***Tobacco mosaic virus* 126-kDa Protein Increases the Susceptibility of *Nicotiana tabacum* to Other Viruses and Its Dosage Affects Virus-Induced Gene Silencing**

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The *Tobacco mosaic virus* (TMV) 126-kDa protein is a suppressor of RNA silencing previously shown to delay the silencing of transgenes in *Nicotiana tabacum* and *N. benthamiana*. Here, we demonstrate that expression of a 126-kDa protein–green fluorescent protein (GFP) fusion (126-GFP) in *N. tabacum* increases susceptibility to a broad assortment of viruses, including *Alfalfa mosaic virus*, *Brome mosaic virus*, *Tobacco rattle virus* (TRV), and *Potato virus X*. Given its ability to enhance TRV infection in tobacco, we tested the effect of 126-GFP expression on TRV-mediated virus-induced gene silencing (VIGS) and demonstrate that this protein can enhance silencing phenotypes. To explain these results, we examined the poorly understood effect of suppressor dosage on the VIGS response and demonstrated that enhanced VIGS corresponds to the presence of low levels of suppressor protein. A mutant version of the 126-kDa protein, inhibited in its ability to suppress silencing, had a minimal effect on VIGS, suggesting that the suppressor activity of the 126-kDa protein is indeed responsible for the observed dosage effects. These findings illustrate the sensitivity of host plants to relatively small changes in suppressor dosage and have implications for those interested in enhancing silencing phenotypes in tobacco and other species through VIGS.

Additional keywords: pathogenicity determinants, post-transcriptional gene silencing, small RNAs, synergy determinant

Post-transcriptional gene silencing (PTGS) constitutes an important component of the plant defense response against viral pathogens (English 1996; Goodwin et al. 1996; Smith et al. 1994). PTGS is triggered by double-stranded RNA (dsRNA) and results in the eventual degradation of cellular RNA containing sequences identical to the dsRNA (Brodersen and Voinnet 2006; Hammond et al. 2000; Szitty et al. 2002; Vance and Vaucheret 2001). The induction of PTGS by viral-derived dsRNA is termed virus-induced gene silencing (VIGS). VIGS has become a commonly used tool for knocking down the expression of specific genes in plants (Burch-Smith et al. 2006; Lu et al. 2003; Senthil Kumar 2008). In this approach, plant sequences of interest are inserted into a virus-based vector. Upon infection with the virus, plant genes containing sequence homologous to the insert are silenced along with the viral

RNA. In particular, *Potato virus X* (PVX) and *Tobacco rattle virus* (TRV) have been highly utilized as vectors for efficient VIGS in *Nicotiana benthamiana* and *Arabidopsis* (Burch-Smith et al. 2006; Ratcliff et al. 2001; Ruiz et al. 1998).

To counteract these plant defenses, many viruses encode so-called silencing suppressors which are capable of inhibiting specific steps in the PTGS pathway (Roth et al. 2004; Voinnet 2005; Voinnet et al. 1999). For example, the 2b protein from *Cucumber mosaic virus* was recently shown to interact directly with Argonaute 1, a core component of the RNA-induced silencing complex (RISC), and suppressors from several viruses have been found to suppress silencing by binding to siRNAs and inhibiting the assembly of the RISC (Diaz-Pendon et al. 2007; Lakatos et al. 2006; Valli et al. 2008; Zhang et al. 2007).

Tobacco mosaic virus (TMV) is a member of the well-studied *Tobamovirus* genus which also includes *Tomato mosaic virus* (ToMV) and *Turnip vein clearing virus* (TVCV). TMV infection restored green fluorescent protein (GFP) fluorescence in *N. benthamiana* plants expressing a silenced GFP transgene, presumably by inhibiting the host plant's PTGS response (Voinnet et al. 1999). This suppressor activity was later found to map to the 126- and 130-kDa replicase proteins in TMV and ToMV, respectively (Ding et al. 2004; Kubota et al. 2003). Recently, the ability of TMV infection to interfere with small RNA methylation, a critical step in PTGS, was linked to the 126-kDa protein's suppressor activity (Vogler et al. 2007). It seems likely that small RNA methylation may be physically blocked by TMV 126-kDa protein because the homologue from the closely related *Tobamovirus* sp., TVCV, was shown to bind small RNA duplexes and prevent their incorporation into the RISC (Csorba et al. 2007; Kurihara et al. 2007).

Because viral suppressors are capable of inhibiting PTGS, it is not surprising that several of these proteins were first identified as viral pathogenicity determinants (Brigneti et al. 1998; Cronin et al. 1995; Ding et al. 1995a). Also, many suppressors have been shown to be capable of interfering with the VIGS response (Anandalakshmi et al. 1998; Deleris et al. 2006; Qiu et al. 2002). Specifically, *Arabidopsis* expressing the p38 suppressor from *Turnip crinkle virus* fail to give a silencing phenotype when inoculated with a TRV VIGS vector, despite high levels of TRV accumulation (Deleris et al. 2006). In addition, the *Potyvirus* suppressor helper-component proteinase (HC-Pro) was shown to be responsible for the synergistic effect observed when potyviruses were co-inoculated with other viruses (Pruss et al. 1997). A synergistic effect was also observed when plants were co-infected with PVX and TMV, although the synergy determinant has not been identified (Balogun et al. 2002; Goodman and Ross 1974).

