Phenotypes and Functional Effects Caused by Various Viral RNA Silencing Suppressors in Transgenic *Nicotiana benthamiana* and *N. tabacum*

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RNA silencing suppressor genes derived from six virus genera were transformed into *Nicotiana benthamiana* and *N. tabacum* plants. These suppressors were P1 of Rice yellow mottle virus (RYMV), P1 of Cocksfoot mottle virus, P19 of Tomato bushy stunt virus, P25 of Potato virus X, HcPro of Potato virus Y (strain N), 2b of Cucumber mosaic virus (strain Kin), and AC2 of African cassava mosaic virus (ACMV). HcPro caused the most severe phenotypes in both *Nicotiana* spp. AC2 also produced severe effects in *N. tabacum* but a much milder phenotype in *N. benthamiana*, although both HcPro and AC2 affected the leaf tissues of the two *Nicotiana* spp. in similar ways, causing hyperplasia and hypoplasia, respectively. P1-RYMV caused high lethality in the *N. benthamiana* plants but only mild effects in the *N. tabacum* plants. Phenotypic alterations produced by the other transgenes were minor in both species. Interestingly, the suppressors had very different effects on crucifer-infecting Tobamovirus (crTMV) infections. AC2 enhanced both spread and brightness of the crTMV-green fluorescent protein (GFP) lesions, whereas 2b and both P1 suppressors enhanced spread but not brightness of these lesions. P19 promoted spread of the infection into new foci within the infiltrated leaf, whereas HcPro and P25 suppressed the spread of crTMV-GFP lesions.

Additional keywords: leaf and flower malformation, transgenic tobacco plants, viral suppressors

RNA silencing is a versatile and complex gene regulation and defense mechanism occurring in a broad range of eukaryotic organisms. It is activated in cells by double-stranded (ds)RNAs, followed by cleavage of the inducing RNAs into short (21- to 24-nucleotide) fragments. These, in turn, mediate multiple different regulatory and defense functions in the cells (Brodersen and Voinnet 2006). In post-transcriptional gene silencing (PTGS), they target the RNA-induced silencing complex (RISC) to degrade homologous RNA transcripts or to arrest their translation. Many transcription factors mediating the differentiation of multicellular organisms are regulated via these pathways, and severe developmental disturbances are associated with malfunctions of the silencing pathways (Bartel 2004; Deleris et al. 2006). Small RNAs function also as epigenetic agents targeting de novo cytosine or histone methylation

to their homologous DNA sequences to induce transcriptional gene silencing (TGS) and chromatin silencing (Chan et al. 2004, 2006; Xie et al. 2004). Both PTGS and TGS processes mediate effective defense mechanisms against invading genetic elements, such as viruses, transposons, and transgenes (Buchon and Vauray 2006). In plants, the small RNAs are involved in both cell-to-cell and long-distance movement of the silencing signal together with other proteins from the diverse RNA silencing pathways, such as DCL4 and CLASSY 1 (Dunoyer et al. 2005; Smith et al. 2007).

Virus-encoded silencing suppressors.

RNA silencing pathways function as inducible defense reactions against viral pathogens and produce a sequence specific, single-strand (ss)RNA-specific ribonuclease (Omarov et al. 2007). To counteract this defense mechanism, many plant viruses encode for specific silencing suppressors which allow the viruses to proliferate in their specific hosts. The importance of these suppressors is reflected by the fact that many of them previously have been identified as pathogenicity factors or as viral cell-to-cell or long-distance movement proteins, essential for infectivity in indicated hosts (Voinnet 2005; Xie and Guo 2006). Silencing suppressor proteins encoded by unrelated RNA and DNA viruses bear no similarity to each other in either coding sequence or protein structure, suggesting separate origins and variable functional mechanisms for each suppressor type. In recent years, the interactions of different silencing suppressors with the RNA silencing pathways have been studied intensively. In transgenic *Arabidopsis*, severe developmental disturbances are induced by many suppressors (e.g., P1/HcPro of Turnip mosaic virus [TuMV, Potyvirus], P19 of Tomato bushy stunt virus [TBSV, Tombusvirus], P15 of Peanut clump virus [PCV, Pecluvirus], and P21 of Beet yellows virus [BYV, Closterovirus]), and the malformed phenotypes appear related to the inhibition of the miRNA-mediated cleavage of their target mRNAs (Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003). On the other hand, P25 cell-to-cell movement protein of Potato virus X (PVX, Potexvirus), P38 coat protein of Turnip crinkle virus (TCV, Carmovirus), and P50 movement protein (MP) of the Apple chlorotic leaf spot virus (ACLSV, Trichovirus) cause no phenotypic changes in the plant, and primarily prevent the short- or long-distance spread of the silencing signal (Bayne et al. 2005; Deleris et al. 2006; Dunoyer et al. 2004; Yaegashi et al. 2007). The phenotype caused by the 2b protein of Cucumber mosaic virus (CMV, Cucumovirus) varies from none to severe depending on the viral strain from which the transgene was isolated, and is related to binding of

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this protein to Argonaute 1 (Lewsey et al. 2007; Zhang et al. 2006). It has been suggested that the silencing suppressor activities of many viral proteins are related to their 21-nucleotide short-interfering (si)RNA-binding activity, while the suppressors of other viruses, even closely related, do not show such binding activity (Meraï et al. 2005, 2006). However, thus far, direct relation between siRNA-binding and silencing suppression activity has been shown in vivo only for P19 of different tombusviruses, P21 of BYV, and HcPro of *Tobacco etch virus* (TEV) (Lakatos et al. 2006; Silhavy et al. 2002).

