CHAPTER 5: IDENTIFICATION OF PLANT GENES INVOLVED IN TYLCV REPLICATION

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1. OVERVIEW

Since there are still no chemicals that can be applied routinely to control plant virus diseases, TYLCV control strategies have been mainly focused on methods to prevent the occurrence of infection and on genetic resistance. Attempts to reduce the incidence of TYLCV by eliminating the sources of inoculum or controlling vector transmission are often ineffective (Picó et al., 1996). Attempts to derive TYLCV resistant tomato cultivars constituted the main effort of extended breeding programmes to introgress resistance from wild Lycopersicon species.

Although some wild relatives of tomato are resistant, introduction of resistance traits into commercial tomatoes is however complicated by several factors. Some tolerant cultivars have been released (Lapidot et al., 1997; Friedmann et al., 1998), but no fully resistants Lycopersicon esculentum are still available.

The identification of plant genes involved in the viral life cycle may offer the opportunity to disrupt the interaction between the virus and the plant cell, thus preventing infection without introducing foreign genes in the plant. Despite differences in the properties of their genomes, all plant viruses face the same two fundamental challenges during the establishment of systemic infections in their plant hosts. The first necessity is to replicate in the infected cells. The second requirement is to move through adjacent plant cells to the vascular system, before spreading throughout the plant. Both processes depend on highly specific interactions with host proteins. Protein–protein interactions are the underpinnings of a vast number of these cellular processes. In recent years, the convergence of biochemistry, cellular and molecular biology has made available a number of powerful techniques for studying such interactions. These techniques vary in their sensitivity, efficiency and rapidity, but judicial deployment of acombination of them has proved to be effective and reliable.

The viruses of the Tomato yellow leaf curl complex that include Tomato yellow leaf curl virus (TYLCV) and Tomato yellow leaf curl Sardinia virus (TYLCSV) isolates are monopartite members

of the Begomovirus genus. Like other begomoviruses, TYLCV virus infection is initiated by whitefly-mediated transmission of virions from an infected plant to a recipient plant. The single-stranded DNA is converted to a double-stranded form, which then serves as a transcription template for the production of the viral replication proteins Rep (also designated AL1, AC1, and C1) and REn (also named AL3, AC3, and C3).

The Rep protein acts as a rolling circle initiator to catalyse a site-specific cleavage and rejoining reaction in a conserved hairpin loop in the viral replication origin (Laufs et al., 1995). The REn protein greatly enhances viral DNA accumulation and symptoms in infected plants (Hanley-Bowdoin et al., 2000; Hanley-Bowdoin et al., 2004; Settlage et al., 2005). Geminiviruses do not encode their own DNA polymerases and rely on the nuclear DNA replication machinery, like many mammalian DNA tumour viruses do. They replicate in nuclei of mature cells, which are inactive in DNA replication.

Accumulating evidence strongly supports the notion that geminivirus proteins have a significant impact on a variety of host cell pathways (reviewed in Gutierrez et al., 2004; Hanley-Bowdoin et al., 2004), including cell differentiation, cell cycle control, DNA replication, plasmodesmata function, RNA silencing, etc. Several studies have shown that the begomovirus Rep and REn proteins bind to viral and host proteins (Table 1). It has been demonstrated that besides the interaction with themselves and with each other (Lucioli et al., 2003; Settlage et al., 2005), Rep and REn interact with the proliferating cell nuclear antigen (PCNA), an essential component of the DNA replisome (Castillo et al., 2003; Settlage et al., 2005), and with the host protein pRBR, the plant retinoblastoma homologue (reviewed in Hanley-Bowdoin et al., 2004). Rep also interacts with a novel protein kinase (GRIK), a kinesin, the histone H3 (Kong & Hanley- Bowdoin, 2002), and with the SUMO conjugating enzyme NbSCE1/Ubc9, a component of the sumoylation pathway (Castillo et al., 2004). Finally, REn from a related virus, Tomato leaf curl virus (TLCV) was recently shown to interact with a transcription factor in the NAC family (Selth et al., 2005).

We highlight here the use of the yeast two-hybrid assay (see Fields, 2005; Gietz, 2006, for recent reviews) to identify plant proteins that interact with geminivirus proteins. The two-hybrid method detects the interaction of two proteins by their ability to reconstitute the activity of a split transcription factor, thus allowing the use of a simple growth selection in yeast to identify new interactions.

In spite of some problems (large number of false positives), two-hybrid is a powerful technique, widely used for the last 15 years and it has been recently scale-up in combination with computational analysis to a high-throughput analysis of protein interaction.

In this chapter, we summarized the results obtained using yeast two-hybrid technique, to identify plant proteins that interact with TYLCV/TYLCSV proteins involved in the replication of the virus (Rep and REn). We also described a system recently developed to easily identify if a host factor is required for geminivirus replication using Virus induced gene silencing (VIGS).

