RNA silencing is an important defence mechanism against virus infection, and many plant viruses encode counter-silencing proteins (Voinnet, 2001; Moissiard & Voinnet, 2004). The trigger for silencing is the presence of double-stranded (ds) RNA. For viruses with single-stranded RNA genomes, dsRNAs comprise an essential replication intermediate. dsRNAs are not obligatory replication intermediates for viruses with DNA genomes but may nevertheless accumulate during infection (Blevins et al., 2006). Several geminiviruses undergo silencing, and several also encode silencing suppressors (Bisaro, 2006; Cui et al., 2005; Trinks et al., 2005). Cauliflower mosaic virus (CaMV), the type member of the family Caulimoviridae, is a pararetrovirus with an 8 kbp dsDNA genome and replicates by reverse transcription (Haas et al., 2002). In Brassica napus, CaMV undergoes silencing (Al Kaff et al., 1998), and CaMV-infected Arabidopsis accumulate siRNAs that are derived mainly from the viral 35S RNA leader (Moissiard & Voinnet, 2006; Blevins et al., 2006).

To test whether CaMV is also capable of suppressing silencing, we infected a transgenic Arabidopsis line in which expression of GFP is silenced (Dalmay et al., 2000). GxA (obtained from Professor D. C. Baulcombe, Sainsbury Laboratory, Norwich, UK) contains both a 35S::GFP transgene and one expressing a potato virus X (PVX) amplicon from a phloem-specific promoter. The amplicon, which is silenced, contains GFP sequences and these co-suppress the 35S::GFP transgene. GxA allows analysis of both local silencing in the vascular tissue (where the amplicon is expressed), and systemic movement of the silencing signal into the remaining leaf tissue (Dalmay et al., 2000).

Groups of approximately ten plants were inoculated with CaMV, either manually at the two-leaf stage (Cecchini et al., 1998), or by particle bombardment at the eight-leaf stage (Love et al., 2007). Inoculated plants began to show typical systemic symptoms of infection (distortion and stunting of the central rosette leaves) at about 12 days post-infection (d.p.i.). Under UV illumination, infected plants showed GFP fluorescence at the centre of the rosette and in expanded leaves that were showing vein-clearing symptoms; mock-inoculated controls showed no GFP fluorescence (Fig. 1a and b). Northern blots of total RNA extracted from infected, but not uninfected plants and probed with 32P-labelled GFP-cDNA, revealed four strong bands corresponding to the expected sizes (Dalmay et al., 2000) of amplicon genomic RNA, the two amplicon subgenomic mRNAs and the 35S::GFP transcript (Fig. 2a).

By 15 d.p.i., all inoculated leaves and some symptomatic expanded leaves had developed a severe necrosis that we never observed in infections of non-transgenic controls. To confirm that fluorescence was a consequence of GFP expression and not necrosis-related autofluorescence, we examined infected leaves in a Zeiss LSM510 confocal microscope using a dual-channel approach to distinguish between autofluorescence and authentic GFP fluorescence.
areas of necrosis (false-coloured in red and indicated by arrows in Fig. 3a). Under higher magnification (Fig. 3b), we could identify fluorescent subcellular structures in the green-fluorescent cells demonstrating that these were living cells, expressing GFP. We never observed GFP fluorescence in uninfected GxA plants. Thus, CaMV-infection reversed silencing of GFP in GxA. However, it also triggered a necrotic response that is never observed in wild-type Arabidopsis (Cecchini et al., 1998).

CaMV gene VI encodes a 66 kDa polypeptide P6 which is essential for replication (Haas et al., 2002; Covey et al., 2000) and is also the major genetic determinant of symptom severity (Stratford & Covey, 1989). Transgene-mediated expression of P6 in Arabidopsis results in a chlorotic dwarf phenotype and alterations in leaf morphology (Zijlstra et al., 1996; Cecchini et al., 1997) reminiscent of those induced by silencing suppressors, e.g. tobacco etch potyvirus HC-Pro (Kasschau et al., 2003). This raises the question whether P6 might be suppressing silencing in infected GxA.

