V2 from a curtovirus is a suppressor of post-transcriptional gene silencing

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Keywords: Geminivirus; BCTV; RNA silencing; silencing suppressor; V2; RDR6. Abbreviations: AGO, Argonaute protein; AMP, Arabidopsis line containing a construct to express a GFP-PVX AMPLICON; BCTV, Beet curly top virus; DCL, Dicer-like ribonucleases; ER, endoplasmic reticulum; PTGS, post-transcriptional gene silencing; PVX, Potato virus X; RDRs, RNA-dependent RNA polymerases; RFP-HDEL, red fluorescent protein targeted to the ER by way of a C-terminal HDEL sequence; SGS3, suppressor of gene silencing 3; siRNAs, small interfering RNAs; SS, Arabidopsis line containing a construct to express the SUC:SUL-hairpin; TGS, transcriptional gene silencing; vsiRNAs, viral small interfering RNAs.

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ABSTRACT:

The suppression of gene silencing is a key mechanism for the success of viral infection in plants. DNA viruses from the Geminiviridae family encode several proteins that suppress transcriptional and post-transcriptional gene silencing (TGS/PTGS). In Begomovirus, the most abundant genus of this family, three out of six genome-encoded proteins, namely C2, C4 and V2, have been shown to suppress PTGS, with V2 being the strongest PTGS suppressor in transient assays. Beet curly top virus (BCTV), the model species for the Curtovirus genus, is able to infect the widest range of plants among geminiviruses. In this genus, only one protein, C2/L2, has been described as inhibiting PTGS. We show here that, despite the lack of sequence homology with its begomoviral counterpart, BCTV V2 acts as a potent PTGS suppressor, possibly by impairing the RDR6 (RNA-dependent RNA polymerase 6)/suppressor of gene silencing 3 (SGS3) pathway.

RESULTS AND DISCUSSION:

Geminiviruses constitute a group of plant viruses with circular, single-stranded DNA genomes packaged within geminate particles that infect a wide range of plants [1]. Among the family Geminiviridae, the genera Mastrevirus, Begomovirus and Curtovirus comprise most of the viral species infecting dicotyledonous plants. Monopartite begomoviruses and curtoviruses possess similar genome structures, encoding six and seven multifunctional proteins, respectively [2]. In both cases, the virion-sense strand contains two open reading frames (ORFs) (V2 and the coat protein, CP), while a third one (V3) is only present in curtoviruses; four ORFs are present in the complementary sense strand (Rep, C2, C3 and C4) (Fig. S1a, available in the online Supplementary Material).

In plants, RNA silencing is the main antiviral mechanism. RNA silencing is initiated when viral double-stranded RNA (dsRNA) is recognized by a set of Dicer-like (DCL) ribonucleases and processed into 21 to 24-nt primary viral small interfering RNAs (vsiRNAs). In Arabidopsis thaliana DCL4, which produces 21-nt vsiRNAs, is the primary sensor of. viral dsRNAs; DCL2 produces 22-nt vsiRNAs, while DCL3 targets viral dsRNA to generate 24-nt vsiRNAs. DCL1 is only a minor contributor to vsiRNA formation for RNA and ssDNA viruses [3]. In the amplification step, one or more host-encoded RNA-dependent RNA polymerases (RDRs) use viral single-stranded RNA to synthesize dsRNA, which serves as a substrate for the generation of secondary vsiRNAs by DCL proteins. In Arabidopsis RDR1, RDR6 and possibly RDR2 have been implicated in vsiRNA production [4]. Both primary and secondary vsiRNAs support the systemic silencing that spreads throughout the plant. vsiRNAs associate with distinct Argonaute (AGO)-containing effector complexes, where they provide targeting specificity for RNA or DNA through a sequence homology-dependent mechanism. For a comprehensive picture of the silencing pathways there are several excellent reviews [3, 5].

All of the plant viruses examined to date encode at least one protein that suppresses antiviral silencing (viral suppressors of RNA silencing) by targeting different steps in the pathway, such as the hindrance of small interfering RNA(siRNA) production, interference with siRNA loading into silencing effectors, or direct or indirect inhibition of the activity of silencing-related proteins [4, 6]. Geminiviruses must confront both transcriptional (TGS) and posttranscriptional gene silencing (PTGS) to achieve successful infections [7, 8]. Since the first description of the begomovirus C2 protein as a silencing suppressor [9], several reports have shown that C4, V2 and Rep can also suppress gene silencing in this genus [7]. In curtoviruses, only the C2/L2 protein has been described as acting as a PTGS and TGS suppressor [10–12].

V2 from Old World begomoviruses has been described as a PTGS and TGS suppressor [13–20]. This protein is proposed to suppress PTGS by interfering with siRNAs produced via the RDR6-mediated amplification pathway, either through direct interaction with the RDR6 interactor suppressor of gene silencing 3 (SGS3), or through competition for dsRNA substrates [21, 22]. Additionally, it has been suggested that V2 could also sequester siRNAs [18]. Begomovirus V2 is also involved in viral movement, is required for full infection, and elicits hypersensitive response (HR)-like cell death when expressed from a Potato virus X (PVX)-derived vector [15, 16, 19, 23–25]. Less is known about the function of curtovirus V2. Although begomovirus and curtovirus V2 ORFs seem to be orthologous, based on genome location and length, their homology at the protein level, which is high within each genus, is extremely poor (Fig. S1). As in begomoviruses, V2-defective curtoviruses produce low levels of viral DNA in infected tissues [26, 27], but the role of curtovirus V2 in gene silencing suppression has not yet been investigated.

As a first step to characterize the V2 protein from the model species of the Curtovirus genus, Beet curly top virus (BCTV), we determined its subcellular localization upon transient expression of GFP-fused versions in Nicotiana benthamiana. To obtain the plasmids to express the GFP-V2 or the V2-GFP fusion proteins, a fragment containing the GFP ORF was amplified from pBINGFP [28] and cloned into the binary plasmid pBINX1 [29] to yield pBINX1-GFP1 or pBINX1-GFP2, respectively. Afterward, a fragment containing the V2 ORF was amplified from pBIN1.2 [30] and cloned into pBINX1-GFP1 or pBINX1-GFP2, yielding pBI-GFP-V2BC or pBI-V2BC-GFP, respectively. The primers, primer combinations and cloning details are shown in Tables S1–S3, respectively. N. benthamiana leaves were agroinfiltrated as described in [15], collected 2 days later and visualized using a Leica TCS SP8 confocal microscope. V2-GFP and GFP-V2 localized in the nucleus and in the cellular periphery (Fig. S2a). These results are in agreement with the subcellular targeting of begomovirus V2 (14, 18, 19, 31–33). A close-up of the images showed a subcellular structure that was reminiscent of that for the endoplasmic reticulum (ER) (Fig. S2b). To confirm whether BCTV V2 localizes in the ER, as described for the begomoviruses Tomato yellow leaf curl virus and Tomato leaf curl Java virus [31, 32], GFP-V2 was co-infiltrated with the ER marker ‘red fluorescent protein targeted to the ER by way of a Cterminal HDEL sequence’ (RFP-HDEL) [34]. Upon coexpression, overlap of the fluorescent signals was detected (Fig. S2a), suggesting localization of V2-GFP in the ER (the Pearson’s coefficient values and line intensity profiles are shown in Fig. S2a). These results indicate that the BCTV V2 localizes in the ER network, from the perinuclear region to the cell periphery, besides accumulating in the nucleus.

To determine whether BCTV V2 acts as a PTGS suppressor, we carried out transient expression assays in N. benthamiana. Leaves were co-infiltrated with two Agrobacterium cultures containing constructs to express GFP (35S:GFP) [28] and BCTV V2. (A PCR fragment containing the full V2 ORF was amplified from pBIN1.2 and cloned into pBSSKII+ to yield pV2BC, while a restriction fragment containing the V2 ORF was cloned into pBINX1 to yield pBIV2BC.) As controls, leaves were co-infiltrated with 35S:GFP and either the empty vector (C-) or a plasmid expressing the P19 silencing suppressor from Tomato bushy stunt virus [35] as a positive control. As expected, leaves co-infiltrated with P19 showed stronger green fluorescence compared to tissues co-infiltrated with the empty vector, in which fluorescence decreased as a result of GFP RNA silencing activation [36] (Fig. 1a). V2 expression produced an increase in fluorescence similar to that produced by P19, which correlated with an increase of GFP protein (Fig. 1b). To assess whether the expression of V2 alters siRNAs accumulation, the relative levels of the GFP-specific siRNAs were determined by Northern blot as described in [15] (Fig. 1c). A decrease in GFP siRNAs that was similar to that obtained by expressing V2 from different begomoviruses [15] was observed when the BCTV V2 protein was coexpressed with GFP (Fig. 1c). The expression of V2 in the analysed tissues was confirmed by RT-semi-quantitative PCR (Fig. 1d).