2. TWO-HYBRID SCREENINGS

To identify cellular proteins that interact with Rep and REn, several screening have been carried out on Arabidopsis thaliana, Nicotiana benthamiana, and L. esculentum cDNA prey libraries using Rep or REn fused to the GAL4 DNA-binding domain as bait.

To clone the viral proteins we used three different plasmids pAS2, pBDGAL4, and pGBKT7 all containing the GAL4 DNA-binding domain. We selected the yeast strain PJ696 for the screening as it has three reporter genes, HIS3, ADE2, and lacZ, to detect the interaction. Positive interactions were indicated by yeast growth in the absence of histidine and adenine. Once, we confirmed that none of the viral-protein-fusions activated histidine/adenine auxotrophy by themselves yeast cells were then cotransformed with the Rep or REn bait plasmid and the plant cDNA, prey libraries. Among transformants that appeared 5–10 days after transformation, we selected those for which the 3 yeast reporter genes were activated. Next, plasmids from the cDNA library were isolated from the selected colonies and reintroduced into the yeast by transformation. Empty bait plasmids, as well as bait plasmid expressing a non-related virus sequence are used to confirm the specificity of the interaction.

Finally, we sequenced the cDNA clone, to confirm that it was cloned in frame, and identify it.

2.1. REn interacting proteins

REn screening lighted up four proteins: one from tomato, one from N. benthamiana, and two from Arabidopsis cDNA libraries. Sequence and databases analysis from the positive clones isolated from Arabidopsis, revealed the existence of three different partial cDNA clones of the same protein, PCNA, an essential component of the eukaryotic replication machinery. PCNA is a ring-like protein that tethers DNA and functions as a moving platform that modulates the interactions of other proteins with DNA. This sliding clamp interacts with many proteins that are involved in important cellular processes like replication and repair of DNA, DNA methylation, cell cycle control,

and chromatin assembly (Warbrick, 2000). The interaction between TYLCSV REn and PCNA was confirmed using PCNA from plant species infected by this virus (N. benthamiana and L. esculentum) (Castillo et al., 2003). PCNA-binding proteins can be divided into two groups: enzymatic proteins that participate in DNA metabolism and regulatory proteins that are involved in cell cycle progression, checkpoint control, and cellular differentiation. Most of the interactions are located in three loop structures protruding on the C-side that are very well conserved in all eukaryotic PCNAs (Tsurimoto, 1999). In tomato PCNA (LePCNA) those structures correspond to residues Asp41 to His44 (central loop), Leu 118 to Glu124 (long loop), and Lys254 to Glu256 (C-terminal tail). REn-binding domain of LePCNA was mapped on using a truncated LePCNA, to a region between Lys132 and Thr187, where no other interactions have been previously described (Castillo et al., 2003). This region is located at the end of the PCNA monomer with amino acids placed to the C-side and loop side (Figure 1).

By analysing the solvent accessibility of the amino acids and identifying possible receptor-binding domains, we mutagenized three amino acids residues (Arg172, Arg183, and Glu184) of the REn-binding region that could be involved in protein–protein interactions. Unexpectedly, none of the mutations reduced the interaction with REn, and moreover four of them increased protein binding, indicating that none of those residues is involved in the interaction (Collinet, unpublished data).

Although the PCNA interaction domain of TYLCSV REn was also analysed by two-hybrid assays using truncated forms of the protein the results were not clear enough to identify it (Castillo et al., unpublished data). A detailed analysis of TYLCV REn domains involved in the interaction with PCNA has been published recently (Settlage et al., 2005). In this work the authors have examined the impact of mutations in amino acids that are conserved across the REn protein family on protein interactions, and replication enhancement of REn.

Analysis of the mutated proteins by two-hybrid assays has localized the PCNA interaction domain between residues 7 and 95.

Notwithstanding its small size (134 amino acids), TYLCV REn also interacts with Rep, with itself and with and the retinoblastoma-related protein (pRBR). The REn-binding domain with PCNA overlaps with the REn region that binds Rep, and itself, but not with the one interacting with pRBR (Settlage et al., 2005).

The mechanism by which REn enhances viral DNA accumulation may reside in its ability to interact with Rep. The REn protein sequence shows no homology to any known enzymatic motifs. Thus, it is more likely that the structure of the REn/Rep complex is important for replication rather

than a catalytic activity of REn that could affect Rep. The results obtained using mutated versions of REn protein of TYLCV, established the importance of REn–REn, REn–Rep, and REn–PCNA interactions in geminivirus replication. While REn–pRBR interaction is not required for viral replication in cycling cells, it may play a role during infection of differentiated cells in intact plants (Settlage et al., 2005).

