The cysteine-rich proteins of beet necrotic yellow vein virus and tobacco rattle virus contribute to efficient suppression of silencing in roots

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INTRODUCTION

RNA silencing is an important defence mechanism against virus infection, and many viruses encode RNA silencing suppressors as a counter-defence (Díaz-Pendón & Ding, 2008). The mechanism has been studied mostly on the basis of the phenotypes appearing in the aerial parts of plants. In fact, many viruses infect the aerial parts of plants by invertebrate vectors and spread systemically through vascular tissue to young shoots and roots of plants. However, soil-borne viruses are directly transmitted to roots by soil-inhabiting organisms such as fungi, protists and nematodes, and thus the virus accumulates first in the roots, moves through the vascular systems and then spreads into the shoots. The extent of systemic movement from infected roots to shoots depends greatly on the combination of virus and host species. For example, viruses such as beet necrotic yellow vein virus (BNYVV; genus Benyvirus) and potato mop-top virus (PMTV; genus Pomovirus) propagate mainly in the root systems and induce severe diseases in roots or tubers (Sandgren, 1995; Tamada, 1999). Thus, the root is an important organ for plant virus life cycle. Nevertheless, there have been only a few studies of silencing suppression activity in roots compared with leaves. Our previous studies demonstrated that RNA silencing is less active in roots than in leaves, and that roots have less of an activity that acts at the step of generation of small interfering RNAs (siRNAs) (Andika et al., 2005, 2006). Thus, lower the silencing level in the roots of plants is considered to be a universal phenomenon (De Wilde et al., 2001; Marjanac et al., 2009; Tomita et al., 2004). Moreover, BNYVV p31 is involved in efficient vector transmission and silencing suppression in roots (Rahim et al., 2007). Therefore, it is presumed that soil-borne viruses may have evolved their own strategy for counteracting host antiviral silencing.

Among viruses that are transmitted by soil-inhabiting organisms, rod-shaped viruses belonging to the genera Furovirus, Pecluvirus, Pomovirus and Benyvirus (all protist vectors) and Tobravirus (nematode vector) have the
common features of a multi-partite positive-sense, single-stranded RNA genome and encoding small cysteine-rich proteins (CRPs) at the 3’-proximal end of one of their genomic RNAs. An exception is the genus Hordeivirus, for which the viruses are not soil transmissible. CRPs encoded by these plant viruses are grouped into several classes based on amino acid sequence similarities. For examples, CRPs encoded by members of the genera Hordeivirus, Pecluvirus, Furovirus and Tobravirus share an amino acid sequence similarity (Savenkov et al., 1998; Te et al., 2005). The proteins yb of barley stripe mosaic virus (BSMV; genus Hordeivirus) (Yelina et al., 2002), p15 of peanut clump virus (genus Pecluvirus) (Dunoyer et al., 2001), 19K of soil-borne wheat mosaic virus (genus Furovirus) (Te et al., 2005) and 16K of tobacco rattle virus (TRV; genus Tobravirus) (Ghazala et al., 2008; Martínez-Priego et al., 2008; Martín-Hernández & Baulcombe, 2008) have been identified as pathogenicity determinants and silencing suppressors. BNYVV p14 and burdock mottle virus (BdMoV; a possible pathogenicity factor (Lukhovitskaya et al., 2007)) have been identified as a silencing suppressor (Guilley et al., 2008). 20K of soil-borne wheat mosaic virus (BSMV; genus Hordeivirus) p13 comprise another class of CRPs and function as silencing suppressors (Guilley et al., 2009; Kondo et al., 2005; Kozlowska-Makulska et al., 2010). PMTV p8 belongs to a further different CRP class (Savenkov et al., 2003; Scott et al., 1994) and has been identified as a pathogenicity factor (Lukhovitskaya et al., 2005).

In this study, we examined the differences in silencing suppression activity of different plant viruses in the shoots and roots of Nicotiana benthamiana plants. Silencing activities were assessed by transgene-silencing reversal assays, by synergistic effects on double-virus infections and by the effects on viral RNA accumulation levels when viral genes or proteins were expressed from a potato virus X (PVX) vector. The results showed that the two soil-borne viruses BNYVV and TRV enhanced transgene silencing suppression in roots, and that their virus-encoded CRPs contributed to this root-specific suppression.