To find out the effect of BCTV V2 on PTGS short- and longdistance movement, leaves of N. benthamiana 16 c plants [37] were co-infiltrated with constructs expressing GFP and eitherV2, P19 or the empty vector (C-). As in the previous assay,co-infiltration with BCTV V2 produced an increase in GFPfluorescence that was comparable to that produced by P19 Fig. 1e). In order to determine whether BCTV V2 can block the cell-to-cell spread of RNA silencing, GFP expression in the cells around the agroinfiltrated area was monitored. As described previously [38], in plants infiltrated with the emptyvector, a red fluorescent ring caused by the decrease in GFP expression produced by local cell-to-cell movement of the silencing signal was formed around the infiltrated area. This ring was also observed in leaves infiltrated with V2, whereas no red ring was detected in the leaves expressing P19 [38] (Fig. 1f). These results suggest that V2 from BCTV cannot suppress short-range (cell-to-cell) spread of gene silencing. Infiltrated 16 c plants were also monitored for the initiation of systemic silencing in the newly emerging leaves. At 19 days post infiltration (p.i.), while systemic silencing was almost complete in plants infiltrated with the empty vector, GFP expression persisted in all newly emerging leaves of plants infiltrated with P19, as previously reported [39]. In contrast, in plants co-infiltrated with V2, apical leaves maintained GFP expression in some areas but showed a vein-centred pattern of GFP silencing (Fig. 1g). At 30 days p.i., GFP silencing was complete in plants infiltrated with V2, whereas plants infiltrated with

P19 kept GFP expression in all tissues (Fig. 1g). These results demonstrate that the curtovirus V2 delays the long-distance spread of RNA silencing, but does not block the process entirely.

To gain more insight into the gene silencing suppression mechanism of V2, we took advantage of the Arabidopsis lines containing constructs to express the SUC:SUL-hairpin(SS) [38] or the GFP-PVX amplicon (AMP) [40]. In these transgenic lines, the silencing of the endogenous SUL gene (SS) or a GFP reporter gene (AMP), is dependent upon the activity of DCL4 and AGO1, but only the latter is sensitive to the inactivation of RDR6 function [41–44]. Arabidopsis SS and AMP plants were transformed using the floral dip method [45], with the same V2 expression cassette used for the gene silencing assays (pBIV2BC) (Fig. 1), and transgenic lines expressing BCTV V2 were selected (Fig. 2b, e; transgene expression was analysed by RT-qPCR).

Visual analysis of the transgenic lines showed that the expression of V2 did not produce a change in the chlorotic phenotype of SS plants (Fig. 2a). By contrast, when V2 was expressed in the AMP line, reactivation of GFP expression in multiple foci was evident in the leaves (Fig. 2c), along with an increase in GFP siRNA accumulation (Fig. 2d, quantified in Fig. S3a), resembling the effect of the introduction of the rdr6-15 mutation in the AMP line [41]. RTqPCR analysis using three reference genes (actin, EF1a and SAND) confirmed that the reactivation of GFP expression correlated with V2 levels in the transgenic lines tested (Figs 2e and S3b). To test whether the presence of V2 also phenocopies the rdr6 mutation in trans-acting RDR6 activity, we used the AMPxGFP plant system generated by crossing AMP lines with a transgenic line expressing GFP from a 35S promoter (GFP142 line). In the AMPxGFP system, PVX-GFP replication triggers the RDR6-dependent silencing of the GFP transgene. Introducing the rdr6 mutation into the AMPxGFP line led to a homogenous green fluorescent phenotype, owing to resumed GFP transgene expression (Fig. 2f) [40, 41]. We crossed four independent V2-AMP lines with the GFP142 line. For each of the four V2-AMP lines used, several replicate crosses with the GFP142 plants were made, and progeny were selected with Basta. As shown in Fig. 2(f), V2 expression produced a reversion of GFP silencing comparable to that produced by mutation of RDR6. Similar results were obtained from the crosses with all V2-AMP lines tested (data not shown). Taken together, these results support the view that V2- mediated silencing suppression operates via hindrance of the RDR6 function either directly or indirectly, but is unable to alleviate the silencing of a plant endogenous gene (SUL) triggered by a hairpin in a RDR6-independent manner.

As an additional approach to analyse whether RDR6 and other components of the antiviral silencing pathway, such as DCL2, DCL4 or RDR2, are genetic targets of V2, we infected Arabidopsis mutants deficient in those genes with wild-type and a V2 BCTV mutant (BCTV-DV2)

previously shown to produce a symptomless infection in N. benthamiana, associated with low levels of viral DNA [27]. Infective BCTV clones for the wild-type (pBIN1.2) and BCTV-DV2 mutant were described in [30] and [27]. Arabidopsis Col-0 plants infected with BCTV were clearly symptomatic, whereas plants infected with BCTV-DV2 did not develop symptoms (Fig. 3a). Quantitative-PCR analysis showed that viral DNA accumulation was significantly reduced in plants infected with BCTV-DV2 (Fig. 3b), indicating that V2 is also required for a full infection in Arabidopsis.

The results from the infections also showed that the amount of viral DNA accumulated in rdr6-15, but not in rdr2-1 plants infected with the wild-type virus, is higher than in the wild-type, indicating that RDR6, but not RDR2, plays a role in mediating defence against BCTV. Similar results have been reported for a bipartite begomovirus, Cabbage leaf curl virus [46]. Whether the RDR6-dependent mechanism of action against geminiviruses is based on the generation of secondary viral siRNA (vsiRNA) or siRNA from plant defence gene families [5] has to be further analysed.

Our data strongly indicate that V2 from BCTV suppresses PTGS by interfering with the RDR6-dependent amplification pathway. However, this suppression seems to only be partial, since higher amounts of wild-type viral DNA accumulated in rdr6 plants compared to Col-0 plants. The partial suppression of RDR6 activity by V2 is also supported by the limited reduction in the accumulation of endogenoustasiRNAs (tasiR255) detected in most of the transgenic plants expressing BCTV V2 (Fig. S3c). Interestingly, the viral DNA content in rdr6 plants infected with BCTV-DV2 was significantly lower than that in these plants when infected with wild-type virus, indicating that V2 is required to achieve higher viral accumulation, even when RDR6 is not present. This suggests that V2 interferes with other RDR6-independent defence mechanisms, or that it is required for some other function. The fact that the wildtype and mutant virus spawn similar amounts of viral DNA in a dcl2/4 double-mutant indicates that this additional defence mechanism that is suppressed by V2 depends on DCL2/4 function. One possibility is that since V2 only suppresses PTGS when it is established through RNA amplification, it will also impair the RDR1-dependent antiviral RNA-silencing pathway, which, as with the RDR6 pathway, is dependent on DCL2/4 (5). The symptoms of rdr6 and dcl2/4 plants infected with wild-type or BCTV V2 mutant are shown in Fig. S4.

Taking into account all the results obtained in this work, we conclude that in spite of limited sequence homology, BCTV V2, as with its begomovirus counterpart: (i) is required for a systemic infection, (ii) accumulates in the nucleus and the ER, and (iii) is a strong suppressor of intracellular

PTGS by impairing the RDR6/SGS3 pathway without impacting on local cell-to-cell silencing movement, and producing a delay in the spread of systemic silencing.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

1. Zerbini FM, Briddon RW, Idris A, Martin DP, Moriones E et al. ICTV virus taxonomy profile: Geminiviridae. J Gen Virol 2017;98:131–133.

2. Fondong VN. Geminivirus protein structure and function. Mol Plant Pathol 2013;14:635–649.

3. Bologna NG, Voinnet O. The diversity, biogenesis, and activities of endogenous silencing small RNas in Arabidopsis. Annu Rev Plant Biol 2014;65:473–503.

4. Pumplin N, Voinnet O. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. Nat Rev Microbiol 2013;11:745–760.

5. Borges F, Martienssen RA. The expanding world of small RNAs in plants. Nat Rev Mol Cell Biol 2015;16:727–741.

6. Csorba T, Kontra L, Burgyan J. Viral silencing suppressors: tools forged to fine-tune host-pathogen coexistence. Virology 2015;479– 480:85–103.

7. Hanley-Bowdoin L, Bejarano ER, Robertson D, Mansoor S. Geminiviruses: masters at redirecting and reprogramming plant processes. Nat Rev Microbiol 2013;11:777–788.

8. Pooggin MM. How can plant DNA viruses evade siRNA-directed DNA methylation and silencing? Int J Mol Sci 2013;14:15233– 15259.

9. Raja P, Wolf JN, Bisaro DM. RNA silencing directed against geminiviruses: post-transcriptional and epigenetic components. Biochim Biophys Acta 2010;1799:337–351.

10. Buchmann RC, Asad S, Wolf JN, Mohannath G, Bisaro DM. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. J Virol 2009;83:5005–5013.

11. Wang H, Buckley KJ, Yang X, Buchmann RC, Bisaro DM. Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. J Virol 2005;79:7410–7418.

12. Zhang Z, Chen H, Huang X, Xia R, Zhao Q et al. BSCTV C2 attenuates the degradation of SAMDC1 to suppress DNA methylation-mediated gene silencing in Arabidopsis. Plant Cell 2011;23: 273–288.

13. Amin I, Hussain K, Akbergenov R, Yadav JS, Qazi J et al. Suppressors of RNA silencing encoded by the components of the cotton leaf curl begomovirus-betasatellite complex. Mol Plant Microbe Interact 2011;24:973–983.

14. Chowda-Reddy RV, Achenjang F, Felton C, Etarock MT, Anangfac MT et al. Role of a geminivirus AV2 protein putative protein kinase C motif on subcellular localization and pathogenicity. Virus Res 2008;135:115–124.

