, S.B., and Robertson, D. (2004). Reprogramming plant gene expression: A prerequisite to geminivirus DNA replication. Mol. Plant Pathol. **5:** 149–156.

Hao, L., Wang, H., Sunter, G., and Bisaro, D.M. (2003). Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. Plant Cell **15:** 1034–1048.

Harmon, F.G., and Kay, S.A. (2003). The F box protein AFR is a positive regulator of phytochrome A-mediated light signaling. Curr. Biol. **13:** 2091–2096.

Hartitz, M.D., Sunter, G., and Bisaro, D.M. (1999). The tomato golden mosaic virus transactivator (TrAP) is a single-stranded DNA and zinc-binding phosphoprotein with an acidic activation domain. Virology **263:** 1–14.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., and Mullineaux, P.M. (2000). pGreen: A versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol. Biol. **42:** 819–832.

Hilson, P., et al. (2004). Versatile gene-specific sequence tags for Arabidopsis functional genomics: Transcript profiling and reverse genetics applications. Genome Res. **14** (10B): 2176–2189.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:** 51–59.

Hotton, S.K., and Callis, J. (2008). Regulation of cullin RING ligases. Annu. Rev. Plant Biol. **59:** 467–489.

Hsieh, Y.H., Su, I.J., Wang, H.C., Tsai, J.H., Huang, Y.J., Chang, W.W., Lai, M.D., Lei, H.Y., and Huang, W. (2007). Hepatitis B virus pre-S2 mutant surface antigen induces degradation of cyclin-dependent kinase inhibitor p27Kip1 through c-Jun activation domain-binding protein 1. Mol. Cancer Res. **5:** 1063–1072.

Hua, Z., Zou, C., Shiu, S.H., and Vierstra, R.D. (2011). Phylogenetic comparison of F-Box (FBX) gene superfamily within the plant kingdom reveals divergent evolutionary histories indicative of genomic drift. PLoS ONE **6:** e16219.

Isaacson, M.K., and Ploegh, H.L. (2009). Ubiquitination, ubiquitin-like modifiers, and deubiquitination in viral infection. Cell Host Microbe **5:** 559–570.

James, P., Halladay, J., and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:** 1425–1436.

Kameda, K., Fukao, M., Kobayashi, T., Tsutsuura, M., Nagashima, M., Yamada, Y., Yamashita, T., and Tohse, N. (2006). CSN5/Jab1 inhibits cardiac L-type Ca^{2+} channel activity through protein-protein interactions. J. Mol. Cell. Cardiol. **40:** 562–569.

Kandan, A., Commare, R.R., Nandakumar, R., Ramiah, M., Raguchander, T., and Samiyappan, R. (2002). Induction of phenylpropanoid metabolism by *Pseudomonas fluorescens* against tomato spotted wilt virus in tomato. Folia Microbiol. (Praha) **47:** 121–129.

Kempema, L.A., Cui, X., Holzer, F.M., and Walling, L.L. (2007). Arabidopsis transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs. Similarities and distinctions in responses to aphids. Plant Physiol. **143:** 849–865.

Kertbundit, S., De Greve, H., Deboeck, F., Van Montagu, M., and Hernalsteens, J.P. (1991). In vivo random beta-glucuronidase gene fusions in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **88:** 5212–5216.

Komatsu, K., Hashimoto, M., Ozeki, J., Yamaji, Y., Maejima, K., Senshu, H., Himeno, M., Okano, Y., Kagiwada, S., and Namba, S. (2010). Viral-induced systemic necrosis in plants involves both programmed cell death and the inhibition of viral multiplication, which are regulated by independent pathways. Mol. Plant Microbe Interact. **23:** 283–293.

Kovac, M., Müller, A., Milanovic Jarh, D., Milavec, M., Düchting, P., and Ravnikar, M. (2009). Multiple hormone analysis indicates involvement of jasmonate signalling in the early defence of potato to potato virus Y^{NTN}. Biol. Plant **53:** 195–199.

Kuroda, H., Takahashi, N., Shimada, H., Seki, M., Shinozaki, K., and Matsui, M. (2002). Classification and expression analysis of Arabidopsis F-box-containing protein genes. Plant Cell Physiol. **43:** 1073–1085.

Kwok, S.F., Solano, R., Tsuge, T., Chamovitz, D.A., Ecker, J.R., Matsui, M., and Deng, X.W. (1998). *Arabidopsis* homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic cop/det/fus mutations. Plant Cell **10:** 1779–1790.

Lageix, S., Catrice, O., Deragon, J.M., Gronenborn, B., Pélissier, T., and Ramírez, B.C. (2007). The nanovirus-encoded Clink protein affects plant cell cycle regulation through interaction with the retinoblastoma-related protein. J. Virol. **81:** 4177–4185.

Liu, Y., Schiff, M., and Dinesh-Kumar, S.P. (2004). Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to tobacco mosaic virus. Plant J. **38:** 800–809.

Liu, Y., Schiff, M., Serino, G., Deng, X.W., and Dinesh-Kumar, S.P. (2002). Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to Tobacco mosaic virus. Plant Cell **14:** 1483–1496.

Lurin, C., et al. (2004). Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16:** 2089–2103.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Shevchenko, A., and Deshaies, R.J. (2001). Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. Science **292:** 1382–1385.

Mahalingam, S., Ayyavoo, V., Patel, M., Kieber-Emmons, T., Kao, G.D., Muschel, R.J., and Weiner, D.B. (1998). HIV-1 Vpr interacts with a human 34-kDa mov34 homologue, a cellular factor linked to the G2/M phase transition of the mammalian cell cycle. Proc. Natl. Acad. Sci. USA **95:** 3419–3424.

Marrocco, K., Zhou, Y., Bury, E., Dieterle, M., Funk, M., Genschik, P., Krenz, M., Stolpe, T., and Kretsch, T. (2006). Functional analysis of EID1, an F-box protein involved in phytochrome A-dependent light signal transduction. Plant J. **45:** 423–438.

Matros, A., and Mock, H.P. (2004). Ectopic expression of a UDP-glucose:phenylpropanoid glucosyltransferase leads to increased resistance of transgenic tobacco plants against infection with Potato Virus Y. Plant Cell Physiol. **45:** 1185–1193.

Moon, J., Zhao, Y., Dai, X., Zhang, W., Gray, W.M., Huq, E., and Estelle, M. (2007). A new CULLIN 1 mutant has altered responses to hormones and light in Arabidopsis. Plant Physiol. **143:** 684–696.

Morilla, G., Castillo, A.G., Preiss, W., Jeske, H., and Bejarano, E.R. (2006). A versatile transreplication-based system to identify cellular proteins involved in geminivirus replication. J. Virol. **80:** 3624–3633.

Mundt, K.E., Liu, C., and Carr, A.M. (2002). Deletion mutants in COP9/signalosome subunits in fission yeast *Schizosaccharomyces pombe* display distinct phenotypes. Mol. Biol. Cell **13:** 493–502.

Murray, S.L., Thomson, C., Chini, A., Read, N.D., and Loake, G.J. (2002). Characterization of a novel, defense-related Arabidopsis mutant, cir1, isolated by luciferase imaging. Mol. Plant Microbe Interact. **15:** 557–566.

Nemhauser, J.L., Hong, F., and Chory, J. (2006). Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell **126:** 467–475.

Nordgård, O., Dahle, O., Andersen, T.O., and Gabrielsen, O.S. (2001). JAB1/CSN5 interacts with the GAL4 DNA binding domain: A note of caution about two-hybrid interactions. Biochimie **83:** 969–971.

Oh, W., Yang, M.R., Lee, E.W., Park, K.M., Pyo, S., Yang, J.S., Lee, H.W., and Song, J. (2006). Jab1 mediates cytoplasmic localization and degradation of West Nile virus capsid protein. J. Biol. Chem. **281:** 30166–30174.

Oron, E., Mannervik, M., Rencus, S., Harari-Steinberg, O., Neuman-Silberberg, S., Segal, D., and Chamovitz, D.A. (2002). COP9 signalosome subunits 4 and 5 regulate multiple pleiotropic pathways in *Drosophila melanogaster*. Development **129:** 4399–4409.

Panchuk, I.I., Volkov, R.A., and Schöffl, F. (2002). Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in Arabidopsis. Plant Physiol. **129:** 838–853.

Raja, P., Wolf, J.N., and Bisaro, D.M. (2010). RNA silencing directed against geminiviruses: Post-transcriptional and epigenetic components. Biochim. Biophys. Acta **1799:** 337–351.

Ren, C., Pan, J., Peng, W., Genschik, P., Hobbie, L., Hellmann, H., Estelle, M., Gao, B., Peng, J., Sun, C., and Xie, D. (2005). Point mutations in Arabidopsis Cullin1 reveal its essential role in jasmonate response. Plant J. **42:** 514–524.

Rojas, M.R., Hagen, C., Lucas, W.J., and Gilbertson, R.L. (2005). Exploiting chinks in the plant's armor: Evolution and emergence of geminiviruses. Annu. Rev. Phytopathol. **43:** 361–394.

Sambrook, J., and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Santner, A., and Estelle, M. (2009). Recent advances and emerging trends in plant hormone signalling. Nature **459:** 1071–1078.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M., and Deng, X.W. (2001). Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. Science **292:** 1379–1382.

Schwechheimer, C., Serino, G., and Deng, X.W. (2002). Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. Plant Cell **14:** 2553–2563.

Sharma, P., Ikegami, M., and Kon, T. (2010). Identification of the virulence factors and suppressors of posttranscriptional gene silencing encoded by Ageratum yellow vein virus, a monopartite begomovirus. Virus Res. **149:** 19–27.

Sheard, L.B., et al. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468:** 400–405.

Stenzel, I., Hause, B., Maucher, H., Pitzschke, A., Miersch, O., Ziegler, J., Ryan, C.A., and Wasternack, C. (2003). Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato - Amplification in wound signalling. Plant J. **33:** 577–589.

Stuttmann, J., Lechner, E., Guérois, R., Parker, J.E., Nussaume, L., Genschik, P., and Noël, L.D. (2009). COP9 signalosome- and 26S proteasome-dependent regulation of SCFTIR1 accumulation in Arabidopsis. J. Biol. Chem. **284:** 7920–7930.

Sunter, G., and Bisaro, D.M. (1992). Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. Plant Cell **4:** 1321–1331.

Sunter, G., Sunter, J.L., and Bisaro, D.M. (2001). Plants expressing tomato golden mosaic virus AL2 or beet curly top virus L2 transgenes show enhanced susceptibility to infection by DNA and RNA viruses. Virology **285:** 59–70.

Tanaka, Y., et al. (2006). The hepatitis B virus X protein enhances AP-1 activation through interaction with Jab1. Oncogene **25:** 633–642.

Tanguy, G., Drévillon, L., Arous, N., Hasnain, A., Hinzpeter, A., Fritsch, J., Goossens, M., and Fanen, P. (2008). CSN5 binds to misfolded CFTR and promotes its degradation. Biochim. Biophys. Acta **1783:** 1189–1199.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448:** 661–665.

Tomoda, K., Kato, J.Y., Tatsumi, E., Takahashi, T., Matsuo, Y., and Yoneda-Kato, N. (2005). The Jab1/COP9 signalosome subcomplex is a downstream mediator of Bcr-Abl kinase activity and facilitates cell-cycle progression. Blood **105:** 775–783.

Trinks, D., Rajeswaran, R., Shivaprasad, P.V., Akbergenov, R., Oakeley, E.J., Veluthambi, K., Hohn, T., and Pooggin, M.M. (2005). Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. J. Virol. **79:** 2517–2527.

Valenzuela-Soto, J.H., Estrada-Hernández, M.G., Ibarra-Laclette, E., and Délano-Frier, J.P. (2010). Inoculation of tomato plants (*Solanum lycopersicum*) with growth-promoting *Bacillus subtilis* retards whitefly *Bemisia tabaci* development. Planta **231:** 397–410.

van den Burg, H.A., Tsitsigiannis, D.I., Rowland, O., Lo, J., Rallapalli, G., Maclean, D., Takken, F.L., and Jones, J.D. (2008). The F-box protein ACRE189/ACIF1 regulates cell death and defense responses activated during pathogen recognition in tobacco and tomato. Plant Cell **20:** 697–719.

van Engelen, F.A., Molthoff, J.W., Conner, A.J., Nap, J.P., Pereira, A., and Stiekema, W.J. (1995). pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res. **4:** 288–290.

van Wezel, R., Liu, H., Tien, P., Stanley, J., and Hong, Y. (2001). Gene C2 of the monopartite geminivirus tomato yellow leaf curl virus-China encodes a pathogenicity determinant that is localized in the nucleus. Mol. Plant Microbe Interact. **14:** 1125–1128.

Vierstra, R.D. (2009). The ubiquitin-26S proteasome system at the nexus of plant biology. Nat. Rev. Mol. Cell Biol. **10:** 385–397.

Vigliocco, A., Bonamico, B., Alemano, S., Miersch, O., and Abdala, G. (2002). Stimulation of jasmonic acid production in *Zea mays* L. infected by the maize rough dwarf virus-Río Cuarto. Reversion of symptoms by salicylic acid. Biocell **26:** 369–374.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003). An

enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. **33:** 949–956.

Wang, X., Feng, S., Nakayama, N., Crosby, W.L., Irish, V., Deng, X.W., and Wei, N. (2003). The COP9 signalosome interacts with SCF UFO and participates in *Arabidopsis* flower development. Plant Cell **15:** 1071–1082.

Wang, X., Kang, D., Feng, S., Serino, G., Schwechheimer, C., and Wei, N. (2002). CSN1 N-terminal-dependent activity is required for Arabidopsis development but not for Rub1/Nedd8 deconjugation of cullins: A structure-function study of CSN1 subunit of COP9 signalosome. Mol. Biol. Cell **13:** 646–655.

Wartig, L., Kheyr-Pour, A., Noris, E., De Kouchkovsky, F., Jouanneau, F., Gronenborn, B., and Jupin, I. (1997). Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2, and C2 ORFs in viral pathogenesis. Virology **228:** 132–140.

Wei, N., Serino, G., and Deng, X.W. (2008). The COP9 signalosome: More than a protease. Trends Biochem. Sci. **33:** 592–600.

Yang, J.Y., Iwasaki, M., Machida, C., Machida, Y., Zhou, X., and Chua, N.H. (2008). betaC1, the pathogenicity factor of TYLCCNV, interacts with AS1 to alter leaf development and suppress selective jasmonic acid responses. Genes Dev. **22:** 2564–2577.

Yang, Y.H., and Speed, T. (2002). Design issues for cDNA microarray experiments. Nat. Rev. Genet. **3:** 579–588.

Zhang, Y., Xu, W., Li, Z., Deng, X.W., Wu, W., and Xue, Y. (2008). F-box protein DOR functions as a novel inhibitory factor for abscisic acid-induced stomatal closure under drought stress in Arabidopsis. Plant Physiol. **148:** 2121–2133.

Geminiviruses Subvert Ubiquitination by Altering CSN-Mediated Derubylation of SCF E3 Ligase Complexes and Inhibit Jasmonate Signaling in *Arabidopsis thaliana*

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Identification of Host Genes Involved in Geminivirus Infection Using a Reverse Genetics Approach

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Abstract

Geminiviruses, like all viruses, rely on the host cell machinery to establish a successful infection, but the identity and function of these required host proteins remain largely unknown. *Tomato yellow leaf curl Sardinia virus* (TYLCSV), a monopartite geminivirus, is one of the causal agents of the devastating Tomato yellow leaf curl disease (TYLCD). The transgenic 2IRGFP *N. benthamiana* plants, used in combination with Virus Induced Gene Silencing (VIGS), entail an important potential as a tool in reverse genetics studies to identify host factors involved in TYLCSV infection. Using these transgenic plants, we have made an accurate description of the evolution of TYLCSV replication in the host in both space and time. Moreover, we have determined that TYLCSV and *Tobacco rattle virus* (TRV) do not dramatically influence each other when co-infected in *N. benthamiana*, what makes the use of TRV-induced gene silencing in combination with TYLCSV for reverse genetic studies feasible. Finally, we have tested the effect of silencing candidate host genes on TYLCSV infection, identifying eighteen genes potentially involved in this process, fifteen of which had never been implicated in geminiviral infections before. Seven of the analyzed genes have a potential anti-viral effect, whereas the expression of the other eleven is required for a full infection. Interestingly, almost half of the genes altering TYLCSV infection play a role in postranslational modifications. Therefore, our results provide new insights into the molecular mechanisms underlying geminivirus infections, and at the same time reveal the 2IRGFP/VIGS system as a powerful tool for functional reverse genetics studies.

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Introduction

Geminiviruses are a large family of plant viruses with circular, single stranded DNA genomes packaged within geminate particles [1]. The *Geminiviridae* family [2] is divided into four genera according to their genome organization and biological properties. The genus *Begomovirus* includes members that are transmitted by whiteflies, infect dicotyledonous plants, and may have either bipartite or monopartite genomes. *Tomato yellow leaf curl Sardinia virus* (TYLCSV) is a member of the *Begomovirus* genus, and is one of the causal agents of the Tomato yellow leaf curl disease (TYLCD), which can cause up to 100% yield losses in tomato fields [3,4,5]. TYLCSV has a monopartite genome of 2.8 kb in size, which encodes six proteins and contains an intergenic region (IR) comprising the origin of replication and viral promoters. The open reading frames (ORFs) in the complementary sense orientation encode a replication-associated protein (Rep, also known as C1), a transcriptional activator protein (TrAP, also known as C2), and a replication enhancer protein (REn, also known as C3); a small ORF, C4, is located within the Rep ORF but in a different reading frame. The virion strand contains two ORFs encoding the coat protein (CP) and a small protein named V2 [4,5].

To establish a successful infection, viruses must create a proper environment for viral propagation, which involves hijacking the cellular machinery for viral functions and, at the same time, preventing or counteracting the plant defence mechanisms. To fulfil these requirements, viral proteins trigger changes in the cell at all levels: transcriptional, translational and posttranslational. Identifying the host genes involved in viral replication, movement, and generally all those processes that lead to the establishment of a successful infection, could provide valuable new targets to ultimately generate viral resistance.

The advances in high-throughput technologies and bioinformatics have made possible to assess gene expression massively, providing an insight into the host's transcriptional responses to viral infections in a genome-wide fashion. These transcriptomic studies, together with proteomic studies, are providing an unprecedented vision of the "host-side" of the plant-virus interaction, leading to the identification of host genes whose function or expression is altered as a consequence of the infection. Geminiviruses have also been recently the subject of this kind of study, unveiling host genes differentially expressed either during the infection [6,7,8] or upon expression of a viral protein [8,9,10]. However, despite all this information being available, it is still a

daunting task to determine the exact role of these host genes in the infection process. Notably, this is particularly challenging in the case of monopartite geminiviruses, in which gene replacement with marker genes is not feasible, and thus are more tedious to monitor. In a previous work, we described the generation of *Nicotiana benthamiana* transgenic plants containing a GFP (Green fluorescence protein) expression cassette flanked by two repeats of TYLCSV IR as a tool to monitor TYLCSV replication [11]. These plants, named 2IRGFP, entail an important potential as a tool in reverse genetics studies to identify host factors involved in the viral infection, when used in combination with VIGS (Virus Induced Gene Silencing) technology. Although the feasibility of this approach was previously confirmed by silencing the Proliferating cellular nuclear antigen (PCNA) and Sumo conjugating enzyme (SCE1) genes [11,12], its use in a larger screening required an optimization of the conditions.

In this work, we explore further the potential of 2IRGFP *N. benthamiana* plants in combination with VIGS to identify host genes with a role in geminivirus infection. We have achieved an accurate description of the dynamics of viral replication by monitoring GFP expression in both space and time, explored the limitations of the strategy to be used in a reverse-genetics screening, and unveiled the effect of silencing selected *N. benthamiana* genes, most of them previously identified in transcriptomic or protein-protein interaction studies, in geminivirus infection. Using this strategy, we have identified eighteen genes involved in several cellular processes whose silencing alters TYLCSV infection. Notably, for fifteen of these genes this is the first description of a role in viral infections. Hence, our results provide new insights into the molecular mechanisms underlying geminivirus infections, and at the same time reveal the 2IRGFP/VIGS system as a powerful tool for functional reverse genetics studies.

Results

Dynamics of *Tomato yellow leaf curl Sardinia virus* infection in transgenic 2IRGFP *N. benthamiana* plants is not altered by co-infection with *Tobacco rattle virus*

Traditionally, the development of geminivirus infections has been monitored by symptom evaluation and quantification of viral DNA by nucleic acid hybridization or PCR [13,14]. These methods, however, have important limitations to monitor the infection in both space and time. Symptom evaluation is semi-quantitative at best, and does not necessarily correlate with viral accumulation. Hybridization or PCR studies, on the other hand, are destructive methods that are not able to discriminate if the viral molecules accumulated in a certain plant organ or tissue have been produced *in situ* or, on the contrary, have been originated elsewhere and subsequently transported. Due to these restrictions, a comprehensive study of the dynamics of the geminivirus infection, considering active replication and not merely virus accumulation, is still lacking.

In a previous work [11], we developed *N. benthamiana* transgenic plants that overexpress GFP in those cells where the virus is replicating. During TYLCSV infection, these plants, named 2IRGFP, display a Rep-dependent GFP overexpression driven by the generation of mGFP replicons. Since overproduction of GFP correlates with TYLCSV active replication, these plants provide an unprecedented opportunity to monitor TYLCSV infection. For this purpose, 2IRGFP plants were infected with TYLCSV (three independent experiments, 20 plants each), GFP expression was exhaustively monitored and samples were collected at different times post-infection. For each time point, three plants were sampled (one per independent experiment); for each of the sampled plants, the three most apical leaves were taken, and tissue printing was performed with the main root. Total DNA was extracted from the harvested leaves, and both mGFP replicons and viral DNA were detected by DNA hybridization.

According to the extension and intensity of GFP expression in leaves, we visually distinguished five phenotypes, which we named RAP (for Replication-Associated Phenotype) 0, 1, 2, 3 and 4, as depicted in Figure S1. Leaves from uninfected plants show a low expression of GFP extended through the whole leaf surface (RAP0). In RAP1, which corresponds to the first stage of the virus infection, GFP overexpression appears in some of the vascular bundles and the background GFP expression is not extensively silenced. RAP2 represents the stage of maximum GFP accumulation, in which an intense green fluorescence is observed as a continuous pattern through the leaf vascular bundles, and the GFP expression background in the leaf lamina has faded. RAP3 is the last stage in GFP expression, where GFP can only be detected in distinct areas of the leaf vascular bundles, before it completely disappears (RAP4). The average evolution of GFP expression in the leaves of TYLCSV-infected plants is depicted in Figure 1A. At 7 days post-infection (dpi), GFP over-expression associated to RAP1 phenotype can already be observed in some, but not most, plants, and accumulation of mGFP replicons and viral DNA is already detectable (Figure 1B). One week later, at 14 dpi, the maximum levels of viral replication, monitored as GFP overexpression (RAP2), are reached in the most apical leaves. As expected, this increase in GFP correlates with a higher accumulation of mGFP replicons and viral DNA. The RAP2 phenotype is maintained in the apical leaves up to 28 dpi, while GFP silencing is extensively detected from 21 dpi in the rest of the leaves. The decrease in GFP over-expression observed from 35 dpi onwards (Figure 1A) correlates to the reduction of mGFP replicons (Figure 1B); the viral accumulation, however, is high, most likely due to previous rounds of replication. As seen in this figure, TYLCSV is also replicating in the roots between 14 and 35 dpi, as indicated by GFP overexpression. The appearance of GFP in roots correlated with presence of viral DNA in the tissue printing (Figure 1C) until 42 dpi, when no GFP can be observed but accumulation of viral DNA is detected. This viral DNA is most likely the result of previous viral replication in the root, or even in the aerial parts of the plant. It is noteworthy that viral DNA could be detected in roots as early as 7 dpi, before GFP expression is clearly noticeable; bearing in mind that the root is a sink organ, this is probably the result of transport from leaves where the virus is actively replicating (Figure 1B).

Once an extensive description of the dynamics of TYLCSV infection has been achieved, detecting changes in the timing or pattern of GFP over-expression due to silencing of a given host gene should be easy and reliable. *Tobacco rattle virus* (TRV)-based silencing vectors have been widely used and offer several advantages over other viral vectors, such as their abilities to mediate VIGS in the absence of TRV-derived symptoms and to target host RNAs in the growing points of plants. To accurately evaluate the impact of TRV infection on the evolution of the RAP phenotype, we monitored the GFP expression in 2IRGFP plants co-infected with TRV and TYLCSV (three independent experiments, 20 plants each). TRV/TYLCSV co-infected plants showed the same pattern of RAP phenotypes described for TYLCSV infected plants; the only detectable difference between single and double infected plants is a slight delay of approximately two days in the appearance of RAP phenotypes.

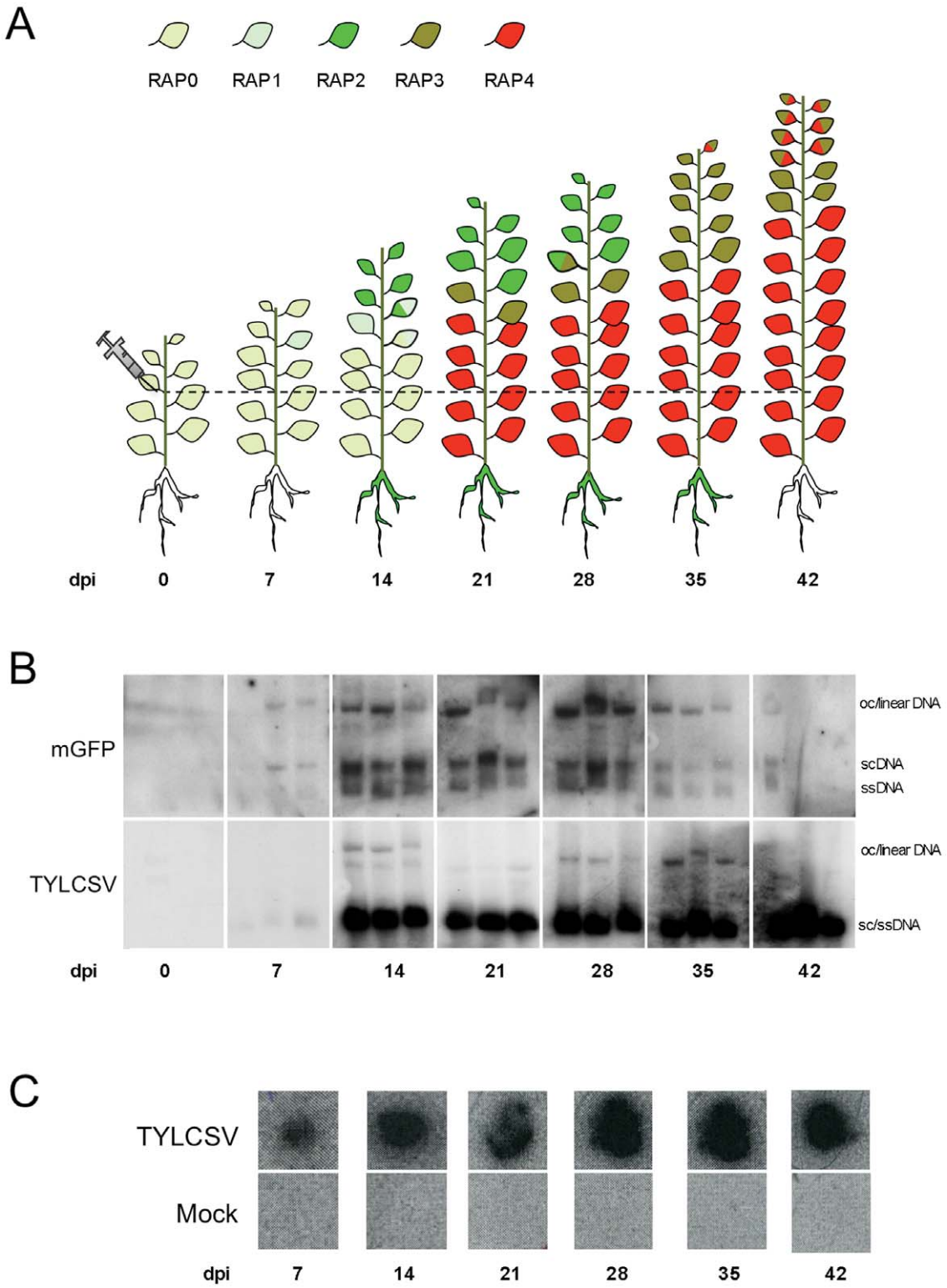

Figure 1. Phenotypic and molecular analysis of TYLCSV-infected 2IRGFP *N. benthamiana* plants. (A) Evolution of RAP phenotypes in TYLCSV-infected transgenic *N. benthamiana* 2IRGFP plants. The diagram displays the average RAP phenotypes of leaves and the induction of GFP in roots at different days post-infection (dpi). Leaves containing areas of two different colours indicate an equivalent coexistence of RAP phenotypes in the population. In roots, green colour indicates GFP overexpression. The depicted results are the average of 60 infected plants. The dashed line marks the inoculation point. (B) Detection of episomal replicons (mGFP) and virus (TYLCSV) in leaves of infected plants. DNA was extracted from the three most apical leaves of three independent plants infected with TYLCSV. Undigested DNA was blotted and hybridized with probes specific for mGFP or TYLCSV. Bands representing open circle (oc), supercoiled (sc) or single-stranded (ss) forms of DNA are indicated. (C) Detection of virus (TYLCSV) in roots of infected plants in tissue printing.
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TYLCSV infection does not revert TRV-induced gene silencing in *N. benthamiana*

Since several proteins encoded by TYLCSV can function as suppressors of gene silencing (A. P. Luna et al., in preparation), TYLCSV infection might interfere with the TRV-induced silencing. To test this possibility, we evaluated the effect of TYLCSV infection on the silencing of either a *GFP* transgene or the endogenous *Sulfur* (*Sul*) gene. To determine the impact of TYLCSV infection on the silencing of the *GFP* transgene, *N. benthamiana* plants constitutively expressing GFP (line 16c) [15,16] were co-infected with TRV:*GFP* and TYLCSV or infected with TRV:*GFP* alone as a control. Infection with TRV:*GFP* triggered the silencing of the transgene, and this silencing was fully extended by 15 dpi (Figure 2A). Co-infection with TYLCSV did not alter this silencing phenotype, indicating that TYLCSV does not interfere with the TRV-induced *GFP* silencing (Figure 2A).

The *Sul* gene was chosen to evaluate the effect of TYLCSV infection on the silencing of an endogenous gene, for it produces a readily visible phenotype when silenced, derived from its involvement in chlorophyll synthesis [17]. 2IRGFP *N. benthamiana* plants were co-infected with TRV:*Sul* and TYLCSV or infected with TRV:*Sul* alone as a control. Once again, co-infection with TYLCSV did not affect the silencing phenotype of TRV:*Sul*

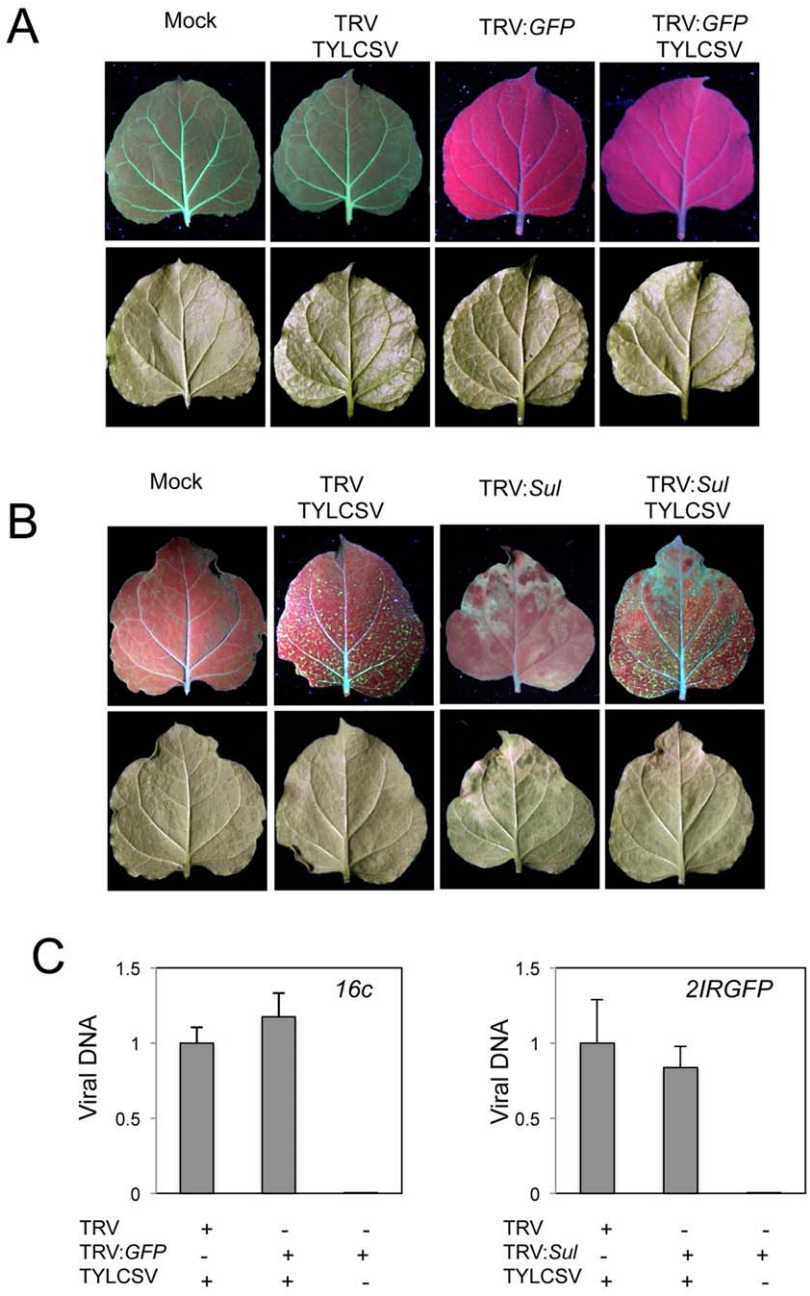

Figure 2. Effect of TYLCSV infection on TRV-induced silencing of *GFP* or *Sul*. Leaves from *N. benthamiana* 16c (A) or 2IRGFP (B) transgenic *N. benthamiana* plants 15 days after inoculation with TRV:*GFP* or TRV:*Sul*, respectively, or co-inoculation with TRV or TRV:*GFP*/*Sul* and TYLCSV. (C) Relative amount of TYLCSV DNA determined by quantitative real-time PCR. Values are the mean of five replicates. Bars represent standard error.
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infected plants (Figure 2B), indicating that TYLCSV does not alter the TRV-induced silencing of this endogenous gene.

Quantification of TYLCSV accumulation using quantitative real-time PCR shows that TRV-induced silencing of either *GFP* or *Sul* does not affect TYLCSV accumulation (Figure 2C).

Simultaneous TRV-induced silencing of multiple genes in *N. benthamiana* plants

One drawback to VIGS is that it very often does not produce a uniform silencing throughout the plant. If the silencing of the gene does not generate a readily visible phenotype, it will be very difficult to distinguish silenced from non-silenced tissues, what would dramatically complicate the interpretation of results. A strategy to compensate for the lack of uniformity of VIGS would incorporate an internal reference to monitor the level of silencing. This system would act as a control for the VIGS vector, marking the silenced areas with a visible phenotype. The use of internal markers for VIGS based in visual phenotypes has been implemented in several plant species and has proven very successful for empowering the method as a tool in reverse genetics. Some works have demonstrated that the simultaneous silencing of several genes is possible by including multiple gene sequences in the same silencing vector [18,19,20]. With the aim of developing a visual reporter system to mark silenced areas in *N. benthamiana* leaves, we decided to follow two different approaches: (*i*) Test if the silencing triggered by two distinct TRV constructs co-localize, and (*ii*) Test if the silencing triggered by two different gene sequences cloned in tandem in the same TRV vector co-localize. For these assays we used two gene sequences whose silencing produces a readily visible phenotype: the *Sul* gene and *PCNA* [11,21].

The results obtained are presented in Figure S2. In our system, silencing of the two marker genes does not significantly co-localize when the two TRV clones are co-inoculated in the plant. Only 13.6% of the new leaves in co-inoculated plants displayed both phenotypes, and the percentages of leaves showing each phenotype considered separately are lower than in single inoculations, indicating that co-inoculation apparently leads to a decreased silencing efficiency. A similar effect is observed when both genes are cloned in tandem in the same TRV vector, although the percentage of leaves showing simultaneous *Sul*- and *PCNA*-silenced phenotypes is slightly higher (20%) (Figure S2). Segregation of the silencing phenotypes warns against the use of this strategy as a marker system for gene silencing in *N. benthamiana* leaves.

Selection and cloning of candidate genes

As a first step in the identification of host genes required for TYLCSV infection, we made a selection of candidate genes following several criteria: (*i*) Genes encoding proteins known to physically interact with geminivirus proteins; (*ii*) Genes exclusively or preferentially expressed in phloematic tissues; (*iii*) Genes transactivated by the C2 homologue from the geminiviruses *Mungbean yellow mosaic virus* and *African cassava mosaic geminivirus*; (*iv*) Genes involved in cellular processes potentially required for geminivirus infection (Table 1). A total of 114 genes were initially included as candidate genes.

silencing could be reached by expressing a DNA fragment of 21 to 23 nucleotides bearing 100% identity to the target gene [22], this is often not efficient at triggering silencing and longer sequences must be used [22,23]. The highest efficiency of VIGS appears to be achieved using fragments in the range of 300–500 nucleotides with multiple stretches of more than 23 nucleotides identity [24,25]. Because of their different sources, our candidate genes belong to different species. Cloning 300–500 bp fragments of the *N. benthamiana* homologous gene would be the strategy of choice; unfortunately, the *N. benthamiana* genome has not been sequenced yet and thus the gene sequences are in most cases not available. To circumvent this difficulty, we carried out homology analyses in all selected genes to identify sequences of 300–500 bp conserved in different plant species, including *Arabidopsis* and tomato. The use of heterologous gene sequences to silence their respective orthologs in *N. benthamiana* has been previously reported [26]. Chosen sequences were further analysed with Invitrogen Block-iT^{TM} RNAi designer (https://rnaidesigner.invitrogen.com/rnaiexpress/) to localize potential efficient siRNAs within the sequence: the fragment of choice was that containing the largest number of proposed siRNA molecules. The selection process is depicted in the flow diagram in Figure 3.