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In this study, we examined the effect of TMV's suppressor, the 126-kDa protein, on the susceptibility of *N. tabacum* to a wide range of plant viruses. We demonstrated that expression of a 126-kDa protein–GFP fusion (126-GFP) strongly enhanced infection by *Alfalfa mosaic virus* (AMV), *Brome mosaic virus* (BMV), PVX, and TRV in this host. We also tested the effect of 126-GFP on TRV VIGS in *Nicotiana* spp. and demonstrated that, whereas high levels of this protein inhibited VIGS, expression at low levels surprisingly enhanced VIGS phenotypes in both *N. tabacum* and *N. benthamiana*. We propose a model to explain this enhanced VIGS response and indicate the potential utility of plants expressing low levels of silencing suppressor proteins for improving VIGS.

RESULTS

The TMV 126-kDa protein, a silencing suppressor, enhances the accumulation and symptom development of a diverse array of viruses in *N. tabacum*.

In order to test the effect of the TMV 126-kDa protein on the infectivity of a wide range of plant viruses, both wild-type (WT) and transgenic *N. tabacum* plants expressing 126-GFP (Ding et al. 2004) were inoculated with AMV, BMV, PVX, and TRV (Fig. 1A). At 15 days postinoculation (dpi), all of the systemic leaves from WT plants (Fig. 1A, top leaves) lacked obvious symptoms of virus infection and were comparable with leaves from mock-inoculated plants. In contrast to WT plants, systemic leaves of 126-GFP-expressing plants (Fig. 1A, bot-

tom leaves) developed strong virus symptoms, including leaf distortion (arrows, PVX and TRV), vein clearing, and rugose crinkling (AMV and BMV). Although uninfected leaves from plants expressing 126-GFP were noticeably smaller than developmentally similar leaves from WT plants (Fig. 1A, Mock), virus infection resulted in additional leaf stunting in the 126-GFP-expressing plants, most notably in plants infected with AMV and BMV. It is pertinent to note that, although TRV and AMV are capable of infecting tobacco (Jaspars and Bol 1980; Semancik and Kajiyama 1967), we observed a pointed lack of virus symptom development in WT *N. tabacum* inoculated with these viruses. This lack of virus infection in WT plants is likely due to the temperature of the greenhouse during our experiments (24°C) because we found that AMV induced disease symptoms on *N. tabacum* leaves when inoculated plants were grown at higher temperatures (27°C) (Supplementary Fig. 1A, for symptom and virus accumulation, and data not shown). However, the difference in symptoms displayed between WT and 126-GFP plants remained at the higher temperature.

To determine if the enhanced virus symptoms observed in the 126-GFP-expressing tobacco plants were associated with increased virus accumulation, we performed semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to determine the level of coat protein (CP) transcript of each virus in the observed systemic leaves (Fig. 1B). For all viruses, the amount of CP transcript present in apical leaves of 126-GFP-expressing *N. tabacum* at both 7 and 15 dpi was comparable with that in the susceptible host *N. benthamiana*. Leaves from

Fig. 1. Expression of *Tobacco mosaic virus* (TMV) 126-green fluorescent protein (GFP) strongly enhances accumulation and symptom development of a wide range of viruses in *Nicotiana tabacum*. **A**, Apical leaves of tobacco 15 days postinoculation (dpi) with the indicated viruses (AMV: *Alfalfa mosaic virus*, PVX: *Potato virus X*, BMV: *Brome mosaic virus*, and TRV: *Tobacco rattle virus*). Each panel shows a representative image of a developmentally similar systemic (noninoculated) leaf from wild-type (WT) (top) or 126-GFP-expressing (bottom) *N. tabacum*. Viral symptoms are not readily apparent in WT leaves whereas 126-GFP-expressing leaves developed a variety of symptoms, including yellowing, stunting, vein-clearing, leaf distortion (arrows PVX and TRV) and rugose crinkling (AMV and BMV). Four individual plants from each genotype were inoculated with each virus. **B**, Accumulation of the indicated viruses in systemic leaves as determined by semiquantitative reverse-transcriptase polymerase chain reaction analysis of coat protein transcript. Mock-inoculated *N. tabacum* (Mock) and inoculated *N. benthamiana* (*N. benth*) served as negative and positive controls, respectively. A representative elongation factor 1α (EF1α) amplification is shown as an internal control. WT and 126-GFP-expressing (126) plants were analyzed at 7 and 15 dpi as indicated.

WT plants showed little (BMV, PVX, and TRV) or no (AMV) CP transcript accumulation at either timepoint. Additionally, there was a noticeable reduction in accumulation of the BMV, PVX, and TRV CP transcripts between 7 and 15 dpi in WT plants, suggesting a suppression of infection between these timepoints. Results from Northern blot analysis for AMV accumulation were similar to those from semiquantitative RT-PCR analysis (Supplementary Fig. 2). In addition, regardless of plant growth temperature, the enhanced symptoms displayed by 126-GFP-expressing plants over those displayed by the WT plants after AMV inoculation correlated with enhanced virus accumulation.

Expression of a silencing suppressor can enhance TRV VIGS in *N. tabacum*.

Because expression of the TMV 126-GFP fusion protein was found to greatly enhance TRV accumulation in *N. tabacum*, we wanted to assess the effect of this protein on TRV VIGS phenotypes. To this end, we inoculated both WT and 126-GFP-expressing *N. tabacum* with a TRV VIGS construct containing a portion of the *N. benthamiana* phytoene desaturase (PDS) gene via an agroinfiltration method (Fig. 2A) (Demmig-Adams and Adams 1992; Ratcliff et al. 2001). *N. benthamiana* plants were inoculated as a positive control. It is pertinent to note that the *N. benthamiana* and *N. tabacum* PDS sequences share 99% identity at the nucleotide level across the fragment utilized in this silencing construct (data not shown). At 15 dpi, systemic leaves of all six *N. benthamiana* plants observed produced a robust photobleaching phenotype typical of PDS silencing, whereas all of the WT *N. tabacum* plants (22/22) failed to produce symptoms of viral infection or yield any VIGS phenotype (Fig. 2A). When we inoculated our 126-GFP-expressing *N. tabacum* line, we were surprised to find that one-half (11/22) of the plants gave a strong photobleached phenotype accompanied by rugose crinkling. The other 11 126-GFP-expressing plants developed TRV-induced symptoms but completely lacked photobleaching (Fig. 2A). The presence or absence of a photobleaching phenotype remained unchanged for all plants between 15 and 30 dpi (data not shown).