15. Luna AP, Morilla G, Voinnet O, Bejarano ER. Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. Mol Plant Microbe Interact 2012;25:1294–1306.

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

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

253 18. Zhang J, Dong J, Xu Y, Wu J. V2 protein encoded by Tomato yellow leaf curl China virus is
254 an RNA silencing suppressor. Virus Res 2012;163:51–58.

255 19. Zrachya A, Glick E, Levy Y, Arazi T, Citovsky V et al. Suppressor of RNA silencing encoded
256 by Tomato yellow leaf curl virus-Israel. Virology 2007;358:159–165.

257 20. Wang B, Li F, Huang C, Yang X, Qian Y et al. V2 of tomato yellow leaf curl virus can suppress
258 methylation-mediated transcriptional gene silencing in plants. J Gen Virol 2014;95:225–230.

259 21. Fukunaga R, Doudna JA. dsRNA with 5' overhangs contributes to endogenous and antiviral
260 RNA silencing pathways in plants. EMBO J 2009;28:545–555.

261 22. Glick E, Zrachya A, Levy Y, Mett A, Gidoni D et al. Interaction with host SGS3 is required for
262 suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. Proc Natl Acad Sci USA
263 2008;105: 157–161.

264 23. Hak H, Levy Y, Chandran SA, Belausov E, Loyter A et al. TYLCV-Is movement in planta does
265 not require V2 protein. Virology 2015; 477:56–60.

266 24. Iqbal Z, Sattar MN, Kvarnheden A, Mansoor S, Briddon RW. Effects of the mutation of selected
267 genes of Cotton leaf curl Kokhran virus on infectivity, symptoms and the maintenance of cotton
268 leaf curl multan betasatellite. Virus Res 2012;169:107– 116.

269 25. Mubin M, Amin I, Amrao L, Briddon RW, Mansoor S. The hypersensitive response induced
270 by the V2 protein of a monopartite begomovirus is countered by the C2 protein. Mol Plant Pathol
271 2010;11:245–254.

272 26. Hormuzdi SG, Bisaro DM. Genetic analysis of beet curly top virus: examination of the roles of
273 L2 and L3 genes in viral pathogenesis. Virology 1995;206:1044–1054.

274 27. Stanley J, Latham JR, Pinner MS, Bedford I, Markham PG. Mutational analysis of the
275 monopartite geminivirus beet curly top virus. Virology 1992;191:396–405.

276 28. Haseloff J, Siemering KR, Prasher DC, Hodge S. Removal of a cryptic intron and subcellular
277 localization of green fluorescent protein are required to mark transgenic Arabidopsis plants
278 brightly. Proc Natl Acad Sci USA 1997;94:2122–2127.

279 29. S_anchez-Dur_an MA, Dallas MB, Ascencio-Ibañez JT, Reyes MI, Arroyo-Mateos M et al.
280 Interaction between geminivirus replication protein and the SUMO-conjugating enzyme is
281 required for viral infection. J Virol 2011;85:9789–9800.

282 30. Briddon RW, Watts J, Markham PG, Stanley J. The coat protein of beet curly top virus is
283 essential for infectivity. Virology 1989;172: 628–633.

284 31. Rojas MR, Jiang H, Salati R, Xoconostle-C_azares B, Sudarshana MR et al. Functional
285 analysis of proteins involved in movement of the monopartite begomovirus, Tomato yellow leaf
286 curl virus. Virology 2001;291:110–125.

287 32. Sharma P, Gaur RK, Ikegami M. Subcellular localization of V2 protein of Tomato leaf curl Java
288 virus by using green fluorescent protein and yeast hybrid system. Protoplasma 2011;248: 281-
289 288.

290 33. Rothenstein D, Krenz B, Selchow O, Jeske H. Tissue and cell tropism of Indian cassava
291 mosaic virus (ICMV) and its AV2 (precoat) gene product. Virology 2007;359:137–145.

292 34. Liu CJ, Dixon RA. Elicitor-induced association of isoflavone Omethyltransferase with
293 endomembranes prevents the formation and 7-O-methylation of daidzein during isoflavonoid
294 phytoalexin biosynthesis. Plant Cell 2001;13:2643–2658.

295 35. Silhavy D, Moln_ar A, Lucioli A, Szittya G, Hornyik C et al. A viral protein suppresses RNA
296 silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J
297 2002;21: 3070–3080.

298 36. Voinnet O, Lederer C, Baulcombe DC. A viral movement protein prevents spread of the gene
299 silencing signal in Nicotiana benthamiana. Cell 2000;103:157–167.

300 37. Ruiz MT, Voinnet O, Baulcombe DC. Initiation and maintenance of virus-induced gene
301 silencing. Plant Cell 1998;10: 937–946.

302 38. Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O. Transitivity-dependent and -
303 independent cell-to-cell movement of RNA silencing. EMBO J 2003;22:4523–4533.

304 39. Hamilton A, Voinnet O, Chappell L, Baulcombe D. Two classes of short interfering RNA in
305 RNA silencing. EMBO J 2002;21:4671–4679.

306 40. Dalmay T, Hamilton A, Mueller E, Baulcombe DC. Potato virus X amplicons in arabidopsis
307 mediate genetic and epigenetic gene silencing. Plant Cell 2000;12:369–380.

308 41. Moissiard G, Parizotto EA, Himber C, Voinnet O. Transitivity in Arabidopsis can be primed,
309 requires the redundant action of the antiviral dicer-like 4 and Dicer-like 2, and is compromised by
310 viral-encoded suppressor proteins. RNA 2007;13: 1268–1278.

311 42. Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC. An RNAdependent RNA
312 polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a
313 transgene but not by a virus. Cell 2000;101:543–553.

314 43. Dunoyer P, Himber C, Voinnet O. DICER-LIKE 4 is required for RNA interference and
315 produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing
316 signal. Nat Genet 2005;37:1356–1360.

317 44. Dunoyer P, Himber C, Ruiz-Ferrer V, Alioua A, Voinnet O. Intra and intercellular RNA
318 interference in Arabidopsis thaliana requires components of the microRNA and heterochromatic
319 silencing pathways. Nat Genet 2007;39:848–856.

320 45. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated
321 transformation of Arabidopsis thaliana. Plant J 1998;16:735–743.

322 46. Blevins T, Rajeswaran R, Shivaprasad PV, Beknazariants D, Si-Ammour A et al. Four plant
323 Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. Nucleic Acids Res
324 2006; 34:6233–6246.

325 47. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
326 quantitative PCR and the 2DDCT method. Methods 2001;25:402–408.

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328 FIGURE LEGENDS

329 Fig. 1. (a–d) Local PTGS suppression activity in wild-type Nicotiana benthamiana. (a) Leaf discs
330 (10mm diameter) infiltrated with a mixture of two Agrobacterium tumefaciens cultures expressing
331 GFP and V2 from Beet curly top virus, under UV light at 4 days postinfiltration (p.i.). P19 and the
332 empty vector (C-) were used as controls. Molecular analysis of infiltrated tissues at 4 days p.i.
333 RNA and proteins were extracted from four to six leaves. (b) Total protein was subjected to
334 immunoblot analysis with an anti-GFP antibody. Coomassie blue staining of the protein blot is
335 shown as a loading control. (c) Total RNA was subjected to Northern blot analysis to detect GFP
336 siRNAs and sRNA U6 as a loading control. (d) Expression of viral protein V2 was confirmed by
337 RT-semi-quantitative PCR using Elongation factor alpha (NbE1Fa) as the reference gene. Similar
338 results were obtained in three independent experiments. (e–g) Systemic and non-autonomous
339 PTGS suppression activity in 16 c N. benthamiana plants. (e) Leaf discs infiltrated with two A.
340 tumefaciens cultures expressing GFP and V2 from Beet curly top virus, under UV light at 4 days
341 p.i. P19 and the empty vector (C-) were used as controls. (f) GFP expression in the cells

surrounding the agroinfiltrated area at 5 days p.i. Bar, 2 mm. (g) Agroinfiltrated 16 c plants under UV light at 19 (top panel) and 30 days p.i. (bottom panel). Four to six plants were agroinfiltrated per experiment. Similar results were obtained in three independent experiments.

Fig. 2. Expression of V2 from BCTV in transgenic SUC:SUL (SS) and amplicon (AMP) Arabidopsis lines. (a) Representative pictures of non-transformed (SS) and T2 kanamycin-resistant plants from the transgenic lines V2-SS1 and V2-SS2 are shown. A similar phenotype was observed in six other independent lines. (b) Total RNA extracted from leaves of transgenic V2-SS lines (1 and 2) was subjected to RT-qPCR to measure the V2 mRNA levels, which were normalized to actin. (c) Representative pictures of non-transformed amplicon plants (AMP) and T2 kanamycin-resistant plants from the transgenic lines V2-AMP1, V2-AMP3 and V2-AMP5 are shown. A similar phenotype was observed in three other independent lines. (d) Total RNA extracted from leaves of transgenic V2-AMP lines 1, 3 and 5 was subjected to Northern blot analysis to detect GFP siRNAs (siGFP) and sRNA U6 as the loading control. RNA from the homozygous AMP line and from the AMP line carrying the rdr6-15 mutation (AMP-rdr6-15) was used as negative and positive controls, respectively. (e) Total RNA extracted from leaves of transgenic V2-AMP lines (1, 3 and 5) was subjected to RT-qPCR to measure the V2 and GFP mRNA levels, which were normalized to actin. RNA from the homozygous AMP line was used as a control. Values are represented as the relative expression compared to that for the V2-AMP5 sample [1]. Bars represent the mean +/- the standard error from three technical replicates obtained from RNA extracted from 15 to 20 plants. (f) GFP expression in the progeny from the cross of the V2-amplicon and GFP-expressing Arabidopsis plants (AMPxGFP). Representative F1 plants resulting from the crosses of the amplicon and GFP lines, the V2-AMP5 and GFP lines (V2-AMP5XGFP) or a homozygous plant AMPxGFP containing the rdr6-15 mutation (AMPxGFP rdr6) are shown. Pictures were taken from plants under visible (left column) or UV light using either the GFP2 filter (allows the chlorophyll autofluorescence, middle column), or the GFP3 filter (only shows GFP fluorescence, right column).