We crossed GxA with two independent transgenic lines A7 and B6 that constitutively express high levels of P6 from CaMV isolate Cabb B-JI under the control of a 35S promoter (Cecchini et al., 1997). To avoid the complication of three segregating transgenes, experiments were carried out on F1 progeny. GFP is still completely silenced in F1 mapping crosses that are hemizygous for both amplicon and 35S::GFP transgenes (D. Baulcombe and M. Jensen, personal communication). Therefore, a single copy of the amplicon is sufficient to trigger silencing. Progeny of GxA and P6 transgenics exhibited the typical P6-dependent chlorotic phenotype, and levels of P6 mRNA, measured by real-time RT-PCR, were at least as high as in the P6 parent, confirming that the P6 transgene was transcriptionally active.

Uninoculated GxA plants did not show any fluorescence (Fig. 3e). Leaves inoculated by bombardment developed distinct local lesions by 12 d.p.i. and extensive necrosis by 15 d.p.i. These emitted strongly in the GFP channel (510–530 nm) and at 560–610 nm (outside the GFP emission spectrum). Therefore, in inoculated leaves much of the fluorescence appeared to be necrosis-related. However, emergent leaves showing symptoms of systemic infection contained extensive areas which emitted only in the GFP channel, plus more limited

Fig. 1. GFP fluorescence in GxA plants under UV illumination. (a) GxA infected with CaMV, 12 d.p.i. Green arrow, inoculated leaf; yellow arrows, systemically infected leaves. (b) GxA mock inoculated, 12 d.p.i. (c) P6 transgenic line A7. (d) P6 transgenic line B6. (e) F1 progeny of A7×GxA cross. (f) F1 progeny of B6×GxA cross.

Fig. 2. (a) Northern blots of RNA from (left to right) CaMV-infected GxA and C24 at 15 d.p.i.; uninfected GxA and C24 grown in parallel, uninfected C24 wild-type (WT) plants. RNA was separated by electrophoresis on 1.0 % agarose gels and hybridized to a 32P-labelled probe from a full-length GFP cDNA. The positions of the amplicon genomic and subgenomic RNAs and the 35S::GFP transcript are shown. The bottom panel shows ethidium bromide (EtBr)-stained rRNA as a loading control. (b) Northern blots of RNA from (left to right) A7×GxA, B6×GxA, GxA and B6. For details of electrophoresis and hybridization see 2(a) above. (c) Northern blots of siRNAs hybridizing to a 32P-labelled siRNA probe from a full-length GFP cDNA. Total RNA was extracted from plants and separated by electrophoresis on 15 % polyacrylamide gels. Electrophoresis, transfer to nylon membranes and hybridization were carried out according to Pall et al. (2007). Lanes contain RNA from (left to right) A7×GxA, B6×GxA, GxA and B6. The expected positions of the 24 and 21 nt siRNAs are indicated. The bottom panel shows EtBr-stained 5S RNA as a loading control. (d) Northern blots of siRNAs hybridizing to a 32P-labelled ssRNA probe complementary to nucleotides 7845–7864 of the Cabb B-JI genomic DNA sequence CaMV 35S RNA leader, a sequence which has been identified as a major component of CaMV siRNAs during infection (Moissiard & Voinnet, 2006). RNA was extracted from A7, B6 and Ler-0 21 days after inoculation with CaMV CM4-184 or Cabb B-JI. RNA samples represent pairs of biological replicates each derived from five plants: lanes 1, 2, A7 infected with CM4-184; lanes 3, 4, B6 CM4-184; lanes 5, 6, Ler-0 CM4-184; lanes 7, 8, A7 Cabb B-JI; lanes 9, 10, B6 Cabb B-JI; lanes 11, 12, Ler-0 Cabb B-JI and lane 13 uninfected Ler-0. The bottom panel shows 5S RNA from the EtBr-stained gel, as a loading control. (e) Levels of 35S RNA in A7, B6 and Ler-0 at 21 days after infection with CaMV CM4-184 or Cabb B-JI. Total RNA was extracted and 35S RNA was quantified by real-time RT-PCR as described by Love et al. (2005). 35S RNA levels were normalized to an ACT2 internal reference and are expressed in arbitrary units. RNA samples correspond to those in (d) above. Arrows indicate samples infected with CM4-184 and Cabb B-JI. (f) Levels of siRNAs in infected P6-transgenic and non-transgenic plants as a proportion of levels of 35S RNA [as shown in (d)]; accumulating at 21 d.p.i. siRNAs in (d) were quantified from the phosphorimager output. Levels of 21+24 nt siRNAs (in arbitrary phosphorimager units) are expressed as a ratio of the levels of 35S RNA (in arbitrary real-time PCR units) for each sample. Error bars show standard deviations for the two biological replicates. White bars, CaMV CM4-184; grey bars, CaMV Cabb B-JI.
We examined the parent lines and progeny under UV illumination. None of the parents showed any GFP fluorescence (Fig. 1b, c and d). In contrast, all of approximately 20 randomly chosen F1 progeny from each cross exhibited strong green fluorescence (Fig. 1e and f), indicating that amplicon-dependent silencing of the 35S::GFP transgene had been reversed. GFP fluorescence was somewhat variable, older leaves in particular exhibiting patches of red tissue, but these were not particularly localized in the veins. Northern blots of total RNA extracted from progeny of the crosses and probed with 32P-labelled GFP-cDNA, revealed the four bands corresponding to amplicon and 35S::GFP transcripts and similar to those in infected GxA plants (Fig. 2a and b). These were undetectable in the GxA parent, demonstrating that P6 had also reversed silencing of the amplicon. RNA was separated by PAGE, and levels of siRNAs were determined by Northern blot hybridization (Pall et al., 2007) using a GFP probe (Fig. 2c). As expected, 21 nt siRNA was the predominant species in the GxA parent. The overall
abundance of this species was little altered in both A7 × GxA and B6 × GxA plants.