In order to compare the accumulation of TRV-PDS between *N. tabacum* expressing or not expressing 126-GFP, we utilized semiquantitative RT-PCR to determine TRV CP transcript levels in developmentally similar apical leaves at 4, 7, and 15 dpi (Fig. 2B). We analyzed four 126-GFP expressing plants with visible PDS silencing and four WT plants at each timepoint. *N. benthamiana* was used as a positive control (+) and plants mock inoculated with phosphate-buffered saline (PBS) were used as negative controls (-). Elongation factor 1 α (EF1 α) transcript served as an internal control for each sample. At 4 dpi, a majority of plants (three of four) from each genotype showed accumulation of TRV CP transcript in apical leaves. However, at 7 dpi, only one of four WT plants accumulated TRV CP in apical leaves, whereas three of four 126-GFP-expressing plants remained positive for TRV CP transcript. By 15 dpi, all 126-GFP plants expressed high levels of TRV CP transcript whereas none was detected in the apical leaves of any of the WT plants.

In order to investigate whether the lack of TRV symptoms on the TRV-PDS-inoculated WT *N. tabacum* was due to a deficiency in the delivery of TRV into cells with the agroinfiltration method used, we analyzed TRV CP minus-strand transcript levels by RT-PCR in infiltrated leaves. We found that TRV CP minus-strand transcript levels increased significantly between 2 and 6 dpi (Supplementary Fig. 3), indicative of active viral replication. The presence of minus-strand TRV RNA in inoculated leaves and the observed decline in TRV CP transcript in systemic leaves of WT *N. tabacum* between 4 and 15 dpi (Fig.

2B) suggests an active surveillance for virus transcript by the plant. Although an active TRV infection was established in *Agrobacterium*-infiltrated leaves followed by a systemic accumulation of virus, the disappearance of TRV CP transcript in the systemic leaves of WT *N. tabacum* between 4 and 15 dpi is likely due to destruction of the virus by the plant's silencing machinery. The finding that plants expressing the TMV 126-kDa protein, a demonstrated silencing suppressor, continue to accumulate TRV CP transcript in systemic leaves between 4 and 15 dpi (Fig. 2B) further supports this possibility.

Fig. 2. Expression of 126-green fluorescent protein (GFP) can enhance Tobacco rattle virus (TRV)-induced gene silencing in *Nicotiana tabacum*. **A**, Phenotypes observed 15 days postinoculation (dpi) with TRV-phytoene desaturase (PDS) in *N. benthamiana* and either wild-type (WT) or 126-GFP-expressing *N. tabacum*. Numbers in the lower right corner of each panel denote the number of plants exhibiting the pictured silencing phenotype out of the total number of plants observed. **B**, Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of TRV coat protein (CP) transcript in apical leaves of four WT or 126-GFP-expressing *N. tabacum* plants at the indicated number of dpi. All 126-GFP plants analyzed by semiquantitative RT-PCR developed visible PDS silencing at approximately 10 dpi. TRV-infected *N. benthamiana* was used as a positive control (+) and mock-inoculated plants as negative controls (-). Elongation factor 1 α (EF1 α) served as an internal control for each sample.

The VIGS response is dependent upon the level of 126-GFP expression in *N. tabacum*.

The finding that expression of 126-GFP in *N. tabacum* resulted in an enhanced VIGS phenotype in approximately half of the plants infected with TRV-PDS led us to question whether variations in 126-GFP expression levels could offer an explanation for the strong dichotomy between phenotypes observed in these plants. To test the role of 126-GFP expression levels on the VIGS phenotype, we identified lines of *N. tabacum* expressing significantly more (30% more; high) and significantly less (40% less; low) 126-GFP than the original line (orig) used in the experiments described above (Fig. 3A). Be-

cause the 126-kDa protein was fused to GFP, we were able to use the average pixel intensity calculated across images from a given line to determine the level of 126-kDa protein expression.

When the three 126-GFP-expressing lines (Fig. 3A) were inoculated with TRV-PDS, all plants developed strong symptoms of virus infection (stunting and rugose crinkling) and we found a clear negative correlation between the level of 126-GFP expressed and the amount of photobleaching observed at 15 dpi (Fig. 3B through E). Although only one of six high expressors developed visible photobleaching (Fig. 3B), one-half of the original line (Fig. 3C) and all of the low expressors (Fig. 3D) developed a visible PDS VIGS phenotype. Our WT controls lacked any symptoms of virus accumulation or photobleaching (Fig. 3E), consistent with earlier findings (Fig. 2A).

In order to determine whether the differences in PDS silencing phenotypes observed corresponded to significant differences in PDS transcript levels, we analyzed PDS transcript extracted from the plants described in Figure 3B through E by semiquantitative RT-PCR at 15 dpi with TRV-PDS (Fig. 3F). Percentage of PDS expression was calculated in comparison with a WT control inoculated with TRV vector alone. Consistent with experiments in Figure 2, in which little or no TRV accumulation and no PDS silencing was observed in WT *N. tabacum*, we found that PDS transcript levels were comparable with the vector control in WT *N. tabacum* inoculated with TRV-PDS. Following inoculation with TRV-PDS, we observed a negative correlation between the amount of 126-GFP expressed and the level of PDS transcript. In comparison with PDS transcript levels in WT plants, we found these transcripts reduced 20, 40, and 70%, on average, in plants expressing high, intermediate (orig), and low levels of 126-GFP, respectively.