RESULTS

BNYVV and TRV suppress transgene silencing in roots but not in shoots

We examined the differences in silencing suppressor activity of different viruses in leaves and roots using GFP transgene-silenced N. benthamiana line 16c plants (Voinnet et al., 1999). GFP-silenced plants were inoculated with seven plant RNA viruses: tobacco mosaic virus (TMV; genus Tobamovirus), potato virus Y (PVY; genus Potyvirus), cucumber mosaic virus (CMV; genus Cucumovirus), BNYVV, TRV, PVX and Odontoglossum ringspot virus (ORSV; genus Tobamovirus). At least six plants were inoculated with each virus, and virus infection was confirmed by symptom expression and, if necessary, by Western blotting. Systemic symptoms developed at 6–8 days post-inoculation (p.i.) for TMV, PVY, CMV, TRV, PVX and ORSV, and at 12–14 days p.i. for BNYVV. At 14–17 days p.i., virus-infected and mock-inoculated plants were transplanted to new test-tubes and newly developed roots were observed for GFP fluorescence by 40 days p.i. At approximately 1 week after the appearance of systemic symptoms, strong green fluorescence was observed in the newly emerging leaves and stems of plants infected with TMV, PVY and CMV, whereas no green fluorescence was observed in any aerial parts of the plants infected with BNYVV, TRV, PVX or ORSV by 40 days p.i. (Fig. 1a). In the virus-infected leaves, GFP mRNA accumulation levels were in accordance with the visual observations (Fig. 1b). However, in newly developed roots, infections of TMV, PVY, CMV, BNYVV and TRV developed obvious green fluorescence, but infections of PVX and ORSV showed only faint green fluorescence in the root tips, which was also seen in mock-inoculated plants (Fig. 1a). The green fluorescence observed in roots infected with TMV and PVY was stronger than that in roots infected with CMV, TRV and BNYVV. In Northern blot analysis, GFP mRNAs were abundant in roots infected with TMV, PVY, CMV, BNYVV and TRV, whereas GFP mRNAs accumulated to low levels in roots infected with PVX and ORSV (Fig. 1b). These results indicated that TMV, PVY and CMV but not PVX and ORSV have a strong silencing suppression activity of a transgene in both leaves and roots, whereas BNYVV and TRV have the ability to suppress transgene silencing specifically in roots.

Differences in viral RNA accumulation in leaves and roots

We compared the differences in viral genomic positive- and negative-strand RNA accumulation levels in the leaves and roots of wild-type N. benthamiana plants infected with different viruses. Leaves and roots were collected on two different days after inoculation, and the experiments were carried out at least twice using six plants each time. Plants infected with TMV, PVY and CMV showed similar levels of viral positive-strand genomic RNA accumulation in roots and leaves at two different stages of infection (Fig. 2a). However, infection by BNYVV, TRV, PVX and ORSV resulted in a great reduction in positive-strand RNA accumulation by 16–21 days p.i. in leaves (recovery phenomenon) but only a slight reduction in roots (Fig. 2a). In contrast, the level of genome-length negative-strand RNA accumulation differed greatly in roots and leaves (Fig. 2a). For most viruses used (except PVY negative-strand RNA, which was not detected in either leaves or roots), viral negative-strand RNAs accumulated at low or undetectable levels in roots, compared with considerable accumulation levels in leaves; for example, for TMV and CMV, negative-strand RNAs were detected at low levels in the later stages of infection, whilst for BNYVV, TRV, PVX and ORSV, they were below the detection limit at all stages of infection. These results suggested that, in roots but not in leaves, negative-strand genomic RNA production was greatly reduced or inhibited, although positive-strand genomic RNAs accumulated considerably. In addition, we noted that, in the case of PVX, the sgRNAs [the triple gene block and coat protein (CP)] accumulated at very low levels in roots,
although positive-strand genomic RNA was detectable (Fig. 2a). However, Western blot analysis showed similar levels of CP accumulation of PVX in roots and leaves (Fig. 2b), and the presence of PVX virions in roots was confirmed by electron microscopy (data not shown).