Another recent study proposed that TLCV REn binding to a transcription factor, SlNAC1, and induction of its expression is the mechanism whereby REn enhances geminivirus replication (Selth et al., 2005). However, it is unlikely that REn–SlNAC1 interactions are sufficient for REn replication enhancement activity. REn mutant replicons are typically constructed by deleting REn sequences that do not overlap with the other geminiviral open reading frame, (Trap), to yield a truncated proteins of 83–111 amino acids. This region of REn retains the capacity to bind to SlNAC, but the REn mutant replicons support little if any detectable replication in protoplasts and are severely attenuated in infectivity assays in planta. Instead, the data suggest that the loss of replication enhancement activity by the truncated REn proteins reflects their inability to form oligomers with themselves, Rep, and/or PCNA. Several explanations for the effect of REn on virus replication have been proposed (Hanley-Bowdoin et al., 2000). There are some experimental observations that suggest that REn might increase the affinity of Rep for the origin of replication.

Another possibility is that REn directs Rep from the Rep/DNA-binding domain to its cleavage site in the origin during the initiation of replication. REn and Rep interact with PCNA and by encircling DNA and interacting with polymerases, PCNA forms a sliding clamp that keeps the polymerases associated with the DNA template during processive DNA synthesis. By interacting with PCNA and Rep, REn could help Rep to recruit the replication machinery necessary to replicate the viral DNA. In the absence of REn, Rep can still bind PCNA although the efficiency of DNA replication decreases. Placing the DNA replication machinery in the viral origin of replication could also involve interactions of Rep and/or REn with other cellular proteins than PCNA.

In fact, it has been demonstrated that Rep from the geminivirus Wheat dwarf virus interacts with RFC-1, the large subunit of PCNA clamp loader (Luque et al., 2002) and the existence of a multimeric REn complex is supported by gel filtration analysis of native Tomato golden mosaic virus (TGMV) REn, which fractionated with a complex of >100 kDa (Hanley-Bowdoin, unpublished data).

155 It is also possible that REn or Rep binding to PCNA prevents the interaction of PCNA with cell
156 cycle regulators such as p21, cyclin D and p57 that inhibit chromosomal replication by interacting
157 with PCNA during cell cycle progression (Kelman, 1997).

158 2.2. Rep interacting proteins

159 Yeast two-hybrid screenings using Rep from TYLCSV and TYLCV as bait, were laborious, as this
160 protein seems to be partially toxic in yeast. We carried out various screenings expressing full
161 length and truncated versions of Rep. We also clone Rep in several bait plasmids that differ in
162 the promoter strength. The best results were obtained when the full-length protein was cloned
163 into the bait plasmid pGBKT7.

164 From the positive clones isolated after screening Arabidopsis and N. benthamiana cDNA libraries,
165 we identified three N. benthamiana proteins able to interact with Rep from TYLCSV. The
166 subsequent procedures to confirm the interactions were similar to those described above for REn.
167 For further studies we selected one of the proteins present in two library clones that specifically
168 interacts with DB-Rep. The two clones contained a single open reading frame of 160 amino acids,
169 named NbSCE1, which had high homology to the AtSCE1a gene, an E2 ubiquitin-conjugating-
170 like enzyme from Arabidopsis, and to UBC9 from Saccharomyces cerevisiae. Like UBC9/SCE1
171 from S.cerevisiae, humans and Arabidopsis, NbSCE1 is predicted to conjugate SUMO, not
172 ubiquitin, to target proteins in a cellular process called sumoylation. Sumoylation is a target protein
173 by the covalent attachment of an Ubiquitin-like polypeptide (Ubl) called SUMO (Bossis & Melchior,
174 2006). Contrary to ubiquitination, SUMO conjugation is mediated by a single E2 enzyme
175 (UBC9/SCE1) that is essential for cell viability and sumoylation in yeast and animals. In yeast,
176 there is just a single gene that codifies for SUMO (SMT3), but in metazoan, SUMO proteins can
177 be divided in two families – SUMO1 and SUMO2/SUMO3.

178 Although SUMO2 and SUMO3 share 50% sequence identity with SUMO1, they are functionally
179 different. In plants little is known about SUMO pathway or the nature of its targets. Many of the
180 core components for sumoylation have been identified in Arabidopsis (Novatchkova et al., 2004).

181 The NbSCE1 protein also interacts with Rep proteins from other bipartite begomoviruses TGMV
182 (AL1) and African cassava mosaic virus-kenya (RepAC or AC1), both of which infect N.
183 benthamiana (Castillo et al., 2004). Many proteins, including some from mammalian viruses,
184 interact with UBC9/SCE1 in yeast two-hybrid assays. Although most of these UBC9/SCE1
185 interacting proteins are also sumoylated, some are not sumoylation substrates. It is not known

yet if Rep/AL1 is a substrate for sumoylation. In plants, it is not easy to prove Rep sumoylation because of the difficulty detecting Rep from infected tissues on immunoblots and problems associated with its overexpression. In mammalian cells and yeast, SUMO is covalently attached to specific lysines in the target protein.