As the silencing suppressors compromise the silencing-mediated host defense, they also may enhance other viral infections, in a way similar to the synergistic effects of double viral infections (Pruss et al. 1997, 2004; Vanitharani et al. 2004). Surprisingly, in transgenic tobacco plants, the HcPro of TEV also enhanced the *N*-gene-mediated resistance against *Tobacco mosaic virus* (TMV) and induced a strong resistance against *Tomato black ring virus* (TBRV, *Nepovirus*) (Pruss et al. 2004). The mechanisms of these induced resistance reactions are not yet well understood.

Most studies concerning the interactions of plants with viral silencing suppressors have been focused on the suppressor effects on the processing of selected miRNA target genes in transgenic *Arabidopsis* plants. Thus, many features of the suppressor functions remain unknown; for instance, what is the degree of host specificity of different silencing suppressors, do they cause similar physiological or phenotypic effects in different hosts, or do they exert similar or different effects upon heterologous viral pathogens. To elucidate such questions, we have produced transgenic *Nicotiana benthamiana* and *N. tabacum* plants which express well-characterized silencing suppressors derived from six different virus genera. The observed features indicate that the two tobacco species react differently to the expression of these silencing suppressors. In addition, the suppressors have different effects on the replication and

spread of a crucifer-infecting *Tobamovirus* (crTMV) amplicon expressing the green fluorescent protein (GFP) marker gene.

RESULTS

N. benthamiana and *N. tabacum* were transformed with silencing suppressor genes derived from six different viral genera (*P1* of *Rice yellow mottle virus* [RYMV], *Cocksfoot mottle virus* [CfMV], *P19* of TBSV, *P25* of PVX, *HcPro* of *Potato virus Y* [PVY], *2b* of CMV, and *AC2* of *African cassava mosaic virus* [ACMV]). To avoid any possible tissue culture effects and to have stable lines, two or three independent homozygote R2 lines for each of the transgene were selected based on their 100% germination on kanamycin (Km)-containing medium. These were used for the observation of plant phenotypes and for analyzing the plant responses to the crTMV-GFP infections. Transgene mRNA expression (Fig. 1A) and variable altered phenotypes were detected in these R2 plants for most of the selected *N. benthamiana* lines. The transgene mRNA remained below detection level in both lines carrying the *P1*-RYMV and *P19* transgenes as well as in one of the lines harboring the *P25* and *AC2* transgenes. However, a clearly altered phenotype also was observed in one of the lines expressing each of the *P1*-RYMV and *P19* genes, and also in the mRNA-negative *AC2* transgenic line, suggesting that these lines were expressing the transgene. For each transgene, the line or lines with the most pronounced phenotype also showed the strongest effect on the spread and accumulation of the crTMV-GFP, as described below, and the transgene-positive status of the selected lines was confirmed by polymerase chain reaction (PCR) (Fig. 1B).

In transgenic *N. tabacum* lines, transgene mRNA was detected in all selected homozygote lines except in one of the lines containing the *P19* transgene, and in either of the two lines carrying the *P25* and *P1*-CfMV transgenes (Fig. 1A). Thus, at least one transgene-expressing *N. tabacum* line was

Fig. 1. A, Northern blot detection of different transgene mRNAs in *Nicotiana benthamiana* (N.b.) and *N. tabacum* (N.t.) and **B**, polymerase chain reaction-mediated detection of the transgene DNA in the selected lines of the transgenic plants. RYMV = *Rice yellow mottle virus* and CfMV = *Cocksfoot mottle virus*.

obtained for each of the transgenes except for the *PI-CfMV* and *P25*; however, these still were maintained for the analysis due to their effects in *N. benthamiana*. One of the *P25*-harboring *N. tabacum* lines showed reduction of the accumulation of PVX RNA in PVX-inoculated plants (data not shown), suggesting that the *P25* sequence was targeted by RNA silencing in this line. The presence of the transgene in all *N. tabacum* lines was confirmed by PCR (Fig. 1B).

Phenotypes of transgenic plants.

The phenotypes observed in the transgenic plants expressing the different silencing suppressor genes varied between the transgenes and also between the two tobacco species (most-pronounced phenotypes are summarized in Tables 1 and 2). The most striking phenotypes were caused by *HcPro* in both *Nicotiana* spp. In *N. benthamiana*, phenotypes caused by this transgene were similar in both selected lines, although more pronounced in one of the lines. In this line, the stems were strongly bending and twisting, causing a creeping growth habit. Leaves were strongly rolled and hairy, with elongated vein organization. Flowers had very short petals and stamens and protruding pistils, and were sterile. In addition, leaves and flowers grew without petioles (Fig. 2A through E; Table 1). Creeping stems and low seed production also were observed in most of the *HcPro* expression lines in the R0 and R1 generations (data not shown). Both *HcPro* transgenic *N. tabacum* lines also had a strong phenotype with distinctly thick, dark, and hairy leaves with moderate blistering. Stems were very hairy with short, thick internodes, and the plants were severely stunted (Fig. 3A). Flowering in these plants was strongly delayed; however, the flowers were fertile (Table 2).