Fig. 3. Arabidopsis infection with BCTV wild-type or V2 mutant (BCTV-DV2). (a) Symptoms induced in Arabidopsis Col-0 plants agroinoculated with BCTV or BCTV-DV2 at 28 days post-inoculation (p.i.). As a negative control, plants were agroinoculated with the empty vector (mock). (b) Analysis of viral DNA accumulation in Arabidopsis wild-type and mutant plants rdr2-1, rdr6-15 and dcl2/4 infected with BCTV or BCTV-DV2. DNA was extracted from five to six plants in each condition at 28 days p.i. and quantified by qPCR. The reaction mixture consisted of approximately 10 ng total DNA, primer mix (10 µM each) and SsoFast EvaGreen Supermix (Biorad, CA, USA) in a total volume of 10 µl. The PCR conditions were: 1 min at 95 C, and 40 cycles of 10 s at 95 C

and 15 s at 60 C. The reactions were performed using a C1000 touch thermal cycler (Biorad). A relative quantification real-time PCR method using the 2DDCT method [47] was performed to compare the amount of BCTV DNA between the different conditions, using actin as reference gene, and represented as the relative level compared to Col-0 plants infected with the wild-type BCTV (set to 1). The primers used in the qPCR are shown in Tables S1 and S2. Bars represent the mean +/- the standard error from three technical replicates obtained from DNA extracted from these five to six plants. Asterisks (*) indicate the BCTV-DV2-infected sample that is statistically different from the BCTV-infected sample on each Arabidopsis background (*P<0.05), as determined by Student's t-test. The plus signs (+) indicate the infected samples that are statistically different from Col-0 plants infected with BCTV (+P<0.05).

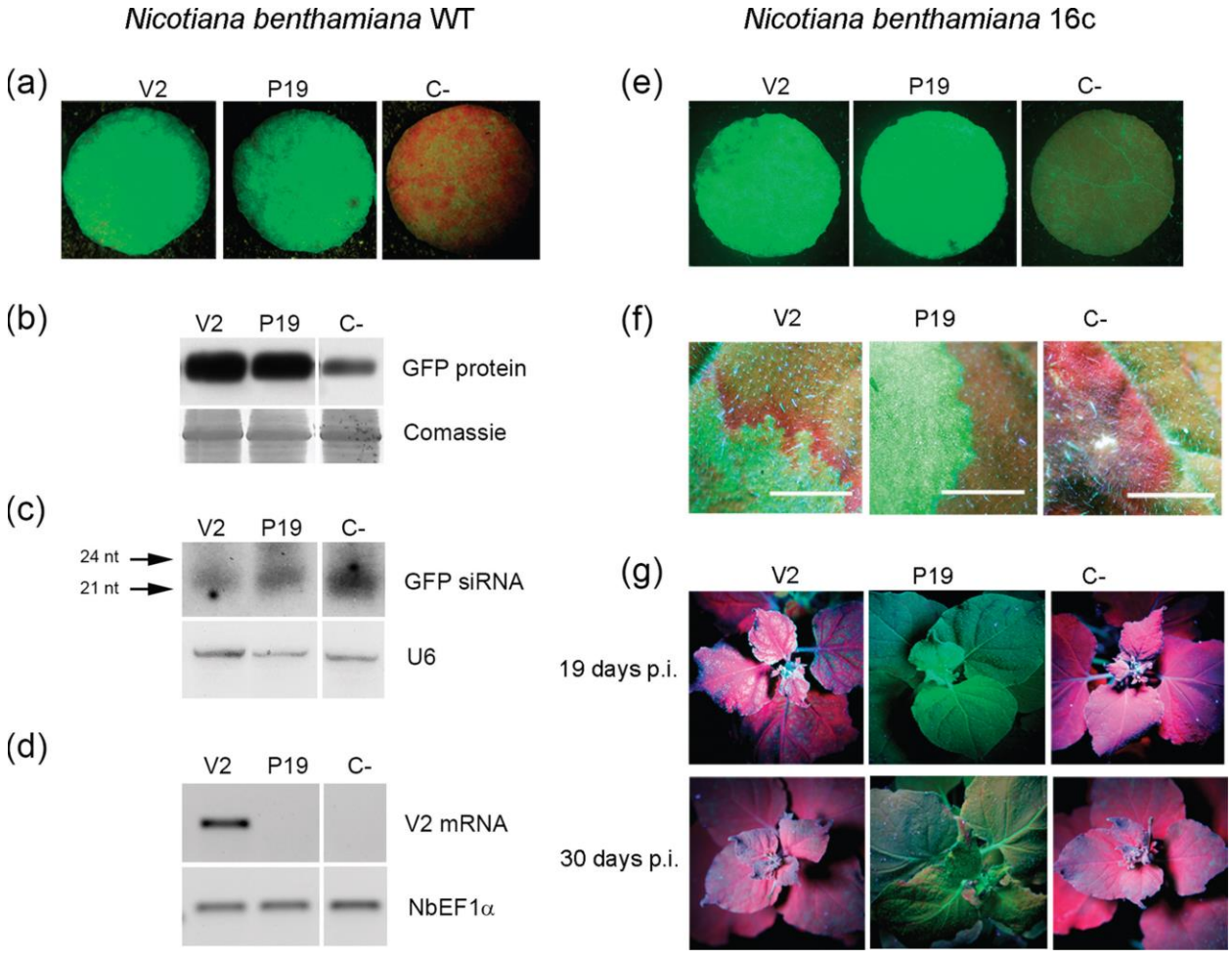
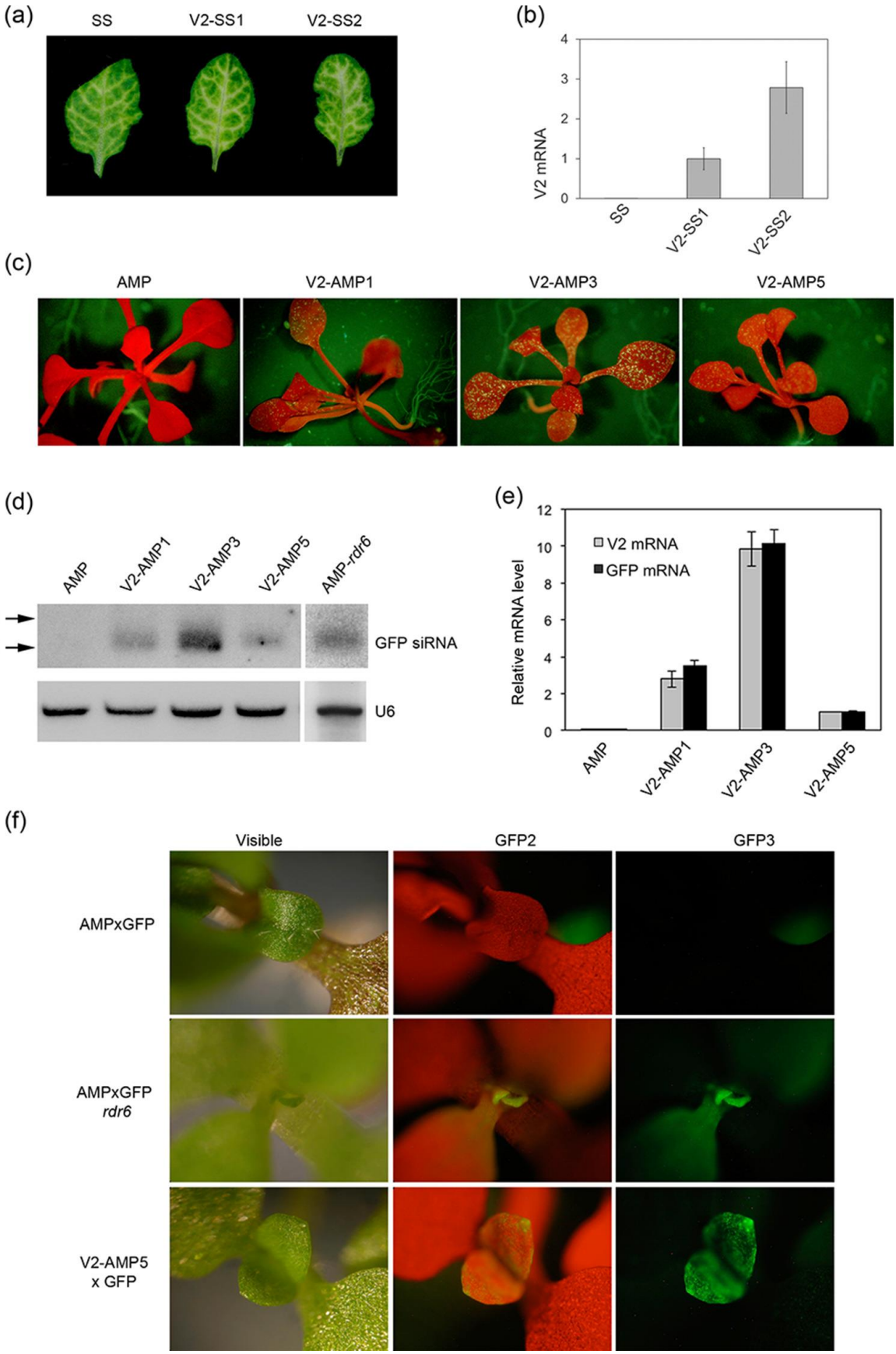