The precise lysine residues modified by SUMO have been identified in more than a dozen known substrates (Muller et al., 2001). The majority of these modification sites conform to a consensus sequence defined by four amino acids “YKXE”, where Y is a large hydrophobic residue and K serves as the acceptor for SUMO. However, there are sumoylated proteins that do not share this exact sequence (Yeh et al., 2000). Rep from TGMV, TYLCSV, or TYLCV does not contain any protein motifs that exactly match the consensus, but has lysine residues surrounded by similar amino acids that are potential sumoylation sites.

Sumoylation plays a role in the geminivirus replication as transgenic tobacco plants showing altered levels of SUMO disturb TGMV replication (Castillo et al., 2004) and silencing of NbSCE1 also reduces TYLCSV replication (see Section 3 of this chapter). It is difficult to provide a simple hypothesis that explains these results, as the biological effects of sumoylation are quite diverse, and the mechanisms and signal pathways involved in most of them remain unclear. SUMO conjugation has been implicated in cellular responses to environmental stress, subcellular protein translocation, nuclear body formation, centromere segregation, protection from ubiquitin-mediated proteolysis, and regulation of transcriptional activity (Hay, 2005)

SUMO may play an important role in pathogen plant defence responses. The tomato SUMO orthologue (LeSUMO) was isolated in a yeast two-hybrid screen by its interaction with ethylene-inducing xilanase (EIX) from the fungus Trichoderma viridae (Hanania et al., 1999). The expression of LeSUMO in tobacco transgenic plants suppressed the induction of the defence response byEIX (a strong elicitor of the rapid defense response in tomato). A virulence factor (AvrBsT) from the plant pathogen Xanthomonas campestris, has SUMO protease activity that interferes with the plant defence response, probably by desumolating a key defence regulator (Gurlebeck et al., 2006). It also has been proposed that SUMO conjugation in Arabidopsis plays a regulatory role in the stress response, modifying the activity or localization of critical effectors.

SUMO conjugation could impact a battery of nuclear regulatory proteins when plants are exposed to stress signals. Potential targets under negative regulation could include factors that promote cell division and other general physiological processes that are repressed while plants cope with adverse environments. Thus, interference with viral DNA replication observed in overexpressing

SUMO transgenic plants or in NbSCE1-silenced plants could be related to changes in the levels and/or the profiles of SUMO conjugates. These changes may repress proteins required for viral replication or induce a stronger defence response.

However, a direct effect on Rep cannot be ruled on virus cell cycle. Viruses have evolved numerous mechanisms to overcome host defences and to utilize host biochemical pathways to their advantage. One type of viral–host interaction that it is well established and widespread is the modulation of viral protein function by post-translational modification systems such as phosphorylation, glycosylation, ubiquitination, and sumoylation. The interaction between viral proteins and the cell sumoylation system has been previously described for mammalian viruses, and the effects are target specific and very diverse (Boggio & Chiocca, 2006). As with sumoylated cellular proteins, the biological effect of sumoylation of viral proteins is target specific. Rep/AL1 interaction with SCE1 could affect geminiviral replication by preventing de novo sumoylation, or enhancing desumoylation of host proteins, or by using SUMO for viral benefit, as long as the outcome is an environment that is more favourable for viral propagation.

3. A VERSATILE TRANSREPLICATION-BASED SYSTEM TO IDENTIFY CELLULAR PROTEINS INVOLVE IN GEMINIVIRUS REPLICATION

In the genomic age, molecular biologists are looking for new alternatives to study gene function on a genome-wide scale. High-throughput techniques for gene discovery and expression analysis, such as whole genome sequencing and micro-arrays, demand efficient procedures to unravel gene functions to render them useful for both basic and applied applications. The information generated by these high-throughput technologies could be combined with post-transcriptional gene silencing (PTGS) approaches to determine gene function on a genome-wide scale.

Traditional gene knock-out techniques use transformation as a delivery system, and they usually require tissue culture procedures to regenerate silenced mutants. PTGS, and particularly virus induced gene silencing systems, can be used as tools to speed up studies of gene function by reverse genetic analysis, since they allow us to bypass the time-consuming transformation and tissue culture procedures. The resulting phenotypes can thus be evaluated within days after the inoculation, vs. the months or even years required when using more traditional transformation methods. VIGS technology has already been used for function analysis of defence-related genes (reviewed in Lu et al., 2003; see e.g., Peart et al., 2002); however, phenotype evaluation remains one of the major constraints to the use of VIGS to identify host genes involved in viral infection.