**Synergistic effects in roots of doubly infected plants**

The synergistic phenomenon that occurs in double infections of different viruses is at least in part due to the complementary effects of suppressors acting at different sites of the RNA silencing pathways (MacDiarmid, 2005; Pruss et al., 1997). The best-known synergistic interaction involves double infection of tobacco with PVY and PVX, resulting in dramatic increases in symptoms and in the accumulation of PVX, which is mediated by the expression of PVY HC-Pro (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau & Carrington, 1998). In these doubly infected leaf tissues, the level of PVX negative-strand RNA increases disproportionately to that of the positive-strand RNA (Vance, 1991).

In our first preliminary experiments, we examined the effect of BNYVV RNA accumulation on the leaves and roots of *N. benthamiana* plants co-inoculated with BNYVV and PVY or PVX. Northern blot analysis showed that double infection with BNYVV and PVY induced a large increase in BNYVV positive- and negative-strand RNA accumulation in leaves and roots (Fig. 3a). By contrast, the double infection with BNYVV and PVX resulted in no apparent increases in BNYVV positive- and negative-strand RNA accumulation in roots, although a higher level of the negative-strand RNA was detected in leaves of doubly infected plants (Fig. 3a). No increase in symptoms was observed following double infection with BNYVV and PVX (data not shown).

As described above, BNYVV and TRV had the ability to increase the transgene silencing suppression in roots of *N. benthamiana* plants, and therefore we next examined whether double infection with PVX and BNYVV or TRV had an effect on PVX RNA accumulation in the roots of *N. benthamiana* plants. To confirm symptom expression and systemic movement of the first virus, PVX was inoculated at 3 and 6 days after inoculation with TRV and BNYVV, respectively. Double infection with PVX and PVY was included as a positive control, and both viruses were inoculated at the same time. Plants co-infected with PVX and PVY showed more severe symptoms than those of singly infected plants, whereas there were no increases in symptoms in plants doubly infected with PVX and either BNYVV or TRV (data not shown). In the leaves, a large increase in PVX negative-strand RNA was observed in double infection with PVX and PVY, whereas no or only a slight increase was found in double infection with PVX and TRV or BNYVV (in another repeated experiment, double infection with BNYVV resulted in a slight increase). Note that these double infections did not show any increase in

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**Fig. 1.** Transgene silencing suppression assays of TMV, PVY, CMV, BNYVV, TRV, PVX and ORSV using GFP-silenced *N. benthamiana* line 16c plants. (a) Leaves and roots were observed under UV light and by fluorescence microscopy, respectively. Photographs were taken at 40 days p.i. (b) Northern blots showing GFP mRNA accumulation in the leaves and roots of the GFP-silenced 16c plants (GFP-sil) shown in (a). Non-transgenic wild-type (WT) and GFP-expressing 16c (GFP-exp) plants were used as controls. Each lane shows a pooled sample from three plants. Ethidium bromide-stained 28S rRNA was used as a loading control.
PVX sgRNA accumulation (Fig. 3b). In contrast, in the roots, double infection with PVX and BNYVV or TRV induced an increase in both PVX negative-strand RNA and sgRNA accumulation compared with single PVX infection (Fig. 3b). Much higher levels of PVX negative-strand RNA and sgRNA accumulation were observed in plants doubly infected with PVX and PVY (Fig. 3b). Thus, double infection with PVX and either BNYVV or TRV showed a synergistic effect to enhance PVX negative-strand RNA and sgRNA accumulation levels in roots.

We next confirmed visually the above synergistic effect of BNYVV and TRV using a PVX vector carrying the GFP gene (PVX-GFP). When PVX-GFP was co-inoculated with either BNYVV or TRV, bright green fluorescence was observed in the upper leaves of singly or doubly infected plants at 7–8 days p.i. (Fig. 3c). The intensity of green fluorescence was similar in the sectioned stems of singly and doubly infected plants (Fig. 3c). However, it was interesting to note that roots (tap and lateral) of the doubly infected plants exhibited bright green fluorescence, whereas those of PVX-GFP singly infected plants were only faintly fluorescent (Fig. 3c). In Western blot analysis, GFP accumulated to a high level in the roots of doubly infected plants, whereas GFP accumulated only at a low level in the roots of PVX-GFP-infected plants (Fig. 3d). These results support the above evidence that both BNYVV and TRV are able to enhance PVX RNA accumulation levels in root tissues.