To determine whether P6 might differentially affect local and systemic silencing, we examined leaves in the confocal microscope. In A7 × GxA plants, vascular, epidermal and mesophyll cells all exhibited fluorescence exclusively in the GFP channel (510–530 nm; Fig. 3c), although some areas of the leaf appeared to be more strongly fluorescent than others (not shown). In B6 × GxA plants, we observed patterns of fluorescence in mesophyll and epidermal cells that were similar to those in A7 × GxA plants (Fig. 3d). However, unlike A7 × GxA plants we did not observe strongly fluorescent cells in the vascular tissue of the B6 × GxA plants (c.f. Fig. 3c and d). Levels of P6 protein in B6 are only about half that in A7 (Smith, 2007). Therefore, although P6 can suppress both local and systemic silencing, it may be less effective against the former.

CaMV-derived siRNAs accumulate in infected Arabidopsis (Moissiard & Voinnet, 2006; Blevins et al., 2006). To determine whether transgene-derived P6 might be able to antagonize RNA-silencing of CaMV during infection, we inoculated A7, B6 and Ler-0 by bombardment with DNA from CaMV, isolates CM4-184 and Cabb B-JI (Delseny & Hull, 1983), and compared the accumulation of CaMV-derived siRNAs in P6-transgenics and wild type.

RNA was isolated at 21 d.p.i., and levels of CaMV 35S RNA, a good indicator of levels of replicating virus (Cecchini et al., 2002; Love et al., 2005), were quantified by real-time RT-PCR (Love et al., 2005) (Fig. 2e). In Ler-0 infected with Cabb B-JI, which causes severe symptoms in Arabidopsis (Cecchini et al., 1998), 35S RNA levels were much higher than with the milder isolate CM4-184. For each isolate, levels of 35S RNA in A7 and B6 were half or less than that in Ler-0. This was counter-intuitive given the potential role of P6 as a silencing suppressor. However, P6 is also essential for correct translation of the 35S RNA (Haas et al., 2002; Bureau et al., 2004), and translational transactivation and pathogenicity have been reported to map to different domains of the protein (Kobayashi & Hohn, 2003). Possibly reduced virus load might be a consequence of aberrant regulation of translation of 35S RNA in P6-transgenic plants.