Traditionally, the level of geminivirus infection has been determined evaluating symptom development, and quantifying viral DNA accumulation by nucleic acid hybridization. Both methods present substantial inconveniences for their use in large-scale VIGS analysis. Geminivirus-induced symptoms could be partially or completely obscured by the phenotype produced as consequence of the host gene silencing, or by the symptoms induced by the viral vector used for the VIGS system. Furthermore, hybridization analysis is time-consuming and difficult to interpret when using VIGS as a gene-silencing method. Suppression of host gene expression by VIGS does not affect the whole plant. Thus, in a plant where a host gene essential for viral replication had been silenced by VIGS, a geminivirus could still replicate in areas where silencing was not implemented.

Viral molecules produced in such non-silenced areas of the plant could then be transported to the silenced parts of the plant and accumulate there, thus producing misleading results when detecting viral DNA accumulation by hybridization analysis. Additionally, several samples from each VIGS-silenced plant should be analysed, since silenced leaves cannot be previously identified.

The use of recombinant viruses containing a reporter gene is a better alternative to evaluate whether gene silencing of a target gene interferes with viral infection. Many RNA viruses have already been labelled with reporter genes such as GFP. One successful example is Potato Virus X (PVX) (Cruz et al., 1996), an RNA virus which like many other rod-shaped viruses does not have limitations in the insert size that is able to package. However, few recombinant geminiviruses tagged with reporter genes have been reported, and GFP targeting has only been successful with Bean dwarf mosaic virus (Sudarshana et al., 1998; Levy & Czosnek, 2003). There are many limiting features, which hinder the tagging of geminiviruses. DNA viruses suffer a stronger DNA packaging size limitation than RNA viruses, actually the smaller viral genomes with partial deletions are selected in the movement through plasmodesmata (Hayes et al., 1989; Gilbertson et al., 2003). Furthermore, in monopartite begomoviruses such as TYLCV all six ORFs are essential for an efficient replication and long distance movement within the plant, making rather difficult the replacement strategy used with bipartite begomoviruses.

Geminiviruses, with their simple genome organization, broad host range, high copy number, and ability to generate infective clones, have many advantages as recombinant virus-based gene amplification system in infected transgenic plants (Timmermans et al., 1992). Extrachromosomal amplification from gemimivirus-based constructs has been exploited for the production of valuable

peptides and proteins (Palmer et al., 1999; Mor et al., 2002; Hefferon et al., 2004), or to analyse the function of Rep in replication (Hong et al., 2003).

We have developed a TYLCSV-based GFP amplification system to identify the plant organs or tissues where viruses replicate in real-time and without their destruction (Morilla et al., 2006). In combination with the VIGS technology, this tool could be an attractive instrument in functional genomics to easily identify host proteins required for geminivirus infection.

3.1. Amplification of mGFP transreplicons during TYLCSV infection

The TYLCSV-based GFP system is based on the construction of N. benthamiana transgenic plants (2IRGFP) that contain a construct with direct repeats of the intergenic region (IR) of TYLCSV flanking a GFP expression cassette (Figure 2A). These transgenic plants developed typical systemic symptoms when infected with TYLCSV or TYLCV and accumulates an episomal DNA (called mGFP replicon) when they were infected with TYLCSV but not with

TYLCV.

The mGFP replicon are circular episomal transreplicons generated from the transgene during viral infection, as a consequence of the interaction between Rep and its cognate origin of replication. TYLCV is unable to mobilize the TYLCSV-derived transreplicon because TYLCV Rep does not recognize TYLCSV IR-binding motifs.

TYLCSV replication is easily detected under long-wavelength UV light in 2IRGFP plants. Three weeks after infection, GFP fluorescence is concentrated at the leaf veins (Figure 2B). An increase on GFP expression is also noticeable in other TYLCSV-infected organs such as stems, roots, or flowers. GFP signal in roots and stems was also concentrated in transport tissues. The changes

observed on the GFP expression pattern of 2IRGFP plants are dependent on: (i) TYLCSV-specific infection, since they were not detected when plants were mock inoculated or infected with TYLCV; and (ii) the presence of the IR in the transgene, since GFP expression of transgenic plants containing a GFP cassette without IR is not affected by infection.

Although induction of GFP expression is noticed already 2–3 days postinfection (dpi), the characteristic GFP expression pattern in the leaves produced by the TYLCSV infection appear several days after (10–12 dpi), reach a maximum between the second and the fourth week postinfection, and slowly decay afterwards. The intensity and spread of the vein expression is stronger in younger leaves. GFP expression is associated with the generation of the episomal replicon. Increase in GFP fluorescence and amplification of mGFP replicon could also be reached

312 expressing only TYLCSV Rep/C4, as Rep is the only viral protein required for IR recognition and
313 virus replication.