Low levels of WT 126-kDa protein but not a silencing-defective mutant can enhance TRV VIGS in *N. benthamiana*.

The TRV-PDS construct was already shown to give a strong VIGS response in the susceptible host *N. benthamiana* (Fig. 2A); therefore, we wanted to determine whether expression of the 126-GFP would have any effect on the VIGS phenotype in this host. To test this possibility, the 126-GFP construct was used to generate stable transformants of *N. benthamiana*. These lines showed an abundant accumulation of 126-GFP-bodies (data not shown) identical to those observed in *N. tabacum* (Ding et al. 2004; Liu et al. 2005). We utilized GFP

Fig. 3. Enhancement of Tobacco rattle virus (TRV)-induced gene silencing in *Nicotiana tabacum* is dependent on the expression level of the 126-green fluorescent protein (GFP) fusion protein. **A**, GFP expression was quantified to identify *N. tabacum* lines that express significantly more (High) and significantly less (Low) 126-GFP than the original line studied (Orig). Bars indicate average pixel intensity across 10 images from five plants $\pm$ standard error. **B** through **E**, Representative TRV-phytoene desaturase (PDS) silencing phenotypes at 15 days postinoculation (dpi) in **B**, High, **C**, Original, **D**, Low, and **E**, wild-type (WT) *N. tabacum* plants. Numbers in the lower right corner of each panel indicate the number of plants that developed a visible photobleaching phenotype out of six plants observed from each line. **F**, PDS expression levels in systemic leaves of the indicated lines of *N. tabacum* 15 dpi with TRV-PDS as determined by semiquantitative reverse-transcriptase polymerase chain reaction. Percentage of transcript level was calculated as the percentage of PDS transcript relative to a WT control inoculated with TRV vector alone. Elongation factor 1 α served as an internal control. WT, High, Low, and Orig refer to the wild-type line and lines expressing high, low, and intermediate levels of 126-GFP, respectively, as determined in **A**. Bars represent the average of six samples from each line $\pm$ standard error.

fluorescence intensity to identify *N. benthamiana* plants whose expression of 126-GFP differed by approximately threefold (Fig. 4A, Low and High). We inoculated four individual plants from each expression group with TRV-PDS and found that silencing phenotypes were entirely consistent within each group. When inoculated with TRV-PDS, WT *N. benthamiana* developed a robust photobleached phenotype at 9 dpi as expected (Fig. 4B). High 126-GFP expressors also developed photobleaching but the amount was visibly reduced in comparison with WT *N. benthamiana* treated similarly (Fig. 4C). Surprisingly, low 126-GFP expressors produced a significantly enhanced VIGS phenotype compared with WT *N. benthamiana* at 9 dpi (Fig. 4D). To confirm the role of the 126-kDa protein's silencing suppressor activity in the observed silencing phenotypes, we utilized transgenic *N. benthamiana* plants expressing a mutant version of the 126-GFP ($M^{IC1,3}$) which contains two amino acid substitutions that have been previously demonstrated to significantly reduce the protein's suppressor capability (Ding et al. 2004). Quantification of GFP expression revealed a level of $M^{IC1,3}$ 126-GFP expression similar to the level of 126-GFP in the low expressors (Fig. 4A, Low and $M^{IC1,3}$). The TRV-PDS-inoculated $M^{IC1,3}$ transgenic plants photobleached slightly less than WT plants

and much less than plants expressing low levels of 126-GFP (Fig. 4B, D, and E).

In order to determine the molecular basis of the observed silencing phenotypes, we analyzed PDS expression levels in systemic leaves of the *N. benthamiana* plants described in Figure 4B through E at 9 dpi with TRV-PDS by semiquantitative RT-PCR (Fig. 4F). Compared with a WT *N. benthamiana* control inoculated with TRV vector alone, we found that PDS transcript was reduced by an average of 47% in WT *N. benthamiana* inoculated with TRV-PDS. We observed an almost identical reduction in PDS transcript (46%) in plants expressing the $M^{IC1,3}$ mutant version of 126-GFP. By contrast, PDS transcript was reduced by only 13% in *N. benthamiana* expressing high levels of 126-GFP. Plants expressing a low level of 126-GFP showed the strongest reduction in PDS transcript levels at 59% below the vector control.

In addition to the observed differences in silencing phenotypes in systemic leaves, we found dramatic variations in the amount of necrosis in infiltrated leaves at 7 dpi (Fig. 4G). Specifically, we observed that infiltrated leaves from WT, low 126-GFP expressors, and the $M^{IC1,3}$ mutant 126-GFP expressors developed a relatively mild necrosis while high 126-GFP expressors were severely necrotic at 7 dpi.