One of the *AC2* transgenic lines in *N. tabacum* exhibited a phenotype very similar to the *HcPro* lines (Fig 3B) except that,

occasionally, the leaves were rolled into cup-shaped forms (Fig. 3C) and the flowers were moderately malformed, with some stamens transformed into petals (Fig. 3D). The other *AC2*-expressing *N. tabacum* line showed less leaf malformation; however, in this line, the flowers were severely malformed, having all stamens transformed into petals (Fig. 3D through G). The seed set was strongly reduced in both *AC2* lines. In the *AC2*-expressing *N. benthamiana*, only a mildly altered phenotype with some blistering of the leaf surfaces was observed in one line; however, the mRNA-negative line also displayed occasional rolling of the leaf blades into a funnel- or cup-shaped form (Fig. 2F). Cup-shaped leaves also were observed in the *AC2*-expressing *N. benthamiana* R1 generation (data not shown), and seed set was severely reduced.

Distinct phenotypes also were observed in plants expressing the other transgenes. *PI-RYMV* caused high lethality in *N. benthamiana* in the R0 generation, and only 3 of the 10 regenerated lines produced seed in this generation. It also caused malformations in the R1 generation (data not shown) and stunting in the R2 generation (Fig. 2G), although the transgene mRNA did not accumulate to detectable level in the surviving R2 progeny lines (Table 1). Occasionally, severe leaf rolling or cup-shaped leaves occurred in the R2 plants (Fig. 2H). In one of the lines, flower stalks were bent and flowers occasionally malformed (Fig. 2I). The seed set of this line and the seedling growth were poor. In *N. tabacum*, this transgene caused no specific leaf or growth phenotype, although the gene was expressed in these plants at high levels (Fig. 1B). In contrast to *PI-RYMV*, the *PI-CfMV* caused no specific phenotype, except that some flowers of one of the *N. benthamiana* lines had bending stalks.

P19-expressing *N. benthamiana* plants had blistered leaf epidermis, hairy and serrated leaves in one of the lines, and

Table 1. Most pronounced phenotypes observed in different transgenic lines of *Nicotiana benthamiana*

Lines ^a	Leaf	Flower	Stunting	Others
Wild type (WT)	Normal	Normal	No	...
pBin61	Normal	Normal	No	...
<i>HcPro</i>	Severe curling, changed vein pattern, no petioles, hairy	Short petals and stamens, protruding pistils, no petioles	Moderate, quantification difficult due to the creeping growth	Hairy creeping stems
<i>AC2</i>	Occasionally cup-shaped, mild blistering	Normal	Moderate, final height approximately 75% of the WT plants	...
<i>PI-RYMV</i>	Occasionally cup-shaped	Few flowers, sterile, bending stalks, malformed petals	Severe, final height 30–40 % of the WT plants	Poor seedling growth (50% impaired)
<i>PI-CfMV</i>	Normal	Bending stalks	No	...
<i>P19</i>	Mild blistering, mildly serrated, hairy	Bending stalks	No	...
<i>P25</i>	Normal	Smaller, not fully opened	Mild, final height 80–90% of the WT plants	Early senescence
<i>2b</i>	Normal	Normal	No	...

^a RYMV = Rice yellow mottle virus and CfMV = Cocksfoot mottle virus.

Table 2. Most pronounced phenotypes observed in different transgenic lines of *Nicotiana tabacum*

Lines ^a	Leaf	Flower	Stunting	Others
Wild type (WT)	Normal	Normal	Normal	...
pBin61	Normal	Normal	No	...
<i>HcPro</i>	Thick and hairy, short internodes, blistering	Normal	Severe, final height approximately 50% of the WT plants	Late flowering (2 months later)
<i>AC2</i>	Thick and hairy, short internodes, blistering, cup-shaped	Moderate malformation, stamens transformed into petals, some sepals transformed into petals, less stamens (4)	Severe, final height approximately 50% of the WT plants	Late flowering (1 month later)
<i>PI-RYMV</i>	Normal	Normal	No	...
<i>PI-CfMV</i>	Normal	Normal	No	...
<i>P19</i>	Normal	Severe malformation	No	...
<i>P25</i>	Normal	Normal	No	...
<i>2b</i>	Normal	Normal	No	Late flowering (1 month later)

^a RYMV = Rice yellow mottle virus and CfMV = Cocksfoot mottle virus.

Fig. 2. Phenotypes observed in *Nicotiana benthamiana* plants expressing different transgenes. **wt**, Wild-type, nontransgenic *N. benthamiana*; **pBin61**, *N. benthamiana* transformed with empty pBin61 vector; **A through E**, *HcPro*-transgenic plants with **A**, strongly curled leaves; **B**, emergence of flowers and leaves without petioles; and **C through E**, malformed flowers with short petals and protruding pistils. **F**, *AC2* transgenic plant showing rolling of the leaf blades into mild cup-shaped form. **G through I**, *P1-Rice yellow mottle virus* transgenic plants showing **G**, stunting compared with wild-type plant on the left; **H**, cup-shaped leaf; and **I**, a malformed flower. **J and K**, *P19* transgenic plants showing **J**, blistered leaf epidermis and **K**, occasional bending of the flower stalks and mild serration. **L and M**, *P25* transgenic plants, showing **L**, small, malformed flowers and **M**, early senescence.