314 3.2. 2IRGFP plants as a tool to detect plant proteins require for TYLCSV replication

315 The distribution of the green fluorescence in infected 2IRGFP transgenic plants highlights the
316 plants organs or tissues where the virus has expressed Rep, and is therefore actively replicating
317 its DNA. So, these transgenic plants could be used to identify plant genes required for TYLCSV
318 replication. If a cell function necessary for virus replication is silenced in TYLCSV-infected
319 transgenic plants, we could readily detect it by illuminating the plants with UV light, as green
320 fluorescent vein-pattern will not be produced. Silencing of host functions could be obtained by
321 dsRNA-mediated suppression of genes through the production of sense or antisense transcripts,
322 or even more efficiently, using single-stranded self-complementary (hairpin) RNA containing an
323 intron (Lee & Roth, 2003).

324 However, both strategies rely on the generation of transgenic plants, which is a time-consuming
325 task. Alternatively, temporal silencing of the genes could be obtained by VIGS. This technology
326 has been widely used for analysis of gene function, particularly for genes involved in defence
327 against pathogens (reviewed in Lu et al., 2003)

328 Hence, the 2IRGFP plants were assessed by VIGS-silencing two host genes: PCNA (Morilla et
329 al., 2006) and NbSCE1 (Lozano et al., unpublished results).

330 PCNA was used to test the system, since it was already known that this protein is required for
331 begomivirus replication (Peele et al., 2001). NbSCE1 was selected an example of functional
332 assay to determine if a candidate gene identified by its ability to interact with Rep was required
333 for virus replication

334 1. PCNA: N. benthamiana PCNA was silenced using a Tobacco rattle virus (TRV) vector (Ratcliff
335 et al., 2001) designed to induce VIGS. Several time lapses between TRV and TYLCSV infections
336 were previously assayed and finally the best results were obtained when transgenic 2IRGFP
337 plants were agroinfected TYLCSV immediately after the infection with TRV-PCNA.

338 Ten days after the infection a phenotype described for PCNA silencing in N. benthamiana was
339 observed. Primary growth is interrupted at the apical meristem, and new leaves show
340 progressively reduced expansion. Although transgenic plants infected with TRV-PCNA seemed
341 to develop less symptoms of TYLCSV infection than control plants, the PCNA-silenced phenotype
342 displayed by these plants somehow obscured the analysis of this difference.

However, a difference is clearly noticed when the plants were illuminated with UV, since GFP vein-pattern fluorescence is almost undetectable on most leaves of PCNA-silenced plants located above the inoculation point, only isolated dots of green fluorescence were noticed (Morilla et al., 2006). The absence of fluorescence in these plants indicated that TYLCSV replication is impaired when the level of PCNA in the cell is reduced. This result was confirmed by Southern blot, TYLCSV DNA accumulation showed a significantly decrease but not completely suppressed. This is not an unexpected result, as silencing of an endogenous gene by VIGS is not uniform and it does not occur in whole plant.

2. SCE1: A 345 bp fragment of N. benthamiana NbSCE1 was cloned in a TRV vector to infect 2IRGFP plants 2 days before that the same plants were agroinfected with TYLCSV. As it was mentioned above, the timing of both infections was selected based on the results obtained with different timing lapses. Plants infected with TRV-NbSCE1 showed a distinctive phenotype with reduction in internodes distance and in the expansion of leaves. When TYLCSV infected leaves from SCE1-silenced plants are observed under UV light and compare with non-silenced leaves, a clear difference in green fluorescence is noticed, lighting up that SCE1 expression is required for TYLCSV infection (Figure 2C)

The experiments achieving PCNA and NbSCE1 silencing demonstrate the great potential of the system to perform a wide screening to identify plant proteins required for viral infection, as well as to determine the effect of the suppression of a certain host gene in viral replication or movement. Although the specificity of the interaction between Rep and the IR limit the use of the plant to TYLCSV, it is expected that most of the host factors identified with TYLCSV will be also needed for other TYLCV and geminivirus infections. The search with other geminivirus will require the construction of new transgenic plants carrying the construct with its own IR.

Several points should be borne in mind when interpreting a VIGS phenotype to avoid any misreading of the results. First, the absence of a phenotype does not necessarily rule out the involvement of the target gene in the trait of interest. VIGS is never complete, thus some silencing phenotypes might be missed due to some residual level of mRNA might still be supporting target-gene function in the viral vector-transformed plants. Second, it is conceivable that fortuitous sequence similarity between the cloned insert and an unknown mRNA might be responsible for the phenotype. To rule out this kind of artefact, a second non-overlapping insert from the same target gene should be used: if the target gene has been correctly identified this second insert would reproduce the original VIGS phenotype. Finally, the development of pleiotropic effects is an issue that needs to be addressed when interpreting VIGS experiments.