Fig. 4. Enhancement of *Tobacco rattle virus* (TRV)-induced phytoene desaturase (PDS) silencing in *Nicotiana benthamiana* is dependent on the level and suppression capability of the 126-green fluorescent protein (GFP) fusion protein. **A**, GFP expression was quantified to determine levels of 126-kDa protein-GFP fusion (Low and High) or the $M^{IC1,3}$ -derived 126-kDa protein-GFP fusion mutant ($M^{IC1,3}$) in *N. benthamiana* plants. Bars indicate average pixel intensity from eight images representing four individual replicates $\pm$ standard error. **B** through **E**, TRV-PDS silencing phenotypes at 9 days postinoculation (dpi) in systemic (noninoculated) leaves of **B**, wild-type (WT) *N. benthamiana*; plants expressing **C**, high or **D**, low levels of 126-GFP; or **E**, a mutant version ($M^{IC1,3}$) of 126-GFP whose ability to suppress silencing is strongly reduced. **F**, PDS expression levels in systemically infected leaves of the indicated lines of *N. benthamiana* at 9 dpi with TRV-PDS were determined by semiquantitative reverse-transcriptase polymerase chain reaction. Percentage of PDS transcript was calculated as the percentage of PDS expressed in treatments relative to a WT control inoculated with TRV vector alone. Elongation factor 1 α served as an internal control. Bars represent the average of four samples from each line $\pm$ standard error. **G**, Infiltrated leaves from the indicated lines at 7 dpi with TRV-PDS. All images (B through E and G) are representative images from four individual replicates.

Different levels of 126-GFP expression do not correspond to significant differences in systemic TRV accumulation.

Given the dramatic disparity observed between TRV VIGS phenotypes in plants expressing different amounts of the TMV 126-GFP, we wanted to determine whether these phenotypic differences could be attributed to persistent variations in the accumulation of TRV-PDS in systemically infected leaves. We examined TRV CP transcript levels in systemically infected leaves of *N. benthamiana* and *N. tabacum* at 9 and 15 dpi, respectively. This analysis was performed on the same plants which were used to analyze silencing phenotypes and target transcript levels for these species (Figs. 3 and 4). Surprisingly, TRV CP transcript levels varied by 2% or less between *N. benthamiana* plants expressing high or low levels of 126-GFP, the mutant 126-GFP (M^{IC}1,3) or no 126-GFP fusion (Fig. 5A). Similarly, TRV CP transcript levels varied by 3% or less in *N. tabacum* plants expressing high, low, or intermediate (original) levels of 126-GFP (Fig. 5B). As noted in earlier experiments (Fig. 2B), TRV CP transcript was undetectable in systemic leaves of WT *N. tabacum* at 15 dpi and, thus, was not included in this analysis.

Fig. 5. Levels of Tobacco rattle virus (TRV) coat protein (CP) transcript are similar in plants expressing different levels of the Tobacco mosaic virus (TMV) 126-kDa protein–green fluorescent protein (GFP) fusion. TRV CP transcript expression levels were determined by semiquantitative reverse-transcriptase polymerase chain reaction on extracts from systemic leaves of plants of the indicated lines of **A**, *N. benthamiana* or **B**, *N. tabacum* at **A**, 9 or **B**, 15 days postinoculation (dpi) with TRV-phytoene desaturase (PDS). Different levels of 126-kDa protein–GFP fusion expression are indicated as high, low, or original in accordance with expression levels determined in earlier experiments. Samples from plants expressing the M^{IC}1,3-derived 126-kDa protein–GFP fusion are indicated (M^{IC}1,3). Percent transcript level was determined in comparison with the line with the highest band intensity (**A**, wild-type and **B**, original). Elongation factor 1 α served as an internal control. Bars represent the means of four replicates from each line $\pm$ standard error.

DISCUSSION

TMV 126-kDa protein increases the susceptibility of *N. tabacum* to a diverse array of viruses.

Because they can act to suppress an important plant defense response, a number of silencing suppressor proteins were first characterized as viral pathogenicity determinants (Brigneti et al. 1998; Cronin et al. 1995; Ding et al. 1995a). One of these suppressors, HC-Pro, at least in part, was also shown to increase the susceptibility of host plants to infection by heterologous viruses (Pruss et al. 1997). When *N. tabacum* plants expressing 126-GFP were inoculated with viruses from a diverse range of genera, we observed strongly enhanced virus symptom development and accumulation for all viruses tested (Fig. 1A and B). These findings are consistent with the ability of the TMV 126-kDa protein to suppress RNA silencing and demonstrate that this protein alone enhances the accumulation and symptom development of an assortment of viruses in *N. tabacum*. Interestingly, despite the widespread use of *N. tabacum* as a host for AMV (Jaspars and Bol 1980; Loesch-Fries et al. 1987; Tumer et al. 1987; Van Dun et al. 1987), we found that AMV CP transcript was entirely absent at both 7 and 15 dpi in WT *N. tabacum* but accumulated to levels comparable with those found in the susceptible host, *N. benthamiana*, in 126-GFP-expressing transgenic *N. tabacum* (Fig. 1B). The absence of AMV infection in WT *N. tabacum* is likely due to the temperature at which the experiment was carried out (24°C) because we observed an enhancement of AMV infection in *N. tabacum* at a higher temperature (27°C). However, even at the higher temperature, infection was significantly enhanced in 126-GFP-expressing plants over WT plants.

Surprisingly, 126-GFP also conferred upon BMV the ability to produce a strong systemic infection in *N. tabacum*. Although certain strains of BMV have been found to infect the dicotyledonous host, cowpea, this virus is primarily known to infect monocotyledonous grasses and has not been shown to infect *N. tabacum* (De Jong and Ahlquist 1991; De Jong et al. 1995; Valverde 1987). We were unable to detect BMV by enzyme-linked immunosorbent assay in WT *N. tabacum* (data not shown) but we were able to detect some systemic movement of BMV in WT plants by the more sensitive RT-PCR assay (Fig. 1B). However, the accumulation of high levels of BMV CP transcript and strong systemic symptoms in *N. tabacum* expressing 126-GFP demonstrates that the 126-kDa protein is capable of significantly extending the penetration of BMV in this host (Fig. 1A and B).