After this analysis, 54 out of the initial 114 genes were maintained as candidate genes (Table 1). Since the sequence of these selected genes was highly conserved, we decided to use the *Arabidopsis* cDNAs to generate the VIGS constructs, with the aim of rendering this strategy faster and more homogeneous. We ordered the 42 *Arabidopsis* cDNA clones that were available at NASC (European *Arabidopsis* Stock Centre) (Table S1) and the selected 300–500 bp fragment for each cDNA was PCR-amplified and cloned in the TRV RNA2-based VIGS vector pTV00 [27]. The primers used to amplify each fragment are included in Table S1.

Screening of candidate genes in *N. benthamiana* 2IRGFP plants

Once the time course of TYLCSV infection in 2IRGFP plants had been established, we followed the strategy depicted in Figure 4A to test the potential effect of candidate gene silencing on TYLCSV infection (Table 1). Summing up, we induced gene silencing for each candidate host gene in 2IRGFP plants using TRV constructs, and subsequently infected these plants with TYLCSV. Plants infiltrated with the empty TRV vector and infected with TYLCSV were used as a control; plants infiltrated with the *Sul*-containing TRV vector were used as a control of VIGS efficiency. GFP overexpression was monitored daily from 9 to 15 dpi under UV light.

According to the effect of their silencing on TYLCSV infection, measured as time of appearance and intensity of GFP expression, we grouped the tested host genes into three classes: those whose silencing did not cause changes in GFP expression (group A), or those whose silencing promoted earlier (group B) or later/lower/null (group C) GFP expression (Table 1; examples of each class are shown in Figure 4B).

Representative genes belonging to groups A (*SKL2*, *ECR1*), B (*UBA1*, *GLO1* and *RPA32*) and C (*HSC70*, *ASK2*, and *deltaCOP*) were chosen to evaluate the impact of their silencing on TYLCSV infection, measured as viral DNA accumulation. For this purpose, 2IRGFP *N. benthamiana* plants were co-inoculated with the TRV derivative clones and TYLCSV. At 15 dpi, total DNA was extracted from the pooled three most apical leaves of each plant and the relative amount of viral DNA was determined using quantitative real-time PCR (two independent experiments, 5 plants each). The mean values of TYLCSV accumulation are represented in Figure 4C. As expected from the GFP overexpression data, silencing of *UBA1* or *GLO1* and silencing of *RPA32* tripled and doubled TYLCSV accumulation, respectively. On the other hand, silencing of *HSC70* and *ASK2* reduced TYLCSV accumulation by 70 and 30%, respectively. Strikingly, silencing of the *deltaCOP* subunit completely abolished TYLCSV accumulation.

Table 1. List of candidate genes.

Identity	Function	Selection criteria	Reference	ACC *A. thaliana*
Group A (no detected effect on infection)				
A-type cyclin-dependent kinase (*CDK2*)	Cell cycle control	Cellular process	[6]	AT3G48750
Cullin-associated and neddylation-dissociated (*CAND1*)	Protein metabolism	TrAP/C2 interaction	Hericourt *et al.* (in preparation)	AT2G02560
DNA polymerase alpha 2 (*POLA2*)	DNA metabolism	Cellular process	[80]	AT1G67630
DNA polymerase delta small subunit (*POLD2*)	DNA metabolism	Cellular process	[80]	AT2G42120
E2F transcription factor 1 (*E2FB*)	Transcription	Cellular process	[6]	AT5G22220
Geminivirus Rep-interacting kinase (*GRIK1*)	Signal transduction	Rep interaction	[81]	AT3G45240
Histone 3 K4-specific methyltransferase SET7/9	Unknown	TrAP/C2 interaction	Hericourt *et al.* (in preparation)	AT1G21920
Homologue to co-chaperone DNAJ-like protein (*ATJ3*)	Protein folding	C3 interaction	Hericourt *et al.* (in preparation)	AT3G44110
NSP interacting kinase 2 (*NIK2*)	Signal transduction	Phloem over-expression	[82]	AT3G25560
Putative nucleic acid binding/transcription factor (*JDK*)	Unknown	TrAP/C2 interaction	Hericourt *et al.* (in preparation)	AT5G03150
Putative transcriptional activators with NAC domain (*ATAF1*)	Transcription	C3 interaction	[83]	AT1G01720
Putative shikimate kinase (*SKL2*)	Unknown	CP interaction	Hericourt *et al.* (in preparation)	AT2G35500
Retinoblastoma-related protein (*RBR*)	Cell cycle control	Rep interaction	[84,85]	AT3G12280
RUB-activating enzyme subunit (*ECR1*)	Protein modification	Cellular process	[6,86]	AT5G19180
Scarecrow-like protein (*SCL13*)	Transcription	Phloem over-expression	[82]	AT4G17230
SNF1-related protein kinase (*AKIN11*)	Signal transduction	TrAP/C2 interaction	[87]	AT3G29160
SUMO activating enzyme (*SAE1B*)	Protein metabolism	Cellular process	[88]	AT5G50580
Transcription factor IIA gamma chain (*TFIIA-S*)	Transcription	Phloem over-expression	[89]	AT4G24440
Wound inducive gene (*F14P1.1*)	Stress	C4 interaction	Hericourt *et al.* (in preparation)	AT1G19660
Group B (promote earlier infection)				
Bearskin 2 (*BRN2*)	Transcription	Phloem over-expression	[89]	AT4G10350
Importin alpha isoform 4 (*IMPA-4*)	Transport	CP interaction	[71]	AT1G09270
Lactoylglutathione lyase (*GLO1*)	Stress	C3 Interaction	Hericourt *et al.* (in preparation)	AT1G15380
Replication protein A32 (*RPA32/RPA2*)	DNA metabolism	Rep interaction	[32]	AT3G02920
Dehydration responsive 21 (*RD21*)	Stress	V2 interaction	Hericourt *et al.* (in preparation)	AT1G47128
RING-type E3 ubiquitin ligase (*RHF2A*)	Protein modification	Transactived by TrAP/C2	[82]	AT5G22000
Ubiquitin activating enzyme (*UBA1*)	Protein modification	TrAP/C2 Interaction	Hericourt *et al.* (in preparation)	AT2G30110
Group C (delay, reduce or prevent the infection)				
4-coumarate:CoA ligase (*AT4CL1*)	Metabolism	Phloem over-expression	[89]	AT1G51680
Allene oxide cyclase (*AOC1*)	Metabolism	Phloem over-expression	[82]	AT3G25760
Barely any meristem 1 *(BAM1)*	Protein modification	C4 interaction	Hericourt *et al.* (in preparation)	AT5G65700
Coatomer delta subunit (*deltaCOP*)	Protein transport	C3 interaction	Hericourt *et al.* (in preparation)	AT5G05010
COP9 signalosome subunit 3 (*CSN3*)	Protein modification	Cellular process	[90]	AT5G14250
Geminivirus Rep A-binding (*GRAB2*)	Transcription	Rep interaction	[29]	AT5G61430
Heat shock protein cognate 70 (*HSC70*)	Protein modification	Phloem over-expression	[82]	AT5G02500
Nuclear acetyltransferase (*NSI*)	Signal transduction	NSP Interaction	[31]	AT1G32070
Patatin-like protein 2 (*PLP2*)	Stress	Phloem over-expression	[82]	AT2G26560
Shaggy-related kinase kappa (*SK4-1/SKK*)	Protein modification	C4 interaction	Hericourt *et al.* (in preparation)	AT1G09840
SKP1-like 2 (*ASK2*)	Protein modification	Transactived by TrAP/C2	[9]	AT5G08590

The criterion for selection is indicated in each case. The accession numbers (ACC) of the homologous *Arabidopsis* gene used in the VIGS experiments are indicated in this case.
doi:10.1371/journal.pone.0022383.t001

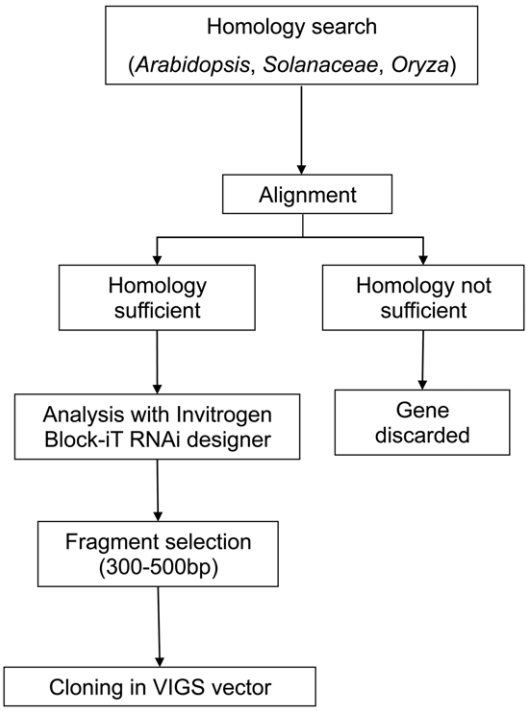

Figure 3. Gene selection strategy. Flow diagram depicting the strategy used for selecting the candidate genes to be tested using the 2IRGFP plants/TRV-based system.
doi:10.1371/journal.pone.0022383.g003

Discussion

Replication dynamics of TYLCSV

Transgenic 2IRGFP *N. benthamiana* plants have proven to be an accurate and sensitive tool that allows monitoring TYLCSV infection real-time and in a non-destructive manner. Using these transgenic plants, we have been able to describe the dynamics of TYLCSV infection in great detail, determining in which tissues the virus is replicating on an average infection at a certain time. To our knowledge, this is the first description of the replication dynamics of a geminivirus infection in both space and time, as most of the previous studies reflect viral DNA accumulation but not active replication.

According to our results, TYLCSV replication can be detected in leaves placed above the inoculation point at 7 dpi. One week later (14 dpi), viral replication is taking place in the apical leaves of all inoculated plants, where it is maintained at a high level until 28 dpi. From that moment onwards, the rate of viral replication decreases, and eight weeks after the inoculation it is only detectable in limited areas of apical leaves. These observations suggest that the virus is able to maintain the replication of its genome, in the aerial parts of the host plant, only in certain leaves and during a limited period of time. Additionally, the virus is also able to replicate in roots between 14 and 35 dpi. Interestingly, while we observe a direct correlation between the changes in GFP expression and the accumulation of episomal replicons (mGFP), the amount of viral DNA seems to be maintained even when viral replication can no longer be detected. These data suggest that, although both DNA molecules are produced by the same mechanism, mGFP replicons must be degraded whereas the viral DNA is not, maybe as a result of its encapsidation.

Double infection with TYLCSV and TRV does not significantly affect TYLCSV infection or TRV-induced silencing

We have demonstrated that co-infection with TRV does not dramatically affect TYLCSV infection in *N. benthamiana*. This fact makes it feasible to use TYLCSV in combination with TRV-mediated VIGS as a tool in reverse genetics studies to identify host factors involved in the geminivirus infection. We observed, however, a slight delay in the development of TYLCSV infection when in combination with TRV. This delay makes the use of appropriate controls (co-infection with the empty TRV vector) of special importance for this type of analysis. Although TYLCSV, like all geminiviruses, encodes suppressors of gene silencing (A. P. Luna et al., in preparation), it does not noticeably affect TRV-induced gene silencing in *N. benthamiana* plants. In agreement with these results, TRV-mediated VIGS has been successfully used in combination with geminiviral infections in tomato in a recent work [28].

Despite our efforts, the attempt to establish a visual reporter system based on the silencing of the *Sulfur* gene has been fruitless. Although simultaneous silencing of two genes is achieved by both co-infiltration of independent TRV-based constructs or by infiltration with a TRV construct harbouring multiple gene sequences (Figure S2), silencing does not significantly co-localize in any case, and the extension of the silencing of each gene considered independently diminishes (Figure S2). Even though the reasons for this outcome remain obscure, the absence of significant co-localization makes it impractical to use this co-silencing approach as a marker for VIGS. A similar effect of simultaneous silencing in *N. benthamiana* had been previously described [21].

Identification of host genes involved in TYLCSV infection

Using our reverse genetics approach, based on the use of transgenic 2IRGFP *N. benthamiana* plants, we have been able to demonstrate that silencing of 18 out of 37 analysed host genes alters TYLCSV infection.

Bearing in mind the limitations of VIGS, and since we have not tested the silencing of those candidate genes in which no effect on TYLCSV infection could be detected (group A), we cannot rule out the possibility that we may have false negatives: some of the tested genes might not have been efficiently silenced, and thus their potential impact on the viral infection would go unnoticed. For this reason, we cannot assess that those tested candidate genes without an obvious effect on TYLCSV infection do not play a role in the viral infection. False positive results, on the other hand, would be more difficult to obtain in our experimental system, and as long as the proper controls are being used we consider the positive results as reliable. In this context, a reasonable concern would be the possibility of silencing unwanted host genes as a consequence of sequence homology with the target host gene. In order to evaluate this undesired effect, we performed a BLAST homology search with every sequence used for VIGS, confirming that the only hit in each case was the selected target gene. However, and since the *N. benthamiana* genome has not been sequenced yet, this is a possible risk that should be kept in mind.

Additionally, it is noteworthy that this screening method tests the candidate gene in the context of the infection, and consequently those genes identified should be biologically relevant.

Out of the eighteen genes whose silencing alters TYLCSV infection, seven have a potential anti-viral effect, since TYLCSV replication is enhanced when they are silenced (group B), whereas the expression of the other eleven is required for a full infection, for their silencing negatively impacts this process (group C).

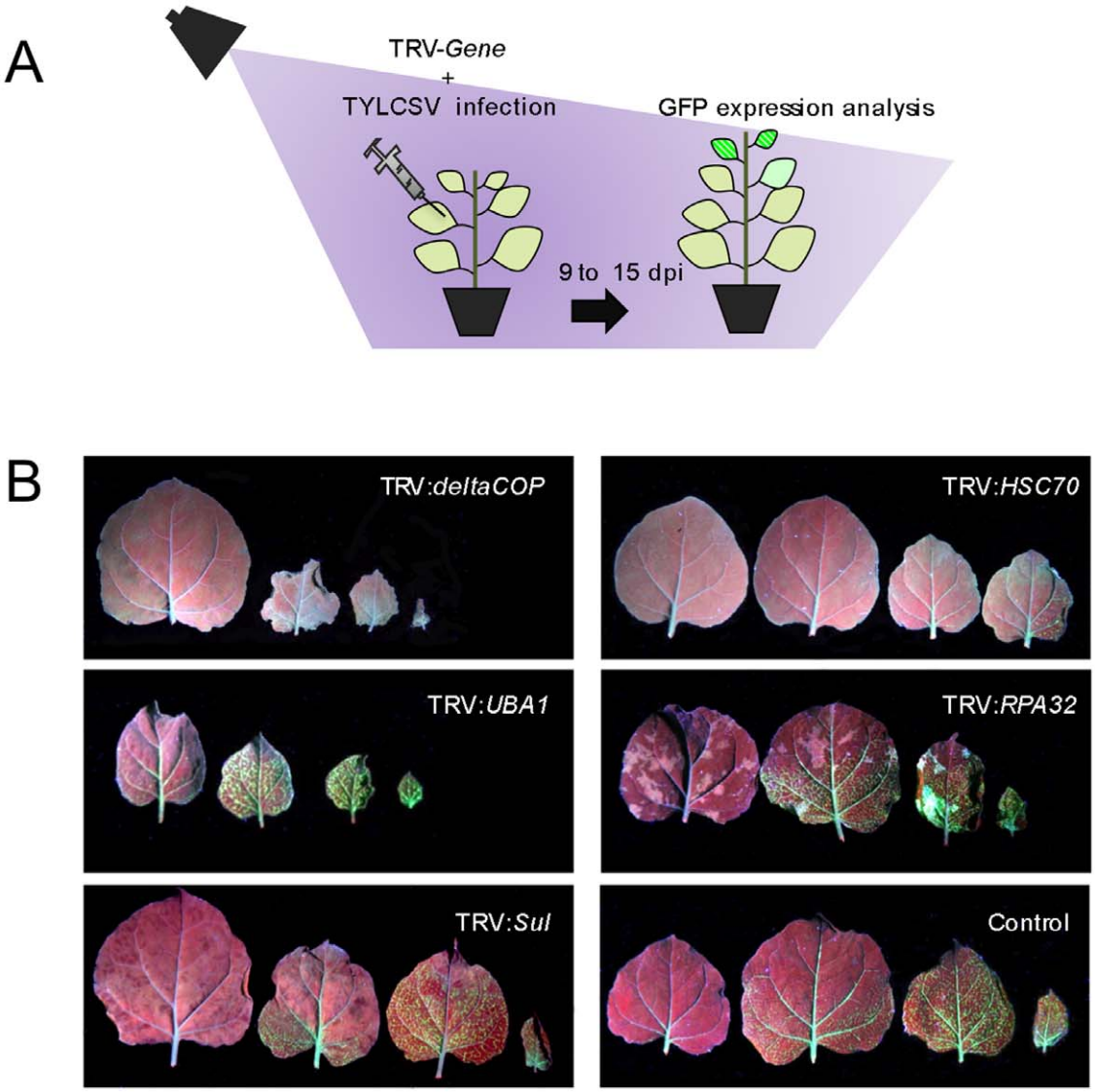

Figure 4. Screening of candidate genes in 2IRGFP transgenic *N. benthamiana* plants. (A) Plants were co-inoculated with a TRV:*Gene* construct and TYLCSV. GFP expression was monitored daily from 9 to 15 dpi. Five plants were used per construct; experiments were repeated at least

twice. (B) GFP expression in the four most apical leaves of 2IRGFP transgenic plants co-infected with TYLCSV and representative TRV constructs. (C) Relative amount of TYLCSV DNA in leaves of plants co-infected with TYLCSV and TRV constructs to induced the silencing of either Coatomer delta subunit (*deltaCOP*), Heat shock cognate 70 (*HSC70*), SKP1-like 2 (*ASK2*), Ubiquitin activating enzyme 1 (*UBA1*), Lactoylglutathione lyase (*GLO1*), Putative shikimate kinase (*SKL2*), RUB-activating enzyme subunit (*ECR1*), Replication associated protein A (*RPA32*), *Sulfur* (*Sul*) or no gene (empty vector, as control). Viral DNA was quantified by quantitative real-time PCR. Values are the mean of five replicates. Bars represent standard error. The sample of TYLCSV and pTV00 co-infected plants was used as the calibrator, with the expression level of the TYLCSV capsid protein gene set to 1.
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Among the genes affecting TYLCSV infection, there are three (*NSI*, *GRAB2* and *RPA32*) whose deregulation was previously shown to modify the geminivirus infection or replication [29,30,31,32].

An earlier work showed that overexpression of the nuclear acetyltransferase NSI, a protein that interacts with the Nuclear shuttle protein (NSP) of the geminivirus *Cabbage leaf curl virus* (CaLCuV), enhances the efficiency of infection [30], suggesting a role for protein acetylation in coordinating replication of the viral genome with its export from the nucleus. This positive effect of NSI in the geminivirus infection is supported by the data obtained with TYLCSV, which demonstrate that silencing of NSI negatively affects viral infection. On the other hand, silencing of the Geminivirus RepA binding gene (*GRAB2*) during TYLCSV infection has an opposite effect on viral propagation to that previously reported for a different geminivirus species [29]. This gene encodes a NAC-containing protein isolated in wheat for its interaction with *Wheat dwarf virus* (WDV) RepA [29]. Even though GRAB2 overexpression inhibits WDV replication in wheat cells, the reason for this remains unclear, and could be ascribed to different roles of GRAB2 on the viral DNA cycle [29]. Our results show that reduction in gene expression of GRAB2 has a deleterious effect on TYLCSV infection, suggesting that correct GRAB2 expression is required for full infectivity. Replication Protein A (RPA32) has been shown to interact with *Mungbean yellow mosaic India virus* (MYMIV) Rep [32] and modulate the functions of Rep by enhancing its ATPase, but down-regulating its nicking and closing activities. Strikingly, even though RPA32 seems to promote the transient replication of a plasmid bearing MYMIV origin of replication *in planta* [32], in our system its silencing seems to enhance the viral infection. We do not have a feasible explanation for this contradictory phenotype at the moment, and further work will be needed to decipher it.

The roles of other host genes whose silencing affects TYLCSV infection might be deduced from their known cellular functions. Therefore, we will briefly discuss below the potential roles of a group of identified host factors with known cellular functions in postranslational modifications, stress responses, metabolism or intracellular transport.

It is noteworthy that 8 out of these 18 genes are involved in processes related to protein modifications or protein metabolism, such as ubiquitination, rubylation, phosphorylation, acetylation or protein folding.

Four of these genes encode components or regulators of the ubiquitin or ubiquitin-like pathways: Ubiquitin activating enzyme (*UBA1)*, RING-type E3 ubiquitin ligase (*RHF2A*), SKP1-like 2 (*ASK2*) and a subunit of the de-rubylating CSN complex (*CSN3*).

Ubiquitination has been shown to contribute to multiple levels of plant defence, including resistance to viruses (reviewed in [33] and [34]). Specifically, several recent works have suggested the existence of links between ubiquitination and geminivirus infection [6,10,35,36]. Since the tomato UBA1 interacts with TYLCSV C2 (F. Hèricourt et al., in preparation), the finding that silencing of this host gene leads to an earlier TYLCSV infection suggests that the interaction with the viral C2 protein might lead to the inhibition of the enzyme, which would be consistent with the previously described general negative impact of C2 on the ubiquitination in the host [10]. On the other hand, the expression of the RING-type E3 ubiquitin ligase *RFH2A* silenced in this work is up-regulated following CaLCuV infection [6] or infiltration with virulent *Pseudomonas syringae* (*Arabidopsis* eFP browser: http://esc4037-shemp.csb.utoronto.ca/efp/cgi-bin/efpWeb.cgi), which may indicate an involvement in plant defence. Such a hypothetical role would explain why the silencing of this gene promotes the viral infection.

The SCF complex seems to be an important target during geminivirus infection, since several geminiviral proteins interfere with or hijack the SCF function [10,37]. The fact that three of the genes whose silencing alters TYLSCV infection are components or regulators of these complexes supports this idea. *ASK2* is a member of a gene family encoding SKP1-like proteins that can be assembled into distinct SCF complexes, and plays a role in a large number of cellular processes such as cell division, development, osmotic stress or drought tolerance [38,39,40]. *ASK2* expression is down-regulated by challenge with bacteria, fungi or elicitors (*Arabidopsis* eFP browser) but transactivated by geminivirus C2 in *Arabidopsis* protoplasts [9], suggesting a possible involvement in plant defence acting as a negative regulator. If this is the case, it could explain the adverse effect of its silencing on TYLCSV infection.

CSN3 is one of the eight subunits of the CSN complex, which derubylates cullins and thus regulates the activity of ubiquitin Cullin RING Ligases (CRLs). Recently, geminivirus C2 protein was shown to interfere with the activity of this complex over CULLIN1, most likely through the interaction with CSN5, the catalytic subunit, therefore altering ubiquitination in the host cell [10]. Given that geminivirus infection on *Arabidopsis csn5a* mutant plants takes place less efficiently that in wild-type plants (Lozano-Durán and Bejarano, submitted), it might be feasible that geminiviruses could be redirecting the activity of the CSN complex, rather that generally impairing it. Since depletion of any of the CSN subunits results in the loss of the complex (reviewed in [41]), it would not be surprising that silencing of CSN3 results in a hindered infection.

Among the host genes that seem to be required for the viral infection, since their silencing delay or suppress TYLCSV replication, we identified two encoding protein kinases that interact with TYLCSV C4 (Héricourt et al., in preparation): *BAM1* (Barely any meristem 1) and *SK4-1/SKK* (Shaggy-related kinase kappa). *BAM1* encodes a CLAVATA1-related receptor kinase-like protein required for both shoot and flower meristem function, which is also involved in leaf and gametophyte development [42,43,44]. Interestingly, *BAM1* expression is down-regulated after challenge with fungi, bacteria or elicitors (*Arabidopsis* eFP browser). In such a scenario, silencing of this gene might lead to an activation of defence responses in the plant. Alternatively, since this protein interacts with TYLCSV C4 (Héricourt et al., in preparation), this gene product might be required for some viral function.

Shaggy-like protein kinases like *SK4-1/SKK* have been shown to interact with other geminiviral C4 proteins, and this interaction is required to trigger disease symptoms [45,46] and for C4 function

to suppress gene silencing [46]. Our results confirm the previous idea that these kinases might be required for geminivirus infection, since silencing of *SK41/SKK* negatively impacts TYLCSV infection.

Five of the identified genes potentially involved in TYLCSV infection have a role in stress responses: *HSC70-1* (Heat shock protein cognate 70), *RD21* (responsive to dehydration 21), *PLP2* (patatin-like protein), *GLO1* (lactoylglutatione lyase) and *AOC1* (allene oxide cyclase 1). HSC70-1 is one of the five cytosolic members of the heat shock protein 70 family in *Arabidopsis* [47]. Infection with several plant viruses, such as the geminivirus *Beet curly top virus*, induce the expression of members of this gene family in systemically infected tissues [48,49] HSC70 is a major interactor of SGT1 [50], which has proven required for resistance to viruses [51,52]. A chloroplastic HSC70 from *Arabidopsis*, CPHSC70-1 (At4g24280), has been recently shown to interact with *Abutilon mosaic virus* movement protein, and this interaction seems to be important for viral transport and symptom induction [53]. Although the role of HSC70 induction in plant-virus interaction is uncertain, it might be expected to fulfil a requirement for rapid protein maturation and turnover during a short virus multiplication cycle. Alternatively, there is evidence that HSC70 may play a role in virus cell-to-cell movement. Our results show that silencing of HSC70-1 results in an impaired TYLCSV infection, supporting that over-production of this protein is required for a full viral infection.

RD21 is a cysteine protease whose homologue in tomato is able to interact with TYLCSV V2 (F. Héricourt et al., in preparation). RD21 has been recently shown to be the target protease of the serpin AtSerpin1 [54]. In animals, serpins are protease inhibitors involved in several physiological processes, including innate immunity. The expression of *RD21* is up-regulated following inoculation with *Botrytis cinerea* or *Pseudomonas syringae (Arabidopsis* eFP browser*)*, or upon CaLCuV infection [6], suggesting a possible role of RD21 in plant defence, which would in turn explain why the silencing of this gene promotes the viral infection.

PLP2 encodes a lipid acyl hydrolase that accumulates upon infection with CaLCuV [6], fungi and bacteria and negatively affects resistance to the last two types of pathogens [55]. On the contrary, it has been shown to contribute to resistance to *Cucumber mosaic virus* by inducing HR [56]. Since this gene product is proposed to positively regulate the biosynthesis of oxylipins providing fatty acid precursors [56], silencing of this gene might result in increased salicylic acid signalling, which could explain the impairment of TYLCSV infection.

GLO1 is part of the glyoxalase system, involved in detoxification of methylglioxal (MG), a cytotoxic byproduct of glycolysis (reviewed in [57]). Overexpression of the glyoxalase pathway in transgenic tobacco and rice plants has been found to keep in check the increase of ROS and MG under stress conditions by maintaining glutathione homeostasis and antioxidant enzyme levels (reviewed in [57]), and overexpression of *GLO1* has been related to enhanced tolerance to abiotic stresses [58,59]. A possible role for reactive oxygen species as a requirement for virus replication [60] and for antioxidative mechanisms as antagonizing viral infection [59] has been proposed. Moreover, viral infections have been shown to induce oxidative stress in plants [61,62,63,64,65,66] and geminivirus infection alters the expression of oxidative stress-related genes [6]. Given that silencing of *GLO1* triggers an earlier TYLCSV infection, it would be feasible that its interaction with C3 might be interfering with this enzyme to promote pathogenicity.

AOC1 is one of four genes that encode this enzyme in *Arabidopsis*, which catalyzes an essential step in jasmonic acid biosynthesis. This gene is repressed upon CaLCuV infection [6], maybe as a consequence of the opposite regulation between jasmonate and salicylic acid signalling pathways, since the latter is activated in this geminivirus-host interaction. Due to this counter-regulation, silencing of this gene might result in activation of the salicylic acid pathway in response to TYLCSV, explaining its negative effect on the viral infection.

Viruses heavily rely on cytoplasmic transport systems for their propagation. Among the host factors involved in TYLCSV infection, we have identified one gene required for vesicular trafficking (Coatomer delta subunit, *deltaCOP*) and another one involved in transport between the cytoplasm and the nucleus (Importin alpha isoform 4, *IMPAA-4*).

deltaCOP encodes a component of the polymeric coatomer coat complexes COPI. The precise role of the COPI remains unclear, although it has been associated with vesicular transport within the Golgi apparatus and from the Golgi apparatus to the ER [67]. Vesicular trafficking has been previously shown to play a role in geminivirus infection, since interaction with synaptotagmin SYTA has proven required for CaLCuV cell-to-cell movement and systemic spread [68]. Interestingly, silencing of this gene completely abolishes TYLCSV infection in our system, suggesting that vesicular trafficking in essential for viral infection.

IMPAA-4 is one of the members of the importin α gene family in eukaryotes. Importin α is a component of the nuclear pore-targeting complex (PTAC) that acts as an adaptor by recognizing the nuclear localization signal (NLS) sequences and binding to importin β. Importin β is the carrier component of PTAC, and targets the complex to the nuclear pore by binding to nuclear pore proteins [69,70]. Importin α has been shown to interact with the CP from the geminivirus MYMV [71], and this interaction might serve for docking of viruses to the nucleus and facilitating nuclear localization of the CP during encapsidation. In this context, the finding that silencing of *IMPA-4* favours the viral infection seems counterintuitive; however, the fact that this gene is overexpressed in response to several pathogens and elicitors (*Arabidopsis* eFP browser) suggests that this host factor might also play a role in plant defence, providing a possible explanation for the observed phenotype. Additionally, TYLCSV CP could rely on the interaction with a different host protein for its nuclear import.

Besides the aforementioned cellular processes, others seem to be involved in TYLCSV infection. Silencing of genes selected because of their specific expression or overexpression in phloem tissue and required for phenylpropanoid metabolism (4-coumarate:CoA ligase1, *4CL1*) or secondary cell wall synthesis (Bearskin2B, *BRN2*) delay or promote TYLCSV infection, respectively. BRN2 is a member of the Class IIB NAC transcription factor family. In *Arabidopsis*, this protein has been suggested to regulate cell maturation in cells that undergo terminal differentiation with strong cell wall modifications [72]. *4CL1* is involved in the last step of the general phenylpropanoid pathway, channeling carbon flow into branch pathways of the phenylpropanoid metabolism. Interestingly, silencing of this gene leads to increased cellulose content and reduced amounts of total lignin [73].

As illustrated in the examples above, the use of this approach has allowed the identification of novel plant genes with a role in the geminivirus infection, which sheds light on the underlying biological processes, therefore paving the way for the development of strategies to counteract these devastating diseases. Given the previously mentioned advantages of this 2IRGFP/VIGS system, it can be considered an easy, fast and effective tool to determine the role of host genes in geminivirus infections, and might be of great assistance to speed up this kind of functional studies. However, using VIGS to target a specific gene requires information about its

nucleotide sequence. This is a limitation when working with *N. benthamiana*, as there is only a relatively small sequence database available for this species (http://www.tigr.org). We have tried to circumvent this difficulty by using nucleotide sequence information from *Arabidopsis* and closely related species. In a genome era, full sequencing of the *N. benthamiana* genome should hopefully be fulfilled in the near future, providing full potential to the VIGS/2IRGFP strategy to identify host factors involved in geminivirus infection.

Materials and Methods

Microorganisms, plants and general methods

Manipulations of *Escherichia coli* and nucleic acids were performed according to standard methods [74,75]. *E. coli* strain DH5-α was used for subcloning. All PCR-amplified fragments cloned in this work were fully sequenced. *Agrobacterium tumefaciens* GV3101 strain was used for the delivery of *Tobacco rattle virus* (TRV) RNA2-based vectors and TYLCSV infective clone; *A. tumefaciens* C58c1 was used for the delivery of the TRV RNA1-based construct pBINTRA6 [27].

2IRGFP *N. benthamiana* plants were grown in soil at 22°C in short day conditions (8 h light/16 h dark photoperiod).

Plasmids and cloning

cDNA clones of the selected candidate genes were obtained from the *Arabidopsis* Information Resource (TAIR) (Table S1). Fragments (300–500 bp) from the selected genes were generated by PCR with specific primers (Table S1) and cloned in pGEMT-easy (Promega). *Spe*I/*Apa*I fragments from the pGEMT clones containing the selected sequenced were subcloned into *Spe*I/*Apa*I sites of TRV RNA2-based vector pTV00 [27] to yield the correspondent TRV used to silencing the plants genes.

To yield the TRV:*GFP* construct, a 383 bp *BamH*I-*Cla*I fragment from pSMGFP [76] was cloned into *BamH*I-*Cla*I of pTV00. To yield the TRV:*Sul* construct, a 450 bp fragment of the *Sulfur* gene amplified from *Arabidopsis* cDNA using At*Sulfur* primers (Table S1) was digested with *Kpn*I and cloned into the *Kpn*I site of pTV00. To yield the TRV:*SulPCNA* construct a 450 bp *Kpn*I fragment from TRV:*Sul* was subcloned into *KpnI* site of TRV:*PCNA* [11].

Geminivirus infection assays and detection of viral and mGFP DNA

Viral infections of 2IRGFP *N. benthamiana* plants were performed by the agroinoculation technique as previously described [77]. Plants were agroinoculated with plasmid pGreen-TYA14 (binary vector containing a partial dimer of TYLCSV-ES[2] [10]) in the axilary bud of the fourth/fifth leaf of 3-week-old wild-type or transgenic 2IRGFP *N. benthamiana* plants. For control, plants were mock inoculated with *A. tumefaciens* culture harbouring the empty binary vector pGreen-0229 [78].

Viral and mGFP DNAs were detected by gel blot hybridization. Total plant DNA was extracted from *N. benthamiana* leaves at different days postinfection. Two micrograms of undigested total DNA per sample were used. As probe for TYLCSV detection, we used a *BamH*I DNA fragment from pGreenTYA14 [10] containing a full-length genome of TYLCSV-ES. For mGFP detection we used a *BamH*I-*Sac*I DNA fragment from pSMGFP comprising the complete GFP open reading frame [76].

For quantitative real-time PCR, total plant DNA was extracted from *N. benthamiana* leaves at 15 dpi. The reaction mixture consisted of approximately 10 ng total DNA, primer mix (3 µM each) and SYBR Green Master Mix (TaKaRa, Kyoto, Japan) in a total volume of 25 µl. The PCR conditions were: 10 minutes at 95°C, and 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. The reactions were performed using a Rotor-Gene real time cycler (QIAGEN, Hamburg Germany). A relative quantification real-time PCR method using the $2^{\Delta\Delta CT}$ method [79] was used to compare the amount of the TYLCSV capsid protein gene (amplified using primers GGAGGCTGAACTTCGACAGC and GGACTTTCAATGGGCCTTCAC) between different infections/experiments. The 25S ribosomal DNA interspacer (ITS) (amplified using primers ATAACCGCATCAGGTCTCCA and CCGAAGTTACGGATCCATTT) was used as the internal control.

Virus Induced Gene Silencing assay

Virus induced gene silencing with TRV in *N. benthamiana* plants were performed according the method described by [27]. Briefly, independent cultures of *A. tumefaciens* GV3101 carrying pTV00 or pTV00-based constructs and *A. tumefaciens* C58c1 carrying pBINTRA6 were grown overnight in LBroth medium plus appropriate antibiotics. Cultures were resuspended in VIGS buffer (10 mM morpholineethanesulfonic acid pH 5.6, 10 mM $MgCl_2$, and 100 µM acetosyringone) adjusting optical density to $OD_{600} = 1$, and incubated overnight at room temperature in the dark. Cultures containing pBINTRA6 plasmid and pTV00 or pTV00-derived plasmid were mixed at a 1:1 ratio. Approximately 1 mL of this mixed culture was used to infiltrate the underside of two leaves of each 3-week-old 2IRGFP *N. benthamiana* plant.

Supporting Information

Figure S1 Phenotypes of TYLCSV-infected 2IRGFP *N. benthamiana* plants. Extension and intensity of GFP expression in the leaves of TYLCSV-infected plants corresponding to RAP phenotypes (for Replication-Associated Phenotype) 0, 1, 2, 3 and 4.
(TIF)

Figure S2 Simultaneous TRV-induced silencing of *PCNA* and *Sul*. Percentage of leaves located above the infiltration point displaying the silencing phenotype of either *PCNA*, *Sul* or both in *N. benthamiana* plants inoculated with TRV:*Sul*, TRV:*PCNA* or TRV:*SulPCNA*, or co-inoculated with TRV:*Sul* and TRV:*PCNA*. For each inoculation, n = 10 plants. The data correspond to leaves collected approximately 28 days after the infection.
(TIF)

Table S1 Oligonucleotides used for amplifying and cloning fragments of the selected genes.
(DOC)

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Author Contributions

Conceived and designed the experiments: RL-D TR-D ERB. Performed the experiments: RL-D TR-D APL. Analyzed the data: RL-D TR-D ERB. Contributed reagents/materials/analysis tools: ERB. Wrote the paper: RL-D TR-D ERB.