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482

483 FIGURE LEGEND

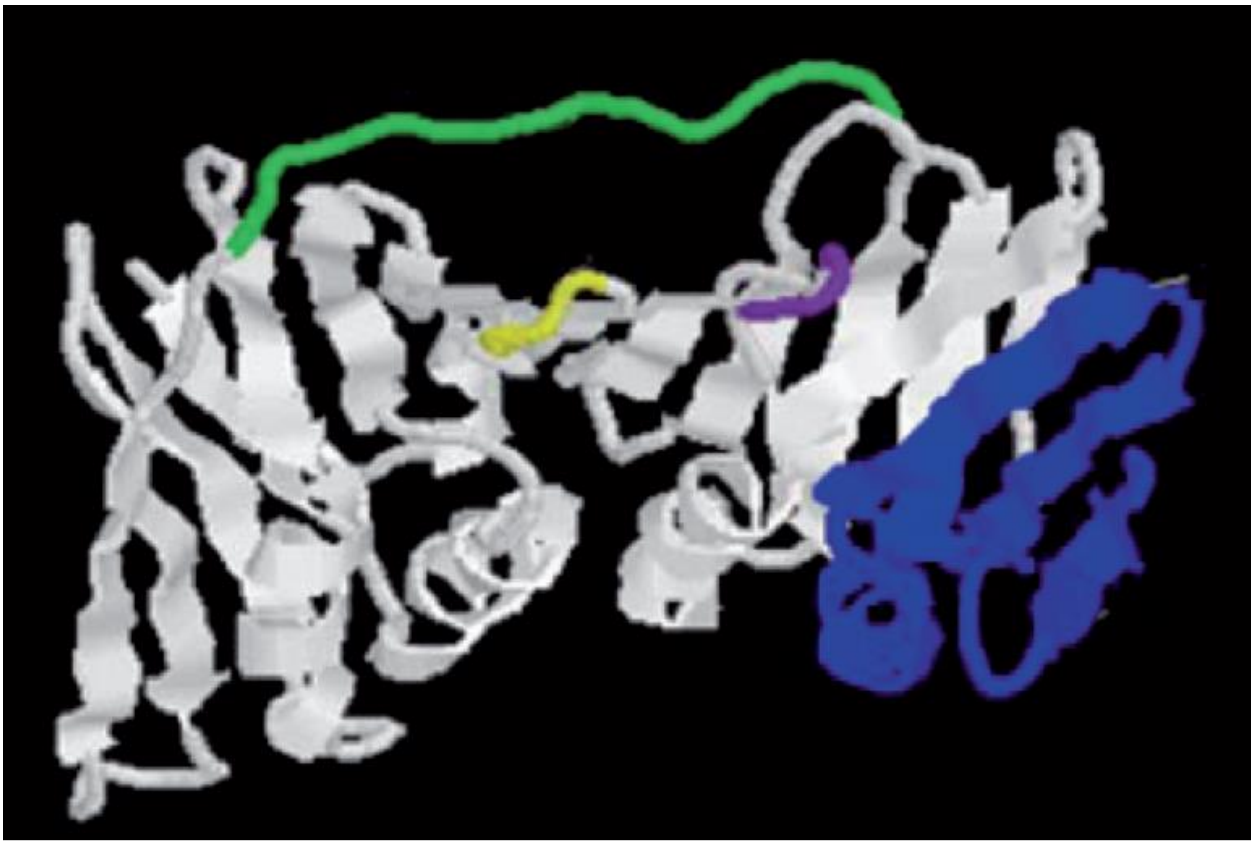
484 Figure 1. 3D model of the L. esculentum PCNA monomer. Loop structures are indicated: central
485 loop in yellow, long loop in green, and C-terminal tale in purple. The minimal interacting domain
486 of L. esculentum PCNA (amino acids 132–187) is in blue.

487 Figure 2. (A) Formation of episomal replicon in 2IRGFP transgenic plants infected with TYLCSV.
488 Transgen contains a direct repeat of IR encompassing a GFP expression cassette. Rep
489 expressed from the virus is able to recognize IR in the transgen and induce the formation of

episomal replicons to express more GFP. (B) Leave from a 2IRGFP transgenic plants 3 weeks after infection with TYLCSV. (C) Effect of SCE1 Tobacco rattle virus (TRV)-induced silencing on GFP expression. Transgenic plants were infected with TYLCSV and TRV or with TYLCSV and the TRV vector containing the SCE1 fragment (TRV-SCE1). Leaves were photographed under visible (left) or UV light (right) 3 weeks after infection.