Although PVX and TRV have been previously shown to infect *N. tabacum* (González-Jara et al. 2004; Semancik and Kajiyama 1967), expression of TMV 126-GFP protein also dramatically increases symptom development (Fig. 1A) and accumulation of these viruses (Fig. 1B). The enhancement of PVX infectivity in the presence of TMV 126-GFP is interesting given that co-infection with PVX and TMV has been shown to yield increased PVX accumulation in both tobacco and tomato (Balogun et al. 2002; Goodman and Ross 1974). In particular, the synergistic effect of TMV on PVX infection was shown to result in higher levels of PVX accumulation per cell rather than an increase in the number of infected cells (Goodman and Ross 1974). Our results suggest that the suppressor activity of the TMV 126-kDa protein is likely responsible for this synergism.

Although viral suppressor proteins may generally enhance susceptibility of host plants to many viruses, the potyviral suppressor HC-Pro was also shown to conversely increase resistance to specific pathogens in tobacco (Pruss et al. 2004). It will be interesting to determine whether expression of the TMV 126-kDa protein might also have a similar effect. Given

the enhanced virus infections observed, expression of the TMV 126-kDa protein will potentially serve as a useful tool, enabling the study of a broad range of viruses in *N. tabacum* that may have previously been limited to other hosts.

TRV VIGS can be enhanced by low levels of TMV 126-kDa protein expression in *Nicotiana* spp.

The enhanced accumulation of TRV in 126-GFP-expressing *N. tabacum* (Fig. 1B) led us to test the effect of the 126-kDa protein and its silencing suppressor activity on TRV-enabled VIGS in *N. tabacum*. Considering the finding that the TCV suppressor P38 inhibited TRV VIGS in *Arabidopsis* (Deleris et al. 2006), we expected that the suppression of the host plant's silencing pathway by the 126-kDa protein would result in virus accumulation with little or no VIGS phenotype. Indeed, this is what we observed in half (11/22) of the 126-GFP-expressing plants upon inoculation with a TRV-PDS VIGS vector (Fig. 2A). We were surprised to find, however, that the remainder of the 126-GFP transgenic plants displayed a very strong VIGS-associated photobleached phenotype (Fig. 2A). The fact that the silencing phenotypes, or lack thereof, observed at 15 dpi remained unchanged at 30 dpi (data not shown) suggests that the opposing phenotypes found in 126-GFP-expressing *N. tabacum* represent persistent differences in VIGS response rather a simple delay of VIGS development in individual plants.

Inoculation of lines expressing different levels of 126-GFP with TRV-PDS revealed a clear negative correlation between the amount of 126-GFP expressed and the severity of the PDS-silenced phenotype (Fig. 3B through E). Specifically, in the plants expressing the highest level of 126-GFP, only one of six developed visible photobleaching (Fig. 3B). In contrast, all of the plants expressing the lowest level of 126-GFP and one-half of the original line developed photobleaching (Fig. 3C and D). Analysis of PDS transcript confirmed that low levels of 126-GFP clearly enhance the VIGS response (Fig. 3F). The significant difference in PDS transcript levels between low and intermediate 126-GFP expressors appears to account for the differences in the penetrance of the silencing phenotypes observed between these lines. These findings are consistent with the idea that slight variations in the expression levels of 126-GFP in our original line (intermediate level of 126-GFP) likely resulted in the split silencing phenotypes observed between plants. It will be interesting to determine whether the ability of a low level of viral suppressor to enhance VIGS is specific to the TMV 126-kDa protein or whether the same effect can be obtained with other viral silencing suppressors. It was suggested that silencing by the *Tomato bushy stunt virus* suppressor p19 is influenced both by its own dosage and the dosage of the silencing inducer, but further work is required to determine the effect of multiple levels of p19 on silencing phenotypes (Qiu et al. 2002).

Unlike *N. tabacum*, WT plants of the susceptible host *N. benthamiana* gave a strong VIGS response following agroinfiltration with the TRV-PDS VIGS construct (Fig. 2A). In order to assess the effect of 126-kDa protein expression on VIGS in this host, we identified plants expressing two different levels of 126-GFP (Fig. 4A). Following inoculation with TRV-PDS, we found that, as in *N. tabacum*, *N. benthamiana* plants expressing the lowest level of 126-GFP (Fig. 4B) developed an enhanced VIGS phenotype compared with WT plants (Fig. 4D). The silencing phenotypes observed correlated well with PDS transcript levels in plants inoculated with TRV-PDS (Fig. 4F). Plants expressing the M^{IC}1,3 126-GFP mutant, defective in its ability to suppress silencing, displayed a VIGS phenotype and PDS transcript levels comparable with those observed for WT plants. However, the VIGS phenotype was less com-

plete and the PDS transcript level was greater in these plants than in plants expressing low levels of 126-GFP (Fig. 4B, D, and E). This result and the finding that M^{IC}1,3 126-GFP levels from plants expressing this protein were comparable with those observed in plants expressing low levels of 126-GFP strongly suggests that it is the 126-kDa protein's silencing suppressor activity that is responsible for the observed effects on the VIGS phenotype in these studies. In addition, because the movement function of the M^{IC}1,3 126-kDa protein mutant was previously shown to be unaltered (Liu et al. 2005), that function is not regulating the VIGS phenotype observed in these studies.

Interestingly, plants expressing a low level of 126-GFP displayed a 12% reduction in PDS transcript after inoculation with TRV-PDS compared with WT *N. benthamiana*, which establishes a substantial silencing response in its own right. The fact that this relatively small difference in PDS transcript levels produces a visible enhancement of silencing phenotypes highlights the potential sensitivity of VIGS phenotypes to relatively minor variations in target transcript level.