Fig. 1. (a–d) Local PTGS suppression activity in wild-type Nicotiana benthamiana. (a) Leaf discs (10mm diameter) infiltrated with a mixture of two Agrobacterium tumefaciens cultures expressing GFP and V2 from Beet curly top virus, under UV light at 4 days postinfiltration (p.i.). P19 and the empty vector (C-) were used as controls. Molecular analysis of infiltrated tissues at 4 days p.i.

RNA and proteins were extracted from four to six leaves. (b) Total protein was subjected to immunoblot analysis with an anti-GFP antibody. Coomassie blue staining of the protein blot is shown as a loading control. (c) Total RNA was subjected to Northern blot analysis to detect GFP siRNAs and sRNA U6 as a loading control. (d) Expression of viral protein V2 was confirmed by RT-semi-quantitative PCR using Elongation factor alpha (NbE1Fa) as the reference gene. Similar results were obtained in three independent experiments. (e–g) Systemic and non-autonomous PTGS suppression activity in 16 c N. benthamiana plants. (e) Leaf discs infiltrated with two A. tumefaciens cultures expressing GFP and V2 from Beet curly top virus, under UV light at 4 days p.i. P19 and the empty vector (C-) were used as controls. (f) GFP expression in the cells surrounding the agroinfiltrated area at 5 days p.i. Bar, 2 mm. (g) Agroinfiltrated 16 c plants under UV light at 19 (top panel) and 30 days p.i. (bottom panel). Four to six plants were agroinfiltrated per experiment. Similar results were obtained in three independent experiments.


(a)
SS
V2-SS1
V2-SS2
(b)
V2 mRNA
0
1
2
3
4
SS
V2-SS1
V2-SS2
(c)
AMP
V2-AMP1
V2-AMP3
V2-AMP5
(d)
AMP
V2-AMP1
V2-AMP3
V2-AMP5
AMP-rdr6
24 nt
21 nt
GFP siRNA
U6
(e)
Relative mRNA level
V2 mRNA
GFP mRNA
0
2
4
6
8
10
12
AMP
V2-AMP1
V2-AMP3
V2-AMP5
(f)
Visible
GFP2
GFP3
AMPxGFP
AMPxGFP
rdr6
V2-AMP5
x GFP

402

Fig. 2. Expression of V2 from BCTV in transgenic SUC:SUL (SS) and amplicon (AMP) Arabidopsis lines. (a) Representative pictures of non-transformed (SS) and T2 kanamycin-resistant plants from the transgenic lines V2-SS1 and V2-SS2 are shown. A similar phenotype was observed in six other independent lines. (b) Total RNA extracted from leaves of transgenic V2-SS lines (1 and 2) was subjected to RT-qPCR to measure the V2 mRNA levels, which were normalized to actin. (c) Representative pictures of non-transformed amplicon plants (AMP) and T2 kanamycin-resistant plants from the transgenic lines V2-AMP1, V2-AMP3 and V2-AMP5 are shown. A similar phenotype was observed in three other independent lines. (d) Total RNA extracted from leaves of transgenic V2-AMP lines 1, 3 and 5 was subjected to Northern blot analysis to detect GFP siRNAs (siGFP) and sRNA U6 as the loading control. RNA from the homozygous AMP line and from the AMP line carrying the rdr6-15 mutation (AMP-rdr6-15) was used as negative and positive controls, respectively. (e) Total RNA extracted from leaves of transgenic V2-AMP lines (1, 3 and 5) was subjected to RT-qPCR to measure the V2 and GFP mRNA levels, which were normalized to actin. RNA from the homozygous AMP line was used as a control. Values are represented as the relative expression compared to that for the V2-AMP5 sample [1]. Bars represent the mean +/- the standard error from three technical replicates obtained from RNA extracted from 15 to 20 plants. (f) GFP expression in the progeny from the cross of the V2-amplicon and GFP-expressing Arabidopsis plants (AMPxGFP). Representative F1 plants resulting from the crosses of the amplicon and GFP lines, the V2-AMP5 and GFP lines (V2-AMP5XGFP) or a homozygous plant AMPxGFP containing the rdr6-15 mutation (AMPxGFP rdr6) are shown. Pictures were taken from plants under visible (left column) or UV light using either the GFP2 filter (allows the chlorophyll autofluorescence, middle column), or the GFP3 filter (only shows GFP fluorescence, right column).

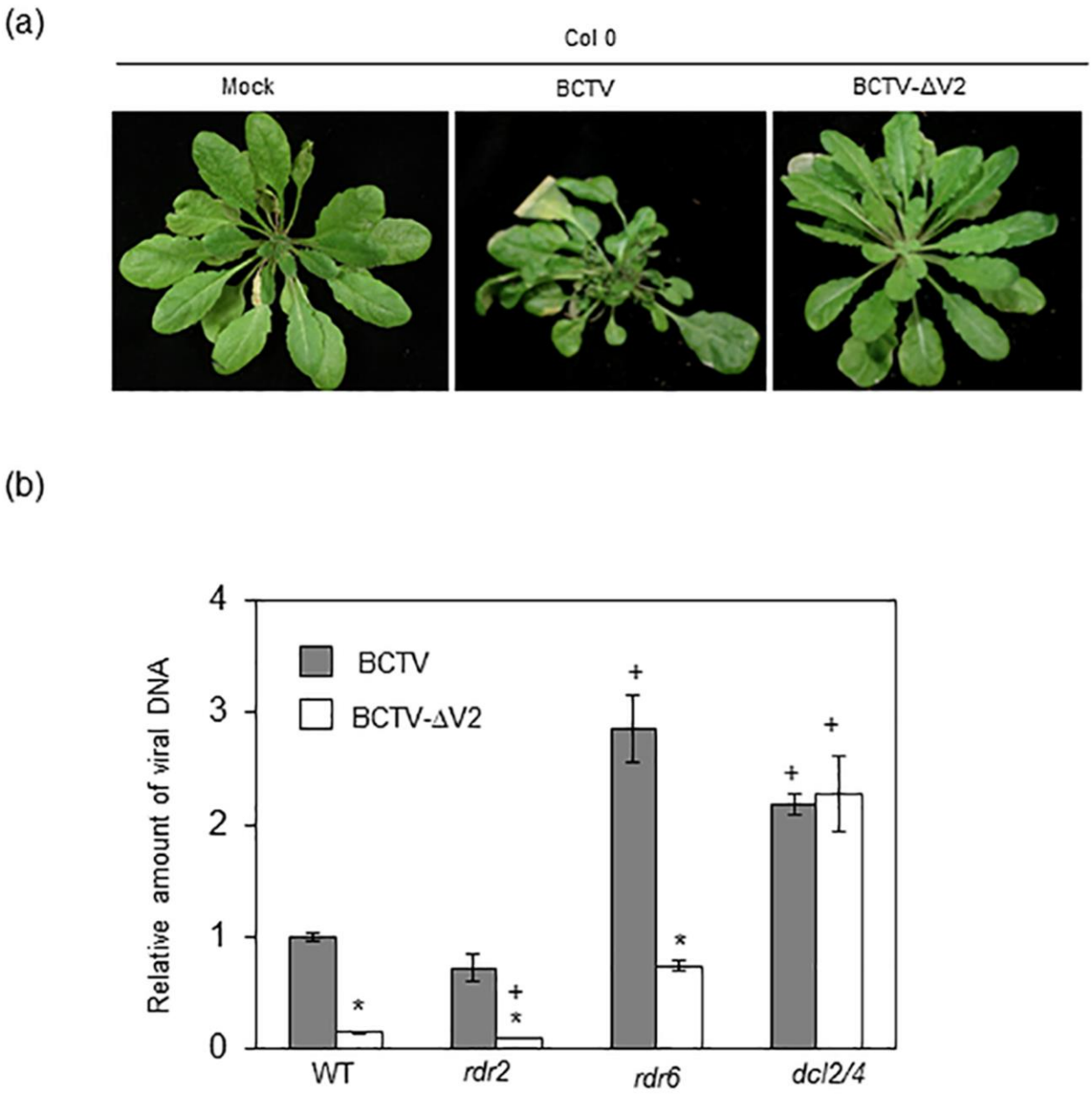


Fig. 3. Arabidopsis infection with BCTV wild-type or V2 mutant (BCTV-DV2). (a) Symptoms induced in Arabidopsis Col-0 plants agroinoculated with BCTV or BCTV-DV2 at 28 days post-inoculation (p.i.). As a negative control, plants were agroinoculated with the empty vector (mock). (b) Analysis of viral DNA accumulation in Arabidopsis wild-type and mutant plants rdr2-1, rdr6-15 and dcl2/4 infected with BCTV or BCTV-DV2. DNA was extracted from five to six plants in each condition at 28 days p.i. and quantified by qPCR. The reaction mixture consisted of approximately 10 ng total DNA, primer mix (10 µM each) and SsoFast EvaGreen Supermix (Biorad, CA, USA) in a total volume of 10 µl. The PCR conditions were: 1 min at 95 C, and 40 cycles of 10 s at 95 C and 15 s at 60 C. The reactions were performed using a C1000 touch thermal cycler (Biorad). A