Table 1. Plants proteins that interact with Rep and/or REn proteins from begomoviruses. IMYMV: Indian Mung bean yellow mosaic virus; CaLCuV: Cabbage leaf curl virus

Plant proteins	Virus	Gene	Reference
pRBR (maize)	TGMV	Rep	Ach et al., 1997
""	TYLCV	Rep	Arguello-Astorga et al., 2004
""	CaLCuV	Rep	Arguello-Astorga et al., 2004
""	TGMV	REn	Settlage et al., 2001
""	TYLCV	REn	Settlage et al., 2005
PCNA (tomato)	TYLCSV	Rep	Castillo et al.,. 2003
""""	TYLCV	REn	Settlage et al., 2005
"(tomato/ N. benthamiana)	TYLCSV	REn	Castillo et al., 2003
"(pea)	IMYMV	Rep	Bagewadi et al., 2004
SCE1(Arabidopsis/N.benthamiana)	TYLCSVT	Rep	Castillo et al., 2004
"	GMV	Rep	Castillo et al., 2004
	ACMV	Rep	Castillo et al., 2004
Histone H3 (Arabidopsis)	TGMV	Rep	Kong and Hanley-Bowdoin, 2002
Mitotic kinase (Arabidopsis)	TGMV	Rep	Kong and Hanley-Bowdoin, 2002
GRIK kinase (Arabidopsis)	TGMV	Rep	Kong and Hanley-Bowdoin, 2002
SINAC1 (tomato)	TYLCV	REn	Selth et al., 2005



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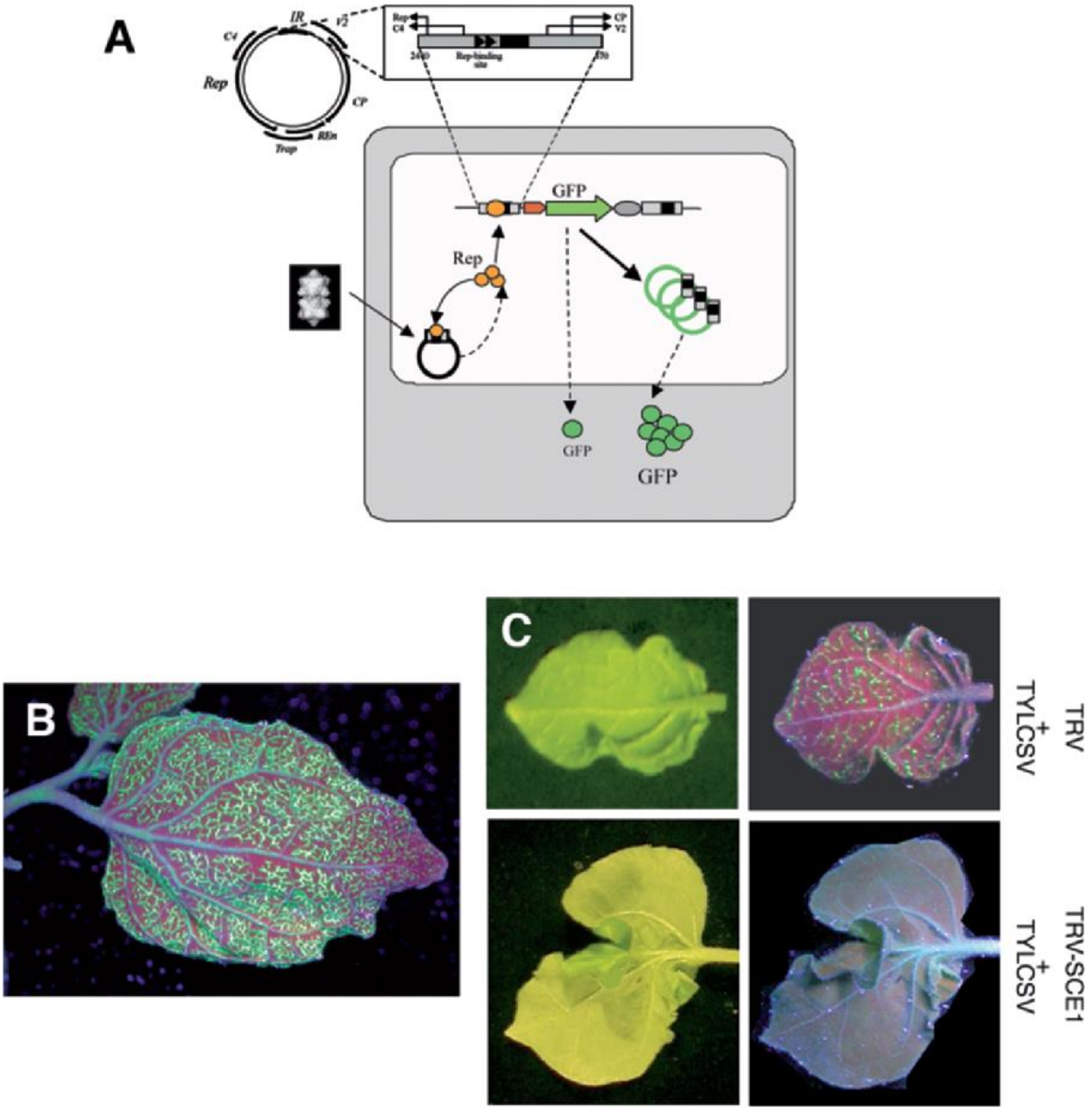

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