Given the increased TRV VIGS response in both *N. tabacum* and *N. benthamiana* expressing a low level of the 126-GFP, expression of this or other suppressors may provide a powerful tool for enhancing VIGS phenotypes. This may be particularly useful for genes that give especially weak VIGS phenotypes in WT plants. In these instances, even a slight increase in silencing may reveal critical phenotypic subtleties that might otherwise be missed. It is also pertinent to note that, despite the recent improvements in methods for TRV-based silencing (Burch-Smith et al. 2006; Ryu et al. 2004), VIGS experiments have traditionally been carried out in *N. benthamiana*, primarily due to this host's enhanced susceptibility to virus infection. The improvement of TRV-based VIGS reported here for *N. tabacum* may provide an additional tool for the enhancement of VIGS phenotypes in this species.

It was surprising to find that the dramatic differences between VIGS phenotypes in both *N. tabacum* and *N. benthamiana* plants expressing different levels of 126-GFP were not reflected in TRV CP transcript levels in systemic leaves of either host (Fig. 5A and B). Although we presume that the strong necrosis observed in the infiltrated leaves of *N. benthamiana* expressing high levels of 126-GFP (Fig. 4F) is reflective of high accumulation of virus early in the infection, our data from systemic leaves suggest that the observed variations in VIGS phenotypes are not due to persistent systemic differences in virus accumulation.

A model for the enhancement of VIGS by low levels of 126-kDa protein.

Recent evidence suggests that the 126-kDa protein functions to suppress silencing by binding to small RNAs and, thus, preventing their incorporation into the RISC (Csorba et al. 2007; Kurihara et al. 2007; Vogler et al. 2007). We propose that the variation in TRV-VIGS-silencing phenotypes observed in *N. tabacum* plants expressing different levels of 126-GFP can be explained by the action of this protein on the host plant's silencing pathway (Fig. 6). In both high and low 126-GFP expressors, dicer-like enzymes will cleave the dsRNA containing PDS sequence and generate small RNAs. In *N. tabacum* plants expressing the highest level of 126-GFP (Fig. 6, left), the small RNAs generated against both virus and PDS sequences are prevented from being loaded into the RISC and, thus, the silencing machinery is effectively suppressed. This results in a normal TRV infection but a lack of visible PDS silencing. Conversely, in *N. tabacum* plants expressing a low level of 126-GFP (Fig. 6, right), only some subset of the siRNAs are prevented from being incorporated into the RISC. The remain-

der of the siRNAs then would proceed through RISC loading and subsequent slicing, resulting in a strong VIGS phenotype. Silencing activity may be somewhat reduced as compared with WT plants, but our results (Fig. 5) show that this compromised defense did not measurably enhance virus accumulation above the normal level. It seems likely that the phenotypic split we observed in our original line of *N. tabacum* expressing 126-GFP (Fig. 2A) was due to fluctuations in the level of transgene expression influenced by unknown environmental factors. The fact that a change in 126-GFP expression of approximately 30% in either direction resulted in absence or presence of VIGS phenotype (Fig. 3) suggests that there may be a threshold level of 126-kDa protein expression above which VIGS is suppressed and below which VIGS is enhanced. We propose that our original 126-GFP-expressing line of *N. tabacum* was very close to this threshold and, thus, relatively small changes in 126-kDa protein levels may have had a very strong effect on silencing phenotypes.

Clearly, the interaction between viral silencing suppressors and the host plant's silencing machinery is complex and may be influenced by as-yet-unknown aspects of the silencing process. Our study provides a direct examination of the relationship between levels of a viral suppressor and the establishment of VIGS phenotypes in two different hosts and demonstrates

that the VIGS response is sensitive to relatively small fluctuations in the cellular dosage of this viral protein.

MATERIALS AND METHODS

Plant growth and materials.

N. benthamiana and *N. tabacum* cv. Xanthi-nn were germinated and grown as previously described (Ding et al. 1995b). Production of *N. tabacum* expressing the 126-kDa protein-GFP fusion (i.e., M^{IC}2-derived 126-kDa protein) behind an enhanced 35S promoter was described elsewhere (Ding et al. 2004). M^{IC} refers to the infectious clone of the M strain of TMV (Holt et al. 1990). Virus expressing the M^{IC}2-derived 126-kDa protein produced a severe mosaic phenotype and the M^{IC}2-derived 126-kDa protein was shown to have a strong suppressor activity (Ding et al. 2004; Shintaku et al. 1996). The production of *N. benthamiana* expressing either the M^{IC}2-derived TMV 126-kDa protein or the M^{IC}1,3-derived 126-kDa protein mutant fused to GFP is described below. For all virus infections and silencing experiments, greenhouse conditions were kept at 24 ± 2°C with 60% humidity and 16 h of supplemental lighting (450 μmol m⁻² s⁻¹).

Plant transformation.

For production of *N. benthamiana* expressing M^{IC}2-derived and M^{IC}1,3-derived 126-kDa protein-GFP fusions, *Agrobacterium* strain LBA4404 containing the appropriate construct was transformed as previously described (Dickey et al. 1998; Ding et al. 2004). Lines expressing differing levels of 126-kDa protein-GFP fusion were initially screened utilizing a fluorescent stereoscope (Olympus, Melville, NY, U.S.A.). Identification of individual plants expressing specific levels of transgene is described below.

Semiquantitative RT-PCR.