References

1. Rojas MR, Hagen C, Lucas WJ, Gilbertson RL (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annu Rev Phytopathol 43: 361–394.
2. Stanley J, Bisaro DM, Briddon RW, Brown JK, Fauquet CM, et al. (2005) Geminiviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, eds. In Virus Taxonomy VIIIth Report of the International Committee on Taxonomy of Viruses. London: Elsevier/Academic Press. pp 301–326.
3. Moriones E, Navas-Castillo J (2000) Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. Virus Res 71: 123–134.
4. Czosnek H (2007) Tomato Yellow Leaf Curl Disease. Management, molecular biologya, breeding for resistance Czosnek H, ed. Dordretch: Spinger. pp 441.
5. Diaz-Pendon JA, Canizares MC, Moriones E, Bejarano ER, Czosnek H, et al. (2010) Tomato yellow leaf curl viruses: menage a trois between the virus complex, the plant and the whitefly vector. Mol Plant Pathol 11: 441–450.
6. Ascencio-Ibanez JT, Sozzani R, Lee TJ, Chu TM, Wolfinger RD, et al. (2008) Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant Physiol 148: 436–454.
7. Sahu PP, Rai NK, Chakraborty S, Singh M, Chandrappa PH, et al. (2010) Tomato cultivar tolerant to Tomato leaf curl New Delhi virus infection induces virus-specific short interfering RNA accumulation and defence-associated host gene expression. Mol Plant Pathol 11: 531–544.
8. Andleeb S, Amin I, Bashir A, Briddon RW, Mansoor S (2010) Transient expression of betaC1 protein differentially regulates host genes related to stress response, chloroplast and mitochondrial functions. Virol J 7: 373.
9. Trinks D, Rajeswaran R, Shivaprasad PV, Akbergenov R, Oakeley EJ, et al. (2005) Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. J Virol 79: 2517–2527.
10. Lozano-Duran R, Rosas-Diaz T, Gusmaroli G, Luna AP, Taconnat L, et al. (2011) Geminiviruses Subvert Ubiquitination by Altering CSN-Mediated Derubylation of SCF E3 Ligase Complexes and Inhibit Jasmonate Signaling in Arabidopsis thaliana. Plant Cell.
11. Morilla G, Castillo AG, Preiss W, Jeske H, Bejarano ER (2006) A versatile transreplication-based system to identify cellular proteins involved in geminivirus replication. J Virol 80: 3624–3633.
12. Castillo AG, Kong LJ, Hanley-Bowdoin L, Bejarano ER (2004) Interaction between a geminivirus replication protein and the plant sumoylation system. J Virol 78: 2758–2769.
13. Ber R, Navot N, Zamir D, Antignus Y, Cohen S, et al. (1990) Infection of tomato by tomato yellow leaf curl virus: suceptibility to infection, symptom development and accumulation of viral DNA. Arch Virology.
14. Czosnek H, Ber R, Navot N, Zamir D (1988) Detection of tomato yellow leaf curl virus in lysates of plants and insects by hybridization with a viral DNA probe. Plant disease 72: 949–951.
15. Voinnet O, Baulcombe DC (1997) Systemic signalling in gene silencing. Nature 389: 553.
16. Ruiz MT, Voinnet O, Baulcombe DC (1998) Initiation and maintenance of virus-induced gene silencing. Plant Cell 10: 937–946.
17. Kjemtrup S, Sampson KS, Peele CG, Nguyen LV (1998) Gene silencing from plant DNA carried by a Geminivirus. Plant Journal 14: 91–100.
18. Orzaez D, Medina A, Torre S, Fernandez-Moreno JP, Rambla JL, et al. (2009) A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation. Plant Physiol 150: 1122–1134.
19. Spitzer B, Zvi MM, Ovadis M, Marhevka E, Barkai O, et al. (2007) Reverse genetics of floral scent: application of tobacco rattle virus-based gene silencing in Petunia. Plant Physiol 145: 1241–1250.
20. Chen J, Jiang C, Gookin T, Hunter D, Clark D, et al. (2004) Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence. Plant Molecular Biology 55: 521–530.
21. Peele C, Jordan CV, Muangsan N, Turnage M, Egelkrout E, et al. (2001) Silencing of a meristematic gene using geminivirus-derived vectors. Plant J 27: 357–366.
22. Thomas CL, Jones L, Baulcombe DC, Maule AJ (2001) Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in Nicotiana benthamiana using a potato virus X vector. Plant J 25: 417–425.
23. Ekengren SK, Liu Y, Schiff M, Dinesh-Kumar SP, Martin GB (2003) Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. Plant J 36: 905–917.
24. Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J 39: 734–746.
25. Liu E, Page JE (2008) Optimized cDNA libraries for virus-induced gene silencing (VIGS) using tobacco rattle virus. Plant Methods 4: 5.
26. Senthil-Kumar M, Hema R, Anand A, Kang L, Udayakumar M, et al. (2007) A systematic study to determine the extent of gene silencing in Nicotiana benthamiana and other Solanaceae species when heterologous gene sequences are used for virus-induced gene silencing. New Phytol 176: 782–791.
27. Ratcliff F, Montserrat A, Martin-Hernandez, Baulcombe D (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. The Plant Journal 25: 237–245.
28. Eybishtz A, Peretz Y, Sade D, Gorovits R, Czosnek H (2010) Tomato yellow leaf curl virus infection of a resistant tomato line with a silenced sucrose transporter gene LeHT1 results in inhibition of growth, enhanced virus spread, and necrosis. Planta 231: 537–548.
29. Xie Q, Sanz-Burgos AP, Guo H, Garcia JA, Gutierrez C (1999) GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Mol Biol 39: 647–656.
30. McGarry RC, Barron YD, Carvalho MF, Hill JE, Gold D, et al. (2003) A novel Arabidopsis acetyltransferase interacts with the geminivirus movement protein NSP. Plant Cell 15: 1605–1618.
31. Carvalho MF, Turgeon R, Lazarowitz SG (2006) The geminivirus nuclear shuttle protein NSP inhibits the activity of AtNSI, a vascular-expressed Arabidopsis acetyltransferase regulated with the sink-to-source transition. Plant Physiol 140: 1317–1330.
32. Singh DK, Islam MN, Choudhury NR, Karjee S, Mukherjee SK (2006) The 32 kDa subunit of replication protein A (RPA) participates in the DNA replication of Mung bean yellow mosaic India virus (MYMIV) by interacting with the viral Rep protein. Nucleic Acids Res 35: 755–770.
33. Dreher K, Callis J (2007) Ubiquitin, hormones and biotic stress in plants. Ann Bot 99: 787–822.
34. Citovsky V, Zaltsman A, Kozlovsky SV, Gafni Y, Krichevsky A (2009) Proteasomal degradation in plant-pathogen interactions. Semin Cell Dev Biol 20: 1048–1054.
35. Eini O, Dogra S, Selth LA, Dry IB, Randles JW, et al. (2009) Interaction with a host ubiquitin-conjugating enzyme is required for the pathogenicity of a geminiviral DNA beta satellite. Mol Plant Microbe Interact 22: 737–746.
36. Lai J, Chen H, Teng K, Zhao Q, Zhang Z, et al. (2008) RKP, a RING finger E3 ligase induced by BSCTV C4 protein, affects geminivirus infection by regulation of the plant cell cycle. Plant J 57: 905–917.
37. Lozano-Durán R, Bejarano ER (2011) Geminivirus C2 protein might be the key player for geminiviral co-option of SCF-mediated ubiquitination. Plant Signaling & Behavior, In press.
38. Umezawa T, Yoshida R, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K (2004) SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in Arabidopsis thaliana. Proc Natl Acad Sci U S A 101: 17306–17311.
39. Boudsocq M, Barbier-Brygoo H, Lauriere C (2004) Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. J Biol Chem 279: 41758–41766.
40. Liu F, Ni W, Griffith ME, Huang Z, Chang C, et al. (2004) The ASK1 and ASK2 genes are essential for Arabidopsis early development. Plant Cell 16: 5–20.
41. Serino G, Deng XW (2003) The COP9 signalosome: regulating plant development through the control of proteolysis. Annu Rev Plant Biol 54: 165–182.
42. Guo Y, Han L, Hymes M, Denver R, Clark SE (2010) CLAVATA2 forms a distinct CLE-binding receptor complex regulating Arabidopsis stem cell specification. Plant J 63: 889–900.
43. DeYoung BJ, Bickle KL, Schrage KJ, Muskett P, Patel K, et al. (2006) The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in Arabidopsis. Plant J 45: 1–16.
44. Deyoung BJ, Clark SE (2008) BAM receptors regulate stem cell specification and organ development through complex interactions with CLAVATA signaling. Genetics 180: 895–904.
45. Piroux N, Saunders K, Page A, Stanley J (2007) Geminivirus pathogenicity protein C4 interacts with Arabidopsis thaliana shaggy-related protein kinase AtSKeta, a component of the brassinosteroid signalling pathway. Virology 362: 428–440.
46. Dogra SC, Eini O, Rezaian MA, Randles JW (2009) A novel shaggy-like kinase interacts with the Tomato leaf curl virus pathogenicity determinant C4 protein. Plant Mol Biol 71: 25–38.
47. Sung DY, Vierling E, Guy CL (2001) Comprehensive expression profile analysis of the Arabidopsis Hsp70 gene family. Plant Physiol 126: 789–800.
48. Escaler M, Aranda MA, Thomas CL, Maule AJ (2000) Pea embryonic tissues show common responses to the replication of a wide range of viruses. Virology 267: 318–325.
49. Aparicio F, Thomas CL, Lederer C, Niu Y, Wang D, et al. (2005) Virus Induction of Heat Shock Protein 70 Reflects a General Response to Protein Accumulation in the Plant Cytosol. Plant Physiol 138: 529–536.
50. Noel LD, Cagna G, Stuttmann J, Wirthmuller L, Betsuyaku S, et al. (2007) Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates Arabidopsis immune responses. Plant Cell 19: 4061–4076.
51. Komatsu K, Hashimoto M, Ozeki J, Yamaji Y, Maejima K, et al. (2010) Viral-induced systemic necrosis in plants involves both programmed cell death and the inhibition of viral multiplication, which are regulated by independent pathways. Mol Plant Microbe Interact 23: 283–293.
52. Dielen AS, Badaoui S, Candresse T, German-Retana S (2010) The ubiquitin/26S proteasome system in plant-pathogen interactions: a never-ending hide-and-seek game. Mol Plant Pathol 11: 293–308.

53. Krenz B, Windeisen V, Wege C, Jeske H, Kleinow T (2010) A plastid-targeted heat shock cognate 70kDa protein interacts with the Abutilon mosaic virus movement protein. Virology 401: 6–17.
54. Lampl N, Budai-Hadrian O, Davydov O, Joss TV, Harrop SJ, et al. (2010) Arabidopsis AtSerpin1, crystal structure and in vivo interaction with its target protease RESPONSIVE TO DESICCATION-21 (RD21). J Biol Chem 285: 13550–13560.
55. La Camera S, Geoffroy P, Samaha H, Ndiaye A, Rahim G, et al. (2005) A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in Arabidopsis. Plant J 44: 810–825.
56. La Camera S, Balague C, Gobel C, Geoffroy P, Legrand M, et al. (2009) The Arabidopsis patatin-like protein 2 (PLP2) plays an essential role in cell death execution and differentially affects biosynthesis of oxylipins and resistance to pathogens. Mol Plant Microbe Interact 22: 469–481.
57. Yadav SK, Singla-Pareek SL, Sopory SK (2008) An overview on the role of methylglyoxal and glyoxalases in plants. Drug Metabol Drug Interact 23: 51–68.
58. Mustafiz A, Sahoo KK, Singla-Pareek SL, Sopory SK (2010) Metabolic engineering of glyoxalase pathway for enhancing stress tolerance in plants. Methods Mol Biol 639: 95–118.
59. Sun W, Xu X, Zhu H, Liu A, Liu L, et al. (2010) Comparative transcriptomic profiling of a salt-tolerant wild tomato species and a salt-sensitive tomato cultivar. Plant Cell Physiol 51: 997–1006.
60. Clarke SF, Guy PL, Burritt DJ, Jameson PE (2002) Changes in the activities of antioxidant enzymes in response to virus infection and hormone treatment. Physiol Plant 114: 157–164.
61. Garcia-Marcos A, Pacheco R, Martianez J, Gonzalez-Jara P, Diaz-Ruiz JR, et al. (2009) Transcriptional changes and oxidative stress associated with the synergistic interaction between Potato virus X and Potato virus Y and their relationship with symptom expression. Mol Plant Microbe Interact 22: 1431–1444.
62. Amari K, Diaz-Vivancos P, Pallas V, Sanchez-Pina MA, Hernandez JA (2007) Oxidative stress induction by Prunus necrotic ringspot virus infection in apricot seeds. Physiol Plant 131: 302–310.
63. Diaz-Vivancos P, Clemente-Moreno MJ, Rubio M, Olmos E, Garcia JA, et al. (2008) Alteration in the chloroplastic metabolism leads to ROS accumulation in pea plants in response to plum pox virus. J Exp Bot 59: 2147–2160.
64. Diaz-Vivancos P, Rubio M, Mesonero V, Periago PM, Barcelo AR, et al. (2006) The apoplastic antioxidant system in Prunus: response to long-term plum pox virus infection. J Exp Bot 57: 3813–3824.
65. Rimmer J, Singh A, Banwell P, Clarke PM, Rhys Evans P (2006) The use of octyl-2-cyanoacrylate (Dermabond) tissue adhesive for skin closure in head and neck surgery. Ann R Coll Surg Engl 88: 412–413.
66. Song XS, Wang YJ, Mao WH, Shi K, Zhou YH, et al. (2009) Effects of cucumber mosaic virus infection on electron transport and antioxidant system in chloroplasts and mitochondria of cucumber and tomato leaves. Physiol Plant 135: 246–257.
67. Lee MC, Miller EA, Goldberg J, Orci L, Schekman R (2004) Bi-directional protein transport between the ER and Golgi. Annu Rev Cell Dev Biol 20: 87–123.
68. Lewis JD, Lazarowitz SG (2010) Arabidopsis synaptotagmin SYTA regulates endocytosis and virus movement protein cell-to-cell transport. Proc Natl Acad Sci U S A 107: 2491–2496.
69. Cook A, Bono F, Jinek M, Conti E (2007) Structural biology of nucleocytoplasmic transport. Annu Rev Biochem 76: 647–671.
70. Terry LJ, Shows EB, Wente SR (2007) Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. Science 318: 1412–1416.
71. Guerra-Peraza O, Kirk D, Seltzer V, Veluthambi K, Schmit AC, et al. (2005) Coat proteins of Rice tungro bacilliform virus and Mungbean yellow mosaic virus contain multiple nuclear-localization signals and interact with importin alpha. J Gen Virol 86: 1815–1826.
72. Bennett T, van den Toorn A, Sanchez-Perez GF, Campilho A, Willemsen V, et al. (2010) SOMBRERO, BEARSKIN1, and BEARSKIN2 regulate root cap maturation in Arabidopsis. Plant Cell 22: 640–654.
73. Yang J, Chen F, Yu O, Beachy RN (2010) Controlled silencing of 4-coumarate:CoA ligase alters lignocellulose composition without affecting stem growth. Plant Physiol Biochem 49: 103–109.
74. Ausubel F, Brent R, Kingston R, Moore D, Seidman J, et al. (1998) Current protocols in molecular biology New YorkN.Y: John Wiley & Sons, Inc..
75. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring HarborNew York: Cold Spring Harbor Laboratory Press.
76. Davis SJ, Vierstra RD (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol 36: 521–528.
77. Elmer JS, Sunter G, Gardiner WE, Brand L, Browing CK, et al. (1988) Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNAs. Plant Mol Biol 10: 225–234.
78. Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol Biol 42: 819–832.
79. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.
80. Shultz RW, Tatineni VM, Hanley-Bowdoin L, Thompson WF (2007) Genome-wide analysis of the core DNA replication machinery in the higher plants Arabidopsis and rice. Plant Physiol 144: 1697–1714.
81. Kong LJ, Hanley-Bowdoin L (2002) A geminivirus replication protein interacts with a protein kinase and a motor protein that display different expression patterns during plant development and infection. Plant Cell 14: 1817–1832.
82. Vilaine F, Palauqui JC, Amselem J, Kusiak C, Lemoine R, et al. (2003) Towards deciphering phloem: a transcriptome analysis of the phloem of Apium graveolens. Plant J 36: 67–81.
83. Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW, et al. (2005) A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. Plant Cell 17: 311–325.
84. Ach RA, Durfee T, Miller AB, Taranto P, Hanley-Bowdoin L, et al. (1997) RRB1 and RRB2 encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. Mol Cell Biol 17: 5077–5086.
85. Kong LJ, Orozco BM, Roe JL, Nagar S, Ou S, et al. (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. EMBO J 19: 3485–3495.
86. Woodward AW, Ratzel SE, Woodward EE, Shamoo Y, Bartel B (2007) Mutation of E1-CONJUGATING ENZYME-RELATED1 decreases RELATED TO UBIQUITIN conjugation and alters auxin response and development. Plant Physiol 144: 976–987.
87. Wang H, Hao L, Shung CY, Sunter G, Bisaro DM (2003) Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. Plant Cell 15: 3020–3032.
88. Lois LM (2010) Diversity of the SUMOylation machinery in plants. Biochem Soc Trans 38: 60–64.
89. Asano T, Masumura T, Kusano H, Kikuchi S, Kurita A, et al. (2002) Construction of a specialized cDNA library from plant cells isolated by laser capture microdissection: toward comprehensive analysis of the genes expressed in the rice phloem. Plant J 32: 401–408.
90. Schwechheimer C, Isono E (2010) The COP9 signalosome and its role in plant development. Eur J Cell Biol 89: 157–162.

Transient Transcriptional Regulation of the *CYS-C1* Gene and Cyanide Accumulation upon Pathogen Infection in the Plant Immune Response[1][C][W]

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Cyanide is produced concomitantly with ethylene biosynthesis. Arabidopsis (*Arabidopsis thaliana*) detoxifies cyanide primarily through the enzyme β-cyanoalanine synthase, mainly by the mitochondrial CYS-C1. *CYS-C1* loss of function is not toxic for the plant and leads to an increased level of cyanide in *cys-c1* mutants as well as a root hairless phenotype. The classification of genes differentially expressed in *cys-c1* and wild-type plants reveals that the high endogenous cyanide content of the *cys-c1* mutant is correlated with the biotic stress response. Cyanide accumulation and *CYS-C1* gene expression are negatively correlated during compatible and incompatible plant-bacteria interactions. In addition, *cys-c1* plants present an increased susceptibility to the necrotrophic fungus *Botrytis cinerea* and an increased tolerance to the biotrophic *Pseudomonas syringae* pv *tomato* DC3000 bacterium and *Beet curly top virus*. The *cys-c1* mutation produces a reduction in respiration rate in leaves, an accumulation of reactive oxygen species, and an induction of the alternative oxidase *AOX1a* and pathogenesis-related *PR1* expression. We hypothesize that cyanide, which is transiently accumulated during avirulent bacterial infection and constitutively accumulated in the *cys-c1* mutant, uncouples the respiratory electron chain dependent on the cytochrome *c* oxidase, and this uncoupling induces the alternative oxidase activity and the accumulation of reactive oxygen species, which act by stimulating the salicylic acid-dependent signaling pathway of the plant immune system.

The gaseous hormone ethylene is known to regulate multiple physiological and developmental processes in plants, such as seedling emergence, leaf and flower senescence, climacteric fruit ripening, and organ abscission. Ethylene is also involved in the response of plants to abiotic and biotic stresses (Wang et al., 2002; Broekaert et al., 2006; van Loon et al., 2006). Enhanced ethylene production is an early, active response of plants to the perception of pathogen attack and is associated with the induction of defense reactions. During ethylene biosynthesis, *S*-adenosyl-L-Met is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC is finally oxidized by ACC oxidase to form ethylene, carbon dioxide, and cyanide (Hartley et al., 1998; Wang et al., 2002). Hydrogen cyanide is a colorless and highly volatile liquid. The anion cyanide is toxic and renders the cells of an organism unable to use oxygen, primarily through the chelation of divalent and trivalent metal ions in the prosthetic groups of several metalloenzymes, including copper/zinc superoxide dismutase, catalase, nitrate and nitrite reductase, nitrogenase, peroxidases, and the mitochondrial cytochrome *c* oxidase (Isom and Way, 1984; Donato et al., 2007).

Cyanide must be rapidly detoxified and metabolized by the plant to keep the concentration below toxic levels. Plants detoxify cyanide primarily through the enzyme β-cyanoalanine synthase (CAS), for which considerable levels of activity are constitutively found in many plant species. Rhodanese and mercaptopyruvate sulfurtransferase activities also make minor contributions to the cyanide detoxification process (Miller and Conn, 1980). CAS is a pyridoxal phosphate-dependent enzyme that converts Cys and cyanide to hydrogen sulfide and β-cyanoalanine, which is later converted to Asn, Asp, and ammonia by NIT4 class nitrilases (Piotrowski, 2008). Arabidopsis (*Arabidopsis thaliana*) plants carry the mitochondrial CAS CYS-C1 (At3g61440; Watanabe et al., 2008), which belongs to the family of β-substituted Ala synthase enzymes. The family also includes the three major *O*-acetyl-serine (thiol)lyase enzymes OAS-A1 (At4g14880), OAS-B (At2g43750), and OAS-C (At3g59760; Watanabe et al., 2008), the L-Cys desulfhydrase DES1 (At5g28030;

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Álvarez et al., 2010), the *S*-sulfocysteine synthase CS26 (At3g03630; Bermúdez et al., 2010), and the functionally unknown cytosolic isoforms CYS-D1 (At3g04940) and CYS-D2 (At5g28020). Mutations in CYS-C1 result in plants that accumulate cyanide and that display abnormal root hair (García et al., 2010), suggesting that cyanide has a signaling role in root development. The lack of the mitochondrial *O*-acetyl-serine(thiol)lyase isoform OAS-C, which is necessary to detoxify the sulfide released by the CAS activity, causes an accumulation of sulfide and cyanide and a root phenotype similar to the *cys-c1* loss-of-function mutant (Álvarez et al., 2012b).

Several authors have suggested that cyanide could act as a regulator of other metabolic processes in addition to performing the described role in plant root development (Siegien and Bogatek, 2006). It has been observed that this molecule is released during seed germination and that exogenously applied hydrogen cyanide breaks seed dormancy in several plants (Cohn and Hughes, 1986; Fol et al., 1989; Bogatek et al., 1991; Bethke et al., 2006). The role of cyanide as a regulatory molecule is not restricted to plants, and it has been demonstrated that cyanide is generated in leukocytes from Gly via a peroxidase (Stelmaszyńska, 1986) as well as in the central nervous system, where it has been hypothesized to act as a neuromodulator (Gunasekar et al., 2000; Cipollone and Visca, 2007). Cyanide production can be stimulated by opiates and decreased by treatment with muscarinic receptor agonists (Borowitz et al., 1997; Gunasekar et al., 2004).

Despite the variety of known functions for cyanide in different organisms, the role of cyanide production in plants seems to have been unevaluated to date. In cyanogenic plants, cyanide is produced during the degradation of cyanogenic lipids and from the catabolism of cyanogenic glycosides (Poulton, 1990). Cyanide and cyanogenic compounds play an important role in plant defense against herbivores (Zagrobelny et al., 2008). In noncyanogenic plants, cyanide is a coproduct of ethylene biosynthesis. The molecule is also produced during the biosynthesis of camalexin, a phytoalexin formed in Arabidopsis plants upon infection by a large variety of microorganisms, including bacteria, fungi, and oomycetes (Glawischnig, 2007). During camalexin biosynthesis, the Trp-derived intermediate indole-3-acetonitrile is conjugated with Cys and serves as a substrate for the cytochrome P450 enzyme CYP71B15. This enzyme catalyzes the formation of the thiazoline ring as well as the release of cyanide and subsequent oxidative decarboxylation of dihydrocamalexic acid to camalexin (Glawischnig, 2007; Böttcher et al., 2009). Since both cyanide sources, camalexin and ethylene, are produced after pathogen attack, cyanide should be produced at significant levels during the plant response to pathogens. It has been shown that exogenous cyanide can enhance the resistance of tobacco (*Nicotiana tabacum*) and Arabidopsis leaves to *Tobacco mosaic virus* and *Turnip vein clearing virus*, respectively (Chivasa and Carr, 1998; Wong et al., 2002). Recently, it has been demonstrated that exogenously applied cyanide increases the resistance of young rice (*Oryza sativa*) plants to blast fungus infection, suggesting that cyanide rather than ethylene contributes to plant resistance (Seo et al., 2011).

This work aims to further investigate the role of endogenously produced cyanide in the plant immune response by analyzing the behavior of Arabidopsis knockout mutants of the mitochondrial CAS CYS-C1 and the regulation of CYS-C1 in response to pathogen attack.

RESULTS

The *cys-c1* Mutant Transcriptome Shows a High Correlation with Biotic Stresses

The loss of function of the CYS-C1 enzyme has previously been characterized in root tissues, but its function in leaves has not been studied to date (García et al., 2010). Phenotypic analysis of the *cys-c1* null mutant shows no obvious alterations in the aerial parts of the plant whether grown in long- or short-day photoperiods. To analyze the effect of the loss of function of the CYS-C1 enzyme at the molecular level, we performed a comparative transcriptomic analysis of leaves of *cys-c1* and wild-type plants grown under identical long-day conditions on Murashige and Skoog (MS) medium for 14 d. Total RNA was prepared and analyzed using the Affymetrix Arabidopsis ATH1 GeneChip array. Three biological replicates were performed for each genotype. Restricting the analysis to the genes whose expression was changed at least 2-fold as a threshold and at a significance level of $P < 0.05$, we identified 51 genes that exhibited alterations in transcription level. Among them, 31 genes were up-regulated in the *cys-c1* mutant plant compared with the wild-type plant, and 20 genes were down-regulated (Microarray Gene Expression Omnibus database accession no. GSE19242; Supplemental Table S1). To detect physiologically relevant patterns, the genes with altered expression were assigned to functional categories based on classification by the Bio-Array Resource for Arabidopsis Functional Genomics (Toufighi et al., 2005). The resulting group lists revealed that a high proportion of both up- and down-regulated genes in the *cys-c1* mutant were associated with the plant's responses to biotic and abiotic stress and signaling (Supplemental Fig. S1). The induction of selected genes such as *WRKY33* (encoding a WRKY transcription factor), *ERF6* (encoding an ethylene response transcription factor), *CYP81F2* (encoding a cytochrome P450 involved in glucosinolate biosynthesis), and *GSTU24* (coding for a putative glutathione-*S*-transferase) was confirmed by real-time reverse transcription (RT)-PCR, thus validating the data obtained by the array (Supplemental Fig. S2)

A meta-analysis of the *cys-c1* transcript profile data was performed by comparison with the available Affymetrix Arabidopsis ATH1 GeneChip array databases and the analytical tools of Genevestigator (Hruz et al., 2008). Biclustering and hierarchical clustering

analysis of the up- and down-regulated genes in *cys-c1* showed that 80% were coregulated with genes that were deregulated in wild-type seeds of the ecotype Columbia (Col-0) after treatment with 0.1% oxygen for 6 d (GSE14420; Christianson et al., 2009; Supplemental Fig. S3; Supplemental Table S2). In comparing microarray data for the gene subset categorized as biotic, 54% of the genes identified overlapped with those already shown to be affected by fungal pathogens or altered in *Pseudomonas syringae* pv *tomato* (*Pst*)-infected Arabidopsis plants or elicitor-treated plants (Supplemental Figs. S4 and S5; Supplemental Table S2). Among the genes identified in these groups are several transcription factors related to the biotic defense response, such as WRKY18, WRKY33, WRKY40, and the gene coding for FLG22-INDUCED RECEPTOR-LIKE KINASE1 (AT2G19190). No correlation was found with ACC treatments or mutants in ethylene signaling (Supplemental Fig. S6).

In the light of this analysis, it is interesting to speculate that cyanide plays a role in signaling and defense against pathogen infection in leaf tissues. We aimed then to investigate this hypothesis further.

Cyanide Accumulates during the Infection of Arabidopsis Plants with *Botrytis cinerea*

B. cinerea is a necrotrophic pathogen that causes gray mold diseases in many crop plants, resulting in significant crop losses. *B. cinerea* and other necrotrophic pathogens promote and benefit from host cell death during pathogenesis, as dead cells and necrotic tissues provide a base for saprophytic growth from which *B. cinerea* further colonizes healthy tissue (AbuQamar et al., 2006). When plants are infected by *B. cinerea*, they produce high levels of ethylene (Cristescu et al., 2002; Han et al., 2010). Figure 1A shows that the ethylene production increases rapidly in Arabidopsis when challenged with *B. cinerea*, reaching a maximum level at 24 h post infection (hpi). We investigated the accumulation of the cyanide coproduced during the *B. cinerea*-Arabidopsis interaction as well as the regulation of the *CYS-C1* gene under these conditions. At the beginning of the interaction, the level of cyanide dropped transiently at 9 hpi and then started accumulating, reaching a maximum of 190% of the basal level at 15 hpi (Fig. 1B); accordingly, *CYS-C1* expression shows a waving curve with expression peaks at 3 and 24 hpi and a valley at 15 hpi, this last level coinciding with the higher level of cyanide (Fig. 1C).

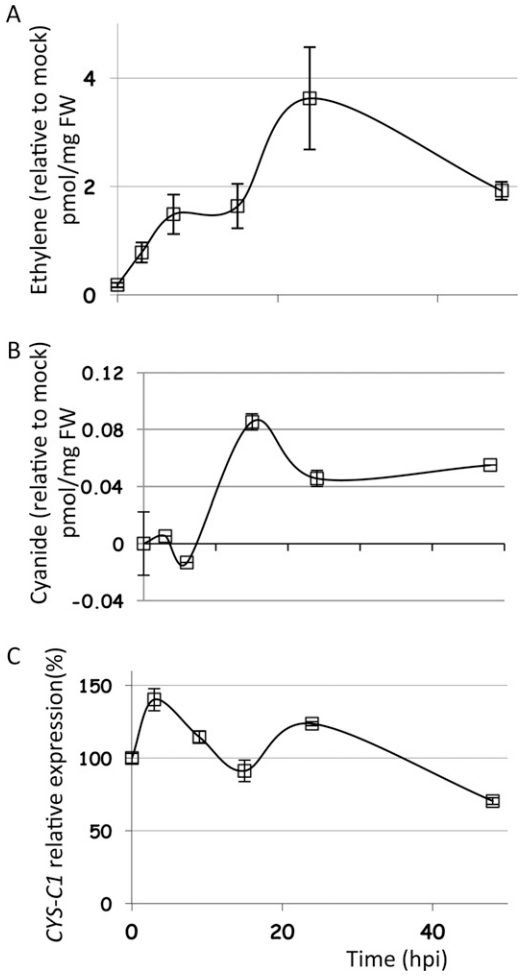

Figure 1. Time course of the accumulation of ethylene and cyanide and the regulation of *CYS-C1* transcript during the Arabidopsis-*B. cinerea* interaction. A and B, Ethylene (A) and cyanide (B) were measured in leaf extracts of wild-type plants grown for 6 to 7 weeks and infected with a spore suspension of *B. cinerea*. The results presented are expressed as means ± SD of a representative experiment in which 12 to 14 leaves from infected plants were pooled and three independent extractions were made from the pooled material. The experiment was repeated three times, with similar results obtained each time. FW, Fresh weight. C, The expression level of *CYS-C1* was analyzed by real-time RT-PCR and referred to the *UBQ10* internal control. The data correspond to means ± SD of three independent analyses using material grown in different batches at different times. For each analysis, five to six plants were pooled, and three independent RNA extractions were made from the pooled material. Two experimental replicates were made for each sample. The data were normalized against the data obtained from plants treated with a mock solution. Nonnormalized data are shown in Supplemental Figures S7A, S8A, and S9A.

Cyanide Accumulation and *CYS-C1* Gene Expression Are Negatively Correlated during Compatible and Incompatible Plant-Bacteria Interactions

The bacterial pathogen *P. syringae* is a hemibiotrophic pathogen that produces bacterial specks in a wide range of plant species. In the early stages of compatible infections, host cell death does not occur. Later stages of infection, however, are associated with host tissue chlorosis and necrosis (Glazebrook, 2005).

Besides the nonhost resistance, plants have the capacity to recognize pathogen-associated molecular patterns by surface pattern-recognition receptors and to induce a response leading to a basal or pathogen-associated

molecular pattern-triggered immunity (PTI; Jones and Dangl, 2006). Some pathogens have evolved to avoid recognition by delivering effectors that suppress PTI, and this results in a compatible plant-pathogen interaction. For their defense, plants have also evolved *RESISTANCE* genes that encode receptors recognizing specific pathogen effectors, resulting in effector-triggered immunity (ETI; Jones and Dangl, 2006). In addition to the PTI response, *Pst* DC3000 can elicit an ETI reaction in Arabidopsis when expressing the type III effector AvrRpm1 (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995). When tobacco plants are infected by *P. syringae*, they produce ethylene. The production is monophasic if the bacteria do not elicit a hypersensitive response (HR) and produce a disease and biphasic if the bacteria induce a HR and do not subsequently produce a disease (Mur et al., 2008). Moreover, transcriptomic data suggest that genes encoding ethylene biosynthetic enzymes were up-regulated in Arabidopsis following challenge with avirulent bacteria (Mur et al., 2008). To investigate this response further, the production of ethylene was monitored during compatible and incompatible interactions. Arabidopsis plants were infected with a virulent *Pst* DC3000 or an avirulent *Pst* DC3000 *avrRpm1* strain. Samples were taken at 1, 3, 6, 9, and 24 hpi. Ethylene was accumulated in the early stages of both interactions, although the accumulation occurred earlier in the incompatible interaction than in the compatible interaction. A second rise occurred at 9 hpi of the avirulent interaction (Fig. 2A). The infection with *Pst* DC3000 induced ethylene accumulation only at the very late stages of the interaction (24 hpi). These data are in agreement with the results already published for the tobacco-*P. syringae* interaction (Mur et al., 2008).

We also determined the kinetics of the accumulation of cyanide in the same samples. Interestingly, cyanide accumulated at different rates in the two Arabidopsis-*Pst* interactions, being detoxified preferentially during the compatible interaction (Fig. 2B). In fact, during ETI, cyanide started accumulating at 3 hpi, and its level did not decrease significantly during the infection. In contrast, during the PTI, cyanide content decreased at 1 hpi, increased to the basal level at 3 and 6 hpi, then decreased and started increasing again after 9 hpi, to reach the basal level of 24 hpi. Accordingly, the transcription of *CYS-C1* was induced during the compatible interaction and was repressed during the ETI, with the curve showing an opposite peak at 3 hpi (Fig. 2C).

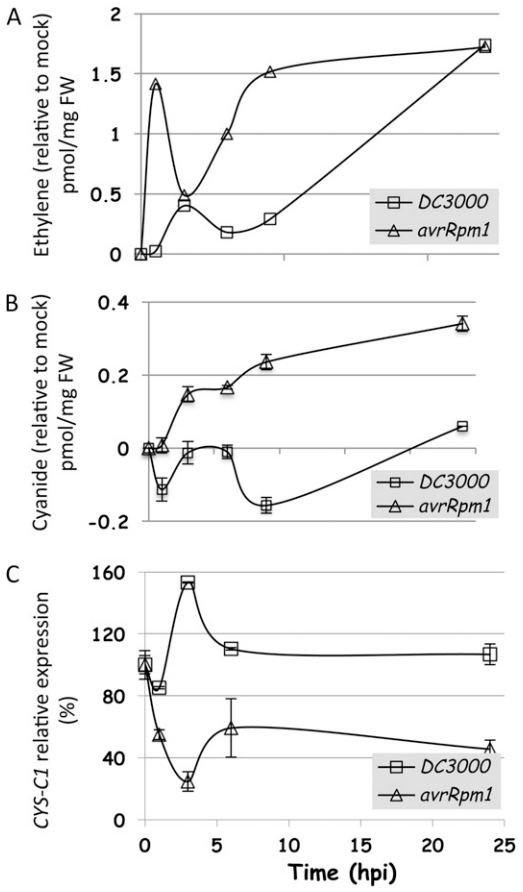

Figure 2. Time course of the accumulation of ethylene and cyanide and the regulation of *CYS-C1* transcript during the Arabidopsis-*P. syringae* interactions. A and B, Ethylene (A) and cyanide (B) were measured in leaf extracts of wild-type plants grown for 6 to 7 weeks and infected with a bacterial suspension of either *Pst* DC3000 or *Pst* DC3000 *avrRpm1* as described in "Materials and Methods." The results presented are expressed as means ± SD of a representative experiment in which 12 to 14 leaves from infected plants were pooled and three independent extractions were made from the pooled material. The experiment was repeated three times, with similar results obtained each time. FW, Fresh weight. C, The expression level of *CYS-C1* was analyzed by real-time RT-PCR and referred to the *UBQ10* internal control. The data correspond to means ± SD of three independent analyses using material grown in different batches at different times. For each analysis, five to six plants were pooled, and three independent RNA extractions were made from the pooled material. Moreover, two experimental replicates were made for each sample. The data were normalized against the data obtained from plants treated with a mock solution. Nonnormalized data are shown in Supplemental Figures S7B, S8B, and S9B.

Mitochondrial Cyanide Differentially Affects the Response to Necrotrophic and Biotrophic Pathogens, and This Effect Is Reversed with Hydroxocobalamin Treatment

Nonlethal concentrations of cyanide can enhance the resistance of plants to fungi (Seo et al., 2011). *cys-c1* mutant plants have been shown to accumulate more cyanide in both root and leaf tissues and to exhibit less ethylene accumulation than wild-type plants (García et al., 2010). To investigate the possible role of mitochondrial cyanide in plant defense against pathogens, *cys-c1* mutant plants defective in the mitochondrial CAS (García et al., 2010) were challenged by a necrotrophic compatible pathogen (*B. cinerea*) and a hemibiotrophic compatible pathogen (*Pst* DC3000). When challenged with the fungus, *cys-c1* showed more severe symptoms than wild-type plants and accumulated six times more

B. cinerea DNA (Fig. 3, A and B). Conversely, the *cys-c1* mutant exhibited a higher tolerance to the infection by *Pst* DC3000 than the Col-0 wild type, as it showed less severe symptoms than wild-type plants and accumulated 12-fold less *Pst* DC3000 colony-forming units (cfu) mg^{-1} fresh weight at 2 d post infection (dpi) than Col-0; the difference was 6-fold at 4 dpi (Fig. 3, C and D). However, the susceptibility to an avirulent strain of *Pst* DC3000 is not affected by the *cys-c1* mutation (Supplemental Fig. S10).

To confirm that the observed phenotype of the *cys-c1* mutant plants was indeed due to the disruption of the *CYS-C1* gene, complementation analysis was performed using the full-length *CYS-C1* genomic fragment including its promoter region (*Pcys-c1*). *cys-c1* plants transformed with the *Pcys-c1-CYS-C1* fragment displayed pathogen sensitivity similar to that of the wild type (Supplemental Fig. S11).