**BNYVV p14 and TRV 16K have the ability to increase PVX RNA accumulation in roots**

BNYVV p14 and TRV 16K CRPs are known to act as suppressors of RNA silencing. First, we compared the silencing suppression activity of BNYVV p14 and TRV 16K by an *Agrobacterium* co-infiltration assay using GFP-transgenic *N. benthamiana* line 16c plants (Voinnet et al., 2000). In this experiment, the CRPs of other viruses – BdMoV p13, PMTV p8 and an unidentified carlavirus 12K – were also included. PVY HC-Pro was used as a positive control. GFP fluorescence observation (Fig. 4a) and RNA
blot analysis (Fig. 4b) showed that BNYVV p14, BdMoV p13 and TRV 16K had similar levels of silencing suppression activity, whereas PMTV p8 and carlavirus 12K exhibited no suppression activity. The silencing suppression activities of p14 and p13 were weaker than that of HC-Pro, in which the GFP mRNA level was five- to sevenfold higher and GFP siRNA was not detected (Fig. 4b).

Next, we examined whether CRPs that act as silencing suppressors are involved in the enhancement of RNA accumulation of PVX in roots. BNYVV p14 and TRV 16K were inserted into a PVX vector (pgR106; Lu et al., 2003), and the resulting PVX vector carrying p14 (PVX-p14) or 16K (PVX-16K) was infiltrated into N. benthamiana plants. PVX-infected plants showed mild mosaic symptoms by 12 days p.i. (Fig. 5a). In contrast, both PVX-p14 and PVX-16K induced strong downward-curled leaves at 7 days p.i., followed by necrosis of the stems and veins of the upper leaves (Fig. 5a), with the plants eventually dying. It was noticed that there was a large difference in PVX RNA accumulation between roots infected with PVX-p14 and the empty vector (PVX-empty); thus, for PVX-p14, the accumulation of PVX negative-strand genomic RNA and positive-strand sgRNAs (but not positive-strand genomic RNA) was detected at much higher levels compared with PVX-empty. By contrast, such an increase in accumulation levels of viral negative-strand RNA and positive-strand sgRNA was not observed in leaves (Fig. 5b). Similar results

![Fig. 3. Synergistic effects of double infections with heterologous viruses on leaves (L) and roots (R) of N. benthamiana plants.](http://vir.sgmjournals.org 1845)
were obtained in the roots and leaves of plants infected with PVX-16K and PVX-empty (Fig. 5c and data not shown). These results indicated that BNYVV p14 and TRV 16K greatly contribute to the enhancement of PVX negative-strand RNA and positive-strand sgRNA accumulation in roots.

Further experiments were carried out to examine the effect of other virus CRPs (BdMoV p13, PMTV p8, BSMV γ-b and an unidentified carlavirus 12K) and the two known suppressors PVY HC-Pro and tomato bushy stunt virus (TBSV) p19. As shown in Fig. 5(a), plants infected with PVX-p13, PVX-γ-b, PVX-p8 and PVX-HC-Pro showed systemic necrosis and wilting of the upper leaves at 7–9 days p.i. These symptoms were similar to those of PVX-p14 and PVX-16K. PVX-p19 and PVX-12K developed systemic necrotic symptoms at 14–17 days p.i., although without the plant death in PVX-12K. The relative levels of PVX sgRNA accumulation levels in roots were as follows: PVX-HC-Pro showed the highest level, PVX-p13, PVX-p19, PVX-p14 and PVX-16K had a moderate level, and PVX-γ-b and PVX-12K had the lowest levels (Fig. 5b, c). PVX-p8 and PVX-GFP had undetectable levels of sgRNA accumulation.

Taken together, these results indicated that the CRPs that act as silencing suppressors have a stimulatory effect on PVX RNA accumulation in roots, although the level of the effect differed among viruses. BNYVV p14, TRV 16K and BdMoV p13, as well as PVY HC-Pro and TBSV p19 (both positive controls), showed a strong effect, whereas BSMV-γ-b and carlavirus 12K had a weak effect, and PMTV p8 had no effect.