relative quantification real-time PCR method using the 2DDCT method [47] was performed to compare the amount of BCTV DNA between the different conditions, using actin as reference gene, and represented as the relative level compared to Col-0 plants infected with the wild-type BCTV (set to 1). The primers used in the qPCR are shown in Tables S1 and S2. Bars represent the mean +/- the standard error from three technical replicates obtained from DNA extracted from these five to six plants. Asterisks (*) indicate the BCTV-DV2-infected sample that is statistically different from the BCTV-infected sample on each Arabidopsis background (*$P<0.05$), as determined by Student's t-test. The plus signs (+) indicate the infected samples that are statistically different from Col-0 plants infected with BCTV (+$P<0.05$).

SUPPLEMENTAL MATERIAL

Fig. S1. Comparison between V2 from curtoviruses and begomoviruses. (a) Genome organization of a monopartite begomovirus and a curtovirus showing the open reading frames coded. (b) Alignment of the aminoacid sequences of the V2 from the curtoviruses *Beet curly top virus* (BCTV) and *Spinach curly top virus* (SpCTV) and the begomoviruses *Cotton leaf curl Multan virus* (CLCuMV), *East African Cassava mosaic Cameroon virus* (EACMCV) and *Tomato yellow leaf curl virus* (TYLCV). Accession numbers for the proteins are indicated. Conserved residues between begomoviruses or curtoviruses are highlighted in light or dark grey respectively. Proteins were aligned with ClustalW (http://www.ebi.ac.uk/clustalw/index.html). Afterwards, the alignment was checked and manually adjusted.

Fig. S2. Subcellular localization of *Beet curly top virus* V2-GFP fusion proteins in epidermal cells of *Nicotiana benthamiana.* (a) Confocal images of transiently transformed *N. benthamiana* epidermal cells co-expressing an ER marker (RFP-HDEL) and V2-GFP, GFP-V2, or free GFP. White bars represent 50 μm. Plots 1, 2, 4, 5, 7 and 8 show fluorescence intensity plots corresponding to the green or yellow lines in the images. Images 3, 6 and 9 show scatter plots of green and red pixels. Pc, Pearson's correlation. Oc, Overlap coefficient. Cr, colocalization rate. (b) Close-up image of transiently transformed *N. benthamiana* epidermal cells co-expressing an ER marker (RFP-HDEL) and GFP-V2. White bar represents 10 μm. (c) Immunoblot analysis of protein extracts obtained from leaves three days after they were agroinfiltrated with constructs to express V2-GFP, GFP-V2, or free GFP, blotted with anti-GFP. Molecular weight of protein markers are indicated.

Fig. S3. (a) Relative GFP siRNAs accumulation in untransformed and AMPLICON *Arabidopsis thaliana* plants expressing V2 from *Beet curly top virus.* Total RNA extracted from leaves of transgenic V2-AMP lines 1, 3, and 5, (T2 kanamycin resistant plants) was subjected to northern blot analysis to detect GFP siRNAs and sRNA U6 as loading control. RNA from homozygous AMPLICON (AMP) was used as control. GFP siRNAs levels were quantified from the northern blot using ImageGauge software (Fuji) and normalised as the relative value to the one obtained in V2-AMP line 5. The data represent the average of three independent experiments each corresponding to RNA extracted from 10-12 plants. Bars represent standard error. (b) Total RNA extracted from leaves of transgenic V2-AMP lines (1, 3 and 5) was subjected to RT-qPCR to measure V2 and GFP. mRNA levels were normalized to two house-

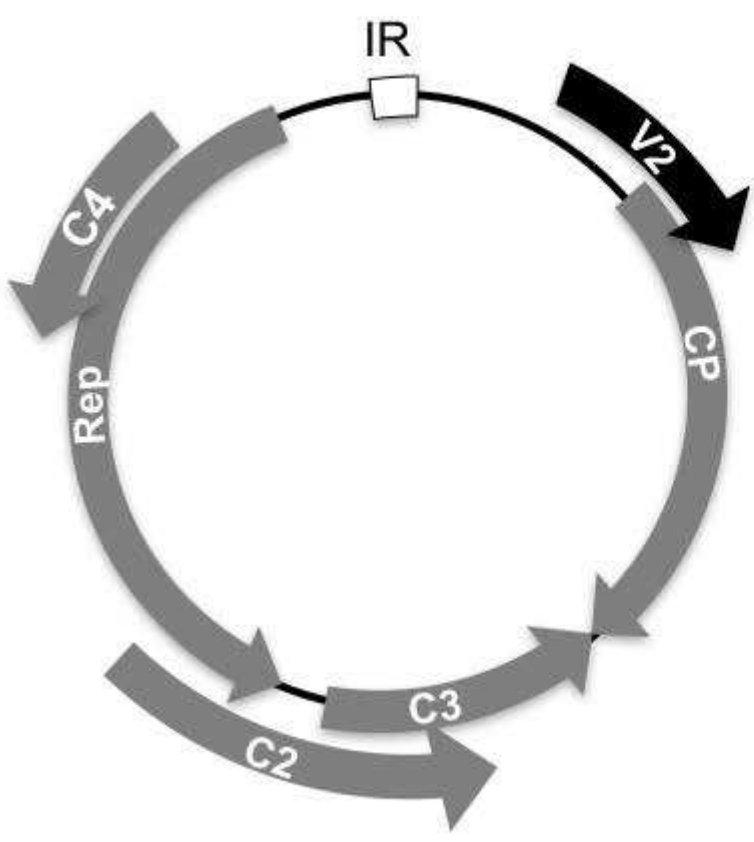
keeping genes: EF1-α and SAND (Table S1 and S2). RNA from the homozygous AMP line was used as a control. Values are represented as the relative expression compared with the V2-AMP5 sample. Bars represent the mean +/- the standard error from three technical replicates obtained from RNA extracted from 15 to 20 plants. (c) Analysis of the accumulation of tasiR255 in untransformed and transgenic *Arabidopsis thaliana* Col-0 lines expressing V2 of *Beet curly top virus* (BCTV). Total RNA isolated from T2 kanamycin-resistant plants expressing V2 (V2BC-1-10) and untransformed plants (C) was subjected to northern blot analysis to detect mRNA V2 and tasiR255 accumulation levels. Et-Br-stained 18S RNA and sRNA U6 were used as loading controls respectively. Relative quantifications of tasiR255/U6 compared to the level found in untransformed plants are indicated below each lane. RNA extracted from *rdr6-15* mutant was used as a negative control for tasiR255 accumulation.

Fig. S4. Symptoms in *Arabidopsis rdr6* and *dcl2/4* plants agroinfected with BCTV wild-type (WT) or V2 mutant (ΔV2) at 28 days after inoculation (dpi).

Table S1. Primers used in this work: (a): Rotenberg et al, 2006; (b): Bertrand et al., 2003; (c) Lilly et al, 2011.

Table S2: Primers used in RT-PCRs, PCRs or real-time qPCRs (qPCR) (a): Genes are indicated by *Arabidopsis* Genome Initiative (AGI) locus identifiers;(b): (Rotenberg *et al*., 2006); (c): (Bertrand *et al*., 2003); (d) Lilly et al, 2011.

Table S3. Plasmids generated in this work. All plasmid names that contain a *Beet curly top virus* ORF are ended in BC or BCTV. The backbone, fragment, primer used, cloning site, and use are shown. Plasmids origins are: (a): Stratagene; (b): (Sanchez-Duran et al., 2011); (c): (Morilla et al., 2006).