Hydroxocobalamin is a natural form of vitamin B12 that is commonly used as an antidote for severe acute cyanide poisoning in humans (Borron et al., 2007; Hall et al., 2007). Hydroxocobalamin can penetrate cells and act at an intracellular level to bind cyanide and form nontoxic cyanocobalamin, which is excreted in the urine (Astier and Baud, 1996). In plants, hydroxocobalamin has been used to antagonize the effect of cyanide in roots, reverting the root hairless phenotype in *cys-c1* lines to that of wild-type plants (García et al., 2010). The addition of 10 mM hydroxocobalamin at the time of infection with *B. cinerea* reverted the sensitivity phenotype exhibited by the *cys-c1* mutant, decreasing the accumulation of *B. cinerea* DNA in infected *cys-c1* leaves to wild-type levels (Fig. 4A). Moreover, this effect was dose dependent, as the treatment with 5 mM hydroxocobalamin partially reverted the susceptibility of the *cys-c1* mutant to *B. cinerea* to levels similar to those of wild-type plants (Supplemental Fig. S12). Similarly, treatment with hydroxocobalamin altered the phenotype of resistance to *Pst* DC3000 exhibited by the *cys-c1* mutant, as bacteria were able to develop even better in *cys-c1* plants treated with the antidote than in wild-type plants in either the presence or absence of hydroxocobalamin (Fig. 4B). To exclude the possibility that the hydroxocobalamin directly affected pathogen growth, we performed growth tests of *Pst* DC3000 in solid culture Luria-Bertani (LB) medium in

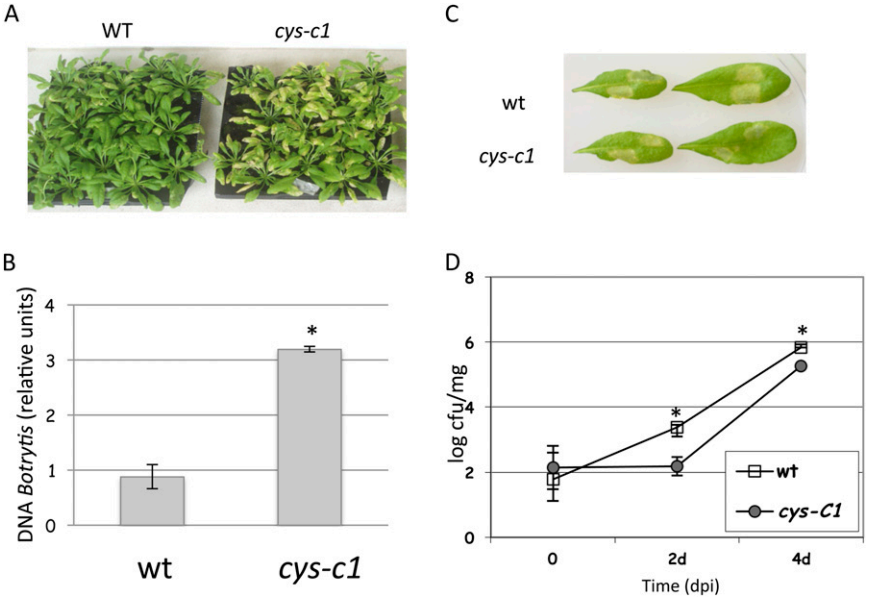

Figure 3. Responses of the *cys-c1* mutant to pathogen infection. A and B, Susceptibility of the wild type (wt) and the *cys-c1* mutant to *B. cinerea* infection. A, Wild-type and *cys-c1* mutant plants after 5 d of *B. cinerea* infection. B, Quantification of fungus growth was performed by real-time PCR amplification of the *B. cinerea creA* gene, which was normalized against the Arabidopsis *UBQ10* gene. DNA was isolated from leaves 5 d after spore inoculation of 6- to 7-week-old wild-type and mutant plants grown in parallel. The data correspond to means ± SD of at least three independent analyses made from material grown in different batches at different times. For each analysis, 20 infected plants were pooled, and six independent DNA extractions were made from the pooled material. Moreover, two experimental replicates were made from each sample. C and D, Susceptibility of the wild type and the *cys-c1* mutant to infection with virulent *Pst* DC3000 bacteria. C, Wild-type and *cys-c1* mutant leaves after 3 d of *Pst* DC3000 infection. *cys-c1* leaves show less severe symptoms than the wild type. D, The cfu were counted at 0, 2, and 4 dpi of 6- to 7-week-old wild-type and mutant plants grown in parallel. At total of 12 to 14 leaves were pooled for each analysis, in which three independent counts were made from the pooled material and two experimental replicates were made from each sample. The data correspond to means ± SD of one representative experiment. $*P < 0.05$. The experiment was performed three times with material grown in different batches at different times; similar results were obtained for each iteration.

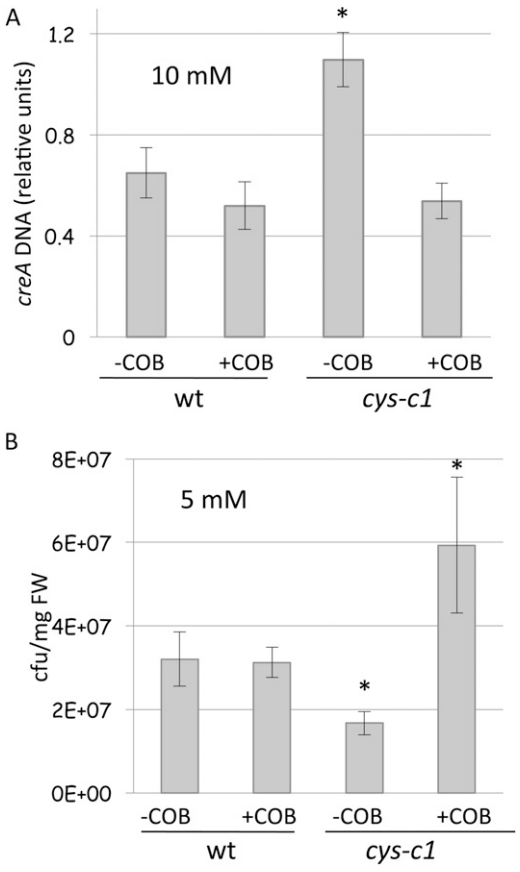

Figure 4. Hydroxocobalamin effect on plant susceptibility to pathogens. Wild-type (wt) and *cys-c1* mutant plants were infected with *B. cinerea* (A) or *Pst* DC3000 (B), as indicated in "Materials and Methods." Pathogens were collected in suspensions containing (+COB) or not containing (−COB) hydroxocobalamin at the concentration indicated and used to perform the susceptibility assays. Quantification of fungus growth was performed by real-time PCR amplification of the *B. cinerea creA* gene, which was normalized against the Arabidopsis *UBQ10* gene. DNA was isolated from leaves 5 d after spore inoculation of 6- to 7-week-old wild-type and mutant plants grown in parallel. The cfu were counted at 3 dpi, with 12 to 14 leaves pooled for each analysis. Three independent determinations were made from the pooled material, and two experimental replicates were made from each sample. The data correspond to means ± SD of one representative experiment. $*P < 0.05$. FW, Fresh weight.

the absence and presence of 5 mM hydroxocobalamin. No differences were observed in either of the two conditions (Supplemental Fig. S13). Therefore, the possibility of a direct effect of hydroxocobalamin in the pathogen's growth rather than rescuing the *cys-c1* phenotype is excluded.

Mitochondrial Cyanide Is Correlated with Plant Resistance to Viral Pathogens

Nonlethal concentrations of cyanide can enhance the resistance of plants to viral infection (Chivasa and Carr, 1998; Wong et al., 2002). Members of the geminivirus family are plant viruses with circular, single-stranded DNA genomes (Rojas et al., 2005) that infect a wide range of plant species and that cause extensive losses in crops. To determine whether mitochondrial cyanide accumulation is involved in the cyanide-related resistance to viruses, wild-type and *cys-c1* mutant plants were challenged with the geminivirus *Beet curly top virus* (BCTV). When infected with the virus, *cys-c1* plants exhibited symptoms less severe than those of respective wild-type plants (Fig. 5, A and B). Plants showing no symptoms (cataloged as 0 by the severity index described by Baliji et al. [2007]) constituted 26.6% in the case of the *cys-c1* mutant and 5.5% in the case of the wild-type plants. Moreover, the sum of plants showing the category 0 (asymptomatic) plus 1 (mild symptoms) was 40% for the *cys-c1* mutant and only 16.6% for the wild-type plants. On the other hand, 33.3% of the *cys-c1* mutant and 61.1% of the wild-type plants showed the most severe symptoms, exhibiting almost no plant growth (categorized as 4 in the severity index). When viral DNA present in infected plants was quantified by real-time PCR, the results clearly showed that the *cys-c1*-infected plants accumulated less viral DNA than did wild-type plants (Fig. 5C). These results indicate that endogenously produced cyanide can protect plants from virus attack just as exogenously applied cyanide does.

The *cys-c1* Mutation Produces a Reduction in Respiration Rate in Leaves and an Induction of Alternative Oxidase and *PR1* Expression

Cyanide binds to the heme iron of the mitochondrial cytochrome *c* oxidase, thereby blocking the cytochrome respiration pathway and the utilization of oxygen in cellular functions (Donato et al., 2007). In higher plants, an alternative cyanide-resistant respiratory pathway is catalyzed by the alternative oxidase (AOX), which is located in the mitochondrial inner membrane and acts as a terminal oxidase in the mitochondrial electron transport chain. AOX branches from the main respiratory chain at the level of the ubiquinone pool and catalyzes the four-electron reduction of oxygen to water, releasing the energy as heat (Millenaar and Lambers, 2003). Much work has revealed that the genes encoding AOX, AOX protein, and the alternative respiratory pathway are frequently induced during plant-pathogen interactions (Hanqing et al., 2010). The *cys-c1* mutant displays a reduction of root (García et al., 2010) and leaf (Fig. 6A) respiration rates. The addition of salicylhydroxamic acid (SHAM), an inhibitor of the AOX pathway, affects the respiration rate of wild-type and mutant plants differently, as it decreases the respiration rate of wild-type leaves only about 8% but alters the respiration rate of the *cys-c1* mutant leaves by about 24% (Fig. 6B). In both wild-type and mutant plants, the addition of potassium cyanide (KCN) reduces the oxygen uptake drastically to about 30% of the maximum respiration rate. The

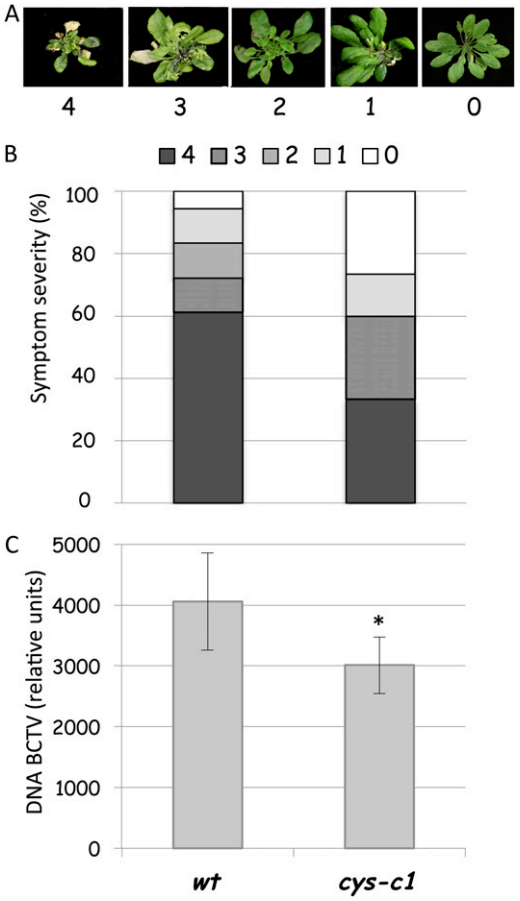

Figure 5. Response of the *cys-c1* mutant to virus. A, Example of the severity index described in "Materials and Methods" and Baliji et al. (2007): 0, no symptoms; 1 to 4, increasing severity of symptoms. B, Susceptibility of the wild type (wt) and the *cys-c1* mutant to BCTV infection. Whole 6- to 7-week-old plants of each genotype were agroinoculated, and the symptom severity was recorded at 28 dpi. C, Quantification of virus growth was performed in the same plants at 28 dpi by real-time PCR amplification of the viral DNA, which was normalized against the Arabidopsis *UBQ10* gene. The data correspond to means ± SD of three independent analyses made from material grown in different batches at different times. For each analysis, at least 10 infected plants were pooled, and six independent DNA extractions were made from the pooled material. Two experimental replicates were performed from each sample. $*P < 0.05$. [See online article for color version of this figure.]

addition of KCN plus SHAM reduces oxygen uptake to levels lower than 10% (Fig. 6B). The increase of the AOX pathway in the *cys-c1* mutant correlates with an increase in transcript abundance of the *AOX1a* gene (Fig. 6C), which is induced by an alteration of the cytochrome respiration pathway (Albury et al., 2009; García et al., 2010). In addition, the expression of *PR1*, a pathogenesis-related protein induced by the salicylic acid-dependent pathway (An and Mou, 2011), is induced in *cys-c1* plants in the absence of stress (Fig. 6C), suggesting that endogenously produced cyanide can modulate this pathway in Arabidopsis plants.

The *cys-c1* Mutant Accumulates Reactive Oxygen Species But Does Not Show Programmed Cell Death Lesions

One of the earliest responses to pathogen infection is the production of reactive oxygen species (ROS; Lamb and Dixon, 1997), which together with nitric oxide and salicylic acid can promote the HR (Delledonne et al., 1998; Álvarez, 2000) and lead to the activation of systemic acquired resistance, a broad-spectrum form of disease resistance (Vlot et al., 2008). Since a reduction of the respiration rate can produce an accumulation of ROS, we compared the accumulation of ROS in *cys-c1* and wild-type seedlings grown under control conditions (Fig. 7). Imaging of ROS in vivo in plant tissues by confocal laser microscopy is a very useful technique (Schneider et al., 1998). We observed a fluorescence emission resulting from the oxidation of the nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate

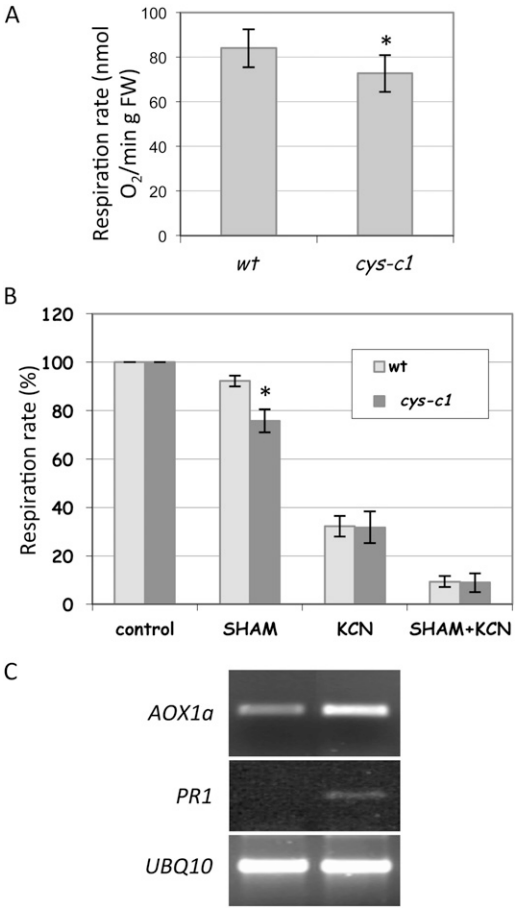

Figure 6. Respiration rates (A and B) and *AOX1a* and *PR1* expression levels (C) in leaves of wild-type (wt) and *cys-c1* mutant plants. Cyanide-independent and AOX respiration were determined in the presence of 0.5 mM KCN or 4 mM SHAM, respectively. The transcription level of the AOX gene *AOX1a*, *PR1*, and the control *UBQ10* was determined by RT-PCR in leaves of noninfected 6- to 7-week-old plants. The data correspond to means ± SD of at least three independent analyses made from material grown in different batches at different times. $*P < 0.05$. FW, Fresh weight.

(H_2DCFDA) to a highly fluorescent product; this signal reflects significant production of hydrogen peroxide (H_2O_2). In roots, this fluorescence was higher in *cys-c1* specimens than in wild-type samples (Fig. 7, A and B). Although chlorophyll autofluorescence interferes with the H_2O_2 detection in green tissues, we were able to observe a higher fluorescence in *cys-c1* than in wild-type cotyledons (Fig. 7, C and D).

Because H_2O_2 is a signaling intermediate molecule in programmed cell death, we stained the leaves of plants grown in long-day and short-day conditions with lactophenol trypan blue. We did not observe lesions characteristic of spontaneous cell death in the leaves of the *cys-c1* mutant (Fig. 7, E–H).

DISCUSSION

Plants synthesize ethylene in response to different environmental stimuli, including pathogen attack (Wang et al., 2002; Pandey and Somssich, 2009). The role of ethylene in defense signaling in plants has been studied extensively, but its involvement remains controversial. Treatment with ethylene increases or decreases resistance to pathogens depending on the plant-pathogen interaction, and the use of mutants defective in ethylene signaling indicates a limited or different role of ethylene in the resistance to some biotrophic and necrotrophic pathogens, including fungi, bacteria, and viruses (Pieterse et al., 1998; Brading et al., 2000; Broekaert et al., 2006; Iwai et al., 2006). Cyanide is produced concomitantly with ethylene biosynthesis. In this work, however, we show different patterns of ethylene and cyanide accumulation during infection of Arabidopsis with both the fungus *B. cinerea* and the virulent and avirulent *P. syringae*. In addition, we show that the lack of mitochondrial CAS of Arabidopsis, which leads to an accumulation of cyanide in plant tissues (García et al., 2010), results in an altered response to plant pathogens. The response is completely dependent on cyanide, as demonstrated by genetic and chemical complementation. All these data suggest that cyanide also acts in the regulation of the plant immune responses. Furthermore, the transcriptional regulation of the *CYS-C1* gene during the three plant-pathogen interactions analyzed allows a differential accumulation of cyanide in each interaction, suggesting that *CYS-C1* is involved in the signaling pathway, leading to resistance or sensitivity depending on the type of pathogen.

The classification of genes differentially expressed in *cys-c1* and wild-type plants reveals that the high endogenous cyanide content of the *cys-c1* mutant is correlated with the biotic stress response. More specifically, the cyanide accumulation is correlated with the induction of genes encoding proteins involved in the plant signaling pathway. Among the induced genes in the *cys-c1* mutant, three WRKY transcription factors, *WRKY18*, *WRKY33*, and *WRKY40*, are involved in the modulation of host defenses toward phytopathogens (Pandey and Somssich, 2009). *WRKY33* in particular was shown to be required for resistance to the necrotrophs *Alternaria brassicicola* and *B. cinerea* (Zheng et al., 2006), while *WRKY18* and *WRKY40* appear to be necessary for the resistance to *P. syringae* (Xu et al., 2006). Often, WRKY factors interact both physically and functionally in a complex pattern of overlapping or antagonistic roles. For instance, the mutation of either *WRKY18* or *WRKY40* does not affect the susceptibility of plants to either necrotrophic or biotrophic pathogens. Double *wrky18wrky40* mutants, however, are more susceptible to *P. syringae* and more resistant to *B. cinerea* than wild-type plants (Xu et al., 2006). The simultaneous activation of *WRKY18*, *WRKY33*, and *WRKY40* in the *cys-c1* mutant, then, does not necessarily lead to an additive

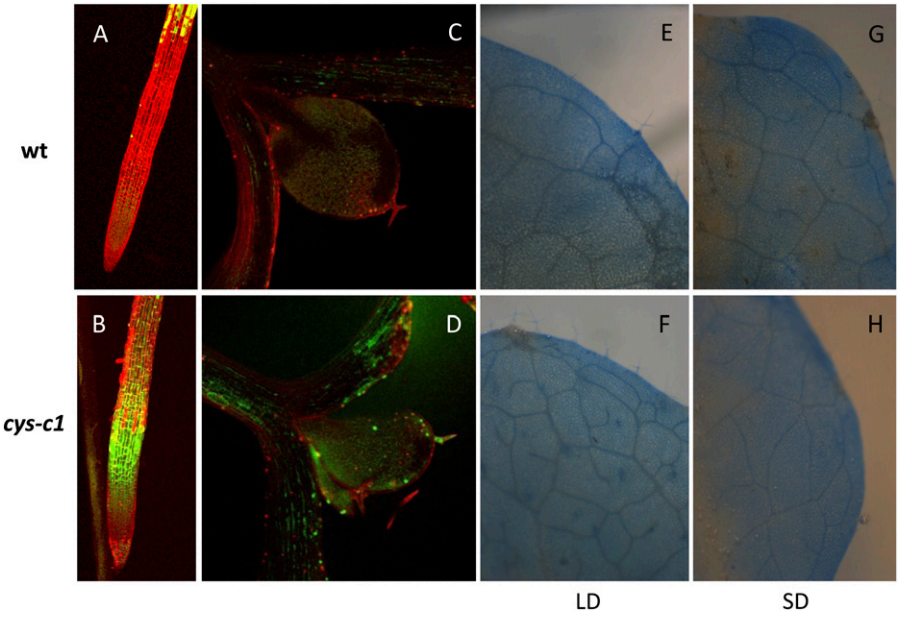

Figure 7. Accumulation of H_2O_2 and lesion formation in the wild type (wt) and the *cys-c1* mutant. A to D, H_2O_2 was detected by H_2DCFDA staining in root (A and B) and cotyledons (C and D) from 5-d-old wild-type and *cys-c1* mutant plants cultured in MS medium. E to H, Lactophenol trypan blue was used to stain spontaneous cell death lesions. Detached leaves of plants grown in soil for 3 weeks in long-day conditions (LD; E and F) or 6 to 7 weeks in short-day conditions (SD; G and H) were used for the assay. All the experiments were repeated at least three times, with similar results obtained each time.

effect for the expression of each WRKY factor separately. In fact, we have found that cyanide accumulation correlates with an increased susceptibility to a necrotrophic pathogen, and this association would probably be due to a deleterious but nonlethal effect of cyanide itself. An intriguing increase of the tolerance to biotrophic pathogens is observed concurrently. This increased tolerance is applicable to both a bacteria and a virus and occurs together with the induction of the pathogenesis-related *PR1* mRNA in the absence of pathogens. Both susceptibility to the necrotrophic fungus and resistance to the biotrophic bacteria are reversed by treatment with the antidote hydroxocobalamin, demonstrating that the effect observed is specifically related to cyanide. Although ROS are more abundant in the *cys-c1* mutant than in wild-type plants, no PCD lesions or microlesions are observed in the mutant, which demonstrates that cyanide does not induce a lesion-mimic phenotype that could be responsible for the resistance to *Pst* DC3000 (Lorrain et al., 2003)

To discriminate between distinctive pathogens and to activate appropriate responses, plants use phytohormones for signaling. In general, responses against biotrophic pathogens include a signaling cascade dependent on salicylic acid, while necrotrophic organisms induce signaling pathways dependent on ethylene and jasmonic acid (Pieterse et al., 2009). Interactions between the different signaling pathways have been demonstrated, indicating a complex network of hormone cross talk (Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Leon-Reyes et al., 2010). Exogenously applied cyanide mimics salicylic acid-induced resistance of tobacco, Arabidopsis, and tomato (*Solanum lycopersicum*) plants to viruses (Chivasa and Carr, 1998; Wong et al., 2002). More recently, this treatment has been shown to confer resistance of rice to the biotrophic fungus *Magnaporthe oryzae*, and it has been suggested that cyanide increases during the HR (Seo et al., 2011). We have demonstrated that Arabidopsis plants accumulate more cyanide when they are infected with an avirulent strain of *Pst* DC3000 than when they are challenged with the virulent strain, suggesting that this molecule has a role in the establishment of the HR. The repression of *CYS-C1* expression during the avirulent interaction and its activation during the virulent interaction further support this hypothesis. Finally, the resistance of the *cys-c1* mutant to biotrophic pathogens indicates that cyanide mimics or induces the salicylic acid signaling pathway in Arabidopsis plants.

Interestingly, *cys-c1* mutant leaves exhibit a reduced respiration rate that is more sensitive to the alternative pathway inhibitor SHAM and an enhanced expression of the *AOX1a* gene, showing that the alternative respiration pathway is activated in the mutant plants. AOX allows flexibility of plant respiratory metabolism, especially under environmental stresses (Vanlerberghe and McIntosh, 1997; Mackenzie and McIntosh, 1999), and it is induced by many adverse conditions (Hanqing et al., 2010). The induction of *AOX1a* in the *cys-c1* mutant in nonstressed conditions could prepare it to better respond to a pathogen attack, probably by inducing a signal transduction dependent on ROS that culminates in the induction of defense proteins such as PR1 and other proteins related to pathogenesis. It has been suggested that tobacco and tomato cyanide-induced resistance to virus is mediated by AOX, which would contribute to the signal transduction pathway leading to resistance (Chivasa and Carr, 1998; Fu et al., 2010). Strikingly, when overexpressing either the native AOX or a version of AOX mutated at its active site, tobacco mosaic virus vectors increase systemic movements in *Nicotiana benthamiana* (Murphy et al., 2004).

In summary, our results suggest that cyanide, a low-M_r and highly hydrophilic molecule, acts as a signal in plants. Nitric oxide and oxygen peroxide are also low-M_r molecules that are toxic at high concentrations but that exhibit a signaling role at low concentrations; their roles have been extensively demonstrated and accepted (Delledonne et al., 1998; Laloi et al., 2004). In our model, cyanide that is produced in the *cys-c1* mutant uncouples the respiratory electron chain dependent on the cytochrome *c* oxidase, and this uncoupling induces the AOX activity and the accumulation of ROS, which act by stimulating the salicylic acid-dependent signaling pathway of the plant immune system.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild-type ecotype Col-0 and the SALK_022479 mutant were used in this work. The plants were grown in soil with a photoperiod of 8 h of white light (120 μE m^{-2} s^{-1}) at 20°C/16 h of dark at 18°C. Plants were cultivated for 6 to 7 weeks. For some experiments, the plants were cultivated in solid MS medium in petri dishes supplemented with 1% Suc with a photoperiod of 16 h of white light (120 μE m^{-2} s^{-1}) at 20°C/8 h of dark at 18°C.

To generate the *cys-c1* complementation line (*cys-c1*::Pcys-c1-*CYS-C1*), a 2,949-bp genomic fragment containing the full-length coding sequence of *CYS-C1* plus the intergenic region between *CYS-C1* and its contiguous *PIP1* gene (At3g61430) was obtained by PCR amplification using the proofreading Platinum Pfx DNA polymerase (Invitrogen) and the primers C1GW-F and C1GW-R (Supplemental Table S3). The fragment was cloned into the pENTR/D-TOPO vector (Invitrogen) and transferred into the pMDC99 vector (Curtis and Grossniklaus, 2003) using the Gateway system (Invitrogen) according to the manufacturer's instructions. The final construct, Pcys-c1-*CYS-C1*, was generated by transformation into *Agrobacterium tumefaciens* and then introduced into *cys-c1* null plants using the floral dip method (Clough and Bent, 1998).

Respiration Measurements in Leaves

Wild-type and mutant plants were grown for 6 to 7 weeks in soil. Approximately 50 mg of leaf tissues was cut and transferred into air-tight cuvettes containing 20 mM HEPES (pH 7.2) and $CaCl_2$, and oxygen uptake was measured as a decrease of oxygen concentration in the dark using a Clark-type electrode. Cyanide-resistant oxygen uptake was measured in the presence of 0.5 mM KCN. The component of the change due to the AOX pathway was determined by measurement in the presence of 4 mM of the inhibitor SHAM.

Bacterial Pathogen Infections

The bacterial strains used in this study were *Pseudomonas syringae* pv *tomato* DC3000 and *Pst* DC3000 bearing a plasmid containing the *avrRpm1* avirulence gene (Grant et al., 1995). For treatment of the plants, bacterial cultures were

collected from plates in 10 mM $MgCl_2$, and their concentrations were adjusted to 5×10^6 bacteria mL^{-1} (optical density at 600 nm = 0.01; *Pst* DC3000 *avrRpm1*) or 2.5×10^6 bacteria mL^{-1} (optical density at 600 nm = 0.005; *Pst* DC3000). Sterile 10 mM $MgCl_2$ was used as a mock solution. The bacterial suspension or the mock solution was then pressure infiltrated into the abaxial side of the leaves of 6- to 7-week-old plants using a syringe without a needle. Wild-type, mutant, and complemented plants were grown at the same time using the same conditions (Swanson et al., 1988).

Bacteria and Growth Tests

Pst DC3000 *avrRpm1* bacteria were collected from LB plates supplemented with rifampicin (50 μg mL^{-1}) in 10 mM $MgCl_2$, and their concentration was adjusted to 5×10^6 bacteria mL^{-1} (optical density at 600 nm = 0.01). To determine whether hydroxocobalamin affects bacterial viability, growth tests were performed as described previously (Álvarez et al., 2012a) by supplementing the growth medium with 5 mM hydroxocobalamin instead of 0.5 mM Cys. Six series of 1:10 dilutions were performed. In all, 10 μL of the resulting suspensions was plated, grown for 48 h at 28°C, and subsequently photographed (Supplemental Fig. S13).

In Planta Growth of Virulent or Avirulent *Pst* DC3000

The protocol for measuring the growth of bacteria was adapted from (Tornero and Dangl, 2001). Wild-type, mutant, and complemented plants were grown for 6 to 7 weeks at the same time and in the same conditions and inoculated with bacterial pathogens as described above. One hour after the inoculation, the samples for day 0 were taken. To determine bacterial growth, 100 mg of leaves was ground in 500 μL of 10 mM $MgCl_2$ and gently vortexed. In all, 20 μL from each sample was added to the wells of a microtiter plate containing 180 μL of 10 mM $MgCl_2$, and serial 10-fold dilutions were plated on petri dishes containing 50 mg mL^{-1} rifampicin. The plates were incubated at 30°C, and the number of colonies was counted 30 h later. The number of cfu mg^{-1} fresh weight was determined by the formula cfu mg^{-1} fresh weight = $k(N \times 10^{d-1})$/(weight of the tissue), where N is the number of colonies counted in the dilution number d and the constant k (500 in our case) represents the number of cfu present in the sample per colony appearing in the first dilution (Tornero and Dangl, 2001).

Fungal Infections

The *Botrytis cinerea* strain ME4 was grown in a solid strawberry broth for 12 d, and spore suspensions were prepared at a concentration of 5×10^5 spores mL^{-1} in 12 g L^{-1} potato dextrose broth. Six- to 7-week-old wild-type, mutant, and complemented plants grown at the same time and in the same conditions were pulverized with a Preval sprayer with spore suspension. Approximately 2 mL of spore suspension per plant was used. The plants were covered with a transparent film to maintain 100% humidity. The samples were collected for PCR analysis after 5 d.

Quantification of *B. cinerea* DNA Accumulation in Infected Plants

DNA from infected plants was quantified by real-time PCR according to a previous study (Calo et al., 2006). DNA from the *B. cinerea creA* gene (Tudzynski et al., 2000) was amplified using the oligonucleotides creABOT-F and creABOT-R (Supplemental Table S3). As an internal standard to normalize the real-time PCR Arabidopsis *UBQ10* DNA was amplified using the oligonucleotides qUBQ10F and qUBQ10R (Supplemental Table S3). Relative quantifications were performed by subtracting the cycle threshold (CT) value of *UBQ10* from the CT value of *creA* (ΔCT). The relative *B. cinerea* DNA was calculated as $2^{-\Delta CT}$.

Geminivirus Infection Assays

Infection of Arabidopsis plants with *Beet curly top virus* was performed by whole plant agroinoculation as described (Briddon et al., 1989; Lozano-Durán et al., 2011). Inoculated plants were scored for the appearance of symptoms typical of a BCTV infection on systemically infected tissue. Symptom severity was evaluated at 28 dpi according to the severity index described by Baliji et al. (2007), where 0 represents symptomless plants and 1 to 4 represent plants showing increasing symptom severity. The infection assay was performed in triplicate.

Quantification of BCTV DNA Accumulation in Infected Plants

Total DNA of infected plants was extracted at 28 dpi using the DNeasy Plant Mini Kit (Qiagen) and digested with *Dpn*I to differentiate between viral DNA originating from a replication in planta, which is not methylated, and viral DNA originating from replication in the inoculum of *A. tumefaciens*, which is methylated. Viral DNA accumulation was quantified by real-time PCR using the primers BCTV-F and BCTV-R (Supplemental Table S2). As an internal standard to normalize the real-time PCR, Arabidopsis *UBQ10* DNA was amplified using the oligonucleotides qUBQ10F and qUBQ10R (Supplemental Table S3). Relative quantifications were performed by subtracting the CT value of *UBQ10* from the CT value of *BCTV* (ΔCT). The relative BCTV DNA was calculated as $2^{-\Delta CT}$.

H_2O_2 Detection

For the fluorimetric detection of H_2O_2, 5-d-old seedlings were incubated with 10 μM H_2DCFDA (Molecular Probes) for 5 min in the presence of 10 μM propidium iodide (López-Martín et al., 2008). Samples were observed using a Leica TCS SP2 spectral confocal microscope with excitation of 488 nm and an emission range of 500 to 550 nm for fluorescein detection and 600 to 650 nm for propidium iodide detection.

Cell Death Staining

Trypan blue staining for dead cells in leaves was performed as described previously (Carol and Dolan, 2006) by incubating the leaves in a lactic acid-phenol-trypan blue solution (2.5 mg mL^{-1} trypan blue, 25% [w/v] lactic acid, 23% phenol, and 25% glycerol), heating them over boiling water for 1 min, and finally destaining them using a 2.5 g mL^{-1} chloral hydrate solution before photographing the leaves.

Ethylene Determination by Gas Chromatography

A total of 100 to 300 mg of infected leaves was collected, weighted, placed inside a 12-mL vial, and finally sealed. The amount of ethylene produced and released to the gas phase during 24 h was determined by gas chromatography by injecting 1 mL of the head space onto a GC2010 apparatus equipped with an activated alumina column and a flame ionization detector. The oven and the detector temperatures were isothermally maintained at 80°C and 150°C, respectively. The results are expressed as means ± SD from at least five replicate samples, and the experiment was repeated three times from independent samples.

Cyanide Determination by HPLC

A total of 100 mg of plant tissue was homogenized using a mortar and pestle with liquid nitrogen and resuspended in cold borate-phosphate extraction buffer (2 mL g^{-1} fresh weight) containing 27 mM sodium borate and 47 mM potassium phosphate, pH 8.0. Homogenates were centrifuged at 15,000*g* for 15 min at 4°C. Extracted cyanide was subsequently quantified by reverse-phase HPLC after derivatization with 2,3-naphthalenedialdehyde to form a 1-cyano-2-alkyl-benz[f]isoindole derivative by previously described methods (Lin et al., 2005; García et al., 2010).

RNA Isolation and Semiquantitative RT-PCR

Total RNA was extracted from Arabidopsis leaves using the RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using an oligo(dT) primer and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions. *AOX1a* and *PR1* expression was determined by semiquantitative PCR using an aliquot of the complementary DNA (cDNA) and the oligonucleotides shown in Supplemental Table S3. The constitutively expressed *UBQ10* gene was used as a control. The PCR conditions were as follows: a denaturation cycle of 2 min at 94°C; 30 amplification cycles of 1 min

at 94°C, 1 min at 60°C, and 1 min at 72°C; and an extension cycle of 5 min at 72°C.

Real-Time RT-PCR

Quantitative real-time RT-PCR was used to validate microarray data and to analyze the expression of the *CYS-C1* gene. First-strand cDNA was synthesized as described above. Gene-specific primers for each gene were designed using the Vector NTI Advance 10 software (Invitrogen; Supplemental Table S3). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad), and the signals were detected on an iCYCLER (Bio-Rad) according to the manufacturer's instructions. The cycling profile consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. A melt curve from 60°C to 90°C was run following the PCR cycling. The expression level of each gene was normalized to that of the constitutive *UBQ10* gene by subtracting the CT value of *UBQ10* from the CT value of the gene (ΔCT). The fold change was calculated as $2^{-(\Delta CT\ mutant\ -\ \Delta CT\ wild\ type)}$.

RNA Extraction and Microarray Hybridization

For microarray studies of the *cys-c1* mutant, plants were grown on MS plates supplemented with 1% Suc under a photoperiod of 16 h of white light (120 μE m^{-2} s^{-1}) at 20°C/8 h of dark at 18°C. Leaves of 15-d-old plants were used for total RNA isolation with TRIzol reagent (Invitrogen) and cleaning with the RNeasy Plant Mini Kit (Qiagen). The resulting material was used to synthesize biotinylated complementary RNA (cRNA) for hybridization to Arabidopsis ATH1 arrays (Affymetrix) using the 3′ Amplification One-Cycle Target Labeling Kit. Briefly, 4 mg of RNA was reverse transcribed to produce first-strand cDNA using a $(dT)_{24}$ primer with a T7 RNA polymerase promoter site added to the 3′ end. After second-strand synthesis, in vitro transcription was performed using T7 RNA polymerase and biotinylated nucleotides to produce biotin-labeled cRNA. The cRNA preparations (15 μg) were fragmented into fragments of 35 to 200 bp at 95°C for 35 min. These fragmented cRNAs were hybridized to the Arabidopsis ATH1 microarrays at 45°C for 16 h. Each microarray was washed and stained in the Affymetrix Fluidics Station 400 following standard protocols. Microarrays were scanned using the Affymetrix GeneChip Scanner 3000.

Microarray Data Analysis

Microarray analysis was performed using the affylmGUI R package (Wettenhall et al., 2006). The Robust Multiarray Analysis algorithm was used for background correction, normalization, and summarizing expression levels (Irizarry et al., 2003). Differential expression analysis was performed using Bayes *t* statistics and the linear models for microarray data (Limma), which are included in the affylmGUI package. *P* values were corrected for multiple testing using the false discovery rate method (Benjamini and Hochberg, 1995; Reiner et al., 2003). Cutoff values of 2-fold change and $P < 0.05$ were adopted to discriminate the expression of genes that were differentially expressed in the mutant plant with respect to the wild type. Gene classification into functional groups was determined using the Bio-Array Resource for Arabidopsis Functional Genomics (Toufighi et al., 2005) and MapMan software (http://gabi.rzpd.de/projects/MapMan/). The microarray data for the *cys-c1* mutant were meta-analyzed using Genevestigator (Hruz et al., 2008).

Statistical Analysis

For all the experiments shown, at least three independent samples were analyzed (for details, see the figure legends). An ANOVA statistical analysis of data was performed using the program OriginPro 7.5 (OriginLab).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers CYS-C1 (At3g61440) and CYS-C1 T-DNA mutant (SALK_022479). The microarray Gene Expression Omnibus accession number is GSE19242.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Analysis of the *cys-c1* transcriptome.

Supplemental Figure S2. Relative expression levels of selected genes in the *cys-c1* mutant plants.

Supplemental Figure S3. Graphic display of the hierarchical clustering of *cys-c1* up- or down-regulated genes in response to hypoxia, performed with Genevestigator (Hruz et al., 2008).

Supplemental Figure S4. Graphic display of the meta-profile analysis of *cys-c1* up- or down-regulated genes in response to biotic stresses, performed with Genevestigator (Hruz et al., 2008).

Supplemental Figure S5. Graphic display of the hierarchical clustering of *cys-c1* up- or down-regulated genes in response to elicitors and pathogens, performed with Genevestigator (Hruz et al., 2008).

