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 (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 post-transcriptional 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]. Little 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 pBIN1.2 [28] and cloned into the binary plasmid pBI121 [29] to yield pBI121-GFP1 or pBI121-GFP2, respectively. Afterward, a fragment containing the V2 ORF was amplified from pBIN1.2 [30] and cloned into pBI121-GFP1 or pBI121-GFP2, yielding pBI-GFP-V2BC or pBI-V2BC-GFP, respectively. The primes, 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 C-terminal HDEL sequence’ (RFP-HDEL) [34]. Upon co-expression, 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 pBISSKII+ to yield pV2BC, while a restriction fragment containing the V2 ORF was cloned into pBIN1X1 to yield pBV2BC.) 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 co-expressed 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 long-distance movement, leaves of N. benthamiana 16 c plants [37] were co-infiltrated with constructs expressing GFP and either V2, P19 or the empty vector (C-). As in the previous assay, co-infiltration with BCTV V2 produced an increase in GFP fluorescence 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 empty vector, 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 transgenic 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
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
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]. RT-qPCR analysis using three reference genes (actin, EF1α and SAND) confirmed that the reactivation of GFP expression 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-ΔV2). (a) Symptoms induced in *Arabidopsis* Col-0 plants agroinoculated with BCTV or BCTV-ΔV2 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-ΔV2. 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 2^ΔΔCT 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-ΔV2-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).
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-ΔV2) 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-ΔV2 mutant were described in [30] and [27]. Arabidopsis Col-0 plants infected with BCTV were clearly symptomatic, whereas plants infected with BCTV-ΔV2 did not develop symptoms (Fig. 3a). Quantitative-PCR analysis showed that viral DNA accumulation was significantly reduced in plants infected with BCTV-ΔV2 (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 (sviRNA) 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 endogenous tasiRNAs (tasiR255) detected in most of the transgenic plants expressing BCTV V2 (Fig. S3c). Interestingly, the viral DNA content in rdr6 plants infected with BCTV-ΔV2 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 wild-type 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.

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