**DISCUSSION**

In this study, we showed that two soil-borne viruses, BNYVV and TRV, which belong to different genera and have different vectors, have the ability to suppress transgene silencing in roots but not in leaves. These results indicate the possibility that CRPs derived from benyviruses and tobraviruses, and possibly from furoviruses and pecluviruses, may suppress RNA silencing more efficiently in roots than in leaves. This property was revealed by an increase in PVX RNA accumulation levels in the roots of *N. benthamiana* plants when in the presence of TRV or BNYVV, or when a TRV- or BNYVV-encoded RNA silencing suppressor gene was expressed by a PVX vector.

A surprising finding in this study was that, unlike in leaves, viral genome-length negative-strand RNAs accumulated at low levels or below the detection limit in roots of *N. benthamiana* plants. This feature was commonly observed for most of the viruses tested, although the differences in the levels of positive- and negative-strand RNA accumulation were not always proportional (Fig. 2a). A further interesting result was that, in the case of PVX, PVX sgRNA accumulation was very low or at undetectable levels in roots. A decrease in sgRNA accumulation was not observed for CMV, TRV and ORSV, regardless of the low accumulation levels of negative-strand RNAs, suggesting that this phenomenon may be specific to PVX.

The reason for the striking differences in negative-strand RNA accumulation levels in shoots and roots is not known, but antiviral silencing may be involved. The capacity of virus replication in roots may be low intrinsically, in which case the negative-strand RNA accumulation (synthesis) would be reduced or prevented. In addition, the viral siRNA-guided RNA-induced silencing complex is reported to target the virus negative-strand RNA more frequently than the positive-strand RNA (Molnár et al., 2005; Pantaleo et al., 2007). Thus, an endogenous silencing mechanism might keep negative-strand RNA accumulation at a low level in roots.

It is known that a large increase in PVX negative-strand RNA and sgRNAs in shoots occurs during double infection
by PVX and PVY (Vance, 1991) and by expression of PVY HC-Pro from the PVX vector; thus, the HC-Pro suppressor prolongs both the accumulation of PVX negative-strand RNA and expression of the sgRNA promoter (Pruss et al., 1997). Likewise, we demonstrated that the accumulation of PVX negative-strand RNA and sgRNAs increased in the roots of *N. benthamiana* plants when PVX was co-inoculated with BNYVV or TRV (Fig. 3b) and when PVY HC-Pro, BNYVV p14 or TRV 16K was expressed from PVX (Fig. 5b, c). Such enhancement of sgRNAs in roots was also observed for other CRPs derived from BdMoV, BSMV and a carlavirus. The extent of PVX sgRNA accumulation seems to reflect the ability of the silencing suppressors; thus, the silencing activity of HC-Pro in roots was much stronger than that of TBSV p19, BNYVV p14, BdMoV p13 or TRV 16K. BSMV γb and a carlavirus 12K seemed to have only a weak activity in roots, and neither virus was transmitted in soil. The PMTV p8 CRP had no silencing suppression activity in leaves (Fig. 4; Lukhovitskaya et al., 2005) or roots (Fig. 5c). We also found that PMTV was not able to restore expression of the silenced GFP transgene in roots in a transgene-silencing reversal assay (data not shown).

BNYVV and TRV are transmitted by soil-inhabiting protist and nematode vectors, respectively. Thus, virus accumulation to high titres in roots is advantageous to virus transmission and completion of the viral life cycle. It is quite plausible, thus, that BNYVV and TRV have a root-specific silencing suppression ability. Further studies will be required to determine whether this is true of other soil-borne viruses. Another interesting finding is that, in the case of BNYVV, the presence of RNA4-encoded p31 is required for both efficient silencing suppression in roots and efficient vector transmission (Rahim et al., 2007). The mechanisms of such a helper effect on silencing suppression are not clear; however, it was shown that p31 had neither silencing suppression activity (data not shown) nor the ability to increase the expression level of the p14 protein (Rahim et al., 2007). The observation that the TRV 16K protein may act with other components of TRV (Martin-Hernández & Baulcombe, 2008) suggests that TRV, like BNYVV, may require other factor(s) for root-specific expression.
As shown in this and other studies (Martin-Hernández & Baulcombe, 2008; Te et al., 2005), the action of CRPs in silencing suppression appeared to be much weaker compared with the well-known suppressors p19 and HC-Pro. The weakness of the CRP suppressors is also supported by the observation that, for BNYVV p14, the fluorescence decreased quickly after 5 days in the Agrobacterium co-infiltration assay, unlike results for other strong suppressors (Kozlowska-Makulska et al., 2010). Furthermore, to our knowledge, there is no evidence for the presence of silencing suppressors in some of the protist-transmitted pomoviruses (Lukhovitskaya et al., 2005) and nematode-transmitted nepoviruses (Jovel et al., 2007; Ratcliff et al., 1997). Thus, we suggest that the presence of no or weak suppressors in leaves may be crucial to soil-borne root-infecting viruses, and that, as if redeeming the weakness, some viruses may have evolved to have an ability to increase the silencing suppression specifically in roots.