Supplemental Figure S6. Graphic display of the hierarchical clustering of *cys-c1* up- or down-regulated genes in response to ACC treatment and in the *etr1-1* mutant, performed with Genevestigator (Hruz et al., 2008).

Supplemental Figure S7. Time course of the accumulation of ethylene during the Arabidopsis-*B. cinerea* interaction (A) and the Arabidopsis-*P. syringae* interactions (B).

Supplemental Figure S8. Time course of the accumulation of cyanide during the Arabidopsis-*B. cinerea* interaction (A) and the Arabidopsis-*P. syringae* interactions (B).

Supplemental Figure S9. Time course of the expression of *CYS-C1* during the Arabidopsis-*B. cinerea* interaction (A) and the Arabidopsis-*P. syringae* interactions (B).

Supplemental Figure S10. Susceptibility of the wild type and the *cys-c1* mutant to infection with avirulent *Pst* DC3000 *avrRpm1* bacteria.

Supplemental Figure S11. Genetic complementation of the pathogen-associated phenotype of the *cys-c1* mutant.

Supplemental Figure S12. Dose-dependent effect of hydroxocobalamin on plant susceptibility to *B. cinerea*.

Supplemental Figure S13. Growth tests of *Pst* DC3000 bacteria grown in LB medium supplemented with rifampicin and 5 mM hydroxocobalamin (COB 5 mM) or with rifampicin alone (−COB).

Supplemental Table S1. List of differentially regulated genes in leaves of the *cys-c1* mutant compared with the wild type.

Supplemental Table S2. Pathogen- and hypoxia-regulated genes in the *cys-c1* mutant.

Supplemental Table S3. Oligonucleotide sequences used in this work.

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LITERATURE CITED

AbuQamar S, Chen X, Dhawan R, Bluhm B, Salmeron J, Lam S, Dietrich RA, Mengiste T (2006) Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to Botrytis infection. Plant J **48:** 28–44

Albury MS, Elliott C, Moore AL (2009) Towards a structural elucidation of the alternative oxidase in plants. Physiol Plant **137:** 316–327

Álvarez C, Bermúdez MA, Romero LC, Gotor C, García I (2012a) Cysteine homeostasis plays an essential role in plant immunity. New Phytol **193:** 165–177

Álvarez C, Calo L, Romero LC, García I, Gotor C (2010) An *O*-acetylserine (thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis. Plant Physiol **152:** 656–669

Álvarez C, García I, Romero LC, Gotor C (2012b) Mitochondrial sulfide detoxification requires a functional isoform O-acetylserine(thiol)lyase C in Arabidopsis thaliana. Mol Plant **5:** 1217–1226

Álvarez ME (2000) Salicylic acid in the machinery of hypersensitive cell death and disease resistance. Plant Mol Biol **44:** 429–442
An C, Mou Z (2011) Salicylic acid and its function in plant immunity. J Integr Plant Biol **53:** 412–428
Astier A, Baud FJ (1996) Complexation of intracellular cyanide by hydroxocobalamin using a human cellular model. Hum Exp Toxicol **15:** 19–25
Baliji S, Sunter J, Sunter G (2007) Transcriptional analysis of complementary sense genes in Spinach curly top virus and functional role of C2 in pathogenesis. Mol Plant Microbe Interact **20:** 194–206
Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B **57:** 289–300
Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ (1994) RPS2 of Arabidopsis thaliana: a leucine-rich repeat class of plant disease resistance genes. Science **265:** 1856–1860
Bermúdez MA, Páez-Ochoa MA, Gotor C, Romero LC (2010) *Arabidopsis*S-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control. Plant Cell **22:** 403–416
Bethke PC, Libourel IG, Reinöhl V, Jones RL (2006) Sodium nitroprusside, cyanide, nitrite, and nitrate break Arabidopsis seed dormancy in a nitric oxide-dependent manner. Planta **223:** 805–812
Bogatek R, Dziewanowska K, Lewak S (1991) Hydrogen-cyanide and embryonal dormancy in apple seeds. Physiol Plant **83:** 417–421
Borowitz JL, Gunasekar PG, Isom GE (1997) Hydrogen cyanide generation by mu-opiate receptor activation: possible neuromodulatory role of endogenous cyanide. Brain Res **768:** 294–300
Borron SW, Baud FJ, Mégarbane B, Bismuth C (2007) Hydroxocobalamin for severe acute cyanide poisoning by ingestion or inhalation. Am J Emerg Med **25:** 551–558
Böttcher C, Westphal L, Schmotz C, Prade E, Scheel D, Glawischnig E (2009) The multifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*. Plant Cell **21:** 1830–1845
Brading PA, Hammond-Kosack KE, Parr A, Jones JD (2000) Salicylic acid is not required for Cf-2- and Cf-9-dependent resistance of tomato to Cladosporium fulvum. Plant J **23:** 305–318
Briddon RW, Watts J, Markham PG, Stanley J (1989) The coat protein of Beet curly top virus is essential for infectivity. Virology **172:** 628–633
Broekaert WF, Delauré SL, De Bolle MF, Cammue BP (2006) The role of ethylene in host-pathogen interactions. Annu Rev Phytopathol **44:** 393–416
Calo L, García I, Gotor C, Romero LC (2006) Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a Trichoderma alpha-1,3-glucanase. J Exp Bot **57:** 3911–3920
Carol RJ, Dolan L (2006) The role of reactive oxygen species in cell growth: lessons from root hairs. J Exp Bot **57:** 1829–1834
Chivasa S, Carr JP (1998) Cyanide restores *N* gene-mediated resistance to tobacco mosaic virus in transgenic tobacco expressing salicylic acid hydroxylase. Plant Cell **10:** 1489–1498
Christianson JA, Wilson IW, Llewellyn DJ, Dennis ES (2009) The low-oxygen-induced NAC domain transcription factor ANAC102 affects viability of Arabidopsis seeds following low-oxygen treatment. Plant Physiol **149:** 1724–1738
Cipollone R, Visca P (2007) Is there evidence that cyanide can act as a neuromodulator? IUBMB Life **59:** 187–189
Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16:** 735–743
Cohn MA, Hughes JA (1986) Seed dormancy in red rice. V. Response to azide, hydroxylamine, and cyanide. Plant Physiol **80:** 531–533
Cristescu SM, De Martinis D, Te Lintel Hekkert S, Parker DH, Harren FJ (2002) Ethylene production by Botrytis cinerea in vitro and in tomatoes. Appl Environ Microbiol **68:** 5342–5350
Curtis MD, Grossniklaus U (2003) A Gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133:** 462–469
Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature **394:** 585–588
Donato DB, Nichols O, Possingham H, Moore M, Ricci PF, Noller BN (2007) A critical review of the effects of gold cyanide-bearing tailings solutions on wildlife. Environ Int **33:** 974–984
Fol M, Frachisse JM, Petel G, Gendraud M (1989) Effect of cyanide on the membrane-potential of Jerusalem artichoke (Helianthus tuberosus L) tuber parenchyma: different responses in relation to dormancy. C R Acad Sci III Sci Vie **309:** 551–556
Fu LJ, Shi K, Gu M, Zhou YH, Dong DK, Liang WS, Song FM, Yu JQ (2010) Systemic induction and role of mitochondrial alternative oxidase and nitric oxide in a compatible tomato-Tobacco mosaic virus interaction. Mol Plant Microbe Interact **23:** 39–48
García I, Castellano JM, Vioque B, Solano R, Gotor C, Romero LC (2010) Mitochondrial β-cyanoalanine synthase is essential for root hair formation in *Arabidopsis thaliana*. Plant Cell **22:** 3268–3279
Glawischnig E (2007) Camalexin. Phytochemistry **68:** 401–406
Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol **43:** 205–227
Grant MR, Godiard L, Straube E, Ashfield T, Lewald J, Sattler A, Innes RW, Dangl JL (1995) Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. Science **269:** 843–846
Gunasekar PG, Borowitz JL, Turek JJ, Van Horn DA, Isom GE (2000) Endogenous generation of cyanide in neuronal tissue: involvement of a peroxidase system. J Neurosci Res **61:** 570–575
Gunasekar PG, Prabhakaran K, Li L, Zhang L, Isom GE, Borowitz JL (2004) Receptor mechanisms mediating cyanide generation in PC12 cells and rat brain. Neurosci Res **49:** 13–18
Hall AH, Dart R, Bogdan G (2007) Sodium thiosulfate or hydroxocobalamin for the empiric treatment of cyanide poisoning? Ann Emerg Med **49:** 806–813
Han L, Li GJ, Yang KY, Mao G, Wang R, Liu Y, Zhang S (2010) Mitogen-activated protein kinase 3 and 6 regulate Botrytis cinerea-induced ethylene production in Arabidopsis. Plant J **64:** 114–127
Hanqing F, Kun S, Mingquan L, Hongyu L, Xin L, Yan L, Yifeng W (2010) The expression, function and regulation of mitochondrial alternative oxidase under biotic stresses. Mol Plant Pathol **11:** 429–440
Hartley SA, Kato K, Salter KJ, Kozlowski RZ (1998) Functional evidence for a novel suramin-insensitive pyrimidine receptor in rat small pulmonary arteries. Circ Res **83:** 940–946
Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W, Zimmermann P (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. Adv Bioinformatics **2008:** 420747
Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:** 249–264
Isom GE, Way JL (1984) Effects of oxygen on the antagonism of cyanide intoxication: cytochrome oxidase, in vitro. Toxicol Appl Pharmacol **74:** 57–62
Iwai T, Miyasaka A, Seo S, Ohashi Y (2006) Contribution of ethylene biosynthesis for resistance to blast fungus infection in young rice plants. Plant Physiol **142:** 1202–1215
Jones JD, Dangl JL (2006) The plant immune system. Nature **444:** 323–329
Koornneef A, Pieterse CM (2008) Cross talk in defense signaling. Plant Physiol **146:** 839–844
Laloi C, Apel K, Danon A (2004) Reactive oxygen signalling: the latest news. Curr Opin Plant Biol **7:** 323–328
Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol **48:** 251–275
Leon-Reyes A, Van der Does D, De Lange ES, Delker C, Wasternack C, Van Wees SC, Ritsema T, Pieterse CM (2010) Salicylate-mediated suppression of jasmonate-responsive gene expression in Arabidopsis is targeted downstream of the jasmonate biosynthesis pathway. Planta **232:** 1423–1432
Lin CC, Wong BK, Burgey CS, Gibson CR, Singh R (2005) In vitro metabolism of a thrombin inhibitor and quantitation of metabolically generated cyanide. J Pharm Biomed Anal **39:** 1014–1020
López-Martín MC, Becana M, Romero LC, Gotor C (2008) Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in Arabidopsis. Plant Physiol **147:** 562–572
Lorrain S, Vailleau F, Balagué C, Roby D (2003) Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? Trends Plant Sci **8:** 263–271
Lozano-Durán R, Rosas-Díaz T, Gusmaroli G, Luna AP, Taconnat L, Deng XW, Bejarano ER (2011) Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes

and inhibit jasmonate signaling in *Arabidopsis thaliana*. Plant Cell **23:** 1014–1032

Mackenzie S, McIntosh L (1999) Higher plant mitochondria. Plant Cell **11:** 571–586

Millenaar FF, Lambers H (2003) The alternative oxidase: in vivo regulation and function. Plant Biol **5:** 2–15

Miller JM, Conn EE (1980) Metabolism of hydrogen cyanide by higher plants. Plant Physiol **65:** 1199–1202

Mindrinos M, Katagiri F, Yu GL, Ausubel FM (1994) The A. thaliana disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell **78:** 1089–1099

Mur LA, Laarhoven LJ, Harren FJ, Hall MA, Smith AR (2008) Nitric oxide interacts with salicylate to regulate biphasic ethylene production during the hypersensitive response. Plant Physiol **148:** 1537–1546

Murphy AM, Gilliland A, York CJ, Hyman B, Carr JP (2004) High-level expression of alternative oxidase protein sequences enhances the spread of viral vectors in resistant and susceptible plants. J Gen Virol **85:** 3777–3786

Pandey SP, Somssich IE (2009) The role of WRKY transcription factors in plant immunity. Plant Physiol **150:** 1648–1655

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol **5:** 308–316

Pieterse CM, van Wees SC, van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, van Loon LC (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell **10:** 1571–1580

Piotrowski M (2008) Primary or secondary? Versatile nitrilases in plant metabolism. Phytochemistry **69:** 2655–2667

Poulton JE (1990) Cyanogenesis in plants. Plant Physiol **94:** 401–405

Reiner A, Yekutieli D, Benjamini Y (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics **19:** 368–375

Rojas MR, Hagen C, Lucas WJ, Gilbertson RL (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annu Rev Phytopathol **43:** 361–394

Schneider K, Mathur J, Boudonck K, Wells B, Dolan L, Roberts K (1998) The ROOT HAIRLESS 1 gene encodes a nuclear protein required for root hair initiation in Arabidopsis. Genes Dev **12:** 2013–2021

Seo S, Mitsuhara I, Feng J, Iwai T, Hasegawa M, Ohashi Y (2011) Cyanide, a coproduct of plant hormone ethylene biosynthesis, contributes to the resistance of rice to blast fungus. Plant Physiol **155:** 502–514

Siegien I, Bogatek R (2006) Cyanide action in plants: from toxic to regulatory. Acta Physiol Plant **28:** 483–497

Spoel SH, Dong X (2008) Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe **3:** 348–351

Stelmaszyńska T (1986) Formation of HCN and its chlorination to ClCN by stimulated human neutrophils. 2. Oxidation of thiocyanate as a source of HCN. Int J Biochem **18:** 1107–1114

Swanson J, Kearney B, Dahlbeck D, Staskawicz BJ (1998) Cloned avirulence gene of *Xanthomonas campestris* pv. *vesicatoria* complements spontaneous race change mutant. Mol Plant Microbe Interact **1:** 5–9

Tornero P, Dangl JL (2001) A high-throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. Plant J **28:** 475–481

Toufighi K, Brady SM, Austin R, Ly E, Provart NJ (2005) The Botany Array Resource: e-northerns, expression angling, and promoter analyses. Plant J **43:** 153–163

Tudzynski B, Liu S, Kelly JM (2000) Carbon catabolite repression in plant pathogenic fungi: isolation and characterization of the Gibberella fujikuroi and Botrytis cinerea creA genes. FEMS Microbiol Lett **184:** 9–15

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: from gene to function. Annu Rev Plant Physiol Plant Mol Biol **48:** 703–734

van Loon LC, Geraats BP, Linthorst HJ (2006) Ethylene as a modulator of disease resistance in plants. Trends Plant Sci **11:** 184–191

Vlot AC, Klessig DF, Park SW (2008) Systemic acquired resistance: the elusive signal(s). Curr Opin Plant Biol **11:** 436–442

Wang KLC, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. Plant Cell (Suppl) **14:** S131–S151

Watanabe M, Kusano M, Oikawa A, Fukushima A, Noji M, Saito K (2008) Physiological roles of the β-substituted alanine synthase gene family in Arabidopsis. Plant Physiol **146:** 310–320

Wettenhall JM, Simpson KM, Satterley K, Smyth GK (2006) affylmGUI: a graphical user interface for linear modeling of single channel microarray data. Bioinformatics **22:** 897–899

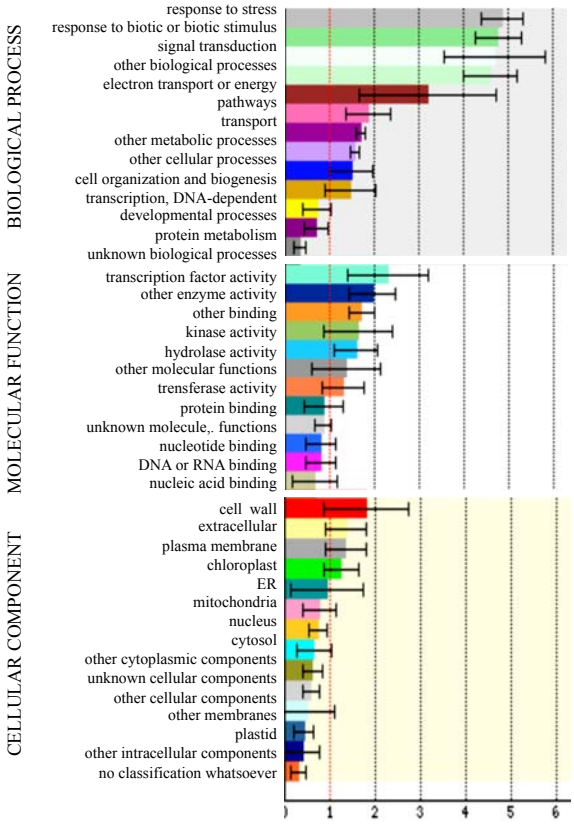
Wong CE, Carson RA, Carr JP (2002) Chemically induced virus resistance in Arabidopsis thaliana is independent of pathogenesis-related protein expression and the NPR1 gene. Mol Plant Microbe Interact **15:** 75–81

Xu X, Chen C, Fan B, Chen Z (2006) Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell **18:** 1310–1326

Zagrobelny M, Bak S, Møller BL (2008) Cyanogenesis in plants and arthropods. Phytochemistry **69:** 1457–1468

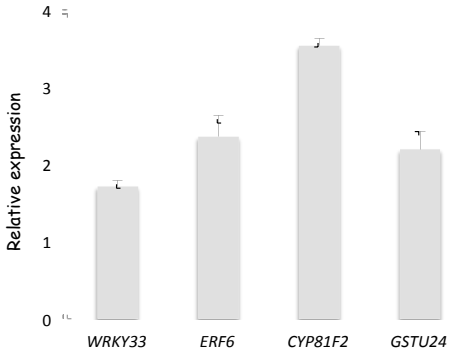
Zheng Z, Qamar SA, Chen Z, Mengiste T (2006) Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J **48:** 592–605

Figure S1



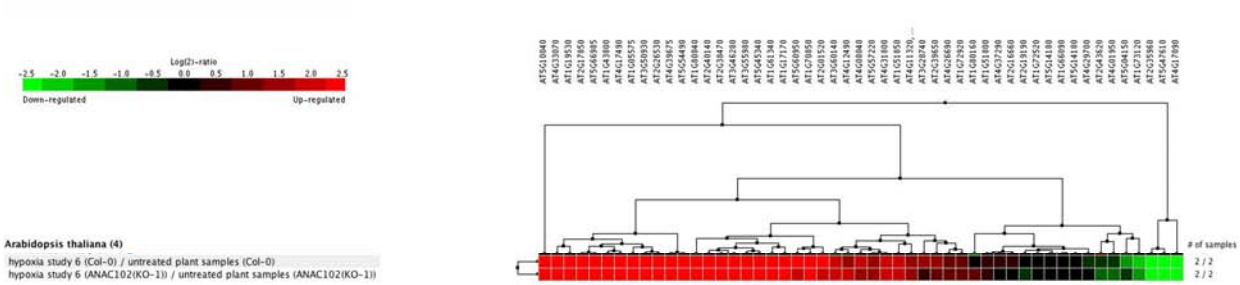
Supplemental Figure S1. Analysis of the *cys-c1* transcriptome. Data were analyzed using the Classification SuperViewer tool of the Bio-Array Resource for Arabidopsis Functional Genomics, BAR (Toufighi et al., 2005). A functional classification of all the deregulated genes in the *cys-c1* mutant based on the GO database and a ranking score for each functional class are shown.

Figure S2



Supplemental Figure S2. Relative expression levels of pathogen-responsive genes in the *cys-c1* mutant type plants compared to wild type Col-0. Real-time RT-PCR analysis of expression of the *WRKY33 (At2g38470), ERF6 (At4g17490), CYP81F2 (At5g57220)* and *GSTU24 (At1g17170)* genes was performed in 15-day-old seedlings. The transcript levels were normalized to the internal control, the constitutive *UBQ10* gene. Data shown are means ± SD of three independent analyses and represent the transcript level of each gene in the *cys-c1* mutant plants relative to the transcript level in the Col-0 plants.

Figure S3

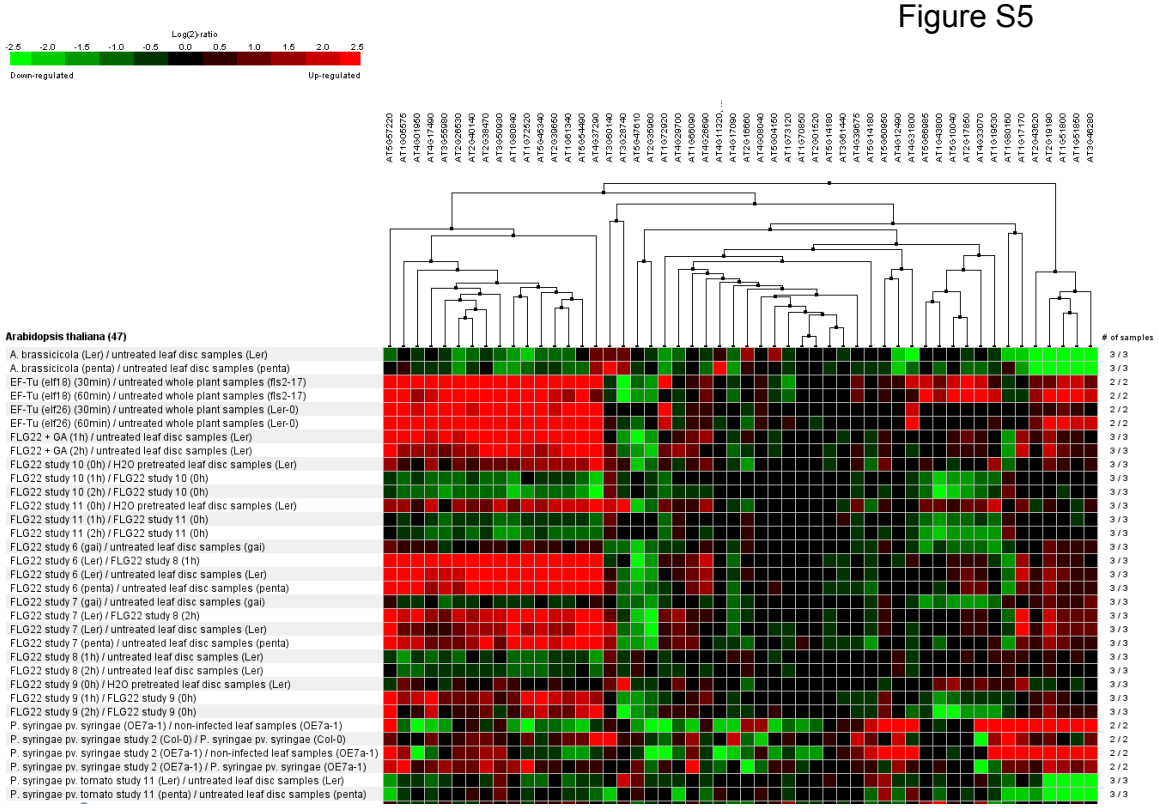


Supplemental Figure S3. Graphic display of the hierarchical clustering of *cys-c1* up- or down-regulated genes in response to hypoxia, performed with Genevestigator (Hruz et al., 2008). Each row represents the treatment indicated, and each column refers to a gene. A dendrogram representing the Euclidian distance between mutants is shown, and the scale to the top marks the correlation coefficient represented by the length of the branches that connect pairs of nodes. The color scale indicates the $\log_2$ level of expression above (red) or below (green) the median.

Figure S4

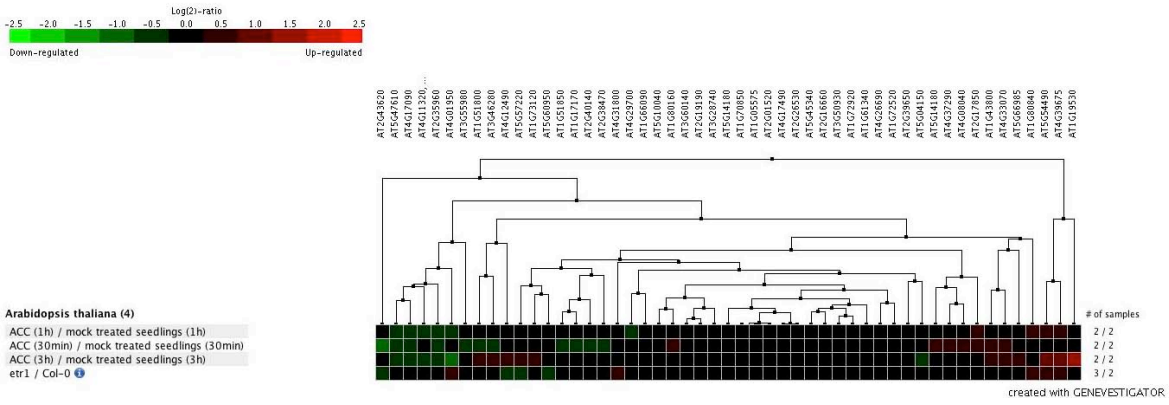
Supplemental Figure S4. Graphic display of meta-profile analysis of *cys-c1* up- or down-regulated genes in response to biotic stresses, performed with Genevestigator (Hruz et al., 2008). See the legend of figure S3 for details.

Figure S5



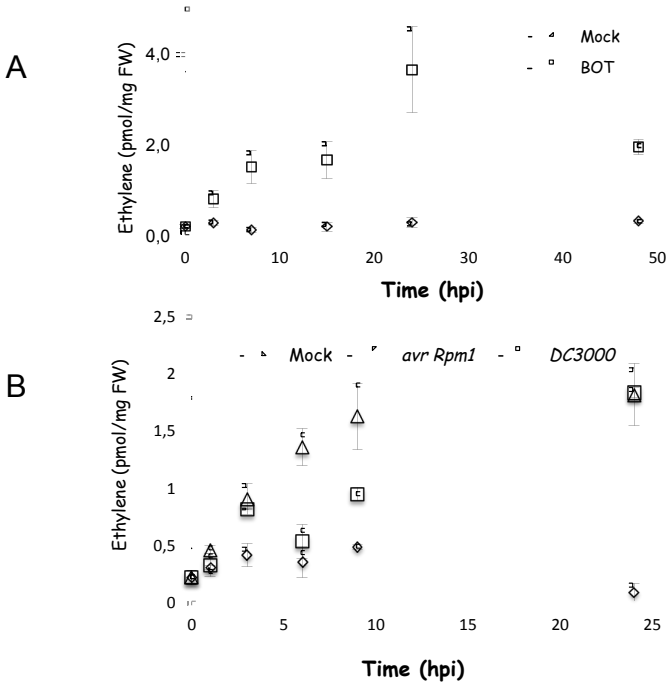
Supplemental Figure S5. Graphic display of hierarchical clustering of *cys-c1* up- or downregulated genes in response to elicitors and pathogens, performed with Genevestigator (Hruz et al., 2008). See the legend of figure S3 for details.

Figure S6



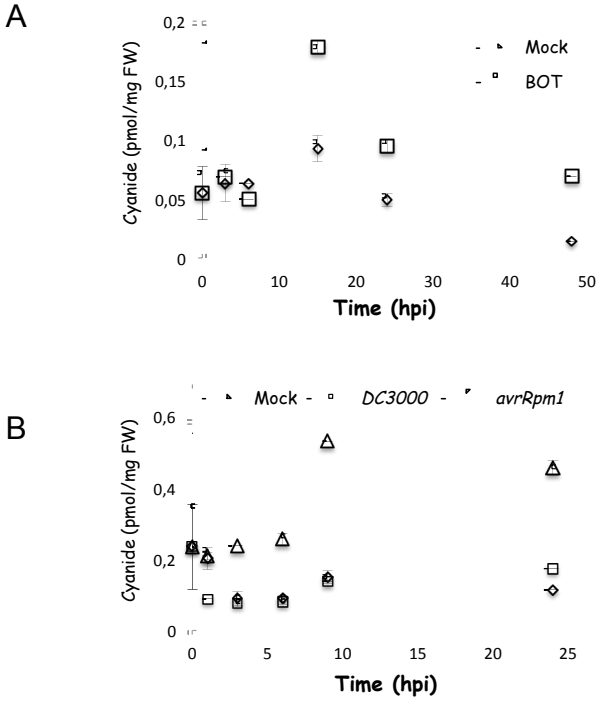
Supplemental Figure S6. Graphic display of hierarchical clustering of *cys-c1* up- or downregulated genes in response to ACC treatment and in the *etr1-1* mutant, performed with Genevestigator (Hruz et al., 2008). See the legend of figure S3 for details.

Figure S7



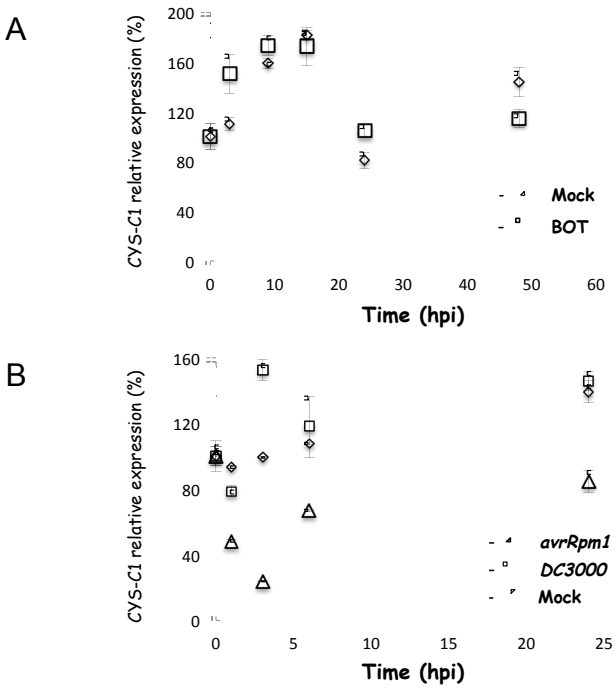
Supplemental Figure S7. Time-course of the accumulation of ethylene during the *A. thaliana-B. cinerea* interaction (A) and the *A. thaliana-P. syringae* interacions (B). Ethylene was measured in leaf extracts of wild type plants grown for 6 to 7 weeks and mock-treated or infected with a spore suspensions of *B. cinerea* (BOT) or a bacterial suspension of either *Pst DC3000* or *Pst DC3000 avrRpm1* as described in Material and Methods. The results presented here are expressed as the mean ± SD of a representative experiment in which 10-12 independent measurements were done. The experiment was repeated three times, with similar results obtained each time. hpi, hours post-infection.

Figure S8



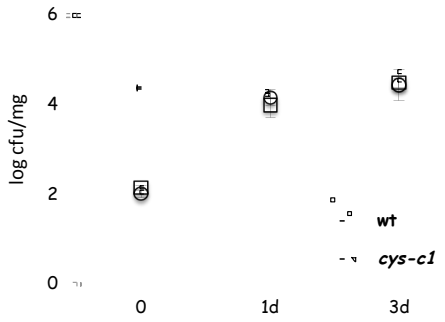
Supplemental Figure S8. Time-course of the accumulation of cyanide during the *A. thaliana-B. cinerea* interaction (A) and the *A. thaliana-P. syringae* interacions (B). Cyanide was measured in leaf extracts of wild type plants grown for 6 to 7 weeks and mock-treated or infected with a spore suspensions of *B. cinerea* (BOT) or a bacterial suspension of either *Pst DC3000* or *Pst DC3000 avrRpm1* as described in Material and Methods. The results presented here are expressed as the mean ± SD of a representative experiment in which 12-14 leaves from infected plants were pooled and three independent extractions were made from the pooled material. The experiment was repeated three times, with similar results obtained each time. hpi, hours post-infection.

Figure S9



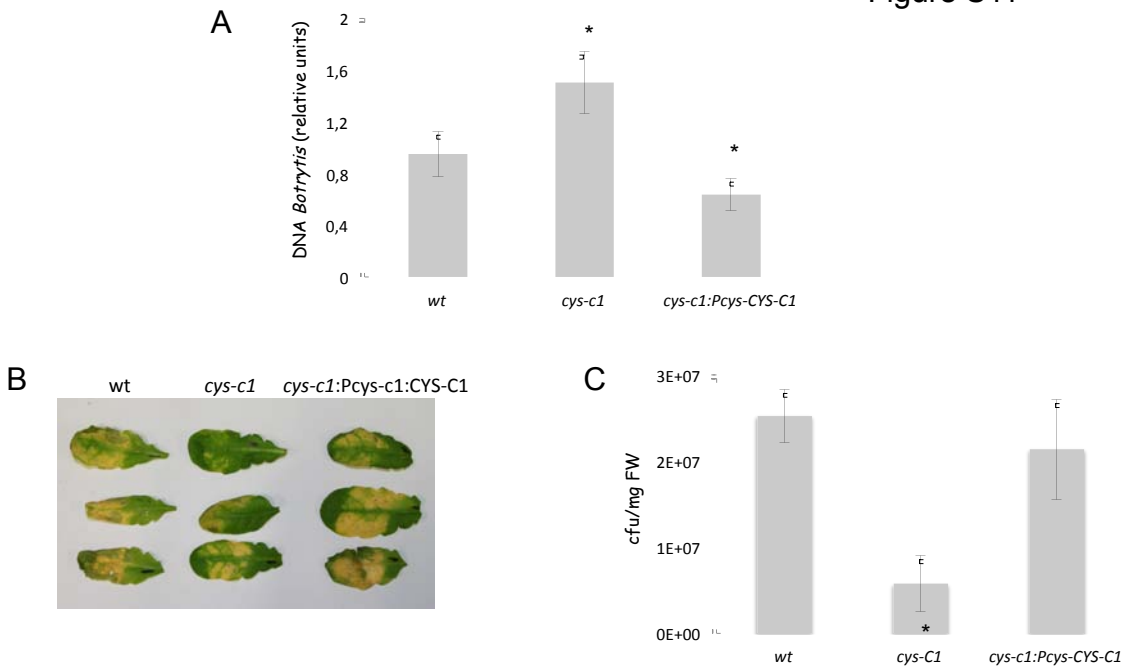
Supplemental Figure S9. Time-course of the expression of *CYS-C1*during the *A. thaliana-B. cinerea* interaction (A) and the *A. thaliana-P. syringae* interacions (B). *CYS-C1* expression was measured in leaf extracts of wild type plants grown for 6 to 7 weeks and mock-treated or infected with a spore suspensions of *B. cinerea* (BOT) or a bacterial suspension of either *Pst DC3000* or *Pst DC3000 avrRpm1* as described in Material and Methods. The expression level of *CYS-C1* was analyzed by real-time RT-PCR and referred to the *UBQ10* internal control. The data correspond to the means ± SD of three independent analysis using material grown in different batches at different times. For each analysis, 5-6 plants were pooled, and three independent RNA extractions were made from the pooled material. Moreover, two experimental replicates were made for each sample. hpi, hours post-infection.

Figure S10



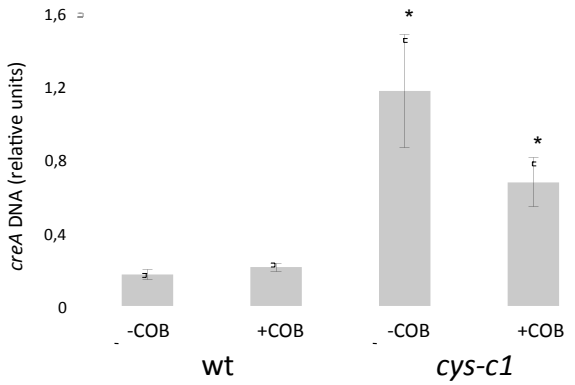
Supplemental Figure S10. Susceptibility of wild type and *cys-c1* mutants to infection with avirulent *Pst DC3000 avrRpm1* bacteria. Colony-forming units (cfu) were counted at 0, 1 and 3 days post-infection of 6- to 7-week-old wild type and mutant plants grown in parallel. At total of 12 to 14 leaves were pooled for each analysis, in which three independent counts were made from the pooled material and two experimental replicates were made from each sample. The data correspond to the mean ± standard deviation (SD) of one representative experiment. The experiment was performed three times with material grown in different batches at different times; similar results were obtained for each iteration.

Figure S11



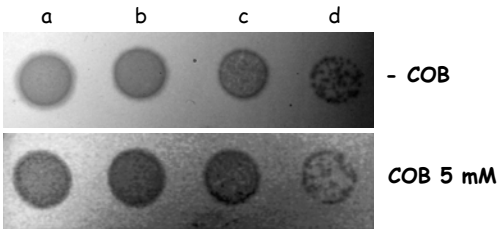
Supplemental Figure S11. Genetic complementation of the pathogen-associated phenotype of the *cys-c1* mutant. Wild type, *cys-c1* mutant and complemented *cys-c1*::Pcys-c1-*CYS-C1* plants were infected with *B. cinerea* (A) and the virulent *Pst DC3000* (B, C) as indicated in Figure 4. A. Quantification of fungus growth was performed by real-time-PCR amplification of the *B. cinerea creA* gene, which was normalized against the Arabidopsis *UBQ10* gene. DNA was isolated from leaves 5 days after spore inoculation of 6- to 7-week-old wild type, mutant and complemented plants grown in parallel. B. Wild type, *cys-c1* mutant and the complemented *cys-c1*::Pcys-c1-*CYS-C1* plant leaves after 3 days of *Pst DC3000* infection. C. Colony-forming units (cfu) were counted at 3 days post-infection (dpi). A total of 12 to 14 leaves were pooled for each analysis. Three independent determinations were made from the pooled material, and two experimental replicates were made from each sample. The data correspond to the mean ± standard deviation (SD) of one representative experiment. *, P<0.05.

Figure S12



Supplemental Figure S12. Dose-dependent effect of the hydroxocobalamin on plant susceptibility to *B. cinerea*. Wild type and *cys-c1* mutant plants were infected with *B. cinerea* as indicated in Figure 4. Pathogens were collected in suspensions containing (+COB) or not containing (-COB) hydroxocobalamin at 5 mM and were used to perform the susceptibility assays. A total of 12 to 14 leaves were pooled for each analysis, in which three independent determinations were made from the pooled material and two experimental replicates were made from each sample. The data correspond to the mean ± standard deviation (SD) of one representative experiment. *, P<0.05

Figure S13



Supplemental Figure S13. Growth tests of *Pst DC3000* bacteria grown in LB medium supplemented with rifampicine and hydroxocobalamin 5 mM (COB 5 mM) or with rifampicine alone (-COB). a to d: 10 µl of serial 10-fold dilutions of a $5x10^6$ bacteria ml^{-1} *Pst DC3000* suspension.