Fig. 3. Phenotypes observed in *Nicotiana tabacum* plants expressing different transgenes. **A**, *N. tabacum* plants transformed with *HcPro* gene compared with a wild type plant on the right. **B**, *AC2* gene-expressing plant, showing the dark and hairy leaves and stem with short and thick internodes, a phenotype very similar to the *HcPro* transgenic plants. **C**, Leaves of *AC2*-expressing plants occasionally turned into funnel- or cup-shaped forms. **D through I**, Malformed flowers with stamens converted to petals, occasional bending of the flower base, or with petals fused or reduced in number, observed frequently in transgenic plants expressing *AC2* (**D through G**) and *P19* (**H and I**). Healthy flower is shown in **G**, on the left.

occasional bending of the flower stalks (Fig. 2J and K). In *N. tabacum*, P19 caused occasional malformation of flowers (Fig. 3H and I).

One of the *P25*-expressing *N. benthamiana* lines had very small, not fully opened flowers (Fig. 2L) with reduced seed set. This line also was moderately stunted and senesced early (Fig. 2M). The other line showed longitudinally rolled leaves.

None of the *2b*-expressing transgenic lines in either of the *Nicotiana* spp. showed any specific phenotypes, although both selected lines expressed transgene mRNA on fairly high levels.

Effects of the HcPro and AC2 silencing suppressors on the structure of the leaf tissues.

The most severe leaf malformations occurred in both *Nicotiana* spp. transformed either with the *HcPro* or the *AC2* genes; therefore, thin sections of these leaves were prepared to investigate how the tissue structures were altered in these transgenic plants. Microscopic analysis of the thin sections indicated that, in both *Nicotiana* spp., *HcPro* transgene caused a significant increase in the numbers of the palisade and spongy mesophyll cells, leading to reduction of the air space between the cells (Fig. 4B and E, *N. benthamiana* sections; H and K, *N. tabacum* sections). Particularly in the *HcPro*-expressing *N. benthamiana* plants, the lower epidermis of the leaves appeared expanded, leading to frequent bulging-out of the epidermis (Fig. 4B and E). Particularly in the thin sections of one of the *HcPro*-expressing lines, the guard cells were frequently observed from the top view (Fig. 4E) in contrast to the control samples, where guard cells typically are seen as cross-sections of the cells, indicating again that the epidermal cells were twisted and crowded. In contrast to the *HcPro*-expressing plants, the mesophyll cells of the *AC2* transgenic plants of both *Nicotiana* spp. were significantly larger than the mesophyll cells of comparable wild-type plants (Fig. 4C and F, *N. benthamiana* sections; I and L, *N. tabacum* sections). Particularly in *N. tabacum*, the cell walls appeared thinner than in the wild-type

cells and the cells appeared to be distorted or wrinkled, with disturbed and clumped chloroplast distribution, suggesting that the cell wall structures were thinner or weaker in these tissues compared with the control samples (Fig. 4I and L).

The thin sections of the *HcPro* and *AC2* transgenic *N. benthamiana* and *N. tabacum* plants indicated that, at the cellular level, the malformations caused by each transgene were similar in both *Nicotiana* spp. Whereas, between the two transgenes, they were associated with different effects (i.e., strong increase of cell number, with distinctly small cells [hyperplasia] in the *HcPro* plants, and very large cells [hypoplasia] in the *AC2* plants).

Interference of the silencing suppressors with the crTMV-GFP spread and accumulation.

The infectious virus derived from the crTMV cDNA, with a GFP gene replacing the coat protein gene, readily caused a local infection in *N. benthamiana* and also slowly spread in this host. This construct has been used successfully to analyze RNA-silencing activity in *N. benthamiana* (Dorokhov et al. 2006) and, therefore, we used it here for further testing the expression of different silencing suppressors in the transgenic plants, as well as to test the suppressor effects on a heterologous virus infection. Two leaves from each of the three plants from each transgenic *N. benthamiana* line were infiltrated with an *Agrobacterium* suspension carrying 35S-controlled infectious crTMV-GFP clone. However, this analysis was not carried out in *N. tabacum* because the virus construct is barely infectious in this host.

The crTMV-GFP lesions spread slightly differently in the sibling plants of different transgenic *N. benthamiana* lines; however, the type of GFP-lesion spread and degree of luminosity still varied clearly between the different transgenic plants (Fig. 5). *AC2* transgene clearly enhanced both the spread and the mean surface brightness (average flux per pixel) of the GFP lesions (Fig. 5). The *2b* transgene and also both of the *P1*

Fig. 4. Microscopic analysis of the leaf structures of the wild-type, *HcPro*, and *AC2* transgenic A through F, *Nicotiana benthamiana* and G through L, *N. tabacum* plants. The size bar in each frame corresponds to 100 μ m. Two typical sites of each leaf type are portrayed. The enlarged detail in E shows one of the guard cells seen from the top view, observed frequently in the *HcPro* samples.

transgenes (Fig. 5) increased the spread of the lesions but did not significantly affect the brightness of the lesions. *P19* transgene did not affect the total infected area but increased its brightness, and also repeatedly caused spread of the infection into new infection foci (Fig. 5). Surprisingly, *HcPro* transgene, and even more strongly the *P25* transgene, reduced the spread of the crTMV-GFP lesions (Fig. 5). The pictures and measured GFP values shown in Figure 5 are derived from one experiment, 15 days after the infiltration of the plants. Very similar results were observed in the other experiments.