To detect siRNAs, Northern blots were hybridized using a probe complementary to nucleotides 7845–7864 of the CaMV sequence, a region of the 35S RNA leader that is a major source of CaMV siRNAs in infected plants (Moissiard & Voinnet, 2006). 24 and 21 nt siRNAs accumulated in Ler-0 and also in A7 and B6, albeit at lower levels (Fig. 2d). However, quantification of siRNAs from the blots demonstrated that, for each isolate, levels of siRNAs relative to 35S RNA were similar or even higher in P6 transgenics than in Ler-0 (Fig. 2f). Thus, the lower absolute levels of siRNAs in P6 transgenic plants is most likely a consequence of reduced accumulation of the virus transcripts from which they are presumably derived.

These results demonstrate that P6 can suppress silencing of an amplicon when expressed from a transgene and is presumably responsible for the suppression of silencing during CaMV infection. This is the first report of a silencing suppressor encoded by a plant pararetrovirus and provides further evidence that silencing suppressors may be a common feature of plant viruses regardless of genome type or evolutionary origin.

Virus-encoded silencing suppressors act through divergent mechanisms. For example, tombusvirus p19 and potyvirus HC-Pro bind siRNAs, sequestering them from the RISC complex (Murai et al., 2006; Lakatos et al., 2006). In contrast, cucumber mosaic virus (CMV) 2b protein inhibits RNA cleavage by the RISC complex (Zhang et al., 2006). Transgene-mediated expression of P6 did not reduce the relative proportion of CaMV leader-derived siRNAs to 35S RNA in virus-infected plants, and the accumulation of 21 nt GFP siRNAs was not significantly reduced in GxA. Although these results are consistent with
the conclusion that P6 does not act by sequestering either transgene- or virus-derived siRNAs, the complex nature of the amplicon system, and in particular the very high levels of amplicon transcripts in the silencing-suppressed plants, mean that levels of siRNA relative to mRNA are lower in these plants and therefore an RNA-binding role should not be excluded.

P6 is the major pathogenicity determinant of CaMV. Suppression of silencing provides a plausible explanation for the role of P6 in host pathogenicity and for the altered leaf morphology and symptom-like phenotype that result from transgene-mediated expression (Cecchini et al., 1997; Yu et al., 2003). Virus-encoded silencing suppressors induce developmental aberrations when expressed from transgenes (Voinnet, 2005; Moissiard & Voinnet, 2004), and these have been attributed to alterations in the activity of miRNA pathways (Kasschau et al., 2003; Lewsey et al., 2007). Moissiard & Voinnet (2006) identified at least one Arabidopsis-encoded transcript that showed micro-homology with CaMV leader-derived siRNAs and which was downregulated by a silencing-dependent mechanism. We have recently found that in addition to suppressing silencing, P6 inhibits both ethylene (Et) signalling (Geri et al., 2004), sensitivity to an auxin transport inhibitor (Smith, 2007) and gene expression in response to salicylic acid (SA) (A. J. Love, A. Sadanandom and J. J. Milner, unpublished), suggesting that it may play multiple roles in defence suppression. Whether its activities as a silencing suppressor and as a suppressor of SA/Et-dependent responses are linked remains to be established.

Acknowledgements

This work was funded by grant BB/D017319 from the Biotechnology and Biological Sciences Research Council to J.J.M. and A.S., A.J.L. and J.L. were supported by this grant. We are grateful to Professor D. C. Baulcombe for the gift of seed for GxA, for allowing us access to unpublished data and for helpful suggestions and discussions. We should like to thank Pawel Schweiger, for assistance with the Northern blots, and an anonymous referee for a helpful suggestion regarding virus titres.

References