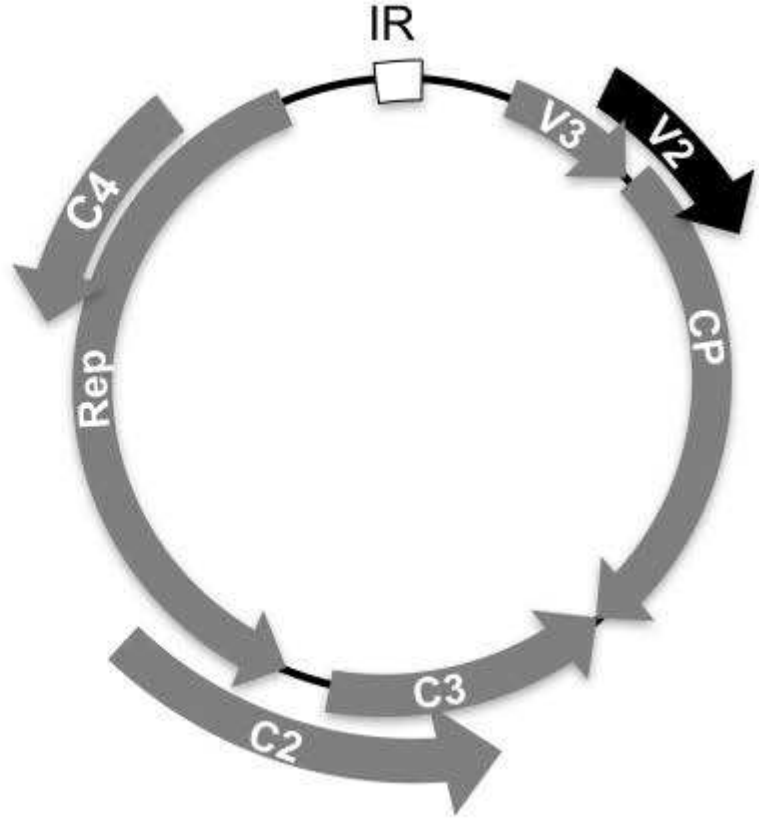


Fig. S1

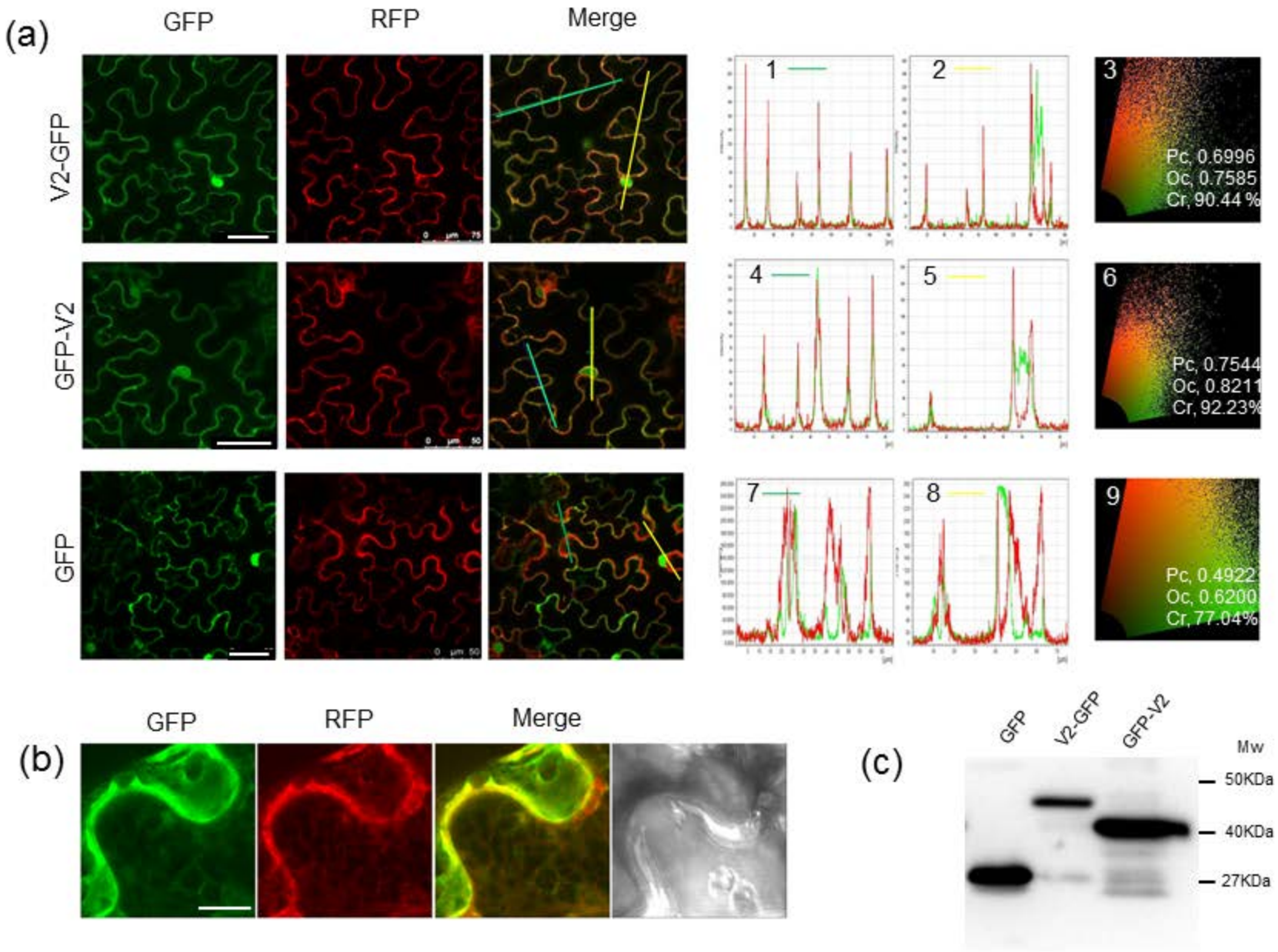
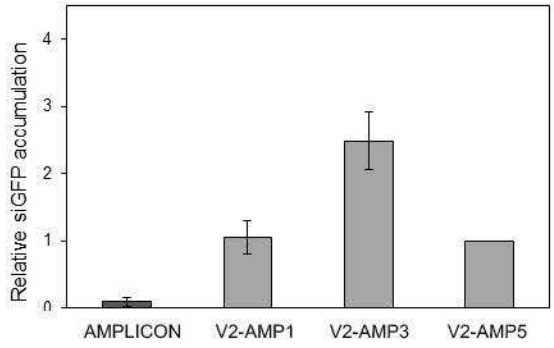
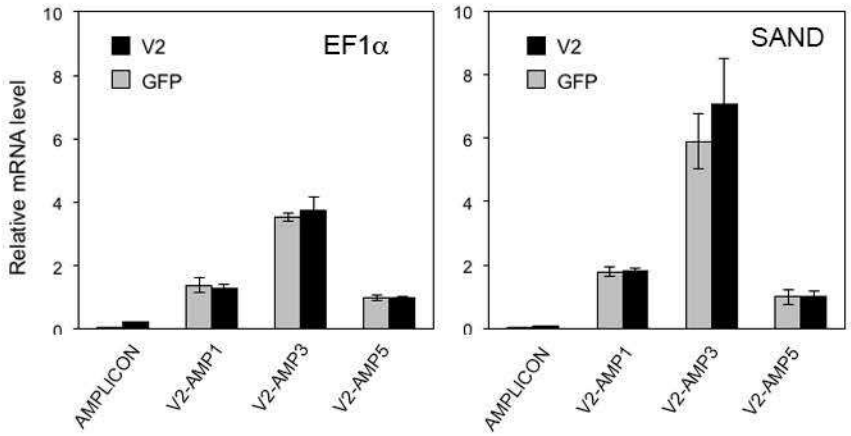

(a)
GFP
RFP
Merge
V2-GFP
GFP-V2
GFP
1
2
3
Pc, 0.6996
Oc, 0.7585
Cr, 90.44 %
4
5
6
Pc, 0.7544
Oc, 0.8211
Cr, 92.23%
7
8
9
Pc, 0.4922
Oc, 0.6200
Cr, 77.04%
(b)
GFP
RFP
Merge
(c)
GFP
V2-GFP
GFP-V2
Mw
50KDa
40KDa
27KDa

Fig. S2

(a)


Relative siGFP accumulation
0
1
2
3
4
AMPLICON
V2-AMP1
V2-AMP3
V2-AMP5

(b)


Relative mRNA level
V2
GFP
EF1α
SAND
0
2
4
6
8
10
AMPLICON
V2-AMP1
V2-AMP3
V2-AMP5

(c)

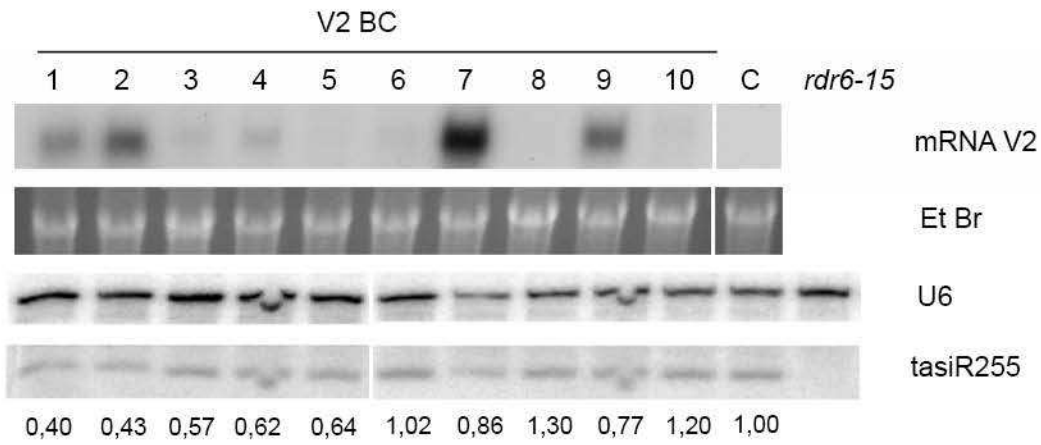

V2 BC
1 2 3 4 5 6 7 8 9 10 C
rdr6-15
mRNA V2
Et Br
U6
tasiR255
0,40 0,43 0,57 0,62 0,64 1,02 0,86 1,30 0,77 1,20 1,00