DISCUSSION

Different viral silencing suppressors are being studied intensively in various laboratories around the world. With these research efforts, a very complex view is emerging, indicating that suppressors encoded by various viruses interfere in different ways with the silencing pathways, affecting the degradation of the target RNAs and the spread of the silencing signal from cell to cell or over long distance (Voinnet 2005). To clarify the various silencing suppressor mechanisms, comparisons

are needed to see how various suppressors function in different plant species.

In this work, we have analyzed for the first time in a comparative way the effects of seven different viral RNA silencing suppressors in *N. benthamiana* and *N. tabacum*. Up to now, similar comparative works have been published only in *Arabidopsis thaliana* (Chapman et al. 2004; Dunoyer et al. 2004). Some of the transgenes used in this study caused different effects in the two *Nicotiana* spp.. For instance, *P1*-RYMV caused high lethality in *N. benthamiana* in the R0 generation and malformations and stunting in the R1 and R2 generations, although the gene was expressed at a very low level in the surviving R2 progeny lines. In *N. tabacum*, this gene did not cause any (observable) adverse effect, even though it was expressed at high levels in the R2 generation. The *N. benthamiana* lines expressing *HcPro* gene had a strongly disturbed growth habit, with creeping stems and strongly malformed flowers as well as leaves. Plants expressing the *AC2* gene showed funnel- or cup-shaped leaf malformations and reduced seed set in both *Nicotiana* spp. In *N. tabacum* plants, both *HcPro* and *AC2* genes caused a similar, rigid growth habit with

Fig. 5. Lesions of the crucifer-infecting Tobacco mosaic virus-green fluorescent protein infection in different transgenic and control *Nicotiana benthamiana* plants, viewed in 366-nm UV light 15 days after infiltration. The upper number under each frame indicates the mean area of three lesions and the lower number indicates the mean of the average surface brightness of three lesions, each from three separate plants, with the indication of the standard deviations. Each of the lesions shown in the figure is the medium representative of the three measured lesions. Each of the frames corresponds to real size of 45 mm in width and 90 mm in height. RYMV = Rice yellow mottle virus and CfMV = Cocksfoot mottle virus.

short internodes and thick, large, hairy leaves. However, at the cellular level, the malformations caused by these transgenes were similar in both species, whereas the effects of the two transgenes were clearly different from each other and were associated with hyperplasia in the *HcPro* transgenic plants, and with hypoplasia in the *AC2* transgenic plants. The most striking flower malformations in *N. tabacum* were caused by the *AC2* transgene; however, in *N. benthamiana*, this gene did not visibly affect the flower morphology, though seed set was severely reduced.

Some of the silencing suppressor transgenes caused very specific phenotypic effects. Particularly, *HcPro* gene caused a very severely distorted growth habit in both *Nicotiana* spp., and the *P25* gene, for example, caused small, not fully opened flowers in *N. benthamiana*. On the other hand, several of the genes caused similar effects in the transgenic plants within the same species. For instance, *P1-RYMV*, *HcPro*, *AC2*, and *P25* genes caused stunting in *N. benthamiana*. In addition, *P1-RYMV*, *P1-CfMV*, and *P19* genes caused bending of the flower base and *P19* and *AC2* genes caused blistering of leaves in *N. benthamiana*. These variable phenotypes suggest that the used transgenes interfere with different steps of the endogenous silencing processes. On the other hand, some of the different effects also may be related to the different expression levels of the transgenes.

It is of interest to compare these observed phenotypes with other silencing suppressor-transgenic plants described in the literature. *HcPro* gene has been extensively studied, and transgenic plants harboring either *HcPro* gene alone or together with *P1* gene from different potyviruses have been produced in *A. thaliana*, *N. benthamiana*, and *N. tabacum* species (Anandalakshmi et al. 2000; Carrington et al. 1990; Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003; Mallory et al. 2001, 2002; Mlotshwa et al. 2002, 2005; Pruss et al. 2004; Savenkov and Valkonen 2002; Shams-Bakhsh et al. 2007). Here, we studied *HcPro* protein (without *P1*) from PVY-N which, similarly to the other potyviral suppressors in *Arabidopsis*, caused severe malformations in the two transgenic *Nicotiana* spp. However, Mlotshwa and associates (2002), Shams-Bakhsh and associates (2007), and Savenkov and Valkonen (2002) reported no phenotypic alterations in transgenic *N. benthamiana* and *N. tabacum* cv. Samsun NN plants expressing *HcPro* protein of PVY-N or PVA, respectively. Also, no phenotype data was reported by Carrington and associates (1990) or Mallory and associates (2001, 2002) for *N. tabacum* expressing the *HcPro* derived from TEV, while severe malformations were reported in such transgenic tobacco plants by Anandalakshmi and associates (2000) and by Pruss and associates (2004). Therefore, the *N. benthamiana* transformed in this work with the *HcPro* gene of PVY-N is the first one reported to display a clear phenotype, including curled leaves with changed vein patterns, soft stems, and strongly malformed flowers as well as moderate stunting. Interestingly, some features described for TuMV *P1/HcPro*-transgenic *Arabidopsis* are similar with these observed phenotypic features (Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003; Mlotshwa et al. 2005). Different phenotypes, or lack of any phenotype observed in various studies in the *HcPro*-expressing transgenic plants, may be due to the high variation in the *HcPro*-coding region between different potyviruses (Flasinski and Cassidy 1998) or different expression levels of the transgenes in the different plants.