**METHODS**

**Viruses, plants and virus inoculations.** BNYVV isolate O11 (Andika et al., 2005), CMV strain Y (Takanami, 1981), PVY (Noda et al., 1988) and ORSV isolate Cy-1 (Ikegami et al., 1995) were used. TRV was derived from the clones of TRV RNA1 (p0081, pCR-TOPO-RNA1) and TRV RNA2 (p0065, pK20-37K-dsRED) (supplied by S. A. Baulcombe, University of Cambridge, UK) (Lu et al., 2003) and pTMV-30B (supplied by W. O. Dawson, University of Florida, Gainesville, FL, USA) (Shivprasad et al., 1999), respectively. Wild-type and transgenic line 16c N. benthamiana plants were used and were all grown in special test tubes (24 × 120 or 30 × 115 mm with a drainage hole) filled with quartz sand. The plants were maintained in a growth cabinet at 24 °C with a 16 h light/8 h dark cycle and watered every day with nutrient solution (Rahim et al., 2007). For foliar rice inoculation, virus-infected N. benthamiana leaves were ground in 0.1 M phosphate buffer (pH 7.4) and the sap was rubbed on to the leaves of N. benthamiana plants. For agro-inoculation with PVX vectors, a simple toothpick method was used (Lu et al., 2003).

**Silencing suppression assays.** A GFP transgene silencing reversal assay using N. benthamiana line 16c plants was carried out as described previously (Rahim et al., 2007). Leaves of N. benthamiana line 16c plants were infiltrated with the bacteria containing the pBin-GFP plasmid. After 20–25 days, when silencing was achieved against GFP transcripts in the whole plants, systemic leaves were inoculated with the various viruses described above. At 14–17 days p.i., when systemic symptoms began to appear, the roots of the infected plants were partially removed and the plants were transplanted into new test tubes to enhance root growth. GFP fluorescence in leaves and new roots was examined under a UV lamp at 3–14 days after infiltration.

**Constructions of PVX vectors carrying viral genes.** The BNYVV p14, BdMoV p13, TRV 16K, PMTV p8, carlavirus 12K, BSMV (Inouye, 1962), B, PVY HC-Pro (from pBin-HC-Pro), TBSV p19 (from pBin-p19) and GFP (from pTXS.GFP) genes were amplified by RT-PCR or PCR and inserted between the pgR106 plasmid.

**Northern and Western blot analyses.** Northern blot analysis of total RNA extracted from leaf or root tissue was carried out as described previously (Andika et al., 2005). For viral RNA detection, blots were hybridized with digoxigenin (DIG)-labelled antisense and sense RNA probes corresponding to the regions of the viral genes used, and prepared using a DIG-RNA Labelling Mix (Roche). For GFP mRNA detection, a DIG-labelled DNA probe was prepared using a PCR DIG Probe Synthesis kit (Roche). For detection of GFP siRNAs, a DIG-labelled sense RNA probe was used. RNA probes were generated by in vitro transcription using SP6 or T7 RNA polymerase (Promega). Western blot analysis was carried out as described previously (Rahim et al., 2007). Approximately equal loading of proteins was confirmed by Coomassie Brilliant Blue staining. An antisera to GFP from the laboratory collection and a mouse anti-GFP mAb (Nakalai Tesque) were used for immunological studies.

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