Fig. S3

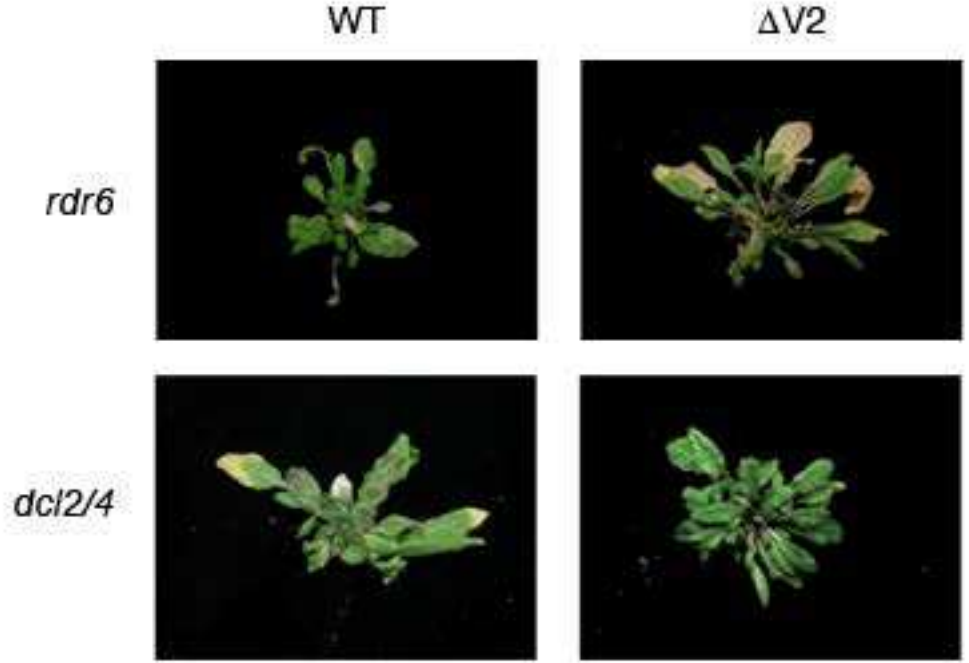


Fig. S4

Primer	Oligonucleotide sequence 5' -> 3'
LowV2BC	GTACTAGTCCTCCTCTTCTTCG
UpV2BC	TTTATGGGACCTTTCAGAGTGG
LowV2BC-RT	GACGAAAGACCTCGCCTTCT
UpV2BC-RT	ATGGGACCTTTCAGAGTGGA
LowV2BC-*BamH*I-A	GAATTCGGATCCCGCGTCCTCCTTTCTTC
LowV2BC-*Sac*I	GAATTCGAGCTCCTAGTCCTCCTCTTCTTC
UpV2BC-*Kpn*I	TAAGCGGTACCATGGGACCTTTCAGAGTG
UpV2BC-*Hpa*I	ATAAGCGTTAACATGGGACCTTTCAGAGTG
UpV2BC-*Nde*I	AACATATGATGGGACCTTTCAGAGTGG
LowGFP-*BamH*I-A	CCCGGATCCTGCTTTGTATAGTTCATCCATGC
LowGFP-*Sac*I	CCCCCCGAGCTCTTATTTGTATAGTTCATCCATGCC
LowGFP5	AAAGGGCAGATTGTGTGGAC
Low RT-GFP5	GATCCTGTTGACGAGGGTGT
UpGFP-*Kpn*I	CCCCCGGTACCATGAGTAAAGGAGAAGAACTTTTCAC
UpGFP-*Hpa*I	CCGCGCGTTAACATGAGTAAAGGAGAAGAAC
UpGFP5	AGTGGAGAGGGTGAAGGTGA
Up-RT-GFP5	GAGGGATACGTGCAGGAGAG
LowBCTV-qRT	CTACACGAAGATGGGCAACCT
UpBCTV-qRT	TGACGTCGGAGCTGGATTTAG
LowEF-1a NB (a)	AGCTTCGTGGTGCATCTC
UpEF-1a NB (a)	GATTGGTGGTATTGGAACTGTC
Actin F(b)	CTAAGCTCTCAAGATCAAAGGCTTA
Actin R(b)	ACTAAAACGCAAAACGAAAGCGGTT
EF1alpha F(c)	CACCACTGGAGGTTTTGAGG
EF1alpha R(c)	TGGAGTATTTGGGGGTGGT
SAND F(c)	GTTGGGTCACACCAGATTTTG
SAND R(c)	GCTCCTTGCAAGAACACTTCA

Table S1. Primers used in this work: (a): Rotenberg et al, 2006; (b): Bertrand et al., 2003; (c) Lilly et al, 2011.

References:

Bertrand, C., Bergounioux, C., Domenichini, S., Delarue, M., & Zhou, DX. (2003). *Arabidopsis* Histone Acetyltransferase AtGCN5 Regulates the Floral Meristem Activity through the WUSCHEL/AGAMOUS Pathway. *J. Biol. Chem.* **278**(30): 28246–28251.

Rotenberg D, Thompson TS, German TL & Willis DK. (2006). Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *J Virol Methods*. **138**(1-2):49-59.

Lilly ST, Drummond RSM, Pearson MN, MacDiarmid RM (2011). Identification and validation of reference genes for normalization of transcripts from virus infected *Arabidopsis thaliana*. *Mol Plant Microbe Interact* **24**: 294–304.

Organism	AGI code (a)	Gene	Forward	Reverse	Use
BCTV	-	*V2*	upV2BC	lowV2BC	RT-PCR/PCR
"	-	$V2_{1\text{-}154}$	upV2BC-RT	lowV2BC-RT	qPCR
"	-	From 2591 to 2774	upBCTV-qRT	lowBCTV-qRT	qPCR
N. benthamiana	-	*NbEF-1*alfa (b)	upEF-1a NB	lowEF-1a NB	RT-PCR
A. thaliana	AT3G18780	actin(c)	Actin F	Actin R	qPCR
"	AT5G60390	EF1alpha(d)	EF1alpha F	EF1alpha R	qPCR
"	AT2G28390	SAND(d)	SAND F	SAND R	qPCR
		mGFP-$ER_{265\text{-}366}$	up-RT-GFP5	low-RT-GFP5	qPCR

Table S2: Primers used in RT-PCRs, PCRs or real-time qPCRs (qPCR) (a): Genes are indicated by *Arabidopsis* Genome Initiative (AGI) locus identifiers;(b): (Rotenberg *et al*., 2006); (c): (Bertrand *et al*., 2003); (d) Lilly et al, 2011.

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Bertrand, C., Bergounioux, C., Domenichini, S., Delarue, M., & Zhou, DX. (2003). *Arabidopsis* Histone Acetyltransferase AtGCN5 Regulates the Floral Meristem Activity through the WUSCHEL/AGAMOUS Pathway. *J. Biol. Chem.* **278**(30): 28246–28251.

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Lilly ST, Drummond RSM, Pearson MN, MacDiarmid RM (2011). Identification and validation of reference genes for normalization of transcripts from virus infected Arabidopsis thaliana. Mol Plant Microbe Interact **24**: 294–304.

Plasmid	Vector backbone	Fragment	Primers or fragment	Cloning site	Used for
35S-expression vectors					
pV2BC	pBSSKII+(a)	BCTV $V2_{1-102}$	upV2BC/lowV2BC	*EcoR*V	Subcloning
pBIV2BC	pBINX1 (b)	BCTV $V2_{1-102}$	pV2BC *BamH*I-*Sal*I	*BamH*I-*Sal*I	Expression in plants
pBINX1-GFP1	pBINX1 (b)	GFP from pBINGFP (c)	UpGFP-*Hpa*I/LowGFP-*BamH*I-A digested with *Hpa*I-*BamH*I	*Hpa*I-*BamH*I	Subcloning
pBINX1-GFP2	pBINX1 (b)	GFP from pBINGFP (c)	UpGFP-*Kpn*I and LowGFP-*Sac*I digested with *Kpn*I-*Sac*I	*Kpn*I-*Sac*I	Subcloning
pBI-GFP-V2BC	pBINX1-GFP1	BCTV $V2_{1-102}$	UpV2BC-KpnI/lowV2BC-SacI *Kpn*I-*Sac*I digested with *Kpn*I-*Sac*I	*Kpn*I-*Sac*I	Expression in plants
pBI-V2BC-GFP	pBINX1-GFP2	BCTV $V2_{1-102}$	upV2BC-*Hpa*I/lowV2BC-*BamH*I-A digested with *Hpa*I-*BamH*I	*Hpa*I-*BamH*I	Expression in plants

Table S3. Plasmids generated in this work. All plasmid names that contain a *Beet curly top virus* ORF are ended in BC or BCTV. The backbone, fragment, primer used, cloning site, and use are shown. Plasmids origins are: (a): Stratagene; (b): (Sanchez-Duran et al., 2011); (c): (Morilla et al., 2006).

References

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.

Sanchez-Duran, M. A., Dallas, M. B., Ascencio-Iba√±ez, 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. *J Virol* **85**: 9789-9800.