There is only one report describing the expression of tombusviral *P19* transgene in *N. benthamiana*. The *P19* transgene expressed in those plants was from *Artichoke mottled crinkle virus* and the described phenotype differed completely from the one observed in this work for TBSV *P19* (Silhavy et al. 2002). Intriguingly, the phenotypes reported for transgenic

Arabidopsis expressing TBSV *P19* are similar to the ones observed here in *N. benthamiana*: serrated leaves and additional trichomes (Chapman et al. 2004; Dunoyer et al. 2004). In both *N. benthamiana* and *N. tabacum*, the altered phenotypes were observed here in plants in which the transgene mRNA accumulation remained below detection level, likewise with the reported transgenic *Arabidopsis*, harboring the *P19* gene of TBSV and showing altered phenotype (Dunoyer et al. 2004).

P25 protein of PVX caused a specific flower malformation and an early senescence phenotype in the *N. benthamiana* plants. *P25* transgenic *N. tabacum* plants showed no altered phenotype; however, this may have been due to the low level of transgene expression in these plants. This transgene has not caused any phenotype in *Arabidopsis* (Dunoyer et al. 2004), whereas the *P25* protein from *Clover mosaic virus* has caused severe abnormalities in *N. benthamiana* leaves (Foster et al. 2002).

The *2b* protein from three different CMV isolates has been used in silencing studies to elucidate its function as a suppressor. It has been shown that the *2b* gene from a severe strain causes more severe developmental abnormalities in *Arabidopsis* (Lewsey et al. 2007; Zhang et al. 2006). The *2b* gene used in this study was derived from a mild strain of CMV (Kin) and caused no developmental abnormalities in the two transgenic *Nicotiana* spp., as also was reported earlier when the corresponding gene of strain CMV-Q (another mild strain) was transformed into either *N. tabacum* (Ji and Ding 2001) or *Arabidopsis* (Lewsey et al. 2007; Zhang et al. 2006).

Up to now, no data about *AC2* gene expression in transgenic plants has been published. In this study, both *N. benthamiana* and *N. tabacum* harboring the *AC2* transgene exhibited altered phenotypes which appeared different on the intact plant level but similar on the cellular structure level. No transgenic plants have been produced from either *P1-CfMV* or *P1-RYMV*; therefore, the observed phenotypes caused by these two viral suppressors cannot be compared with previous reports. *P1-RYMV* caused severe stunting and leaf and flower malformations in the transgenic *N. benthamiana* plants but no abnormalities in the *N. tabacum* plants. On the other hand, *P1-CfMV* did not cause any observable effects on growth, except for some bending of flower stalks occurring in the *N. benthamiana* transgenic plants. The fact that the phenotypic alterations are more pronounced in the case of *P1-RYMV* is in correlation with the results published for the suppression efficiency of both sobemoviral proteins in these two *Nicotiana* spp.; *P1-RYMV* was the stronger of the two suppressors in agroinfiltration experiments (Sarmiento et al. 2007).

The disturbed phenotypes in the silencing suppressor-expressing transgenic plants likely are due to the interference of these suppressors with the endogenous RNA silencing pathways. Previously, it has been shown that viral suppressors interfere with miRNA biosynthesis in *Arabidopsis* and inhibit the cleavage of target genes by specific miRNA in the plant developmental pathway (Alvarez et al. 2006; Chapman et al. 2004; Dunoyer et al. 2004; Jacobsen et al. 1999; Kasschau et al. 2003; Llave et al. 2002; Mallory et al. 2002, 2004; Millar and Gubler 2005; Park et al. 2002; Ray et al. 1996; Vazquez et al. 2004). Such interference, targeted at an early step in the silencing pathways, would impair the regulation of multiple miRNA-regulated target genes, such as *SCL6*, targeted by miR171; NAC-domain proteins (*CUC1* and *CUC2*), targeted by miR164 (Mallory et al. 2004; Rhoades et al. 2002); *AP2*, and *ARF8* and *ARF10*, coding for transcription factors, expressed specifically in inflorescence and leaves, respectively; and regulating their differentiation (Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003; Mallory et al. 2002; Park et al. 2002).