Entire leaves were harvested and ground into a homogeneous powder in liquid nitrogen. RNA was isolated from ground tissue using the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.). DNase-treated RNA (2 μg) was used to generate cDNA with either Superscript (Invitrogen, Carlsbad, CA, U.S.A.) or M-MLV (Promega Corp., Madison, WI, U.S.A.) reverse transcriptase. Fifteen- to eighteen-base Oligo (dT) (Invitrogen) was used for first-strand cDNA synthesis for TRV and host elongation factor 1α (EF1α). For analysis of transcript levels of viruses other than TRV, cDNA was synthesized with CP reverse primers specific to each virus: AMV 2400, 5'-CGTCAGCTTCGTCGAGCA-3'; BMV 12794, 5'-GTGCGCCCGGGTGGTCTCTTTAGAGATTTACAGTGTT-3'; and PVX 23997, 5'-CAGCCTCAGCGGTTGTTG-3'. As an internal control, we generated host-specific cDNA with oligo dT primers as described above for each sample and amplified EF1α with primers 13069, 5'-TGGTGTCTCAAGCCTGGTATGGTTGT-3' and 13070, 5'-ACGCTTGAGATCCTTAACCGCAACATTCTT-3'. TRV minus-strand cDNA was generated with a primer 21039 that was complementary to the TRV RNA2 sequence, 5'-GCACCTATGGTGTGTCCTG-3'. Following synthesis, cDNA was treated with RNase H (Promega Corp.) in order to remove RNA/DNA hybrids. In total, 1/50 of each cDNA reaction was then used as template for PCR. Annealing temperatures and cycle number were optimized for each primer set in order to be in the linear range of amplification. CP sequences were amplified with the following primers: AMV 23998, 5'-GCTGGTGGGAAAGCTGGT-3' and 23999, 5'-GCCCTACCATCCTCAGTCG-3'; BMV 13630, 5'-TGTCGACTTCAGGA ACTGGT-3' and 13631, 5'-GGGGTGAAGAAGTCATCGAA-3'; PVX 23995, 5'-GGACCTCAGCGAGATTGAGG-3' and 23996, 5'-CAGACGGTGGCCGAATGAG-3'; and TRV 18491,

Fig. 6. Model explaining strong and weak virus-induced gene silencing (VIGS) phenotypes observed in *Nicotiana tabacum* in the presence of different levels of the *Tobacco mosaic virus* (TMV) 126-kDa protein silencing suppressor. Dicer-like enzymes cleave the *Tobacco rattle virus* (TRV)-phytoene desaturase (PDS) double-stranded RNA into siRNAs. In the presence of high levels of 126-green fluorescent protein (GFP) (left), the loading of siRNA into the RNA-induced silencing complex (RISC) (black arrows) is effectively suppressed (two blue Xs) by the 126-kDa protein's siRNA-binding capability. This prevents either viral or PDS RNA from being targeted for degradation and results in a plant with obvious virus symptoms but a lack of VIGS phenotype. In the presence of low levels of 126-GFP (right), fewer siRNAs are prevented from being loaded in the RISC (one blue X). This results in a situation where TRV virus still accumulates to significant levels due to suppressor activity and siRNAs against PDS RNA accumulate at sufficient levels to trigger a strong VIGS phenotype.

5'-GCAGTGGCGGGTGTACAA-3' and 18492, 5'-TCCAAA GTCGAGCCAGTGTTC-3'. PDS transcript was amplified with primers 21816, 5'-GTGTGTACGCTGACATGTCTGTT-3' and 21818, 5'-CATCTTCTGGCTCCGGCC-3'. All PCR reactions were performed with Ex-Taq enzyme mix (Takara, Tokyo). For quantification, TIF images of gels were taken on an Epi Chemi II Darkroom imaging system (UVP Bio Imaging, Upland, CA, U.S.A.). Band intensities were calculated with Image J software and were adjusted for differences in an EF1 α internal control. Percent transcript level was calculated relative to the intensity of the highest expresser.

Virus inoculation and *Agrobacterium* spp. infiltration.

For comparison of virus infections between WT and 126-GFP-expressing *N. tabacum*, infections were first established in *N. benthamiana*. At 10 dpi, infected tissue was ground by mortar and pestle in 1 $\times$ PBS (pH 7.0) and equal volumes were inoculated onto developmentally similar leaves of 5-week-old *N. tabacum*. H-BMV contains RNA1 and RNA2 of F-BMV and RNA3 of the Russian strain (Ding et al. 2006). AMV strain 425 (Madison) was a gift of V. Yusibov and PVX was a gift from D. Baulcombe (Baulcombe et al. 1995). Clones containing TRV sequences were a gift from S. P. Dinesh-Kumar. TRV infections were established in *N. benthamiana* by co-agroinfiltration of pTRV1 and pTRV2 containing *Agrobacterium* spp. (Liu et al. 2002). Infectious TRV clones containing a portion of the *N. benthamiana* PDS sequence for VIGS studies were obtained from K. Mysore and are described elsewhere (Ekengren et al. 2003). *Agrobacterium* spp. containing TRV-PDS silencing constructs were grown under selection and infiltrated at a final optical density (600 nm) of 0.5 as described previously (Voinnet et al. 1998).

Quantification of 126-kDa protein expression levels.

Comparisons of 126-GFP expression levels were made between developmentally similar leaves from individual plants at 6 weeks after planting. Leaf tissue was mounted in water under a coverslip and images were acquired on a Bio-Rad 1024ES (Bio-Rad Laboratories, Hercules, CA, U.S.A.) confocal imaging system. The 126-GFP within leaf tissue was excited at 488 nm and emissions were captured at 522 nm. In order to allow comparison between samples, microscope settings remained unchanged for all samples collected. Average pixel intensity across images was determined using Image J software.

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