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Review

Discovering Host Genes Involved in the Infection by the *Tomato Yellow Leaf Curl Virus* Complex and in the Establishment of Resistance to the Virus Using *Tobacco Rattle Virus*-based Post Transcriptional Gene Silencing

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Abstract: The development of high-throughput technologies allows for evaluating gene expression at the whole-genome level. Together with proteomic and metabolomic studies, these analyses have resulted in the identification of plant genes whose function or expression is altered as a consequence of pathogen attacks. Members of the *Tomato yellow leaf curl virus* (TYLCV) complex are among the most important pathogens impairing production of agricultural crops worldwide. To understand how these geminiviruses subjugate plant defenses, and to devise counter-measures, it is essential to identify the host genes affected by infection and to determine their role in susceptible and resistant plants. We have used a reverse genetics approach based on *Tobacco rattle virus*-induced gene silencing (TRV-VIGS) to uncover genes involved in viral infection of susceptible plants, and to identify genes underlying virus resistance. To identify host genes with a role in geminivirus infection, we have engineered a *Nicotiana benthamiana* line, coined 2IRGFP,

which over-expresses GFP upon virus infection. With this system, we have achieved an accurate description of the dynamics of virus replication in space and time. Upon silencing selected *N. benthamiana* genes previously shown to be related to host response to geminivirus infection, we have identified eighteen genes involved in a wide array of cellular processes. Plant genes involved in geminivirus resistance were studied by comparing two tomato lines: one resistant (R), the other susceptible (S) to the virus. Sixty-nine genes preferentially expressed in R tomatoes were identified by screening cDNA libraries from infected and uninfected R and S genotypes. Out of the 25 genes studied so far, the silencing of five led to the total collapse of resistance, suggesting their involvement in the resistance gene network. This review of our results indicates that TRV-VIGS is an exquisite reverse genetics tool that may provide new insights into the molecular mechanisms underlying plant infection and resistance to infection by begomoviruses.

Keywords: Tomato yellow leaf curl disease; geminiviruses; plant-resistance; tomato; VIGS; reverse genetics; plant-virus interaction

1. Introduction

Viral diseases threaten the production of agriculture plant crops. To establish a successful infection, viruses must hijack the cellular machinery and prevent or counteract the plant defenses. On the other hand, plants have developed a variety of resistance mechanisms, either ready to meet incoming pathogens or induced by them. High-throughput technologies allow following changes in gene expression upon virus infection at the genome level and evaluating the functions of these genes during infection [1, 2], in susceptible as well as resistant plants [3]. Begomoviruses (genus *Begomovirus*, family *Geminiviridae*), a major virus family affecting agricultural crops worldwide, have been the subject of such studies [4-6]. Identifying the host genes selectively expressed during infection and determining their role is a pre-requisite to understand the process of begomovirus infection in susceptible and resistant plants. We review here how the use of a reverse genetics approach based on virus-induced gene silencing (VIGS) has allowed the identification of plant genes involved in infection and in resistance to begomoviruses of the *Tomato yellow leaf curl virus* (TYLCV) complex.

2. Analysis of gene expression in plants using a reverse genetics approach based on virus-induced gene silencing

Plant innate response to virus invasion includes triggering resistance gene products, local cell death and systemic acquired resistance [7]. During the last decade, it appeared that RNA silencing is another, sequence-specific, universal plant defense mechanism against virus invasion [8]. It was discovered that replication of RNA and DNA viruses is associated with the accumulation of virus-derived small RNAs that help cleave viral messengers in a sequence specific manner [9,10]. This mode of RNA silencing was referred as post-transcriptional gene silencing (PTGS). Viruses encode suppressors of RNA silencing, which efficiently inhibit host antiviral responses [11]. RNA silencing of viruses led to the

development of an outstanding reverse genetic tool now widely used in plant biology, known as virus-induced gene silencing (VIGS). In plants, VIGS is specifically targeted against the viral genome. However, with virus vectors carrying inserts derived from host genes, the process can be targeted against the corresponding mRNAs [12]. Hence, VIGS has emerged as an efficient tool to study gene silencing in plants [13].

One of the most common vectors currently used is based on the *Tobacco rattle virus* (TRV) [14,15]. This method uses a bipartite vector system designed between left and right borders of the *Agrobacterium* Ti plasmid. *TRVI* contains the RNA-dependant RNA polymerase (RdRp) and the MP components of the virus whereas *TRVII* contains multiple cloning sites (MCS) and the CP sequences. The bipartite plasmids are flanked by the 35S C*auliflower mosaic virus* promoter and a *Nopaline synthase* gene terminator. The MCS in *TRVII* allows ligation of DNA target sequences that will induce PTGS in the plant upon delivery by agroinoculation. The multiplication of the vector in the plant tissue triggers the cleavage of target sequence resulting in loss of expression [14]. Among other features, VIGS has been used to dissect the genetics of floral development and scent production [16], water deficit stress tolerance [17], embryogenesis, chlorophyll biosynthesis and disease resistance [18], and protective acyl sugars in trichomes [19]. The siRNAs-mediated RNA silencing has been exploited to engineer plants resistant to diseases by targeting the genome of viruses, viroids, insects and fungi [20].

TRV is not the only virus used as vector for PTGS studies. More than 30 viruses have been shown to have potential as VIGS vectors [21]. Among others, the tobamovirus *Tobacco mosaic virus* (TMV) and the potyvirus *Potato virus X* (PVX) have been engineered to target the plant phytoene desaturase gene (PDS), frequently used as a reporter gene for efficient silencing (the leaf loses its green color) [22]. The Hordeivirus *Barley stripe mosaic virus* (BSMV) served as vector to silence PDS, magnesium chelatase subunit H and plastid transketolase genes, and the powdery mildew resistance 5 gene *PMR5* in *Nicotiana benthamiana*, barley and wheat [23]. Several geminiviruses have been engineered to serve as VIGS vectors. *Tomato golden mosaic virus* was used to silence the proliferating cell nuclear antigen (PCNA) and a subunit of magnesium chelatase in *N. benthamiana* [24]. *Tomato leaf curl virus* (ToLCV) served to silence tomato PCNA [25]. TYLCV was modified to serve as a gene silencing system in tomato and was applied to silence a viral silencing suppressor of Grapevine virus A (GVA), resulting in GVA-tolerant *N. benthamiana* plants [26]. *Cabbage leaf curl virus* (CaLCuV) was used to dissect the host geminivirus silencing mechanism in *Arabidopsis thaliana* [27]. The DNA1 satellite of the *Tobacco curly shoot virus* has been modified into a VIGS vector to study floral development [28]. *African cassava mosaic virus* (ACMV) was used to silence genes involved in glycoside synthesis in cassava [29]. *Cotton leaf crumple virus* (CLCrV) was used to silence a cotton magnesium chelatase subunit I gene [30].

3. Tomato yellow leaf curl viruses: a complex of begomoviruses infecting tomato plants worldwide

Tomato cultures (*Solanum lycopersicum)* worldwide are under the constant threat of diseases caused by geminiviruses belonging to the TYLCV complex [31]. In nature, the TYLCVs are exclusively transmitted by the whitefly *Bemisia tabaci* [32]. Members of the TYLCV complex have a single 2,700-2,800 nucleotide (n) circular ssDNA genome encapsidated in a geminate particle. The

TYLCVs replicate in the nuclei of infected cells following a rolling-circle strategy, using a double stranded DNA intermediate replicative form as a template [33]. Their genome encodes two genes, V1 and V2; the complementary viral strand encodes four genes, C1 to C4. A 300 n intergenic region (IR) includes a stem-loop structure containing the origin of replication shared by all known begomoviruses and bidirectional promoters. V1 encodes the coat protein (CP); V2 encodes a movement protein (MP) and may also function as a silencing suppressor. C1 encodes a protein (Rep) necessary for replication, C2 a transcription activator (TrAP), C3 a replication enhancer (REn) and C4 a small protein embedded within the Rep that may act as a symptom determinant [34].

Plants have been genetically engineered to resist infection by members of the TYLCV complex. Strategies employed were based on expressing viral proteins, whether wild-type or mutants, of virus-binding proteins, and on viral gene silencing [35]. However, in view of the public reticence regarding genetically modified food crops, breeding remains a method of choice to obtain plants resistant to TYLCV [36]. Wild relatives of domesticated plant species constitute an invaluable reservoir of resistance genes, which have been tapped by plant breeders to improve agricultural crops [37]. It is thought that the expression of these resistances involves sets of genes that interact upon positive and negative signals within an interconnecting network [38]. Along domestication, these networks have been disrupted and resistances lost, probably because resistance alleles were linked with undesired horticultural qualities. Breeding has been instrumental in reconstituting (part of) the resistance gene network(s).

Since the domesticated tomato *S. lycopersicum* is susceptible to TYLCV, breeders have introgressed resistance traits identified in wild tomato species (such as *S. chilense*, *S. peruvianum* and *S. habrochaites*) into *S. lycopersicum* [36,39]. As a result, the resistant tomato lines contain chromosomal fragments from the wild species on a domesticated tomato background, identifiable with polymorphic DNA markers [40]. Several loci from wild tomato species associated with resistance to TYLCV and related begomoviruses (coined Ty-1 to Ty-5) have been identified using such markers. The gene conferring TYLCV-resistance at the Ty-1 (from *S. chilense*) and Ty-5 (from *S. peruvianum*) loci have been identified (unpublished) but their function in the establishment of resistance is not known.

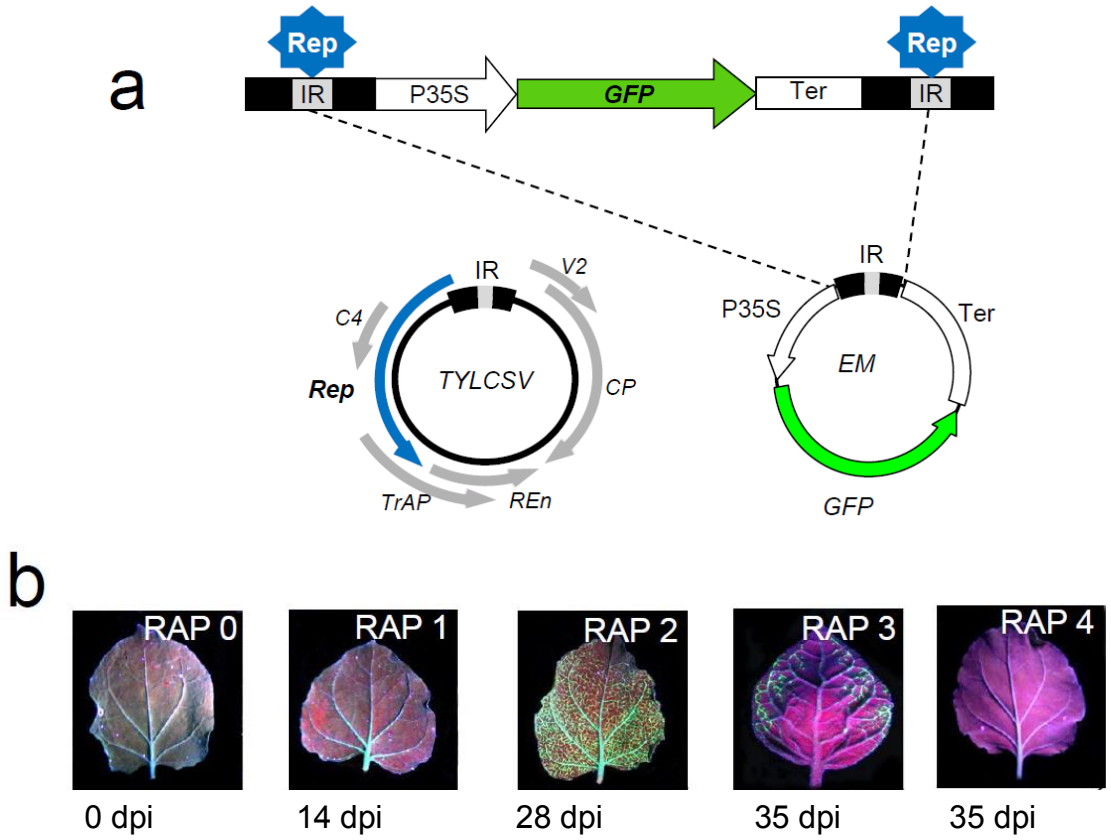
4. Identification of host genes involved in TYLCV infection

4.1. A Nicotiana benthamiana system to monitor TYLCSV infection in combination with host gene silencing

We wished to identify plant genes responding to infection by a close relative of TYLCV, *Tomato yellow leaf curl Sardinia virus* TYLCSV, and to analyze their function. To achieve these goals, we have generated a *N. benthamiana* transgenic line, named 2IRGFP, which allows monitoring virus-induction of host genes and their silencing. 2IRGFP plants contain a green fluorescence protein gene (GFP) expression cassette flanked by two repeats of the TYLCSV intergenic region IR [41]. Uninfected 2IRGFP plants display a basal low level of GFP. During infection, the TYLCSV Rep protein specifically recognizes the IRs flanking the cassette, and initiates replication and strong expression of the GFP transgene (Figure 1a). Therefore, induction of GFP expression directly

correlates with viral replication, allowing monitoring the development of infection in plant tissues in both space and time in a simple visual, reliable and non-invasive manner (Figure 1b) [41]. Since the evaluation and monitoring of the viral infection is extremely straight-forward, we have used 2IRGFP plants as a tool in combination with VIGS to identify host genes with an impact in viral pathogenicity.

Figure 1. Generation and phenotypic analysis of TYLCSV-infected 2IRGFP *N. benthamiana* transgenic plants. **a.** Construct 2IRGFP contains a direct repeat of the TYLCSV IR encompassing a GFP expression cassette that contains the 35S CaMV promoter (P35S), the complete ORF of *GFP* and the NOS terminator (Ter). During TYLCSV infection, the viral Rep protein specifically recognizes the IRs flanking the cassette, and mGFP replicons are generated (EM), which in turn leads to a strong over-expression of the *GFP* transgene and the subsequent accumulation of the fluorescent protein. **b.** Evolution of virus replication-associated phenotype (RAP) in infected 2IRGFP plants at different days post-infection (dpi). A representative photograph of each RAP phenotype showing the extension and intensity of GFP expression is displayed.



Prior to the use of TRV-based VIGS for a reverse genetics screen in 2IRGFP plants, we have shown that co-infection of TYLCSV with TRV did not alter the pattern of TYLCSV-dependent over-expression of GFP, even though TYLCSV accumulation was slightly delayed in plants co-infected with TRV compared to control plants [41]. At least three different proteins encoded by TYLCSV have been described to function as suppressors of gene silencing [42]. The possibility that a TYLCSV suppressor of gene silencing could counteract TRV-mediated silencing in TYLCSV-TRV co-infected plants was tested using either the endogenous *Sulfur* (*Sul*) gene (in 2IRGFP *N. benthamiana* plants) or

a *GFP* transgene. The results indicated that co-infection with TYLCSV did not significantly alter the silencing phenotypes, confirming that TRV-mediated VIGS can be reliably used in combination with TYLCSV.

4.2. Selection and screening of candidate genes involved in TYLCSV infection

Genes potentially involved in TYLCSV infection was established following a literature search according to one of the four criteria: 1) they encode proteins binding geminiviral proteins; 2) they are exclusively or preferentially expressed in phloem tissues, to which TYLCSV is restricted; 3) they are trans-activated by C2 from the begomoviruses *Mungbean yellow mosaic virus* (MYMV) or ACMV [43]; 4) they are involved in cellular processes potentially required for geminivirus infection. A list of 114 genes was established. Since these genes belong to different plant species (the genome sequence of *N. benthamiana* was not available at the time), we performed homology analyses to identify sequence stretches conserved in diverse plant species, including *Arabidopsis* and tomato, which could serve as silencing targets. These sequences were used to design potentially efficient silencing siRNA molecules (Invitrogen Block-iT^{TM} RNAi designer tool). The fragments we chose for TRV-mediated silencing were those containing the largest number of potential siRNAs. Fifty-four of the initially selected 114 candidate genes fitted these pre-conditions; 37 were tested for their potential impact on TYLCSV infection upon silencing. The silencing recombinant TRVs were induced in 2IRGFP *N. benthamiana* plants, which were subsequently infected with TYLCSV. GFP over-expression was monitored daily from 9 to 15 days post-infection (dpi) under UV light; pictures and tissue samples were taken at 15 dpi (Figure 2). TYLCSV co-infection with empty TRV vectors or *Sul*-silencing TRV was used as control.

The effect of silencing the 37 host genes TYLVSV infection was classified into three categories: A- silencing of 7 resulted in an earlier or enhanced infection; B- silencing of 11 delayed, reduced or completely abolished infection; C- silencing of 19 did not induce a noticeable change in the pattern of infection. The identity and associated GO terms (biological process, cellular component and molecular function) for each of these genes are listed in Table 1. The genes identified in this screen can be classified into three functional groups discussed in more detail below: 1) genes with a previously known function in geminivirus infection; 2) genes involved in stress responses; 3) genes involved in posttranslational modifications.

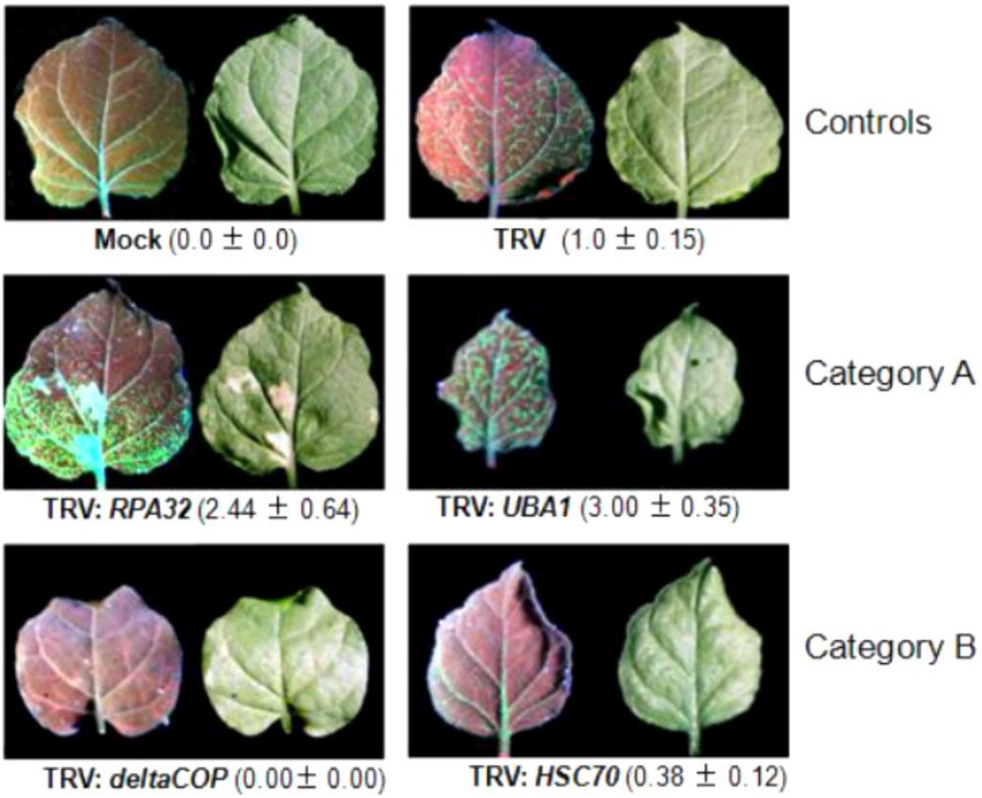
Table 1. List of genes whose silencing enhances (category A) or delays (category B) TYLCSV infection. The criterion for selection is indicated in each case. The accession numbers (ACC) of the homologous *Arabidopsis* gene used in the VIGS experiments are indicated. ND: not determined.

Identity	ACC *A. thaliana*	GO Biological process	GO Cellular component	GO Molecular function	Selection criteria
Category A					
Bearskin 2 (*BRN2*)	AT4G10350	Multicellular organismal development, positive regulation of gene expression, positive regulation of transcription, DNA-dependent, regulation of transcription, root cap development, secondary cell wall biogenesis	ND	Sequence-specific DNA binding transcription factor activity	Phloem over-expression
Importin alpha isoform 4 (*IMPA-4*)	AT1G09270	Host response to induction by symbiont of tumor, nodule or growth in host, protein transport, symbiont intracellular protein transport in host	Cytosol, host cell, intracellular	Protein binding, protein transporter activity	Interaction with CP
Lactoylglutathione lyase (*GLO1*)	AT1G15380	Carbohydrate metabolic process	ND	Lactoylglutathione lyase activity	Interaction with C3
Replication protein A32 (*RPA32*/*RPA2*)	AT3G02920	Unknown	ND	Nucleic acid binding	Interaction with Rep
Dehydration responsive 21 (*RD21*)	AT1G47128	Metabolic process, response to water deprivation	Apoplast, chloroplast, plasmodesma, vacuole	Cysteine-type endopeptidase activity, protein binding	Interaction with V2
RING-type E3 ubiquitin ligase (*RHF2A*)	AT5G22000	Megagametogenesis, microgametogenesis, proteolysis involved in cellular protein catabolic process, regulation of cell cycle	Plasma membrane	Zinc ion binding	Transactived by TrAP/C2
Ubiquitin activating enzyme (*UBA1*)	AT2G30110	Metabolic process, protein ubiquitination, response to cadmium ion, response to other organism, ubiquitin-dependent protein catabolic process	Cytosol, plasma membrane, plasmodesma	Ubiquitin activating enzyme activity, ubiquitin-protein ligase activity	Interaction with TrAP/C2

Category B					
4-coumarate:CoA ligase (*AT4CL1*)	AT1G51680	Metabolic process, phenylpropanoid metabolic process, response to UV, response to fungus, response to wounding	Unknown	4-coumarate-CoA ligase activity	Phloem over-expression
Allene oxide cyclase (*AOC1*)	AT3G25760	Jasmonic acid biosynthetic process, metabolic process, response to desiccation	Chloroplast, chloroplast envelope, chloroplast thylakoid membrane	Allene-oxide cyclase activity	Phloem over-expression
Barely any meristem 1 *(BAM1)*	AT5G65700	Anther development, floral organ development, gametophyte development, protein phosphorylation, regulation of meristem growth, regulation of meristem structural organization, trans-membrane receptor protein tyrosine kinase signaling pathway	Plasma membrane	Kinase activity, protein binding, protein self-association, protein serine/threonine kinase activity, receptor serine/threonine kinase binding	Interaction with C4
Coatomer delta subunit (*deltaCOP*)	AT5G05010	Intracellular protein transport, transport, vesicle-mediated transport	Cytosol, membrane, plasmodesma	ND	Interaction with C3
COP9 signalosome subunit 3 (*CSN3*)	AT5G14250	G2 phase of mitotic cell cycle, cullin deneddylation, photomorphogenesis	Cytosol, signalosome	Protein binding	Cellular process
Geminivirus Rep A-binding (*GRAB2*)	AT5G61430	Multicellular organismal development, regulation of transcription, DNA-dependent	Unknown	sequence-specific DNA binding transcription factor	Interaction with Rep
Heat shock protein cognate 70 (*HSC70*)	AT5G02500	Defense response to bacterium, defence response to fungus, negative regulation of seed germination, protein folding, response to cadmium ion, response to cold, response to heat, response to virus, stomatal closure	Apoplast, cell wall, chloroplast, cytoplasm, cytosol, membrane, nucleus, plasma membrane, plasmodesma	ATP binding,protease binding, protein binding	Phloem over-expression
Nuclear acetyltransferase (*NSI*)	AT1G32070	Pathogenesis, spread of virus in host	Chloroplast, nucleus	N-acetyltransferase activity	Interaction with NSP

Patatin-like protein 2 (*PLP2*)	AT2G26560	Cell death, cellular response to hypoxia, defence response to virus, lipid metabolic process, oxylipin biosynthetic process, plant-type hypersensitive response, response to cadmium ion	Cytoplasm, membrane	Lipase activity, nutrient reservoir activity	Phloem over-expression
Shaggy-related kinase kappa (*SK4-1/SKK)*	AT1G09840	Protein phosphorylation	Plasma membrane	ATP binding, protein serine/threonine kinase activity	Interaction with C4
SKP1-like 2 (*ASK2*)	AT5G08590	Phosphorylation, protein phosphorylation, response to osmotic stress, response to salt stress	Nucleus	Kinase activity, protein binding, protein kinase activity	Transactived by TrAP/C2

Figure 2. Screening of candidate genes in 2IRGFP transgenic *N. benthamiana* plants. Plants were co-inoculated with a TRV:*Gene* construct and TYLCSV. GFP expression was monitored daily up to 15 days post-inoculation (dpi). The picture shows GFP expression in one of the apical leaves under UV (left) and visible light (right) of 2IRGFP *N. benthamiana* transgenic plants 15 days after they were co-infected with TYLCSV and TRV constructs to induced silencing of genes classified in category A (Replication associated protein A, *RPA32*, and Ubiquitin activating enzyme 1, *UBA1*) or category B (Coatomer delta subunit, *deltaCOP,* and Heat shock cognate 70, *HSC70*). Leaves from control 2IRGFP plants are shown: agroinfiltrated with an empty binary vector (Mock) or with the empty TRV vector (TRV). The relative amount of TYLCSV DNA accumulated in co-infected plants was quantified by qPCR; results are shown below the images. Values are the mean of five to ten plants. The numbers correspond to the mean ±standard error. This experiment was repeated three times with similar results.



4.2.1. Genes with a known function in geminivirus infection

Among the candidate genes that were found to exert an effect on TYLCSV infection when silenced, three have been implicated in begomovirus infection.

NSI (Nuclear shuttle interaction). *NSI* encodes a nuclear acetyl-transferase that physically interacts with the Nuclear shuttle protein (NSP) of CaLCuV. Over-expression of *NSI* resulted in enhanced infection [44], indicating that protein acetylation may coordinate replication of the viral genome with its export from the nucleus. This promoting effect of NSI on geminivirus infection is supported by our data, which showed that silencing of NSI negatively affects TYLCSV.

GRAB2 (Geminivirus Rep A-binding). *GRAB2* encodes a NAC-containing protein that interacts with *Wheat dwarf virus* (WDV) RepA in wheat [45]. Over-expression of *GRAB2* inhibits WDV replication in wheat cells. Unexpectedly, our results showed that low levels of GRAB2 enhanced TYLCSV infection. It is possible that GRAB2 has different roles in WDV and TYLCSV infections.

RPA32 (Replication protein A32). The gene product was shown to interact with the Rep protein of *Mungbean yellow mosaic India virus* (MYMIV) [46], repressing the Rep nicking and closing activities while promoting its ATPase activity. In our system, silencing of RPA32 resulted in enhanced TYLCSV infection.

4.2.2. Genes involved in stress responses

Five of the 18 genes identified in the screen as potentially involved in TYLCSV infection have been shown to play a role in plant stress responses.

HSC70-1 (Heat shock protein cognate 70). HSC70-1 is one of five cytosolic members of the heat shock protein 70 family in *Arabidopsis* [47]. Infection with several plant viruses, including the geminivirus *Beet curly top virus* (BCTV), leads to enhanced expression of this gene family [48]. HSC70 interacts with the co-chaperone SGT1, which has been shown to be required for resistance against viruses [49]. The finding that silencing of *HSC70-1* results in impaired TYLCSV infection indicates that high levels of this protein are required for a successful geminivirus replication and spread. HSC70 may promote protein maturation during the virus multiplication cycle, and/or may be involved in virus cell-to-cell movement [50].

RD21 (Responsive to dehydration 21). RD21 is a cysteine protease. Tomato RD21 interacts with TYLCSV V2 in yeast (our unpublished results). Expression of *RD21* is induced following inoculation with *Botrytis cinerea* or *Pseudomonas syringae* (*Arabidopsis* eFP browser), or upon CaLCuV infection [51], pointing to a potential role in plant defense. Since silencing of *RD21* promotes TYLCSV infection, we hypothesize that this gene may also have anti-viral activities.

PLP2 (Patatin-like protein 2). PLP2 is a lipid acyl hydrolase, hydrolyzing membrane glycerolipids to produce monoacyl compounds and free fatty acids. Expression of *PLP2* is induced upon infection by CaLCuV [52]. Upon desiccation, *Arabidopsis* with a *plp2* mutation accumulates high levels of jasmonic acid (JA) [53]. Since in some cases activation of JA signaling negatively impacts geminivirus infection [54], over-production of JA due to *PLP2* silencing may explain the inhibition of TYLCSV infection.

GLO1 (Lactoylglutatione lyase). *GLO1* belongs to the glyoxalase system, which detoxifies methylglioxal (MG), a cytotoxic by-product of glycolysis [55]. Over-expression of *GLO1* results in increased tolerance to abiotic stresses [56]. Enhancement of the glyoxalase pathway in transgenic tobacco and rice helps maintaining low levels of reactive oxygen species (ROS) and MG [55]. Plant virus infection alters the expression of oxidative stress-related genes and induces oxidative stress correlated with the extent of symptoms [57]. In our system, silencing of *GLO1* could result in an increased accumulation of ROS, which would in turn favor viral infection.

AOC1 (Allene oxide cyclase 1). AOC1 catalyzes an essential step in the biosynthesis of jasmonic acid. Exogenous application of JA negatively impact geminivirus infection [54]. Therefore silencing of *AOC1*, which would presumably impair jasmonate biosynthesis, was expected to result in enhanced

viral infection. Surprisingly, *AOC1*-silenced plants were more resistant to TYLCSV. It is possible that due to cross-talk between JA and salicylic acid (SA) signaling pathways, the silenced plants may accumulate high levels of SA, known to impair geminivirus infection [58].

4.2.3. Genes involved in post-translational modifications (PTMs)

Strikingly, 8 of the 18 genes identified in the screen as involved in TYLCSV infection have been ascribed roles in post-translational modification (PTM) pathways: ubiquitination, rubylation, phosphorylation and acetylation. In this section, we will discuss the role of the four genes involved in ubiquitination since the involvement of this PTM in viral infections of plants and animals is well established. Ubiquitination consists in the addition of one (mono-ubiquitination) or more (poly-ubiquitination) ubiquitin moieties to a substrate protein; poly-ubiquitination generally results in the degradation of the modified protein by the 26S proteasome, while mono-ubiquitination can have other, non-fatal effects, such as changes in activity or sub-cellular localization [59]. In plants, ubiquitination contributes to multiple levels of defense [60], including resistance to viruses [61] and in plant-geminivirus interactions [54,62].

UBA1 (Ubiquiting-activating enzyme). UBA1 catalyzes the first step in ubiquitin conjugation. Interestingly, an *uba1* mutant in *Arabidopsis* can revert the constitutive defense response phenotype of *snc1*, which links UBA1 to plant defense [63]. We found that the tomato UBA1 interacts with TYLCSV C2 in yeast (unpublished results). Silencing of *UBA1* promotes TYLCSV infection, suggesting that a viral pathogenicity factor may suppress the activity of this enzyme. This hypothesis is in agreement with the previously described negative impact of C2 on ubiquitination [54,64], and would imply that C2 interferes with this process at multiple levels.

RHF2A (RING-type E3 ubiquitin ligase). RHF2a links ubiquitin to target protein substrates. *RHF2a* is highly expressed in pollen, and to a lower extent, in vegetative tissues. This gene is up-regulated upon CaLCuV infection [4] and following *P. syringae* inoculation (Arabidopsis eFP browser). The potential role of *RHF2a* in plant responses to pathogens fits the findings that *RHF2a* silencing in the VIGS/2IRGFP system results in an enhancement of TYLCSV infection.

SCF (Skp1/Cullin1/F-box protein). SCF is a multi-subunit E3 ligase. Its modular structure allows the incorporation of different substrate-binding subunits (F-box proteins) with more than 700 potential targets in *Arabidopsis* [65]. Interestingly, the C2 protein from several geminiviruses interferes with the SCF machinery [54,64]. In the VIGS/2IRGFP system we found two genes interacting with the SCF complex and involved in TYLCSV infection: *ASK2* and *CSN3*. *ASK2* belongs to a gene family encoding SKP1-like protein in *Arabidopsis*; it plays a role in cell division, development, and abiotic stress response *via* ABA signaling [66, 67]. ASK2 interacts with GALA effectors from *Ralstonia solanacearum* [68] and with the VirF virulence factor from *Agrobacterium tumefaciens* [69], suggesting that ASK2 is a preferential target of pathogens virulence functions. Since V2 has been shown to trigger the degradation of the plant SGS3 in order to counter gene silencing [70], ASK2 may be essential for its efficient assembly into the SCF complexes, a process that may be assisted by C2 [54], ensuring the success of the viral infection. The finding that silencing of *ASK2* has a negative impact on TYLCSV in the VIGS/2IRGFP system is in line with this hypothesis.

CSN3 (subunit of the de-rubylating CSN complex). CSN3 is one of eight subunits of the CSN (COP9 signalosome) complex, which de-rubylates CULLINs and therefore regulates the activity of CULLIN-based ubiquitin E3 ligases, including the SCF complex [71]. Geminivirus C2 was shown to interfere with the CSN de-rubylation activity, most likely through the interaction with CSN5 [64], presumably leading to an alteration of SCF-mediated ubiquitination. Since geminivirus infection is hindered in an *Arabidopsis csn5a* knock-down mutant [72], geminiviruses may be redirecting the activity of the CSN complex, taking over the regulation of SCF complexes rather than suppressing this process. Once again, this hypothesis is supported by the negative effect of *CSN3* silencing on TYLCSV infection. Taken together, the results obtained with *ASK2* and *CSN3* point at the usurpation of the SCF ubiquitination machinery by geminiviruses, involving different viral proteins and lines of attack.

5. Identification of genes involved in resistance to TYLCV

5.1. Genes preferentially expressed in TYLCV-resistant tomatoes and the effect of their silencing on resistance

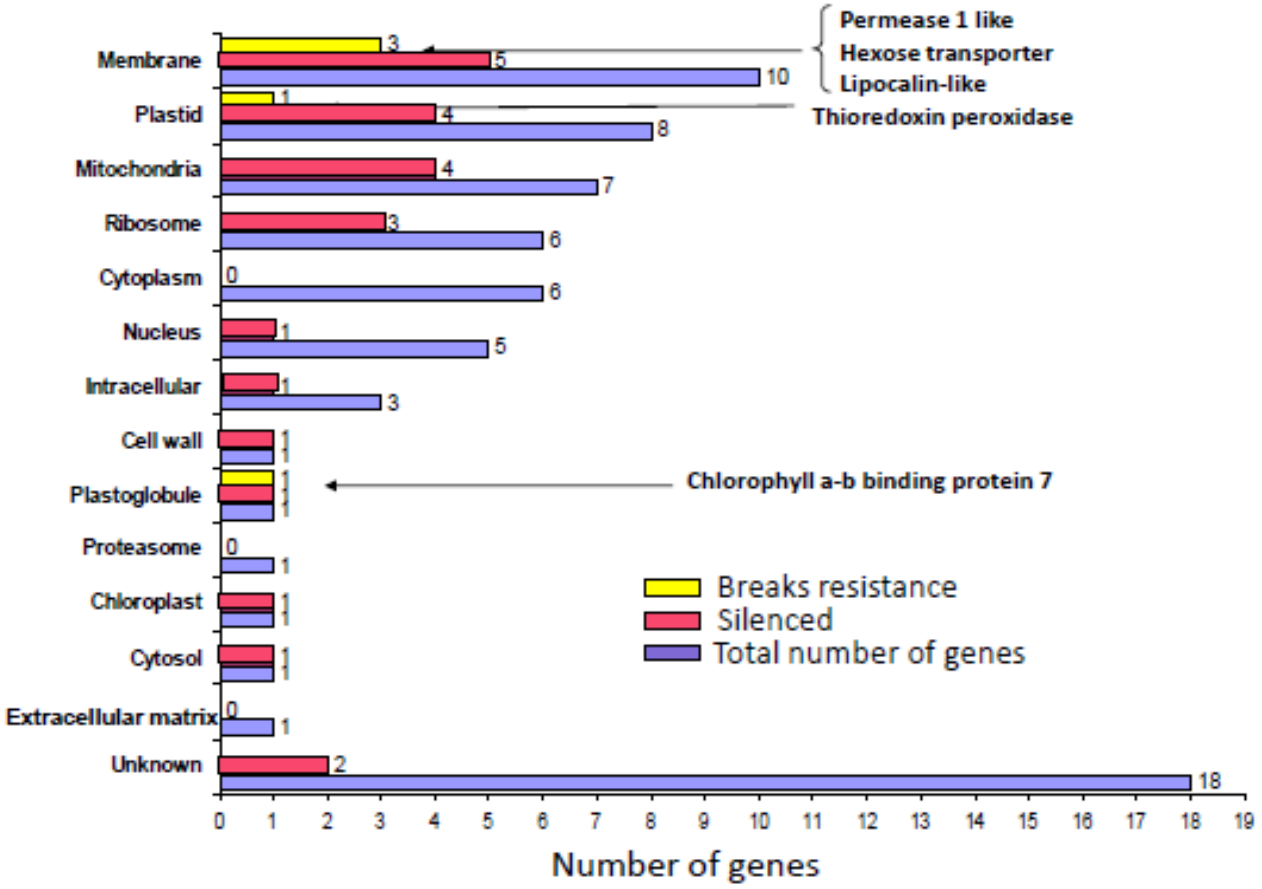
Breeding has allowed not only to develop TYLCV-resistant crops for farmers but the resistant plants have been the object of genetic studies aimed at understanding genes and signals involved in plant response to viruses [5]. To identify these genes, we have compared two inbred tomato lines issued from the same breeding program, which used *S. habrochaites* as a source of resistance: the TYLCV-susceptible line 906-4 and the TYLCV-resistant line 902, hereafter designated S and R, respectively [73]. Upon infection, plants from the S line present the typical disease symptoms of stunting, leaf yellowing and curling, contain large amounts of virus and produce a small number of fruits. In comparison, plants from the R line remain symptomless, yield, and contain several orders of magnitude less virus that S plants.

We have postulated that resistance is sustained by a gene network responding to biochemical triggers induced by virus infection. In addition, we assumed that these genes are preferentially expressed in the R line and that their silencing will lead to the collapse of resistance. Comparing cDNA libraries from R and S plants, before and after infection, allowed the identification of about 70 genes preferentially expressed in R plants. Some of these genes were silenced using the TRV VIGS system. Fragments of 150 to 200 bp of the target genes were cloned in the TRVII vector. The TRVI and recombinant TRVII vectors were delivered to R and S tomato plants by agroinoculation [14] at the day of planting (20 days after sowing). Seven days later, the expression of the target gene was inhibited and the RNAi signal was conspicuous in the plant leaves and remained high for the duration of the experiments. This was the time the plants were caged with viruliferous whiteflies for a three days inoculation period. The effect of silencing was appraised during the next 40 days. TRV expression had no effect on subsequent TYLCV infection, neither enhancing nor depressing the virus spread [5].