In addition to the abovementioned genes, other miRNA-regulated transcription factors also have similar effects, as observed in our transgenic plants. These include the *GAMYB*-like genes (e.g., *MYB33*) which malfunction in *Arabidopsis*, causing stunting, spindly growth, sterility, and reduced petiole lengths (Millar and Gubler 2005), and the *PHABULOSA* gene, which malfunction in *Arabidopsis*, causing leaves with upward curling (Mallory et al. 2004). It is not clear which specific steps or target genes of the endogenous silencing pathways are targeted in the two *Nicotiana* spp. by the silencing suppressors used in this study. The different phenotypes observed in these two species indicate that the effects of these silencing suppressors are, at least to some extent, species specific. In general, more severe developmental disturbances were observed in transgenic *N. benthamiana* than in *N. tabacum* plants. All the transgenes, except for the *2b*, caused various developmental defects in *N. benthamiana*; however, in *N. tabacum*, defects occurred only in the *HcPro*- and *AC2*-expressing plants, and in one line of the *P19* transgenic plants (even though the mRNA level of this line remained below detection level). The high lethality of *P1*-RYMV-transformed *N. benthamiana* lines in the R0 generation and the very low expression level of this transgene in surviving plant lines also were in contrast with the *N. tabacum* lines, where *P1*-RYMV was expressed on high levels without any adverse effect.

In addition to their effects on the endogenous silencing pathways, the viral silencing suppressors also interfere with the plant defense reactions with different viruses. The crTMV-GFP construct is very suitable for quantitating silencing suppression effects because, according to Kurihara and Watanabe (2004), crTMV itself does not suppress silencing, at least in *Arabidopsis* (which is a natural host of crTMV). In this work, we observed that the spread and accumulation of the crTMV-GFP construct was very differently affected in the different transgenic *N. benthamiana* plants. Both the crTMV-GFP lesion spread and brightness were strongly enhanced in the *N. benthamiana* lines expressing the *AC2* gene. This spread also was increased in the *2b*, *P1*-RYMV, and *P1*-CfMV transgenic lines. *P19* transgene specifically caused the infection to spread into new foci within the infiltrated leaves. Surprisingly, both the *HcPro* and *P25* transgenes reduced the spread of the crTMV-GFP construct. These different reactions suggest that the used suppressors affect differently the silencing functions targeted against the accumulation of this virus in cells, or against the cell-to-cell spread of the silencing signal. Some of these results, particularly those related to *HcPro* and *P25* transgenes, contradict the original hypothesis, which assumes that the silencing suppressors should enhance viral infections. However, these results seem to be in good accordance with previous results of Pruss and associates (2004), showing that expression of the *HcPro* of TEV in transgenic tobacco plants enhances their resistance against TMV and against *Tomato black ring virus*. Understanding the mechanisms of this resistance enhancement needs further investigation.

MATERIALS AND METHODS

Viral silencing suppressor constructs and their transformation to *Nicotiana* spp.

P19 of TBSV, *P25* of PVX, *HcPro* of PVY (strain N), *2b* of CMV (strain Kin), *AC2* of ACMV (*Geminivirus*), and *P1* of RYMV (*Sobemovirus*) in pBin61 vector, obtained from the laboratory of D. Baulcombe (through Plant Bioscience Ltd.), and *P1* of CfMV (*Sobemovirus*) in pBin61 vector (Sarmiento et al. 2007) were used for transformation. These constructs and empty vectors were introduced into *Agrobacterium tumefaciens* through electroporation, and transformed into leaf disks

of *N. benthamiana* and *N. tabacum* cv. Xanthi (nn) L. by standard procedures (Smith et al. 1994). The transformants were regenerated on Murashige-Skoog (MS) medium using Km selection (Km at 100 µg/ml, cefotaxime at 250 µg/ml, and vancomycin at 100 µg/ml). Rooted plantlets were transferred to pots and grown to maturity in the greenhouse at 25°C with a 16-h photoperiod. In all, 10 lines with each silencing suppressor construct in both *Nicotiana* spp. were regenerated. All lines produced adequate amounts of seed for propagation, except *P1*-RYMV lines, where only three of the regenerated *N. benthamiana* plants produced seeds.

Propagation of the R1 and R2 generations.

Seed of each of the 10 selected R0 transgenic lines were germinated on Km-containing MS medium, transferred to soil, and grown in the greenhouse, as mentioned above, with observation of the phenotypes and collection of seed. Five independent R1 lines with altered phenotype were selected and their seed were germinated on Km-containing MS medium. Two or three independent lines showing 100% germination, indicating homozygote transgene status, were selected for further analysis. For any further experiments, the seed were germinated in soil and the plants propagated in controlled greenhouse conditions as described above.

PCR and Northern blot analysis.

For PCR amplification of the transgene sequences from the genomic DNA of the selected plant lines, the plant DNAs were extracted with cetyltrimethylammonium bromide procedure (Siddiqui et al. 2007), and amplified using either primers annealing to the 35S promoter and terminator sequences (for *P1*-RYMV gene) or specific primers annealing to the coding regions of the transgenes. Primers used were as follows: for detection of *P1*-RYMV, 5'-ACTCAGCAGACTGTGGCAAA A-3' (forward) and 5'-TACCGGAGTGCTCGGGGATAT-3' (reverse); for detection of *P19*, 5'-AGGGAACAAGCTAACA GTGAAC-3' (forward) and 5'-TACCTTCAGGGCATCCTCT TG-3' (reverse); for detection of *P25*, 5'-GTTTAGGTTATTCT AGGACTTC-3' (forward) and 5'-TCCCTTTGACCTGGTGTA TAGCG-3' (reverse); for detection of *AC2*, 5'-ACTCTACTCA GGTTCGAATCAAAG-3' (forward), and 5'-TCTGAGGCTGT AAGGTTGTCC-3' (reverse); for detection of *HcPro*, 5'-GTT GCATCCGAATGGGAC-3' (forward), and 5'-TGTGAGCCA AACGAGTCAACTAC-3' (reverse); and for detection of *2b*, 5'-TGACAGTAGTGGTGTGCGACC-3' (forward), and 5'-ACG ACCCTTCGGCCATTCGTTAC-3'. For the detection of *P1*-CfMV 35S primer and terminator, specific forward and reverse primers were used (i.e., 5'-GTGGATTGATGTGACATCTCC-3' and 5'-GCTCAACACATGAGCGAAACC-3', respectively).

For Northern blot analysis, total RNA was extracted according to Sijen and associates (1996). Briefly, leaves (0.5 g) were ground in liquid nitrogen and powder was extracted in hot phenol and RNA extraction buffer (100 mM Tris-HCl, pH 8.0; 100 mM LiCl; 10 mM EDTA; and 1% sodium dodecyl sulfate) (1:1) followed by extraction with one volume of chloroform. An equal amount of 4 M LiCl was added to the supernatant and RNA was separated from DNA by incubating on ice overnight at 4°C, followed by centrifugation (13,000 rpm for 20 min at 4°C). Pellets were resuspended in double-distilled water and RNA was recovered by ethanol precipitation. RNA (5 µg) was separated on 1% agarose denaturing gel and transferred to Hybond-N (GE Healthcare, Munich, Germany) membrane via capillary blotting by standard methods (Sambrook and Russell 2001) and fixed by baking at 80°C for 2 h. PCR-amplified digoxigenin-labeled probes were generated for each suppressor from original construct by using PCR primers, 5'-GTGGATTG ATGTGACATCTCC-3' (35S promoter region) and 5'-GCTCA

ACACATGAGCGAAACC-3' (35S-terminator region). Northern blots were probed and developed according to the manufacturer's instructions (Roche, Mannheim, Germany).

Microscopy.

For microscopic analysis, samples were collected from the first fully expanded leaves of young *N. benthamiana* and *N. tabacum* plants and immediately fixed with 3% glutaraldehyde in 0.1 M Na-phosphate buffer, pH 7.0, and postfixed in 1% osmium tetroxide in the same buffer. After dehydration in an alcohol series, the samples were embedded in Epon. Thin sections were cut with Reichert ultramicrotome and examined with a Reichert zetopan microscope, mounted with a Canon EOS 20D digital camera.

Agrobacterium infiltration.

Fresh overnight cultures of *Agrobacterium* cells, carrying a 35S-controlled infectious clone of the crTMV cDNA with a GFP gene replacing the coat protein gene (a gift from Y. Dorokhov), adjusted to an optical density at 600 nm = 1.0 as final density, were induced with acetosyringone as described by Hamilton and associates (2002). Equal volumes (approximately 100 µl) of the cell suspension were infiltrated to the two uppermost, fully expanded leaves of three plants of each transgenic *N. benthamiana* line. In two of three repeating experiments, the infiltrated plants were incubated for 15 days and, in one experiment, for 7 days before detaching the leaves for photography. The infiltrated plants were maintained in the greenhouse under the conditions mentioned above.

GFP imaging.

The GFP was visualized by using a hand-held 366-nm wavelength UV lamp (BLACK RAY, UVL-21, Ultra-Violet Products, Inc., Upland, CA, U.S.A.). Photographs were taken at 15 days postinfiltration with a Canon EOS 20D digital SLR camera. Equal exposures of each leaf sample were obtained by fixing lamps, camera, and sample positions. The pictures were analyzed using Photoshop CS3. Only the green channel images were used. The brightness values of each pixel in every image were checked to ensure that none were saturated. This is important because information about the true brightness is lost if saturation levels are reached. No image had saturated pixels.

The raw format images were transferred to linear tiff format (note: jpeg format images, normal gamma-corrected tiff, and most other formats are not linear). The linearity of this transformation has been verified by astronomical measurements of stars of different magnitudes covering a factor of approximately 100 in brightness. The linearity was better than 3%, which is sufficient for these measurements (H. Lehto, *unpublished results*). The total brightness of the GFP spots was measured with an aperture photometry technique, in which the background level brightness was subtracted automatically. The units in the total brightness are in an arbitrary but linear scale. The surface area was measured using jpg images because, here, the linearity is not critical. The edges of the spots were typically sharp. A suitable cut-off level for the surface area was selected by eye. The result is not sensitive to small differences at this level. From repeated measurements of the same sample the accuracy of the surface area was estimated as $\pm 5\%$ and the integrated brightness within $\pm 5\%$, giving an estimated error of $\pm 7\%$ for the surface brightness. This accuracy is sufficient for detecting significant differences between samples.

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