At present, we have silenced 25 out of the 69 genes preferentially expressed in R plants. Five genes out of the 25 tested led to the collapse of resistance when silenced (Figure 3). Hence, it seems that many genes are involved in the establishment of natural resistance to TYLCV. We summarize here the

behavior of four genes preferentially expressed in R plants upon silencing and TYLCV inoculation. We also show that there seems to be a hierarchy in these genes.

Figure 3. Genes preferentially expressed in R plants (Gene ontology, cellular component). The number of genes silenced so-far and the genes which silencing leads to collapse of resistance are indicated.



Permease I. With the *PermeaseI-like* gene, we have shown for the first time that silencing a single gene can lead to the loss of TYLCV resistance in tomato plants. *Permease I-like protein* was preferentially expressed in non-inoculated R plants (compared to S plants) and was strongly up-regulated upon TYLCV inoculation [5]. Silencing this gene (Figure 4a) led to the collapse of the resistance phenotype: the R plants ceased to grow, developed typical yellowing and curling of leaves and contained amounts of virus similar to those measured in infected S plants (Figure 5). This permease may be involved in trafficking of macromolecules and signaling.

Figure 4. Relative amounts of transcripts of *Permease I*, *Hexose transporter LeHTe1*, and *Lipocalin-like* genes in R tomato plants (Ro:0), infected R tomato plants (Ri:0) and infected R tomato plants with silenced *Permease I* (Ri:*TRV-Perm*), *Hexose transporter LeHTe1* (Ri:*TRV-Hex*), and *Lipocalin-like* (Ri:*TRV-Lip*) genes. Tubulin RNA was used as a reference gene transcript for each of the plants analyzed by qPCR. The amount of transcript immediately before silencing (at day 0) is taken as 1. Average of triplicate measures of three different plants. Bars: standard error.

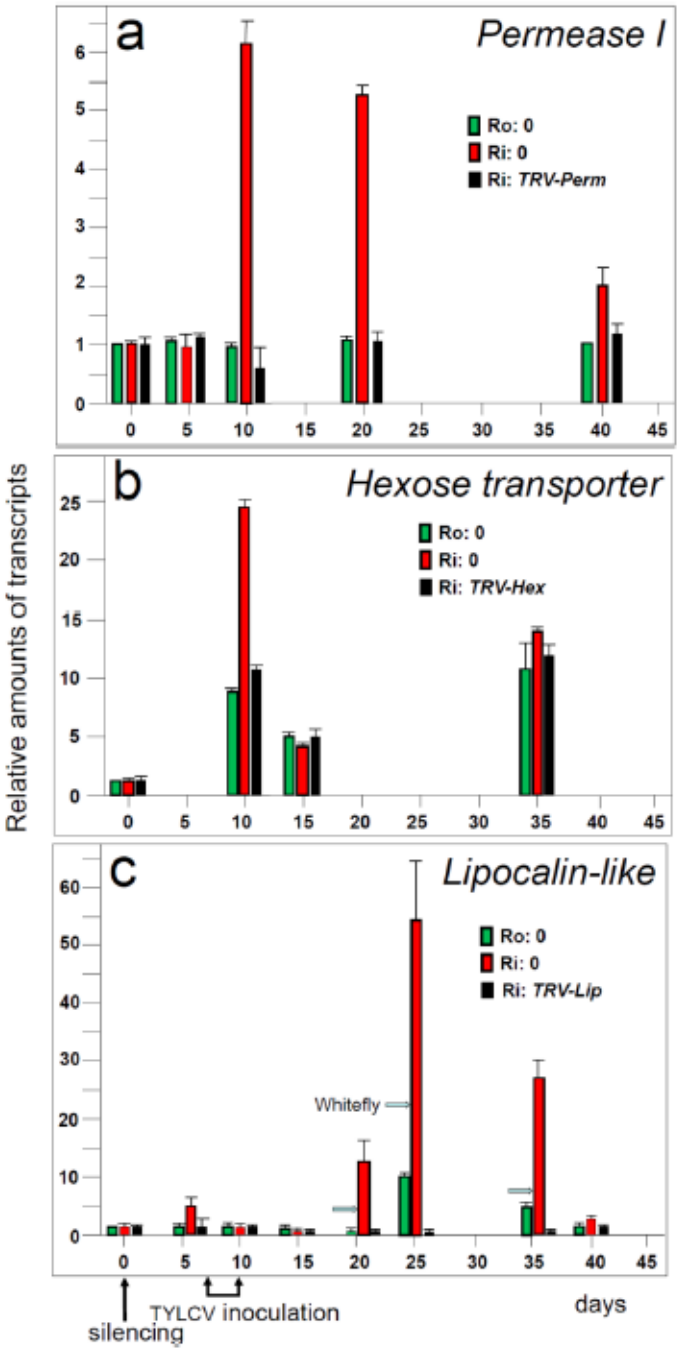
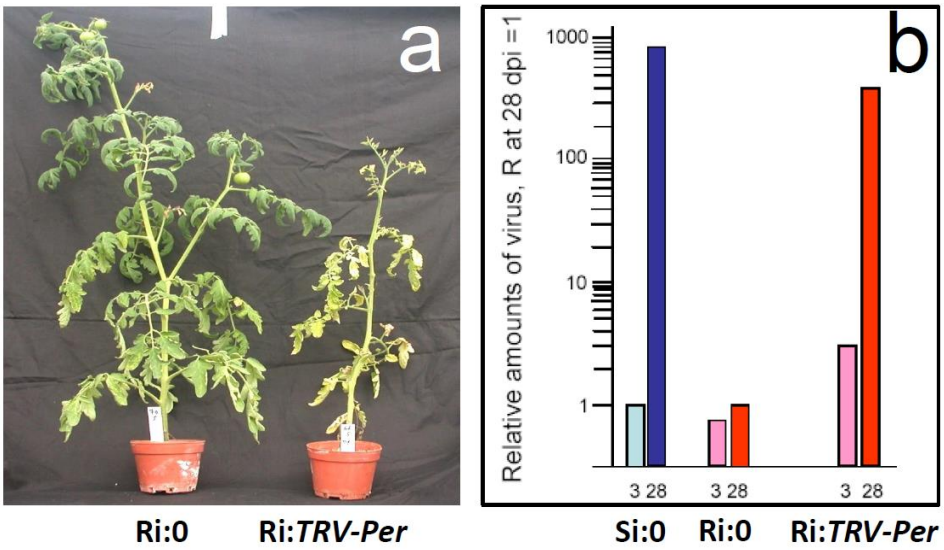


Figure 5. Collapse of resistance in infected R plants where the *Permease I* gene has been silenced. **a**: R tomato plants 8 weeks after TYLCV inoculation; Ri:0, not silenced; Ri:*TRV-Per*, silenced. Note that Ri:0 do not present symptoms and yield fruits, in comparison Ri:*TRV-Per* are symptomatic and present inhibited growth. **b**: Relative amounts of virus (measured by qPCR) in infected tomato plants 3 and 28 days after inoculation; Si:0 is S plants, Ri:0 is R plants and Ri:*TRV-Per* is R plants where the *Permease I* gene has been silenced. The amount of virus in Ri:0 plants at 28 dpi was considered as 1.



Hexose transporter LeHT1. *LeHT1* is one of the three known tomato hexose transporter genes [74]. Plant hexose transporters are plasma membrane carriers, which function as proton/hexose symporters, mediating intercellular and long-distance transport of sugars [75]. They are involved in energy production [76], pathogenesis [77], pathogen defense [78] and programmed cell death (PCD) [79]. *LeHT1* is developmentally regulated, preferentially expressed in R tomato leaves, and upregulated upon TYLCV inoculation of R plants (Figure 4b). Infected *LeHT1*-silenced R plants ceased to growth and their leaves contained large amounts of virus in the vascular tissues and reduced sucrose concentrations [80], emphasizing the role of the hexose transporters and of sugars as part of a defense mechanism limiting virus movement. Necrosis appeared on the stem and petioles of the *LeHT1*-silenced R plants about three weeks after inoculation not only with TYLCV, but also with other viruses such as TMV and CMV [80]. Hence silencing of R plant *LeHT1* revealed a second line of defense associated with PCD features: DNA laddering, increased amounts of MAPKs, and release of reactive oxygen species (ROS) [80]. In most cases, PCD minimizes the pathogen spread [81], however, in the case of infection of *LeHT1*-silenced R plants, the plant defense mechanisms were unable to confine virus infection and the resistance collapsed.

Lipocalin-like protein. A gene encoding a putative lipocalin protein [82] with its typical barrel-shaped architecture [83], was expressed in the leaves of S and R tomatoes during a two week-long window, starting about 40 days after sowing (Figure 4c). This gene, coined *SlVSRLip*, was upregulated in R (but not S) plants upon infection but also, to a lesser extent, following whitefly feeding [82]. The association of lipocalins with virus infection has not been reported before. Following TYLCV

inoculation, *SlVSRLip*-silenced R plants ceased to grow, developed disease symptoms, and contained large amounts of virus. As in the case of *LeHT1*, *SlVSRLip*-silenced R plants presented a PPCD-related necrotic response along the stems and petioles [82]. The role of *SlVSRLip* is not known, as it behaved differently than the known tomato lipocalins [83], which appear to protect plants from temperature-induced stresses [84].

Pectin methylesterase. Another gene preferentially expressed in R plants was a *Pectin methylesterase*. This gene is a member of a large family encoding enzymes that modify plant cell wall pectins. Pectin methylesterases play a role in the plant host defenses against cold, wounding and phloem-feeders [85]. They have also been involved in virus-induced gene silencing [86] and in virus systemic infection [87]. Contrary to the three genes described above, silencing *Pectin methylesterase* did not affect the resistance of R plants. Hence, although *Pectin methylesterase* is more expressed in R than in S plants, this gene is probably not located at a bottleneck of the resistance network. Thus, not all the genes preferentially expressed in R plants play the same role in the establishment of resistance to TYLCV.

5.2. Hierarchy of genes involved in resistance to TYLCV

We hypothesized that the genes conferring resistance in R plants are organized in an interconnected hierarchical network. We therefore tested the hypothesis that the silencing of one gene will cause the down-regulation of genes downstream in the network. Expression of *SlVRSLip* was estimated in R plants in which *LeHT1* had been silenced [82]. In the *LeHT1*-silenced R plants, the expression of *SlVRSLip* was totally inhibited. Conversely, silencing of *SlVRSLip* did not affect the expression of *LeHT1*. Hence, *SlVRSLip* is downstream of *LeHT1* in the hierarchy of the resistance network [82]. Silencing a *Permease* gene did not affect the expression of either *SlVRSLip* or *LeHT1*; conversely, silencing either *SlVRSLip* or *LeHT1* did not affect the *Permease* gene expression, indicating that the later gene does not belong to the *LeHT1*/*SlVRSLip* branch of the network. *SlVRSLip* and *LeHT1* do not seem to be linked by any obvious biochemical or physiological link. However, as a consequence of *LeHT1*-silenced, the concentration of sucrose in leaves was lower of than that in non-silenced R tomatoes [82]. It has been already reported that silencing *LeHT* genes decreased hexose accumulation in tomato fruits by half [88]. Hence the inhibition of sugar transport due to *LeHT1* silencing resulted in a limited level of cellular sucrose, and consequently energy, to activate and maintain the resistance response [78]. Sugars act as secondary messengers [89] and sugar sensing mediates a direct link between carbohydrate metabolism and the defense response [78]. In this context, intracellular sugars may up-regulate the expression of *SlVSRLip* in R plants upon TYLCV infection, contributing to resistance by increasing lipocalin ROS scavenging. A reduction in the intracellular concentration of sugars due to *LeHT1* silencing may inhibit the signal-transduction pathway leading to the activation of *SlVSRLip*.

6. Discussion

We have shown that TRV-VIGS is a tool of choice to discover plant genes responding to TYLCSV infection. Using the 2IRGFP *N. benthamiana* transgenic line, we have been able to demonstrate that

silencing of 18 out of 37 analyzed host genes alters TYLCSV infection. An attractive feature of this screening method is the fact that candidate genes are tested in the context of the infection, hence the genes discovered are likely to be biologically relevant. On the other hand, we cannot rule out that some of the tested genes have not been efficiently silenced, rendering their potential impact on TYLCSV infection undetectable. A strategy similar to VIGS/2IRGFP is more difficult to apply to tomato, since expression of GFP in leaves does not bear green fluorescence. Therefore, the genes discovered in the *N. benthamiana* 2IRGFP plant screen could be validated subsequently in tomato.

It is striking that almost half the genes shown to interfere with TYLCSV infection are involved in processes related to PTMs, such as ubiquitination, rubylation, phosphorylation, acetylation or folding. It has been postulated that PTMs provide means to respond quickly to environmental stimuli in a fast and efficient way critical for the plant survival. Thus, it is not surprising that PTMs affect viral infection and may be preferred targets of viral pathogenicity factors. Increase evident obtained in the last years confirm the central role played by PTMs in virus-host interactions, being both manipulated by viruses to achieve a successful infection and used by the host as an important defense mechanism [59,61].

We have also applied the TRV-VIGS reverse genetics tool to discover genes involved in tomato natural resistance to TYLCV. The current view to plant responses to stress involve integrated transcriptional and cellular changes that result in physiological adaptations expressed as resistance in certain genotypes, which may be regulated by metabolite and hormone signaling pathways [90]. Accordingly, we have postulated that resistance to TYLCV is sustained by a gene network. Indeed, we have identified several genes from R plants which, when silenced, lead to the collapse of resistance. We found a beginning of hierarchy in the TYLCV-resistance network, where *SlVRSLip* is downstream of *LeHT1*. To uncover the genes up- and downstream *LeHT1* in the resistance network we are using a home-made oligonucleotide microarray to analyze the transcriptome re-programming in leaves of *LeHT1*-silenced R plants using a home-designed microarray [91]. Resistance to TYLCV may consist of several layers of defense - a general feature of the plant response to pathogens [38]. This multilayer response starts with a basal response and production of general pathogen-associated molecular pattern molecules (PAMPs), followed by activation of MAPK-signaling cascades and production of antimicrobial compounds [92]. The next layer of resistance usually involves the expression of genes related to the plants response to specific pathogens, in our case *LeHT1* and *SlVSRLip*.

Since the R line's resistance to TYLCV was introgressed from *S. habrochaites*, it would be of interest to determine whether the genes that are preferentially expressed in R tomato plants were introgressed from this wild tomato species. It is worth noting that the three genes we have studied are located on three different chromosomes: *Permease I-like protein* is on chromosome 3, *LeHT1* is on chromosome 2, and *SlVSRLip* is on chromosome 10 (http://solgenomics.net accesed 18 February 2013). If these three genes originate from the wild *S. habrochaites* genitor, they must have been introgressed as three chromosomal fragments during breeding and selection for resistance.

In summary, the results presented here are a good example of the potential of VIGS as a tool for functional studies in plant-virus interactions, providing at the same time new insights into the roles that specific plant genes play during geminivirus infection. In the genomic era, the completion of genome sequences of many important plant species, including *N. benthamiana* and tomato [93.94], together with the efforts made to improve the efficiency and applicability of the VIGS system to different hosts,

are contributing to make this technology an essential tool for high-throughput functional genomics studies in plants.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Marathe, R.; Guan, Z.; Anandalakshmi, R.; Zhao, H.; Dinesh-Kumar, S.P. Study of *Arabidopsis thaliana* resistome in response to cucumber mosaic virus infection using whole genome microarray. *Plant Mol. Biol.* **2004**, *55*, 501–520.
2. Satoh, K.; Kondoh, H.; Sasaya, T.; Shimizu,T.; Choi, I.R.; Omura, T.; Kikuchi, S.. Selective modification of rice (*Oryza sativa*) gene expression by rice stripe virus infection. *J. Gen. Virol.* **2010**, *91*, 294–305.
3. McGregor, C.E.; Miano, D.W.; Hoy, M.; Clark, C.A.; Rosa, G.J.M. Differential gene expression of resistant and susceptible sweetpotato plants after infection with the causal agents of sweet potato virus disease. J. *Amer. Soc. Hort. Sci.* **2009**, *134*, 658–666.
4. Ascencio-Ibanez, J.T.; Sozzani, R.; Lee, T.J.; Chu, T.M.; Wolfinger, R.D.; Cella, R.; Hanley-Bowdoin, L. Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol.* **2008**, *148*, 436–454.
5. Eybishtz, A.; Peretz, Y.; Sade, D.; Akad, F.; Czosnek, H. Silencing of a single gene in tomato plants resistant to Tomato yellow leaf curl virus renders them susceptible to the virus. *Plant Mol. Biol.* **2009**, *71*, 157–171.
6. Lozano-Duran, R.; Rosas-Diaz, T.; Luna, A.P.; Bejarano, E.R. Identification of host genes involved in geminivirus infection using a reverse genetics approach. *PLoS One* **2011**, 6, e22383.
7. Whitham, S.A.; Yang, C.; Goodin, M.M. Global impact: elucidating plant responses to viral infection. *Mol. Plant-Microbe Inter.* **2006**, *19*, 1207–1215.
8. Carrington, J.C.; Kasschau, K.D.; Johansen, L.K. Activation and suppression of RNA silencing by plant viruses. *Virology*, **2001**, *281*, 1–5.
9. Hamilton, A.J.; Baulcombe, D.C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, **1999**, *286*, 950–952.
10. Pratt, A.J.; MacRae, I.J. The RNA-induced silencing complex: a versatile gene-silencing machine. *J. Biol. Chem.*, **2009**, *284*, 17897–17901.

11. Burgyán, J.; Havelda, Z. Viral suppressors of RNA silencing. *Trends Plant Sci*. **2011**, *16*, 265–272.
12. Lu, R.; Martin-Hernández, A.M.; Peart, J.R.; Malcuit, I.; Baulcombe, D.C. Virus induced gene silencing in plants. *Methods*, **2003**, *30*, 296–303.
13. Purkayastha, A.; Dasgupta, I. Virus-induced gene silencing: a versatile tool for discovery of gene functions in plants. *Plant Physiol*. *Biochem*.,**2009**, *47*, 967–976.
14. Liu, Y.; Schiff, M.; Dinesh-Kumar, S.P. Virus-induced gene silencing in tomato. *Plant J*., **2002**, *31*, 777–786.
15. Brigneti, G.; Martin-Hernández, A.M.; Jin, H.; Vhen, J.; Baulcombe, D.C.; Baker, B.; Jones, J.D.G. Virus-induced gene silencing in Solanum species. *Plant J*. **2004**, *39*, 264–272.
16. Spitzer, B.; Moyal Ben Zvi, M.; Ovadis, M.; Marhevka, R.; Barkai, O.; Edelbaum, O.; Marton, I.; Masci, T.; Alon, M.; Morin, S.; Rogachev, I.; Aharoni, A.; Vainstein, A. Reverse genetics of floral scent: application of Tobacco rattle virus-based gene silencing in petunia. *Plant Physiol*. **2007**, *145*, 1241–1250.
17. Senthil-Kumar, M.; Rame Gowda, H.V.; Hema, R.; Mysore, K.S.; Udayakumar, M. (2008). Virus-induced gene silencing and its application in characterizing genes involved in water-deficit-stress tolerance**.** *J*. *Plant Physiol*. **2008**, *165*, 1404–1421.
18. Burch-Smith, T.; Schiff, M.; Liu, Y.; Dinesh-Kumar, S.P. (2004). Efficient virus-induced silencing in *Arabidopsis*. *Plant Physiol*. **2004**, *142*, 21–27.
19. Schilmiller, A.L.; Charbonneau, A.L.; Last, R.L. Identification of a BAHD acetyltransferase that produces protective acyl sugars in tomato trichomes. *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*. **2012**, *109*, 16377–16382.
20. Duan, C.-G.**;** Wang, C.-H.**;** Guo, H.-S**.** Application of RNA silencing to plant disease resistance. *Silence* **2012**, *3*, 3–5.
21. Unver, T.; Budak, H. Virus-induced gene silencing, a post transcriptional gene silencing method. *Intl*. *J*. *Plant Genomics* **2009**, 2009: Article ID 198680, doi:10.1155/2009/198680
22. Qin, G.; Gu, H.; Ma, L.; Peng, Y.; Deng, X.W.; Chen, Z.; Qu, L.J. Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in Arabidopsis by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. *Cell Res*. **2007**, *17*, 471–482.
23. Yuan, C.; Li, C.; Yan, L.; Jackson, A.O.; Liu, Z.; Huan, C.; Li, D. A high throughput *Barley Stripe Mosaic Virus* vector for virus induced gene silencing in monocots and dicots. *PLoS ONE* **2011**, 6: e26468.
24. Peele, C.; Jordan, C.V.; Muangsan, N.; Turnage, M.; Egelkrout, E.; Eagle, P.; Hanley-Bowdoin, L.; Robertson, D. Silencing of a meristematic gene using geminivirus-derived vectors. *Plant J*. **2001**, *27*, 357–366.
25. Pandey, P.; Choudhury, N.R.; Mukherjee, S.K. A geminiviral amplicon (VA) derived from *Tomato leaf curl virus* (ToLCV) can replicate in a wide variety of plant species and also acts as a VIGS vector. *Virol*. *J*. **2009**, *6*, 152.
26. Peretz, Y.; Mozes-Koch, R.; Akad, F.; Tanne, E.; Czosnek, H.; Sela, I. A universal expression/silencing vector in plants. *Plant Physiol*. **2007**, *145*, 1251–1263.

27. Muangsan, N.; Beclin, C.; Vaucheret, H.; Robertson, D. Geminivirus VIGS of endogenous genes requires SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. *Plant J*. **2004**, *38*, 1004–1014.
28. Huang, C.; Xie, Y.; Zhou, X. Efficient virus-induced gene silencing in plants using a modified geminivirus DNA1 component. *Plant Biotech*. *J*. **2009**, *7*, 254–265.
29. Fofana, I.B.; Sangare, A.; Collier, R.; Taylor, C.; Fauquet, C.M. A geminivirus-induced gene silencing system for gene function validation in cassava. *Plant Mol*. *Biol*. **2004**, *56*, 613–624.
30. Tuttle, J.R.; Idris, A.M.; Brown, J.K.; Haigler, C.H.; Robertson, D. Geminivirus-mediated gene silencing from *Cotton leaf crumple virus* is enhanced by low temperature in cotton. *Plant Physiol*. **2008**, *148*, 41–50.
31. Czosnek, H. Editor. *Tomato yellow leaf curl virus disease: management, molecular biology, breeding for resistance*. Springer, Dordrecht, The Netherlands, **2007**.
32. Stansly, P.A., Naranjo, S.E., Eds. *Bemisia*, *Bionomics and Management of a Global Pest*. Springer, Dordrecht, The Netherlands, **2012**.
33. Hanley-Bowdoin, L.; Settlage, S.B.; Orozco, B.M.; Nagar, S.; Robertson, D. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Crit*. *Rev*. *Biochem*. *Mol*. *Biol*. **2000**, *35*, 105–140.
34. Díaz-Pendón, J.A.; Cañizares, M.C.; Moriones, E.; Bejarano, E.R.; Czosnek, H.; Navas-Castillo, J. Tomato yellow leaf curl viruses: ménage à trois between the virus complex, the plant, and the whitefly vector. *Mol*. *Plant Pathol*. **2010**, *11*, 441–450.
35. Shepherd, D.N.; Martin, D.P.; Thomson, J.A. Transgenic strategies for developing crops resistant to geminiviruses. *Plant Sci*. **2009**, *176*, 1–11.
36. Chen, H.; Fu, R.; Li, X. Tomato yellow leaf virus (TYLCV): The structure, ecotypes and the resistance germplasm resources in tomato. *African J*. *Biotech*. **2011**, *10*, 13361–13367.
37. Zamir, D. Improving plant breeding with exotic genetic libraries. *Nature Rev*. *Genetics*, **2001**, *2*, 983–989.
38. Katagiri, F. A global view of defense gene expression regulation—a highly interconnected signaling network. *Curr*. *Opin*. *Plant Biol*. **2004**, *7*, 506–511.
39. Lapidot, M.; Friedman, M. Breeding for resistance to whitefly-transmitted geminiviruses. *Ann*. *Appl*. *Biol*. **2002**, *140*, 109–127.
40. Anbinder, I.; Reuveni, M.; Azari, R.; Paran, I.; Nahon, S.; Shlomo, H.; Chen, L.; Lapidot, M.; Levin, I. Molecular dissection of Tomato yellow leaf curl virus (TYLCV) resistance in tomato line TY172 derived from *Solanum peruvianum*. *Theor*. *Appl*. *Gen*. **2009**, *119*, 519–530.
41. Morilla, G.; Castillo, A.G.; Preiss, W.; Jeske, H.; Bejarano, E.R. A versatile transreplication-based system to identify cellular proteins involved in geminivirus replication. *J*. *Virol*. **2006**, *80*, 3624–3633.
42. Luna, A.P.; Morilla, G.; Voinnet, O.; Bejarano, E.R. Functional analysis of gene silencing suppressors from Tomato yellow leaf curl disease viruses. *Mol*. *Plant Microbe Interact*. **2012**, *25*, 1294–1306.
43. Trinks, D.; Rajeswaran, R.; Shivaprasad, P.V.; Akbergenov, R.; Oakeley, E.J.; Veluthambi, K.; Hohn, T.; Pooggin, M.M. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *J*. *Virol*. **2005**, *79*, 2517–2527.

44. McGarry, R.C.; Barron, Y.D.; Carvalho, M.F.; Hill, J.E.; Gold, D.; Cheung, E.; Kraus, W.L.; Lazarowitz, S.G. A novel Arabidopsis acetyltransferase interacts with the geminivirus movement protein NSP. *Plant Cell* **2003**, *15*, 1605–1618.
45. Xie, Q.; Sanz-Burgos, A.P.; Guo, H.; Garcia, J.A.; Gutierrez, C. GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. *Plant Mol. Biol*. **1999**, *39*, 647–656.
46. Singh, D.K.; Islam, M.N.; Choudhury, N.R.; Karjee, S.; Mukherjee, S.K. The 32 kDa subunit of replication protein A (RPA) participates in the DNA replication of Mung bean yellow mosaic India virus (MYMIV) by interacting with the viral Rep protein. *Nucleic Acids Res*. **2006**, *35*, 755–770.
47. Clément, M.; Leonhardt, N.; Droillard, M.J.; Reiter, I.; Montillet, J.L.; Genty, B.; Lauriere, C.; Nussaume, L.; Noel, L.D. The cytosolic/nuclear HSC70 and HSP90 molecular chaperones are important for stomatal closure and modulate abscisic acid-dependent physiological responses in Arabidopsis. *Plant Physiol*. **2011**, *156*, 1481–1492.
48. Aparicio, F.; Thomas, C.L.; Lederer, C.; Niu, Y.; Wang, D.; Maule, A.J. Virus induction of heat shock protein 70 reflects a general response to protein accumulation in the plant cytosol. *Plant Physiol*. **2005**, *138*, 529–536.
49. Noel, L.D.; Cagna, G.; Stuttmann, J.; Wirthmuller, L.; Betsuyaku, S.; Witte, C.P.; Bhat, R.; Pochon, N.; Colby, T.; Parker, J.E. Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates *Arabidopsis* immune responses. *Plant Cell* **2007**, *19*, 4061–4076.
50. Verchot, J. Cellular chaperones and folding enzymes are vital contributors to membrane bound replication and movement complexes during plant RNA virus infection. *Frontiers Plant Sci*. **2012**, *3*, 1–12.
51. Shindo, T.; Misas-Villamil, J.C.; Horger, A.C.; Song, J.; van der Hoorn, R.A. A role in immunity for Arabidopsis cysteine protease RD21, the ortholog of the tomato immune protease C14. *PLoS One* **2012**, 7, e29317.
52. La Camera, S.; Balague, C.; Gobel, C.; Geoffroy, P.; Legrand, M.; Feussner, I.; Roby, D.; Heitz, T. The Arabidopsis patatin-like protein 2 (PLP2) plays an essential role in cell death execution and differentially affects biosynthesis of oxylipins and resistance to pathogens. *Mol. Plant Microbe Interact*. **2009**, *22*, 469–481.
53. Yang, W.Y.; Zheng, Y.; Bahn, S.C.; Pan, X.Q.; Li, M.Y.; Vu, H.S.; Roth, M.R.; Scheu, B.; Welti, R.; Hong, Y.Y.; Wang, S.M. The patatin-containing phospholipase A pPLAIIalpha modulates oxylipin formation and water loss in *Arabidopsis thaliana*. *Mol. Plant* **2012**, *5*, 452–460.
54. Lozano-Duran, R.; Bejarano, E.R. Geminivirus C2 protein might be the key player for geminiviral co- option of SCF-mediated ubiquitination. *Plant Signal Behav*. **2011**, *6*, 999–1001.
55. Mustafiz, A.; Sahoo, K.K.; Singla-Pareek, S.L.; Sopory, S.K. Metabolic engineering of glyoxalase pathway for enhancing stress tolerance in plants. *Methods Mol. Biol*. **2010**, *639*, 95–118.
56. Sun, W.; Xu, X.; Zhu, H.; Liu, A.; Liu, L.; Li, J.; Hua, X. Comparative transcriptomic profiling of a salt-tolerant wild tomato species and a salt-sensitive tomato cultivar. *Plant Cell Physiol*. **2010**, *51*, 997–1006.

57. Hakmaoui, A.; Pérez-Bueno, M.L.; García-Fontana, B.; Camejo, D.; Jiménez, A.; Sevilla, F.; Barón, M. Analysis of the antioxidant response of *Nicotiana benthamiana* to infection with two strains of *Pepper mild mottle virus*. *J*. *Exp*. *Bot*. **2012**, *63*, 5487–5496.
58. Chen, H.; Zhang, Z.; Teng, K.; Lai, J.; Zhang, Y.; Huang, Y.; Li, Y.; Liang, L.; Wang, Y.; Chu, C.; Guo, H.; Xie, Q. Up-regulation of LSB1/GDU3 affects geminivirus infection by activating the salicylic acid pathway. *Plant J*. **2010**, *62*, 12–23.
59. Dielen, A.S.; Badaoui, S.; Candresse, T.; German-Retana, S. The ubiquitin/26S proteasome system in plant-pathogen interactions: a never-ending hide-and-seek game. *Mol*. *Plant Pathol*. **2010**, *11*, 293–308.
60. Trujillo, M.; Shirasu, K. Ubiquitination in plant immunity. *Curr*. *Opin*. *Plant Biol*. **2010**, *13*, 402–408.
61. Alcaide-Loridan, C.; Jupin, I. Ubiquitin and plant viruses, let's play together! *Plant Physiol*. **2012**, *160*, 72–82.
62. Eini, O.; Dogra, S.; Selth, L.A.; Dry, I.B.; Randles, J.W.; Rezaian, M.A. Interaction with a host ubiquitin-conjugating enzyme is required for the pathogenicity of a geminiviral DNA beta satellite. *Mol*. *Plant Microbe Interact*. **2009**, *22*, 737–746.
63. Goritschnig, S.; AZhang, Y.; Li, X. The ubiquitin pathway is required for innate immunity in Arabidopsis. *Plant J*. **2007**, *49*, 540–551.
64. Lozano-Duran, R.; Rosas-Diaz, T.; Gusmaroli, G.; Luna, A.P.; Taconnat, L.; Deng, X.W.; Bejarano, E.R. Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes and inhibit jasmonate signaling in Arabidopsis thaliana. *Plant Cell* **2011**, *23*, 1014–1032.
65. Hua, Z.; Zou, C.; Shiu, S.; Vierstra, R.D. Phylogenetic comparison of F-Box (FBX) gene superfamily within the plant kingdom reveals divergent evolutionary histories indicative of genomic drift. *PLOS One* **2011**, *6*, 1–20.
66. Liu, F.; Ni, W.; Griffith, M.E.; Huang, Z.; Chang, C.; Peng, W.; Ma, H.; Xie, D. The ASK1 and ASK2 genes are essential for *Arabidopsis* early development. *Plant Cell* **2004**, *16*, 5–20.
67. Li, C.; Liu, Z.; Zhang, Q.; Wang, R.; Xiao, L.; Ma, H.; Chong, K.; Xu, Y. SKP1 is involved in abscisic acid signalling to regulate seed germination, stomatal opening and root growth in *Arabidopsis thaliana*. *Plant Cell Environ*. **2012**, *35*, 952–965.
68. Angot, A.; Peeters, N.; Lechner, E.; Vailleau, F.; Baud, C.; Gentzbittel, L.; Sartorel, E.; Genschik, P., Boucher, C., Genin, S. *Ralstonia solanacearum* requires F-box-like domain-containing type III effectors to promote disease on several host plants. *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*. **2006**, *103*, 14620–14625.
69. Schrammeijer, B.; Risseeuw, E.; Pansegrau, W.; Regensburg-Tuink, T.J.; Crosby, W.L.; Hooykaas, P.J. Interaction of the virulence protein VirF of *Agrobacterium tumefaciens* with plant homologs of the yeast Skp1 protein. *Curr*. *Biol*. **2001**, *11*, 258–262.
70. Glick, E.; Zrachya, A.; Levy, Y.; Mett, A.; Gidoni, D.; Belausov, E.; Citovsky, V.; Gafni, Y. Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*. **2008**, *105*, 157–161.
71. Hotton, S.K.; Callis, J. Regulation of cullin RING ligases. *Annu*. *Rev*. *Plant Biol*. **2008**, *59*, 467–489.

72. Lozano-Duran, R.; Bejarano, E.R. Mutation in *Arabidopsis* CSN5A partially complements the lack of Beet curly top virus pathogenicity factor L2. *J. Plant Pathol. Microbiol.* **2011**, *2*, 2–3.
73. Vidavski, F.; Czosnek, H. Tomato breeding lines immune and tolerant to Tomato yellow leaf curl virus (TYLCV) issued from *Lycopersicon hirsutum*. *Phytopathology* **1998**, *88*, 910–914.
74. Gear, M.; McPhillips, M.; Patrick, J.; McCurdy, D. Hexose transporters of tomato: molecular cloning, expression analysis and functional characterization. *Plant Mol. Biol.* **2000**, *44*, 687–697.
75. Bush, D.R. Proton-coupled sugar and amino acid transporters in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1993**, *44*, 513–542.
76. Büttner, M.; Sauer, N. (2000). Monosaccharide transporters in plants: structure, function and physiology. *Biochim. Biophys. Acta* **2000**, *1465*, 263–274.
77. Wahl, R.; Wippel, K.; Gioos, S.; Kämper, J.; Sauer, N. A novel high-affinity sucrose transporter is required for virulence of the plant pathogen *Ustilago maydis*. *PLoS Biology* **2010**, *8*, e10003030.
78. Bolton, M.D. Primary metabolism and plant defense - fuel for the fire. *Mol. Plant Microbe Interact.* **2009**, *22*, 487–497.
79. Nørholm, M.H.; Nour-Eldin, H.H.; Brodersen, P.; Mundy, J.; Halkier, B.A. Expression of the *Arabidopsis* high-affinity hexose transporter STP13 correlates with programmed cell death. *FEBS Lett.* **2006**, *580*, 2381–2387.
80. Eybishtz, A.; Peretz, Y.; Sade, D.; Gorovits, R.; Czosnek, H. Tomato yellow leaf curl virus (TYLCV) infection of a resistant tomato line with a silenced sucrose transporter gene LeHT1 results in inhibition of growth, enhanced virus spread and necrosis. *Planta* **2010**, *231*, 537–548.
81. Greenberg, J.T.; Yao, N. The role and regulation of programmed cell death in plant-pathogen interactions. *Cell. Microbiol.* **2004**, *6*, 201–211.
82. Sade, D.; Eybishtz, A.; Gorovits, R.; Sobol, I.; Czosnek, H. A developmentally regulated lipocalin-like gene is overexpressed in *Tomato yellow leaf curl virus*-resistant tomato plants upon virus inoculation, and its silencing abolishes resistance. *Plant Mol. Biol.* **2012**, *80*, 273–287.
83. Charron, J.B.F.; Ouellet, F.; Pelletier, M.; Danyluk, J.; Chauve, C.; Sarhan, F. Identification; expression, and evolutionary analyses of plant lipocalins. *Plant Physiol.* **2005**, *139*, 2017–2028.
84. Levesque-Tremblay, G.; Havaux, M.; Ouellet, F. The chloroplastic lipocalin AtCHL prevents lipid peroxidation and protects Arabidopsis against oxidative stress. *Plant J.* **2009**, *60*, 691–702.
85. Thompson, G.A.; Goggin, F.L. Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *J. Exp. Bot.* **2006**, *57*, 755–766.
86. Dorokhov, Y.L.; Frolova, O.Y.; Skurat, E.V.; Ivanov, P.A.; Gasanova, T.V.; Sheveleva, A.A.; Ravin, N.V.; Mäkinen, K.M.; Klimyuk, V.I.; Skryabin, K.G.; Gleba, Y.Y.; Atabekov, J.G. A novel function for a ubiquitous plant enzyme pectin methylesterase: the enhancer of RNA silencing. *FEBS Lett.* **2006**, *580*, 3872–3878.
87. Chen, M.; Citovsky, V. Systemic movement of a tobamovirus requires host cell pectin methylesterase. Plant J. **2003**, *35*, 386–392.
88. McCurdy, D.W.; Dibley, S.; Cahyanegara, R.; Martin, A.; Patrick, J.W. Functional characterization and RNAi-mediated suppression reveals roles for hexose transporters in sugar accumulation by tomato fruit. *Mol. Plant* **2010**, *3*, 1049–1063.
89. Rolland, F.; Moore, B.; Sheen, J. Sugar sensing and signaling in plants. *Plant Cell* **2002**, *2*, S185–S205.

90. Atkinson, N.J.; Urwin, P.E. The interaction of plant biotic and abiotic stresses: from genes to the field. *J. Exp. Bot.* **2012**, *63*, 3523–3543.
91. Sade, D.; Brotman, Y.; Eybishtz, A.; Cuadros-Inostroza, A.; Fernie, A.R.; Willmitzer, L.; Czosnek, H. Involvement of the hexose transporter gene *LeHT1* and of sugars in resistance of tomato to *Tomato yellow leaf curl virus*. *Mol. Plant*, **2013**, accepted.
92. He, P.; Shan, L.; Sheen, J. Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. *Cell Microbiol.* **2007**, *9*, 1385–1396.
93. Bombarely, A.; Rosli, H.G.; Vrebalov, J.; Moffett, P.; Mueller, L.; Martin, G. A draft genome sequence of Nicotiana benthamiana to enhance molecular plant-microbe biology research. *Mol. Plant Microbe Interact.* **2012**, *25*, 1523–1530.
94. Tomato-Genome-Consortium. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **2012**, *485*, 635–641.

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Arabidopsis thaliana, an experimental host for tomato yellow leaf curl disease-associated begomoviruses by agroinoculation and whitefly transmission

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Tomato yellow leaf curl disease is one of the most devastating viral diseases affecting tomato crops worldwide. This disease is caused by several begomoviruses (genus *Begomovirus*, family *Geminiviridae*), such as *Tomato yellow leaf curl virus* (TYLCV), that are transmitted in nature by the whitefly vector *Bemisia tabaci*. An efficient control of this vector-transmitted disease requires a thorough knowledge of the plant–virus–vector triple interaction. The possibility of using *Arabidopsis thaliana* as an experimental host would provide the opportunity to use a wide variety of genetic resources and tools to understand interactions that are not feasible in agronomically important hosts. In this study, it is demonstrated that isolates of two strains (Israel, IL and Mild, Mld) of TYLCV can replicate and systemically infect *A. thaliana* ecotype Columbia plants either by *Agrobacterium tumefaciens*-mediated inoculation or through the natural vector *Bemisia tabaci*. The virus can also be acquired from *A. thaliana*-infected plants by *B. tabaci* and transmitted to either *A. thaliana* or tomato plants. Therefore, *A. thaliana* is a suitable host for TYLCV–insect vector–plant host interaction studies. Interestingly, an isolate of the Spain (ES) strain of a related begomovirus, *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES), is unable to infect this ecotype of *A. thaliana* efficiently. Using infectious chimeric viral clones between TYLCV-Mld and TYLCSV-ES, candidate viral factors involved in an efficient infection of *A. thaliana* were identified.

Keywords: Arabidopsis thaliana, *Bemisia tabaci*, *Tomato yellow leaf curl virus*, tomato yellow leaf curl virus disease, TYLCSV, TYLCV

Introduction

Plant virus diseases involve complex interactions between the host plant, the virus, and its vector. Understanding the basis of this triple interaction is essential to design more robust control strategies to reduce crop damage. The study of the basis of these complex interactions, however, is not always feasible due to the lack of adequate genetic resources and tools for agronomically important crops. The possibility of using *Arabidopsis thaliana* as an experimental host has many advantages. It has a small genome completely sequenced, many mutants have been characterized, insertion mutations for nearly all genes are available and it is an easy plant to transform. All these facts make this model plant an excellent system to study the viral infection cycle and virus–plant–vector interaction mechanisms (Carr & Whitham, 2007; Ouibrahim & Caranta, 2013).

Amongst vector-transmitted viral diseases, tomato yellow leaf curl disease (TYLCD) is one of the most devastating affecting tomato (*Solanum lycopersicum*) worldwide (Moriones *et al.*, 2011). This disease is transmitted in nature by the whitefly (Hemiptera: Aleyrodidae) *Bemisia tabaci*. A complex of mostly monopartite begomovirus (genus *Begomovirus*, family *Geminiviridae*) species has been associated with TYLCD (Navas-Castillo *et al.*, 2011), such as *Tomato yellow leaf curl Sardinia virus* (TYLSV) or *Tomato yellow leaf curl virus* (TYLCV). The latter virus is the most widely distributed and was considered as one of the top 10 most important plant viruses (Lefeuvre *et al.*, 2010; Scholthof *et al.*, 2011). Monopartite begomoviruses have a circular, single-stranded DNA genome that contains six partially overlapping open reading frames (ORFs), two in the virion-sense (V2 and CP), and four in the virion-complementary-sense (Rep, Ren, TrAP and C4) strands, separated by an intergenic region (IR) that contains key elements for initiating replication and transcription of the viral genome (Hanley-Bowdoin *et al.*, 1999).

Due to the limited coding capacity, TYLCD-associated viruses have to rely on plant cellular factors and interact with a wide range of plant proteins to replicate, move within and between cells, and avoid plant defence

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mechanisms (Castillo *et al.*, 2003; Gorovits *et al.*, 2013; Hanley-Bowdoin *et al.*, 2013). On the other hand, for virus disease spread, compatible interactions have to occur between the plant and the vector. Phloem-feeding insects such as *B. tabaci* are highly specialized in their mode of feeding and it is important to understand how plants can perceive and defend themselves against them. During plant–insect interactions, elicitors present in insect oral secretions can activate or suppress a variety of defence signalling pathways (Walling, 2000). Among the major plant hormone defence pathways, *B. tabaci* has been shown to induce the salicylic acid (SA) signalling pathway, while the jasmonic acid (JA)-dependent pathway shows no change or is repressed (Zarate *et al.*, 2007). As a consequence, compounds are produced that can influence insect attraction/deterrence, inhibit insect growth and development, or inhibit insect digestive enzymes and/or decrease the nutritive value of the plant tissue (Ussuf *et al.*, 2001; Baldwin *et al.*, 2002; Chen *et al.*, 2005).

A limited number of begomovirus species have been reported to be able to infect *A. thaliana*, including *Cabbage leaf curl virus* (CaLCuV) (Hill *et al.*, 1998), *Cleome leaf crumple virus* (ClLCrV), *Euphorbia mosaic virus* (EuMV) (Paprotka *et al.*, 2010), *Sri Lankan cassava mosaic virus* (SLCMV) (Mittal *et al.*, 2008), and *South African cassava mosaic virus* (SACMV) (Pierce & Rey, 2013). Nevertheless, a great body of knowledge of geminivirus–plant interactions has been generated by studying viral infections in this model plant. These studies have identified interactions that regulate the infection cycle (e.g. Carvalho & Lazarowitz, 2004), virus movement (Lewis & Lazarowitz, 2010), RNA silencing response (Aregger *et al.*, 2012), and global changes that occur in host gene expression during geminiviral infection (Ascencio-Ibáñez *et al.*, 2008; Pierce & Rey, 2013).

The aim of this study was to determine whether *A. thaliana* could be a host plant for TYLCV, opening the possibility to use this model plant to research basic aspects of the TYLC-like virus–host plant–*B. tabaci* triple interaction. Therefore it was investigated whether isolates of the Mild and Israel strains of TYLCV (TYLCV-Mld and TYLCV-IL, respectively) and an isolate of the Spain strain of TYLCSV (TYLCSV-ES) could replicate and systemically infect *A. thaliana* ecotype Columbia plants, by using *Agrobacterium tumefaciens*-mediated inoculation of infectious clones. In addition, *B. tabaci* transmissibility of both strains of TYLCV to this ecotype of *A. thaliana* was examined. Differences between TYLCSV-ES and TYLCV-Mld were investigated by using chimeric infectious clones to identify candidate viral host specificity factors associated with efficient infection of *A. thaliana*.

Materials and methods

Virus sources and plant materials

Infectious clones of isolates of begomovirus species and strains associated with TYLCD, TYLCV-Mld[ES:72:97], TYLCV-IL[ES:Alm:Pep:99] and TYLCSV-ES[ES:Mur1:92] have been described elsewhere (Noris *et al.*, 1994; Navas-Castillo *et al.*, 1999; Morilla *et al.*, 2005). Isolates of the Mld and IL strains of TYLCV differ genetically, with the latter having a recombination-related genomic fragment exchange with a *Tomato leaf curl*-like virus (Navas-Castillo *et al.*, 2000). Also, biological differences exist, with Mld inducing significantly milder symptoms on some tomato varieties (Antignus & Cohen, 1994). Infectious clones of chimeric viruses Q1, Q5 and Q6 were artificially constructed by genomic fragment exchanges between TYLCV-Mld[ES:72:97] and TYLCSV-ES[ES:Mur1:92] (Fig. S1) (Sánchez-Campos, 2000; Monci, 2004).

Arabidopsis thaliana ecotype Columbia (Col-0) plants were used for inoculation assays. Also, plants of *Nicotiana benthamiana* and/or tomato cultivar Moneymaker were used as controls. Plants were maintained in a growth chamber at 22°C, 70% relative humidity, and short-day conditions (8:16-h light:dark cycle) with photosynthetically active radiation at 250 μmol s^{-1} m^{-2}.

Agrobacterium tumefaciens-mediated inoculation

For *A. tumefaciens*-mediated leaf patch infiltration (agroinfiltration), liquid cultures of *A. tumefaciens* containing the viral clones were grown at 28°C for 2 days with vigorous shaking and resuspended in 10 mM MES, 10 mM $MgCl_2$ and 100 μM acetosyringone to OD_{600} = 1·0 prior to infiltration. The suspensions were then kept at room temperature for 2 h without shaking. Three totally expanded leaves of each *A. thaliana* plant at the 10–12-leaf growth stage were agroinfiltrated using a 1-mL syringe lacking a needle. For *A. tumefaciens*-mediated apex injection (agroinjection) of *A. thaliana* plants, *A. tumefaciens* containing the viral clones were grown at 28°C on solid media agar plates for 2 days. Then, an 18-gauge needle dipped into the *A. tumefaciens* inoculum culture was inserted 6–8 times in the shoot apex of the rosette of plants at the 8–10-leaf growth stage. Cultures of *A. tumefaciens* carrying empty cloning vectors were used for mock-inoculation controls. As positive controls, susceptible *N. benthamiana* or Moneymaker tomato plants at the 4–5-leaf growth stage were agroinoculated. Except where indicated, four plants per virus combination and procedure were inoculated in three replicated experiments.

Whitefly transmission

Whitefly transmission experiments were conducted within insect-proof screened cages using *B. tabaci* Mediterranean species (formerly biotype Q) individuals from a healthy population reared on melon (*Cucumis melo* 'ANC42', IHSM-La Mayora-CSIC seed bank) plants. For transmission to healthy *A. thaliana* plants, TYLCV-IL was used. Viruliferous whiteflies were obtained by allowing adult individuals a 48 h acquisition access period (AAP) on TYLCV-IL-infected Moneymaker plants. Fifty viruliferous whiteflies were then transferred to each of six *A. thaliana* test plants and maintained in individual plastic cages with an opening covered with muslin for ventilation, for a 48 h inoculation access period (IAP). This 48 h IAP was sufficient to allow for the latent period between acquisition and transmission. As a positive control, Moneymaker tomato plants were included. For transmission experiments from *A. thaliana* plants, the strain Mld of TYLCV was used. Systemically infected plants at 21 days post-inoculation (dpi) were used as a virus acquisition source for healthy *B. tabaci* adult whiteflies, which were given a 48 h AAP. After the AAP, the whiteflies were released to four healthy *A. thaliana* or Moneymaker tomato plants (50 viruliferous individuals per plant) for a

48 h IAP. After the IAP, whiteflies were eliminated from test plants by insecticide spraying and plants were maintained, until analysed, in a growth chamber at 22°C day and 18°C night, 70% relative humidity, and a 8:16-h light:dark cycle with photosynthetically active radiation at 250 μmol s^{-1} m^{-2}. Mock-inoculated control plants were obtained with equivalent inoculation procedures but using healthy *B. tabaci* adult individuals and healthy acquisition source plants.

Viral DNA isolation and detection

Three agroinfiltrated and/or five non-inoculated (1–5, with leaf 1 being the youngest) leaves were collected per plant and ground separately (agroinfiltrated and non-inoculated) in liquid nitrogen. Then, aliquots of 100 mg of plant tissue powder were used for total DNA extraction as described by Noris *et al.* (1998) and extracted DNA was quantified in an ND-100 Spectrophotometer (NanoDrop Technologies). Viral accumulation levels in inoculated plants were semiquantified by Southern blot hybridization analysis, loading 5 μg total DNA per lane. For virus detection, a mixture of digoxigenin-labelled DNA probes was used, able to recognize all TYLCD-associated viruses detected in the Mediterranean basin and chimeric viruses (Navas-Castillo *et al.*, 1999). When needed, TYLCV was detected by PCR using primers designed to the TYLCV sequence of GenBank accession number AF071228: forward primer MA272 (5′-CTGAATGTTYGGATGGAAATGTGC-3′), corresponding to nucleotides 2353–2376, and reverse primers MA274 (5′-GCTCGTAAGTTTCCTCAACGGAC-3′), complementary to nucleotides 232–254, or MA167 (5′-ATTGCAAGA CAAACTACTTGGGG-3′), complementary to nucleotides 132–154.

Results

TYLCV can replicate and spread systemically in the model plant *A. thaliana* after *A. tumefaciens*-mediated inoculation

To determine whether TYLCV can replicate in the host plant *A. thaliana*, an agroinfiltration assay was carried out using rosette leaves and an infectious clone of TYLCV-Mld. Agroinfiltrated patches were collected at 5 days post-infiltration and total DNA was extracted and analysed by Southern blotting. As shown in Fig. 1a, single-stranded viral replicative forms were detected in agroinfiltrated leaves, although with lower accumulation levels than for the control *N. benthamiana* plants. This result indicated that *A. thaliana* could support TYLCV-Mld replication. Then, the ability of TYLCV-Mld to accumulate systemically in this host plant was examined. For this, viral DNA accumulation in newly emerged young non-infiltrated rosette leaves was examined at 30 dpi. Although viral DNA forms were not observed by Southern blot analysis, presence of viral DNA could be detected by PCR (Fig. 1b). Taken together, these results indicated that TYLCV can replicate and spread systemically in *A. thaliana* plants, and that the patch assay was a useful methodology to perform studies at the local level in this host.

To evaluate whether more efficient systemic TYLCV accumulation could be obtained in *A. thaliana* plants, an alternative inoculation procedure was used. It has been

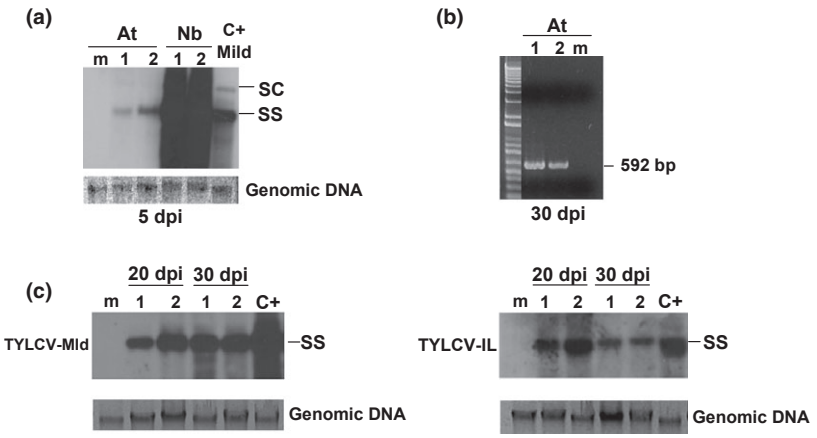

Figure 1 Analysis of *Tomato yellow leaf curl virus* (TYLCV) accumulation in *Agrobacterium tumefaciens*-mediated inoculated plants. (a) Southern blot analysis of DNA from *Arabidopsis thaliana* (At) and *Nicotiana benthamiana* (Nb) leaf patches (results for two plants per combination are shown) agroinfiltrated with an infectious clone of the Mild strain of TYLCV (TYLCV-Mld). DNA was extracted 5 days post-infiltration (dpi) and extracts from mock-inoculated leaf patches of *A. thaliana* (m), and from TYLCV-Mld-infected tomato plants (C+Mld), were included as negative and positive controls, respectively. Ethidium bromide-stained genomic DNA is shown as a loading control at the bottom of the figure. Positions are indicated for the single-stranded genomic (SS) and supercoiled double-stranded (SC) DNA forms of TYLCV DNA. (b) PCR detection of TYLCV-Mld DNA from extracts of non-inoculated leaves of TYLCV-Mld-agroinfiltrated *A. thaliana* (At) plants at 30 days post-inoculation (results for two plants are shown). DNA extracted from leaves of a mock-inoculated *A. thaliana* plant (m) was included as negative control. The position of the 592 bp fragment amplified is indicated. (c) Southern blot analysis of DNA extracted from newly emerged young non-inoculated leaves of *A. thaliana* plants agroinjected in the shoot apex with infectious clones of the Mild or Israel strains of TYLCV (TYLCV-Mld and TYLCV-IL, respectively). The results for two inoculated plants analysed at 20 and 30 days post-inoculation are shown. DNA extracts from mock-inoculated *A. thaliana* (m), and TYLCV-Mld and TYLCV-IL infected tomato (C+) plants, were included as negative and positive controls, respectively. Ethidium bromide-stained genomic DNA is shown as a loading control at the bottom of each panel. The position for the single-stranded genomic DNA (SS) form of viral DNA is indicated.

shown, for some plants recalcitrant to *A. tumefaciens*-mediated transfer, that more effective inoculation is achieved by using meristematic tissues (Grimsley *et al.*, 1988). Thus, agroinjection of TYLCV-Mld was performed at or close to the plant apex by using a needle dipped in *A. tumefaciens* inoculum. Southern blot analysis of total DNA extracted from young newly emerged non-inoculated leaves at 20–30 dpi indicated that by using this methodology, evident TYLCV-Mld viral DNA accumulation was observed in plants (Fig. 1c, left panel). An intense single-stranded viral DNA band was observed in all viral-inoculated plants, whereas no such band was detected in mock-inoculated plants (five replicated and independent experiments, 10 plants per experiment). No noticeable disease symptoms were observed in any infected plant.

Next it was tested whether the IL strain of TYLCV, that is more aggressive in its natural tomato host plant (Antignus & Cohen, 1994), was also capable of infecting *A. thaliana* plants, and if infections with symptoms occurred. As for TYLCV-Mld, Southern blot analysis of total DNA extracted from newly emerged non-inoculated leaves at 20–30 dpi resulted in a clear detection of single-stranded viral DNA replicative forms in all *A. thaliana* plants that had been agroinjected in meristematic tissues (20 plants inoculated in two independent experiments; Fig. 1c, right panel). Thus, systemic infection occurred but, as for TYLCV-Mld, no symptoms were observed in any *A. thaliana*-infected plant.

TYLCV can be transmitted to and from *A. thaliana* plants by using its natural vector *B. tabaci*

The potential use of *A. thaliana* as a model plant for TYLCV studies was further analysed by testing whether infection of plants also occurred when virus was inoculated using the natural vector *B. tabaci*. As shown in Fig. 2a, transmission was achieved in two out of six plants inoculated with the vector carrying TYLCV-IL acquired from an infected tomato source plant. Therefore, these results demonstrated that TYLCV-IL could be transmitted to *A. thaliana* plants through its natural vector *B. tabaci*. Interestingly, it was also demonstrated that TYLCV could be acquired and transmitted from *A. thaliana* plants to both *A. thaliana* and tomato plants, by this insect vector: total DNA was extracted from newly emerged non-inoculated leaves of *A. thaliana* or tomato test plants, which had been exposed to whiteflies that fed on a TYLCV-Mld-infected *A. thaliana* plant; subsequent PCR amplification indicated that one *A. thaliana* and all four tomato plants out of the four inoculated per species had been infected systemically with TYLCV-Mld (Fig. 2b). The efficient transmission rate achieved in tomato plants indicated that the virus could be acquired efficiently by *B. tabaci* from infected *A. thaliana* plants. Therefore, these results supported *A. thaliana* as a suitable host plant for acquisition and transmission of TYLCV-like viruses by using the natural vector *B. tabaci*. As for *A. tumefaciens*-mediated inoculation, no symptoms were observed in any *A. thaliana*-infected plant, whereas characteristic TYLCD symptoms were observed in infected tomato plants.

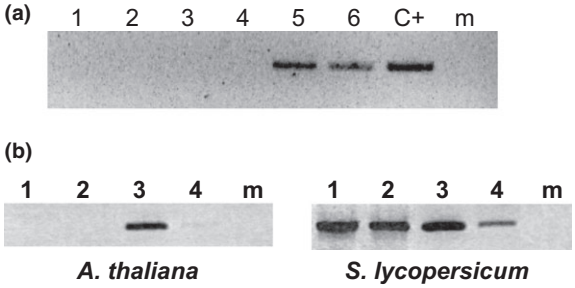

Figure 2 Detection of *Tomato yellow leaf curl virus* (TYLCV) in newly grown young non-inoculated leaves of plants inoculated using *Bemisia tabaci* adult whiteflies. (a) PCR detection of TYLCV on DNA extracts obtained 20 days post-inoculation (dpi) from *Arabidopsis thaliana* test plants inoculated with *B. tabaci* carrying an isolate of the Israel strain of TYLCV (TYLCV-IL) acquired from an infected tomato plant. (b) PCR detection of TYLCV on DNA extracts obtained 24 dpi from *A. thaliana* or tomato test plants inoculated with *B. tabaci* carrying an isolate of the Mild strain of TYLCV (TYLCV-Mld) acquired from an infected *A. thaliana* plant. DNA extracts from mock (m) and TYLCV infected tomato plants (C+) were included as negative and positive controls, respectively.

The determinant of TYLCD-associated viruses for effective infection of *A. thaliana* plants resides in the V2-CP region

Among TYLCD-associated viruses, TYLCSV is another species frequently found associated with epidemics occurring in the Mediterranean basin (Moriones *et al.*, 2011). Therefore, the ability of this virus to infect *A. thaliana* plants by *A. tumefaciens*–mediated inoculation was also studied. Interestingly, in contrast to the efficient ability of TYLCV-Mld and TYLCV-IL to infect *A. thaliana* plants locally by agroinfiltration or systemically by apex agroinjection, the strain ES of TYLCSV could not efficiently infect this host. Thus, in addition to the absence of local virus accumulation observed in agroinfiltrated leaves (data not shown), the apex agroinjection method showed that TYLCSV-ES was unable to infect efficiently non-inoculated leaves of this host. Thus, in four independent experiments (10 plants inoculated per experiment), the inoculated *A. thaliana* plants did not show any systemic virus accumulation, except for two single plants exhibiting a faint single-stranded viral DNA band in Southern blots, compared to the intense band observed in plants inoculated with TYLCV-Mld (Fig. 3a,b).

The contrasting ability of isolates of strains of the related TYLCD-associated species TYLCV and TYLCSV to infect *A. thaliana* plants was further studied. For this, chimeric viruses were artificially constructed by genomic fragment exchanges between TYLCV-Mld and TYLCSV-ES (Fig. S1). Then, chimeras Q1, Q5 and Q6, were compared to their parental viruses for their ability to infect systemically *A. thaliana* plants (10 plants used per virus combination in replicated experiments). Southern hybridization analysis revealed that, similarly to TYLCV-Mld, presence of viral DNA systemically infecting plants was

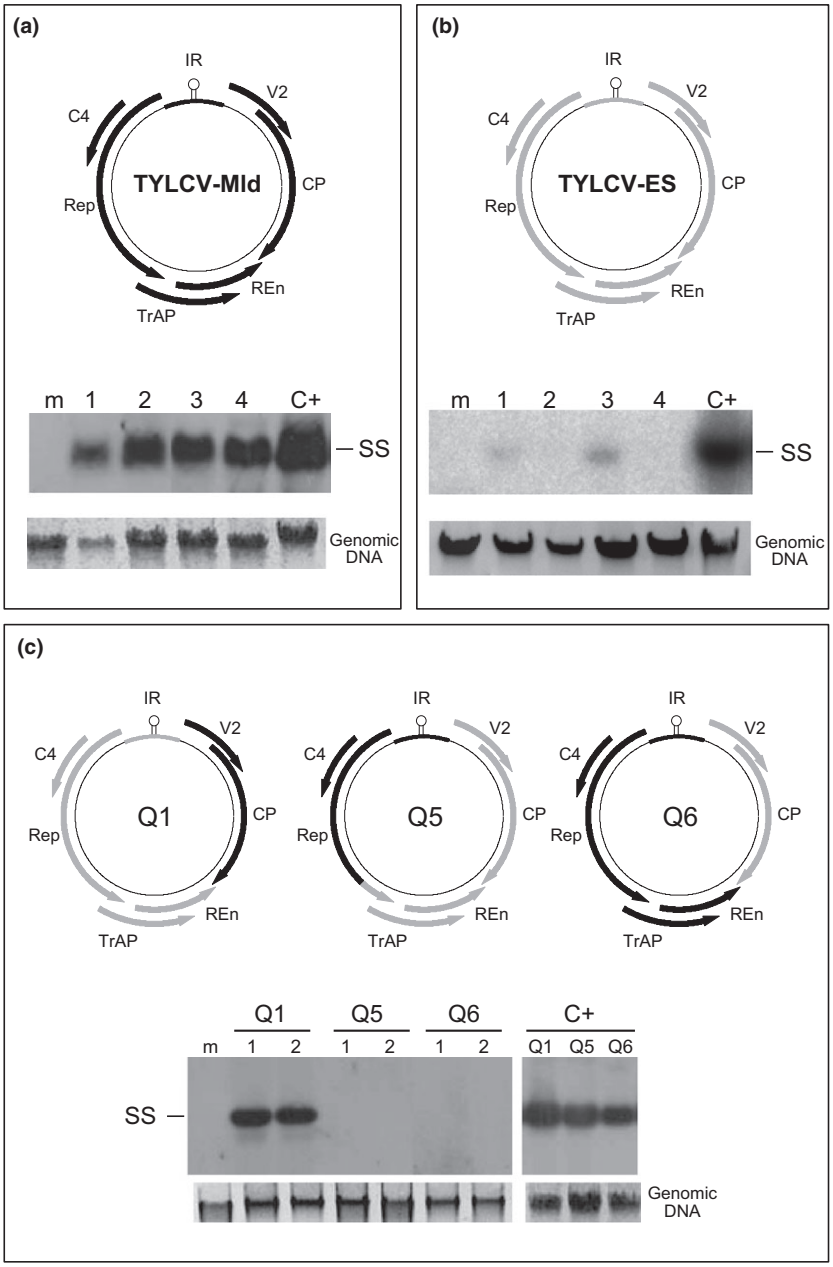

Figure 3 Southern blot analysis of DNA extracted from young newly grown non-inoculated leaves of *Arabidopsis thaliana* plants agroinjected in the shoot apex with infectious clones of (a) the Mild strain of *Tomato yellow leaf curl virus* (TYLCV-Mld), (b) the Spain strain of *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES), or (c) chimeric viruses Q1, Q5 and Q6 artificially-obtained by genomic fragment exchanges between them. The results for four TYLCV-Mld and TYLCSV-ES inoculated plants, and two Q1, Q5 and Q6 inoculated plants analysed at 20 days post-inoculation are shown. In the upper part of each panel a schematic representation is shown of the genome organization of the viruses and chimeras used for inoculation, with the regions involved in the genomic fragment exchanges performed between TYLCV-Mld and TYLCSV-ES to construct the chimeras; the open reading frames (V2, CP, Rep, TrAP, REn, and C4) and the intergenic non-coding region (IR) are shown. DNA extracts from mock-inoculated *A. thaliana* (m), and virus-inoculated tomato (a and b) or *Nicotiana benthamiana* (c) plants (C+), were included as negative and positive controls, respectively. Ethidium bromide-stained genomic DNA is shown as a loading control at the bottom of each panel. Positions are indicated for the single-stranded genomic DNA (SS) form of viral DNA.

detected in all *A. thaliana* plants agroinjected with Q1 (Fig. 3a,c), although, as before, no symptoms were observed. In contrast, similarly to TYLCSV-ES, none of the *A. thaliana* plants agroinjected with Q5 and Q6 exhibited systemic virus accumulation whereas all control plants exhibited virus infection (Fig. 3c). Therefore, based on the differences observed, especially between the symmetric chimeras Q1 and Q6, the ability to efficiently infect *A. thaliana* plants seems to reside in the V2-CP region of TYLCV-Mld.

Discussion

Among the limited number of begomovirus species that have been shown to infect *A. thaliana*, no monopartite begomovirus has been reported (Ouibrahim & Caranta, 2013). This presented a major limitation for performing basic studies with this type of geminivirus. Among monopartite begomoviruses, TYLCD-associated viruses (Navas-Castillo *et al.*, 2011) are excellent candidates for investigating aspects related to begomovirus–plant–vector interactions, due to the large and interesting amount of biological information available for them. This includes virus species and strains that differ in their host range/induction of symptoms (Navas-Castillo *et al.*, 1999; Monci *et al.*, 2002), or the ability to confront resistance genes (García-Andrés *et al.*, 2009; Tomás *et al.*, 2011). The present investigation demonstrated that *A. thaliana* was a suitable host to support local replication and

systemic accumulation of TYLCV by using *A. tumefaciens*-mediated inoculation. It has been reported that geminivirus agroinoculation can sometimes fail to result in infection due to lack of productive interaction of *A. tumefaciens* (Saeed, 2008). It has been demonstrated here, however, that, as observed for some other species previously considered recalcitrant, inoculation of *A. tumefaciens* cells into meristematic tissues at or close to the apex (Grimsley *et al.*, 1988) of the model plant *A. thaliana*, resulted in an effective systemic accumulation of ssDNA molecules of TYLCV. This result opens multiple possibilities to study basic aspects of plant–virus interactions with members of the TYLCD-associated virus group, as demonstrated by the differences detected between isolates of TYLCV-Mld and TYLCSV-ES in their ability to infect *A. thaliana* plants. Interestingly, it was found that the viral determinants associated with efficient infections reside in the V2-CP region of TYLCV. It has been shown that the gene silencing suppressor activity of the V2 proteins from these viral species are host dependent (Luna *et al.*, 2012). An intriguing hypothesis could be that the ability to circumvent the host antiviral defence systems could be connected to the differential ability to infect *A. thaliana* plants, and/or the existence of a compatible interaction of V2 with host factors. Alternatively, the fact that the CP gene of monopartite begomoviruses is required for systemic movement in plants (Rojas *et al.*, 2001) might suggest effective virus movement as the putative limiting factor for *A. thaliana* infection. This is an aspect that will require further study. *Arabidopsis thaliana* tools, such as mutants of genes involved in silencing pathways or cellular trafficking will be very helpful to understand the mechanisms and identify the cellular factors involved in the host specificity differences observed.

Finally, the possibility of transmitting TYLCV-like viruses to and from *A. thaliana* plants using the whitefly vector, offers a unique opportunity to study the basic aspects of plant–virus–vector triple interactions of the TYLCD system in this host plant. The ability of *B. tabaci* to manipulate host environment is already known (Zarate *et al.*, 2007; Puthoff *et al.*, 2010). How this manipulation could determine modification of the ability of TYLCD-associated viruses to infect the host is an open question that can now be approached by using all the tools and technology available for *A. thaliana*.

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References

Antignus Y, Cohen S, 1994. Complete nucleotide sequence of an infectious clone of a mild isolate of *Tomato yellow leaf curl virus* (TYLCV). *Phytopathology* **84**, 707–12.

Aregger M, Borah BK, Seguin J *et al.*, 2012. Primary and secondary siRNAs in geminivirus-induced gene silencing. *PLoS Pathogens* **8**, e1002941.

Ascencio-Ibáñez JT, Sozzani R, Lee T-J *et al.*, 2008. Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiology* **148**, 436–54.

Baldwin IT, Kessler A, Halitschke R, 2002. Volatile signaling in plant–plant–herbivore interactions: what is real? *Current Opinion in Plant Biology* **5**, 351–4.

Carr T, Whitham SA, 2007. An emerging model system: *Arabidopsis* as a viral host plant. In: Waigmann E, Heinlein M, eds. *Viral Transport in Plants*. Berlin, Germany: Springer, 159–83.

Carvalho MF, Lazarowitz SG, 2004. Interaction of the movement protein NSP and the *Arabidopsis* acetyltransferase AtNSI is necessary for cabbage leaf curl geminivirus infection and pathogenicity. *Journal of Virology* **78**, 11161–71.

Castillo AG, Collinet D, Deret S, Kashoggi A, Bejarano ER, 2003. Dual interaction of plant PCNA with geminivirus replication accessory protein (Ren) and viral replication protein (Rep). *Virology* **312**, 381–94.

Chen H, Wilkerson CG, Kuchar JA, Phinney BS, Howe GA, 2005. Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. *Proceedings of the National Academy of Sciences, USA* **102**, 19237–42.

García-Andrés S, Tomás D, Navas-Castillo J, Moriones E, 2009. Resistance-driven selection of begomoviruses associated with the tomato yellow leaf curl disease. *Virus Research* **146**, 66–72.

Gorovits R, Moshe A, Ghanim M, Czosnek H, 2013. Recruitment of the host plant heat shock protein 70 by *Tomato yellow leaf curl virus* coat protein is required for virus infection. *PLoS ONE* **8**, e70280.

Grimsley NH, Ramos C, Hein T, Hohn B, 1988. Meristematic tissues of maize plants are most susceptible to agroinfection with maize streak virus. *Nature Biotechnology* **6**, 185–9.

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D, 1999. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Critical Reviews in Plant Sciences* **18**, 71–106.

Hanley-Bowdoin L, Bejarano ER, Robertson D, Mansoor S, 2013. Geminiviruses: masters at redirecting and reprogramming plant processes. *Nature Reviews Microbiology* **11**, 777–88.

Hill JE, Strandberg JO, Hiebert E, Lazarowitz SG, 1998. Asymmetric infectivity of pseudorecombinants of cabbage leaf curl virus and squash leaf curl virus: implications for bipartite geminivirus evolution and movement. *Virology* **250**, 283–92.

Lefeuvre P, Martin DP, Harkins G, *et al.*, 2010. The spread of *Tomato yellow leaf curl virus* from the Middle East to the World. *PLoS Pathogens* **6**, e1001164.

Lewis JD, Lazarowitz SG, 2010. *Arabidopsis* synaptotagmin SYTA regulates endocytosis and virus movement protein cell-to-cell transport. *Proceedings of the National Academy of Sciences, USA* **107**, 2491–6.

Luna AP, Morilla G, Voinnet O, Bejarano ER, 2012. Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. *Molecular Plant–Microbe Interactions* **25**, 1294–306.

Mittal D, Borah BK, Dasgupta I, 2008. Agroinfection of cloned Sri Lankan cassava mosaic virus DNA to *Arabidopsis thaliana*, *Nicotiana tabacum* and cassava. *Archives of Virology* **153**, 2149–55.

Monci F, 2004. *Búsqueda de Estrategias de Control Frente a los Begomovirus que Afectan a Tomate y Judía en España*. Córdoba, Spain: University of Córdoba, PhD thesis.

Monci F, Sánchez-Campos S, Navas-Castillo J, Moriones E, 2002. A natural recombinant between the geminiviruses *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. *Virology* **303**, 317–26.

Morilla G, Janssen D, García-Andrés S, Moriones E, Cuadrado I, Bejarano E, 2005. Pepper (*Capsicum annuum*) is a dead-end host for *Tomato yellow leaf curl virus*. *Phytopathology* **95**, 1089–97.

Moriones E, Navas-Castillo J, Díaz-Pendón JA, 2011. Emergence of begomovirus diseases. In: Caranta C, Aranda MA, Tepfer M, López-Moya J, eds. *Recent Advances in Plant Virology*. Norfolk, UK: Caister Academic Press, 301–20.

Navas-Castillo J, Sánchez-Campos S, Díaz JA, Sáez-Alonso E, Moriones E, 1999. *Tomato yellow leaf curl virus-Is* causes a novel disease of common bean and severe epidemics in tomato in Spain. *Plant Disease* **83**, 29–32.

Navas-Castillo J, Sánchez-Campos S, Noris E, Louro D, Accotto G, Moriones E, 2000. Natural recombination between *Tomato yellow leaf curl virus-Is* and *Tomato leaf curl virus*. *Journal of General Virology* **81**, 2797–801.

Navas-Castillo J, Fiallo-Olivé E, Sánchez-Campos S, 2011. Emerging virus diseases transmitted by whiteflies. *Annual Review of Phytopathology* **49**, 219–48.

Noris E, Hidalgo E, Accotto G, Moriones E, 1994. High similarity among the tomato yellow leaf curl virus isolates from the West Mediterranean Basin: the nucleotide sequence of an infectious clone from Spain. *Archives of Virology* **135**, 165–70.

Noris E, Vaira A, Caciagli P, Masenga V, Gronenborn B, Accotto G, 1998. Amino acids in the capsid protein of *Tomato yellow leaf curl virus* that are crucial for systemic infection, particle formation, and insect transmission. *Journal of Virology* **72**, 10050–7.

Ouibrahim L, Caranta C, 2013. Exploitation of natural genetic diversity to study plant–virus interactions: what can we learn from *Arabidopsis thaliana*? *Molecular Plant Pathology* **14**, 844–54.

Paprotka T, Metzler V, Jeske H, 2010. The first DNA 1-like α satellites in association with New World begomoviruses in natural infections. *Virology* **404**, 148–7.

Pierce EJ, Rey MC, 2013. Assessing global transcriptome changes in response to South African cassava mosaic virus [ZA-99] infection in susceptible *Arabidopsis thaliana*. *PLoS ONE* **8**, e67534.

Puthoff DP, Holzer FM, Perring TM, Walling LL, 2010. Tomato pathogenesis-related protein genes are expressed in response to *Trialeurodes vaporariorum* and *Bemisia tabaci* biotype B feeding. *Journal of Chemical Ecology* **36**, 1271–85.

Rojas MR, Jiang H, Salati R *et al.*, 2001. Functional analysis of proteins involved in movement of the monopartite begomovirus, *Tomato yellow leaf curl virus*. *Virology* **291**, 110–25.

Saeed M, 2008. Limitations observed in the use of agroinoculation for geminivirus research. *Virus Genes* **37**, 434–5.

Sánchez-Campos S, 2000. *El Rizado Amarillo del Tomate en España: Caracterización de los Geminivirus Implicados y Estudio de las Epidemias*. Málaga, Spain: University of Málaga, PhD thesis.

Scholthof KBG, Adkins S, Czosnek H *et al.*, 2011. Top 10 plant viruses in molecular plant pathology. *Molecular Plant Pathology* **12**, 938–54.

Tomás DM, Cañizares MC, Abad J, Fernández-Muñoz R, Moriones E, 2011. Resistance to *Tomato yellow leaf curl virus* accumulation in the tomato wild relative *Solanum habrochaites* associated with the C4 viral protein. *Molecular Plant–Microbe Interactions* **24**, 849–61.

Ussuf K, Laxmi N, Mitra R, 2001. Proteinase inhibitors: plant-derived genes of insecticidal protein for developing insect-resistant transgenic plants. *Current Science* **80**, 847–53.

Walling LL, 2000. The myriad plant responses to herbivores. *Journal of Plant Growth Regulation* **19**, 195–216.

Zarate SI, Kempema LA, Walling LL, 2007. Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. *Plant Physiology* **143**, 866–75.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Schematic representation of the construction of the chimeras Q1, Q5 and Q6 between the Mld strain of *Tomato yellow leaf curl virus* (TYLCV-Mld) and the ES strain of *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES).