Viral Virulence Protein Suppresses RNA Silencing–Mediated Defense but Upregulates the Role of MicroRNA in Host Gene Expression [W]

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Small interfering RNAs (siRNAs) and microRNAs (miRNAs) are processed by the ribonuclease Dicer from distinct precursors, double-stranded RNA (dsRNA) and hairpin RNAs, respectively, although either may guide RNA silencing via a similar complex. The siRNA pathway is antiviral, whereas an emerging role for miRNAs is in the control of development. Here, we describe a virulence factor encoded by turnip yellow mosaic virus, p69, which suppresses the siRNA pathway but promotes the miRNA pathway in *Arabidopsis thaliana*. p69 suppression of the siRNA pathway is upstream of dsRNA and is as effective as genetic mutations in *A. thaliana* genes involved in dsRNA production. Possibly as a consequence of p69 suppression, p69-expressing plants contained elevated levels of a Dicer mRNA and of miRNAs as well as a correspondingly enhanced miRNA-guided cleavage of two host mRNAs. Because p69-expressing plants exhibited disease-like symptoms in the absence of viral infection, our findings suggest a novel mechanism for viral virulence by promoting the miRNA-guided inhibition of host gene expression.

INTRODUCTION

RNA silencing is a generic term describing related gene silencing processes that include posttranscriptional gene silencing (PTGS) in plants, double-stranded RNA (dsRNA)–mediated RNA interference in animals, and quelling in fungi (Ding, 2000; Matzke et al., 2001; Vance and Vaucheret, 2001). A unifying feature of RNA silencing is the production of small interfering RNAs (siRNAs) that are 21 to 26 nucleotides long and of both sense and antisense polarities (Hamilton and Baulcombe, 1999; Hamilton et al., 2002; Plasterk, 2002). siRNAs are processed from dsRNA precursors by the endoribonuclease Dicer, become incorporated into the RNA-induced silencing complex that contains at least one Argonaute (AGO) protein, and guide specific cleavage of complementary mRNAs to a central position of an siRNA/mRNA duplex (Hammond et al., 2000, 2001; Bernstein et al., 2001; Elbashir et al., 2001). MicroRNAs (miRNAs) are a new class of small RNAs that may control development in animals and plants (Ambros et al., 2003). miRNAs are present in one polarity and are derived from RNA transcripts that have potential to form hairpin structures. miRNAs are also a product of Dicer and found in a complex similar if not identical to the RNA-induced silencing complex, although miRNAs may guide either cleavage or translational arrest of target mRNAs (Ambros et al., 2003).

In plants, PTGS can be induced by transgenes that encode either a highly transcribed sense RNA or an inverted repeat RNA (IR-RNA) that is folded into a long dsRNA. Several genes essential for sense RNA–induced PTGS have been cloned from *Arabidopsis thaliana.* These include a cellular RNA-dependent RNA polymerase (RdRP) homolog (*SGS2/SDE1*), *AGO1*, an RNA helicase gene (*SDE3*), and genes that encode a coiled-coil protein (*SGS3*) and a novel protein (*HEN1*) (Fagard et al., 2000; Mourrain et al., 2000; Boutet et al., 2003). Current models place these genes upstream of dsRNA because none of these genes are required for IR-RNA–induced PTGS (Dalmay et al., 2001; Beclin et al., 2002). In *A. thaliana*, both *Dicer-like 1* (*DCL1*) and *HEN1* are involved in the production of miRNAs (Park et al., 2002; Reinhart et al., 2002), and there is evidence that miRNAs function as siRNAs to specify cleavage of their mRNA targets (Llave et al., 2002; Rhoades et al., 2002; Kasschau et al., 2003).

A biological function for RNA silencing, first established in plants, is as an adaptive defense against RNA and DNA viruses (Covey et al., 1997; Ratcliff et al., 1997; Al-Kaff et al., 1998; Vance and Vaucheret, 2001). Recent work has demonstrated a similar antiviral role for RNA silencing in the animal kingdom (Li et al., 2002, 2004). The antiviral response may be triggered by virus-specific dsRNA intermediates produced during genome replication, by transcription from converging promoters, or through recognition of viral RNAs by host RdRP, as proposed in plants

(Voinnet et al., 2000; Szittya et al., 2002). As a counterdefense, viruses encode proteins such as helper component-proteinase (HC-Pro) and *Cucumber mosaic virus* 2b that suppress RNA silencing at various steps in the pathway (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998; Mlotshwa et al., 2002; Silhavy et al., 2002). The identification of a silencing suppression function typically provides a molecular basis for previously observed defects in viral accumulation and spread of virus mutants that lack a functional suppressor (Li and Ding, 2001; Mlotshwa et al., 2002).

Here, we report the identification of a viral suppressor of RNA silencing that also plays a unique role in the induction of viral disease. The suppressor, p69, is encoded by turnip yellow mosaic virus (TYMV), a plus-strand RNA virus prevalent both in cultivated and wild species of the Brassicaceae family of dicotyledonous plants (Morch et al., 1988; Skotnicki et al., 1992). Previous work has shown that p69 is essential for virus spread and influences viral yields and symptom severity in infected plants but is dispensable for viral replication in single cells (Bozarth et al., 1992; Tsai and Dreher, 1993). Using distinct PTGS systems with defined silencing triggers, we found that p69 represents a unique viral suppressor of RNA silencing by targeting a step upstream of dsRNA production in the cellular RdRP-dependent branch of RNA silencing. However, transgenic *A. thaliana* plants expressing p69 alone displayed disease-like symptoms, indicating a role of p69 in disease induction that is independent of its suppression of the host PTGS mechanism targeting viral RNAs. Further analyses revealed a significantly upregulated miRNA pathway in p69-expressing plants, including elevated levels of miRNAs and enhanced miRNA-guided cleavages of two host mRNAs that encode putative transcriptional factors. Thus, p69 is distinct to the viral PTGS suppressor HC-Pro that was recently shown to inhibit miRNA silencing (Kasschau et al., 2003). We propose that enhanced miRNA silencing is a consequence of a negative feedback regulation of the RNA silencing pathway triggered by p69 suppression and that miRNAs can play a pathogenic role in the induction of viral diseases.

RESULTS

p69 Inhibits PTGS and DNA Methylation Induced by Sense RNA Transgenes

The first indication that TYMV might encode a suppressor of RNA silencing came from TYMV infection of *A. thaliana* plants that carry a silencing *35S-β-glucuronidase* (*GUS*) sense RNA transgene at the L1 locus (Elmayan et al., 1998). We found that TYMV infection of L1 plants resulted in a restored GUS expression in the newly emerged leaves as shown by histochemical staining (Figure 1B).

TYMV contains three genes, two of which are encoded in out-of-frame overlapping open reading frames (Figure 1A). The viral RdRP and p69 are translated from the genomic RNA with their initiation codons seven nucleotides apart, whereas the coat protein (CP) is produced from a subgenomic RNA that is 3′ coterminal with the genomic RNA (Morch et al., 1988; Skotnicki

et al., 1992). We predicted that the suppressor activity of TYMV was encoded by P69 based on its role in viral spread and virulence that is similar to the known viral suppressors (Bozarth et al., 1992; Tsai and Dreher, 1993; Li and Ding, 2001).

To test this possibility, the *P69* gene was introduced into *A. thaliana* ecotype C24 under the control of the 35S promoter of *Cauliflower mosaic virus*. It is striking that 7 of the 11 *35S-P69* transformants obtained showed developmental defects (Figure 2, left; see also Supplemental Figure 1 online). The expression of *P69* was hardly detectable in the remaining four lines that were morphologically indistinguishable from either wild-type plants or any of the eight *35S-ΔP69* transformants (data not shown). The pleiotropic defects of the P69 plants cosegregated with the transgene in backcrosses with wild-type plants and included severe dwarf, pale-colored leaves with reduced cell sizes, late flowering, short siliques, and decreased fertility. For example, line 3 homozygous for a single *35S-P69* transgene (P69c) began flowering 48.5 d after sowing with 13.4 rosette leaves on average ($n = 65$) in contrast with wild-type plants that flowered 42.4 d with 11.8 rosette leaves ($n = 61$) under our growth conditions. The developmental defects observed in the P69 plants mimicked, but were more severe than, the disease symptoms found in wild-type plants systemically infected with TYMV (Figure 2, right). Thus, p69 alone is able to confer pathogenicity independent of TYMV infection, further extending the finding by Tsai and Dreher (1993).

Further analyses confirmed that p69 is a potent suppressor of sense RNA–induced RNA silencing. High levels of GUS activity (Figure 1C) and *GUS* mRNA (Figure 3A, top panel) were detected in the F1 progeny plants of crosses between line P69c and line L1. The distinct GUS staining pattern in the L1xP69c plants (Figure 1C) may be caused by the constitutive expression of *P69* possibly before or during the *GUS* transgene was silenced, as compared with that in TYMV-infected L1 plants (Figure 1B) in which *GUS* silencing was established before virus inoculation. By contrast, *35S-GUS* remained silenced in the F1 progeny of a similar cross between the wild type and L1 (Figures 1C and 3A). Furthermore, whereas the *GUS*-specific siRNAs accumulated to high levels in L1xC24 plants, they were undetectable in L1xP69c plants (Figure 3B). Notably, these RNA analyses showed that suppression of *GUS* RNA silencing in P69c plants was as effective as in the *sgs2* mutant that contains a defective cellular RdRP (Figures 3A and 3B) (Mourrain et al., 2000). Interestingly, silencing suppression as measured by the accumulation of both *GUS* mRNA and siRNAs, was less efficient in L1xP69d plants that expressed *P69* mRNA at a lower level (Figure 3A, cf. lanes 3 and 4). Using 35S constructs directing translation of either the full-length or the first 45 codons of p69 and gene delivery by *Agrobacterium tumefaciens* coinfiltration, we further showed that p69 actively suppressed RNA silencing targeted against a 35S-controlled green fluorescent protein (GFP) sense transgene in *Nicotiana benthamiana* (see Supplemental Figure 2 online).

The autonomous RNA silencing in L1 plants is associated with cytosine methylation within the coding sequence of the *35S-GUS* transgene (Mourrain et al., 2000). As expected, the *GUS* DNA in the L1 plants was partially resistant to digestion by the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I (Figure 3C, left panel). By contrast, the *GUS* DNA from the L1xP69c
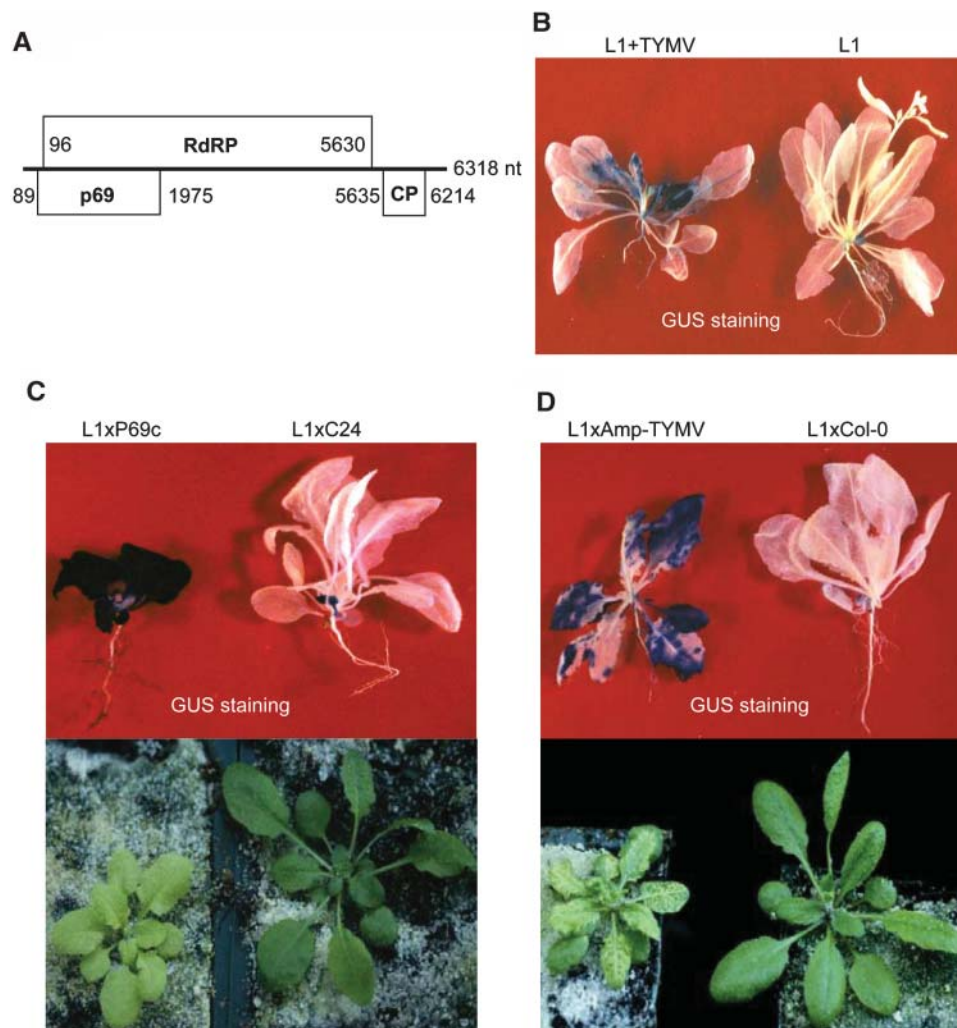
Figure 1. p69 Is a PTGS Suppressor in *A. thaliana*.

(A) TYMV genome organization.
(B) to **(D)** Suppression of the *GUS* RNA silencing in line L1 by TYMV 2 weeks after infection **(B)**, by a *35S-P69* transgene **(C)**, or a TYMV amplicon transgene **(D)**. Photos of the same plants before GUS staining are also shown in the bottom panels in **(C)** and **(D)**.

plants, in which the *GUS* siRNAs were undetectable, was digested completely by *Msp*I, although the digestion by *Hpa*II, sensitive to methylation at both overlapping and nonoverlapping CG sites, was less efficient (Figure 3C, right panel). This pattern of demethylation was similar to that found previously in the *sgs2* mutant in the L1 background (Figure 3C, middle panel) (Mourrain et al., 2000). Thus, p69 expression also prevented the methylation of *GUS* DNA as completely as in the *sgs2* mutant. These results establish p69 as a unique suppressor of RNA silencing because none of the known viral suppressors have been shown to eliminate both siRNA accumulation and transgene methylation associated with a silencing sense RNA transgene (Mallory et al., 2001; Guo and Ding, 2002).

P69 Inhibits PTGS Induced by a Virus-Derived Amplicon Transgene

Viral RNAs are silenced in plants via both the cellular and viral RdRP pathways (Dalmay et al., 2000b; Voinnet et al., 2000). We analyzed p69 suppression of PTGS that targets an amplicon transgene that encodes a replication-competent recombinant potato virus X (PVX) carrying GFP (PVX:GFP) in *A. thaliana* lines A and GxA (Dalmay et al., 2000a). The autonomous silencing of the amplicon requires active replication of PVX:GFP RNA but is *SDE1* independent as shown in line GxA. Line G contains a highly expressed *35S-GFP* transgene that is silenced in line GxA by the amplicon. In contrast with amplicon silencing, silencing of the *35S-GFP* transgene in line GxA requires *SDE1* and is associated

accumulation level was lower for the *GFP* mRNA and higher for the viral RNAs in (GxA)xP69 plants (Figure 4B, lanes 6 to 8) than in the *sde1*(GxA) mutant (Figure 4B, lane 13). Thus, p69 suppression of sense RNA silencing is incomplete, suggesting a differential suppression by p69 and the genetic mutation in *SDE1* of RNA silencing directed against the viral amplicon and sense-transgene transcripts.

We further examined the methylation status of the *35S-GFP* transgene in (GxA)xP69c plants using the methylation-sensitive restriction enzyme *Hae*III (Figure 4D). The *GFP* DNA extracted from (GxA)xP69c plants (lane 3) was completely digested by *Hae*III as found for the *GFP* DNA extracted from either line G (lane 1) or the *sde1*(GxA) mutant (lane 4). This was in contrast with the *GFP* DNA extracted from GxA plants that was partially resistant to the digestion by *Hae*III (Figure 4D, lane 2), as was found previously (Dalmay et al., 2000a). These results indicate that p69 also suppresses PTGS mediated by the viral amplicon either alone or in combination with a homologous transgene and that p69 suppression is associated with elimination of transgene methylation.

Hybridizations using a probe complementary to the *GFP* sequence detected the accumulation of siRNAs both in AxP69c and (GxA)xP69c plants (Figure 4C). In fact, the siRNAs became
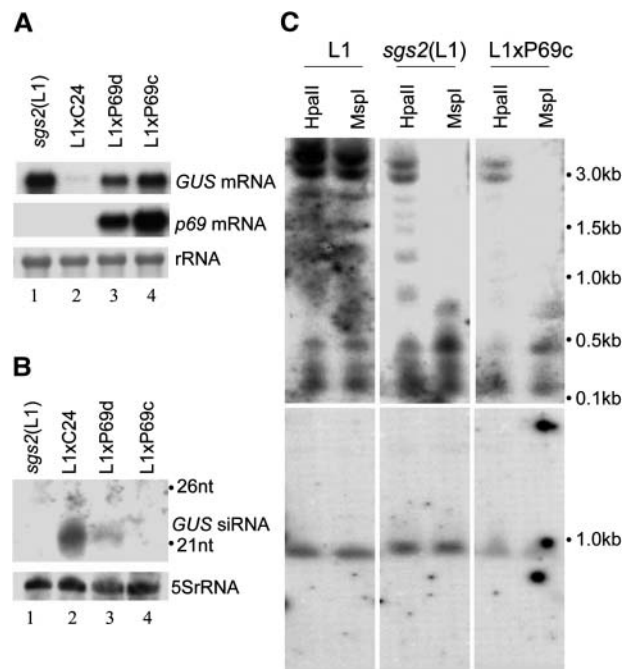

Figure 2. p69-Conferred Virulence in *A. thaliana*.

Transgenic line P69c (left) expressing *P69* causes pleiotropic developmental defects that resemble the phenotype displayed by a plant infected systemically with TYMV (right).

with methylation of the *GFP* and *PVX:GFP* transgenes (Dalmay et al., 2000a).

RNA gel blot hybridizations detected significant accumulation of PVX:GFP genomic and subgenomic RNAs in both A and GxA plants either after systemic infection with TYMV (Figure 4A, lanes 4 and 5) or after the *35S-P69* transgene was introduced from line P69b, P69c, or P69d through genetic crosses (Figure 4B, lane 3 and lanes 6 to 8). Accumulation of *GFP* mRNA was also detected in (GxA)xP69 plants (Figure 4B, lanes 6 to 8) and in GxA plants infected with TYMV (Figure 4A, lane 4). Suppression of the amplicon and *GFP* RNA silencing was weaker in lines (GxA)xP69b and (GxA)xP69d than in (GxA)xP69c and undetectable in (GxA)xP69h and (GxA)xP69i as in lines (GxA)xΔP69a and (GxA)xΔP69b. These results indicate that the silencing suppression activity was determined by the expression levels of the *P69* transgene and by whether or not the *P69* transgene was translatable in these plants (Figure 4B). As expected from previous work (Dalmay et al., 2000b), the *GFP* mRNA and the genomic and subgenomic RNAs from PVX:GFP were detected in the *sde1*(GxA) mutant (Figure 4B, lane 13). Interestingly, the

Figure 3. Molecular Characterization of *P69* Suppression of PTGS Induced by a Sense RNA Transgene in *A. thaliana*.

The detection of the *GUS* mRNA **(A)**, *GUS* siRNAs **(B)**, and *GUS* DNA methylation **(C)** in *sgs2*(L1), L1xC24, L1xP69c, and L1xP69d seedlings. In contrast with *Hpa*II, *Msp*I cleavage is not blocked to methylation at sites with overlapping CG. Equal loading and completion of DNA digestion was demonstrated by rehybridization of the filter for *SPL3* (bottom panel of **[C]**), whereas equal loading of small RNAs was shown by reprobing for 5S rRNA **(B)**. The positions of 21- and 26-nucleotide RNA markers are indicated **(B)**.
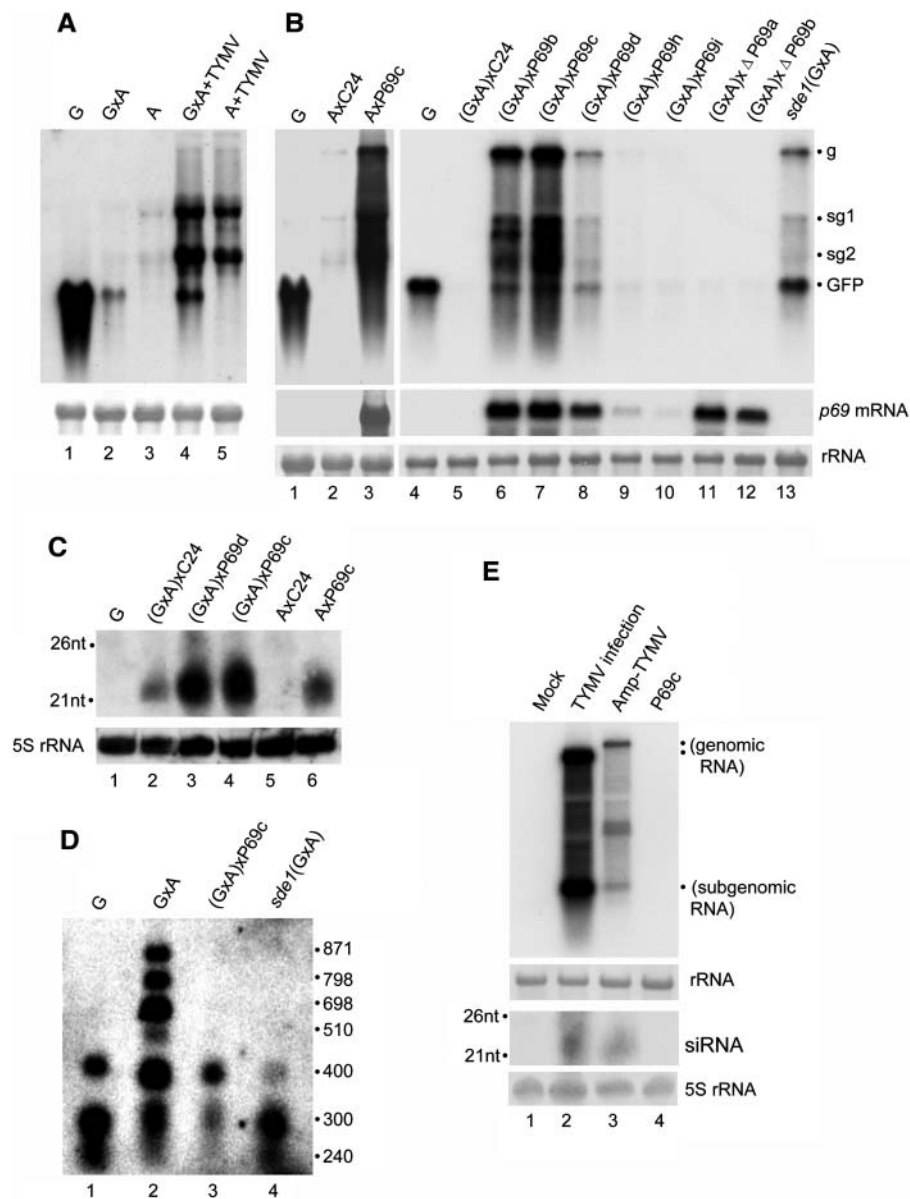
Figure 4. Molecular Characterization of p69 Suppression of PTGS Induced by a Virus-Derived Amplicon Transgene.

(A) Silencing suppression in amplicon lines infected with TYMV (lanes 4 to 5). Samples from mock-inoculated lines were included as controls (lanes 1 to 3). PVX:GFP genomic (g) and subgenomic (sg) RNAs as well as *GFP* mRNA are indicated.

(B) Silencing suppression by the *P69* transgene introduced into lines A and GxA by genetic crosses with the maternal parent listed first (this is used throughout the text). Except for G and *sde1*(GxA), all plants analyzed were hemizygous F1 plants. Total RNA loaded was 5 μg in lanes 1 to 3 and 3 μg in lanes 4 to 13 and equal loading was visualized by methylene blue staining of rRNA.

(C) Detection of siRNAs by a GFP-specific probe in hemizygous F1 amplicon plants crossed with P69c, P69d, or C24. A similar pattern of siRNA accumulation was detected using a PVX-specific probe.

(D) Detection of the *GFP* DNA methylation. Note that *Hae*III cleavage is not blocked to methylation at sites with overlapping CG. The positions of DNA standards (in base pairs) are shown at the right.

(E) Detection of TYMV high and low molecular weight RNAs in either the TYMV-based amplicon plants or wild-type plants infected TYMV. The filters were probed with labeled DNA and RNA sequences corresponding to nucleotides 5591 to 6260, respectively, of the TYMV genome. The amplicon transgene contained an insertion of nonviral sequence (1.6 kb) upstream of the viral CP gene and thus gave rise to a recombinant viral genomic RNA longer than the wild-type TYMV genomic RNA, although the size of the CP subgenomic RNA remained unaltered.

more abundant in these amplicon lines after *P69* was introduced (Figure 4C, cf. lanes 3 and 4 with lane 2 or lane 6 with lane 5). Because Ax P69c plants did not carry the *35S-GFP* transgene, all of the siRNAs detected in these plants must have come from the PVX:GFP amplicon transgene. Thus, p69 suppression of amplicon silencing led to enhanced accumulation of both viral genomic/subgenomic RNAs and siRNA, as found in the *sde1*(GxA) mutant (Dalmay et al., 2000b), rather than siRNA elimination observed in L1xP69c plants. This suggests that p69 expression did not prevent Dicer cleavage of virus-derived dsRNAs into siRNAs.

Unlike the PVX-based amplicon that induces consistent silencing in transgenic tobacco and *A. thaliana* plants (Angell and Baulcombe, 1997; Dalmay et al., 2000a), transgenic *A. thaliana* plants carrying a TYMV-based amplicon transgene displayed the characteristic TYMV symptoms (Figure 1D, bottom left) and suppression of the *GUS* transgene silencing as shown by GUS staining after the amplicon was introduced into line L1 by genetic crosses (Figure 1D, top left), suggesting that p69 also suppresses silencing induced by the TYMV amplicon. Compared with the *35S-P69* transgene (Figure 1C), however, suppression of *GUS* silencing by the TYMV amplicon was less effective (Figure 1D); this was probably caused by silencing of the amplicon transgene as indicated by detection of TYMV-specific siRNAs in the amplicon plants (Figure 4E, bottom panel, lane 3) and much lower levels of the genomic and subgenomic viral RNAs in those plants than in TYMV-infected plants (Figure 4E, top panel, cf. lanes 3 and 4). Consistent with the findings from the PVX amplicon plants, p69 expression from TYMV also did not prevent accumulation of viral siRNAs in TYMV-infected plants (Figure 4E, bottom panel, lane 2).

p69 Does Not Inhibit PTGS Induced by IR-RNA Transgenes

RNA silencing induced by IR-RNA transgenes is distinct to sense and virus RNA silencing because neither the cellular nor viral RdRP is involved in the formation of the dsRNA inducer from a nuclear self-complementary IR-RNA transcript (Beclin et al., 2002). Thus, we next determined if *P69* interfered with silencing of the phytoene desaturase (PDS) gene mediated by an IR-RNA transgene (*IRPDS*), which caused a dominant photobleaching phenotype. An *IRPDS/−* heterozygote (in Columbia [Col-0] background) that contained a single copy of the transgene was crossed with P69c (in C24 background) and C24, respectively, and the F1 progenies were allowed to grow in the MS medium and MS plus hygromycin (for selection of the *IRPDS* and/or *35S-P69* positive individuals). The progeny of C24x*IRPDS/−* segregated 1:1 for hygromycin sensitive (HYG^S) to hygromycin resistant (HYG^R), but all of the 12 HYG^R individuals showed photobleaching (Figure 5B). All of the 19 progenies from P69cx*IRPDS/−* carried the *35S-P69* transgene and were HYG^R; however, only 10 of these F1 plants showed photobleaching (Figure 5C). When grown on MS medium (Figure 5A), both F1 populations from crosses C24x*IRPDS/−* ($n = 29$) and P69cxIRPDS/− ($n = 52$) contained ∼50% individuals displaying the photobleaching phenotype. These results indicate that the presence of *35S-P69* did not suppress the *PDS* silencing phenotype of *IRPDS*.

RNA analysis showed that expression of the *IRPDS* transgene in C24x*IRPDS* plants resulted in the accumulation of *PDS*-specific siRNAs (Figure 5D, lane 4) and an ∼75% knockdown of the *PDS* mRNA (Figure 5D, top panel, cf. lanes 3 and 4). Consistent with lack of *P69* suppression indicated by the phenotypic analysis, *P69* expression in the *IRPDS*-expressing plants was not associated with an increased accumulation of *PDS* mRNA (Figure 5D, cf. lanes 2 and 4). Furthermore, we found that TYMV infection of *A. thaliana* plants that carry the *35S-GFP* transgene targeted by an IR-RNA transgene did not suppress GFP silencing as shown by RNA analyses (see Supplemental Figure 4 online). Thus, potent p69 suppression of RNA silencing triggered by sense RNA transgenes but not by the IR-RNA transgene suggests that p69 disrupts a cellular function leading to dsRNA synthesis.

p69 Enhances miRNA-Mediated Cleavage of Two Target mRNAs

Similar to IR-RNA, the hairpin precursor of miRNAs enters the RNA silencing pathway without new dsRNA synthesis. Thus, we predicted that p69 would not inhibit mRNA cleavage by endogenous miRNAs. We first examined the cleavage of the *SCL6-IV* mRNA by miR171, which occurs at positions centered in the middle of the miRNA–mRNA duplex so that both the full-length mRNA (SCL6-IVf) and a shorter 3′ fragment of ∼1.3 kb (SCL6-IV3′) are detected by a 3′-proximal probe in wild-type *A. thaliana* (Llave et al., 2002). We found that this cleavage indeed was not blocked in the presence of p69 because both of the RNA species were detected in P69c plants (Figure 6A). Notably, a decrease in the accumulation of the full-length mRNA and a corresponding increased accumulation of the 3′ fragment was detected in P69c plants as compared with wild-type plants (Figure 6A). This suggests that p69 may enhance RNA silencing mediated by the miRNA pathway.

We further examined the steady state mRNA levels of SQUAMOSA-promoter binding protein-like gene 3 (*SPL3*), a predicted target of miR156 (Rhoades et al., 2002). *SPL3* transcription is developmentally regulated, and constitutive overexpression of *SPL3* resulted in early flowering (Cardon et al., 1997). RNA gel blotting analysis revealed significant decreases (0.7- to 2-fold) in the accumulation of the *SPL3* mRNA in two independent p69 lines as compared with wild-type plants (Figure 6B). The reduction was more pronounced in line P69c than in line P69d (cf. lanes 2 and 3), which was correlated with a higher expression level of the *P69* transgene in P69c plants as detected by RNA gel blot hybridization (Figure 4B, lanes 7 and 8). A modest decrease in the accumulation of *SPL3* mRNA was also observed in wild-type plants infected with TYMV (Figure 6B, lane 4).

miR156 has mismatches to *SPL3* mRNA in contrast with both miR171 and siRNAs that are perfectly complementary to their target mRNAs (Llave et al., 2002; Plasterk, 2002). Thus, we determined if there was cleavage of *SPL3* mRNA within the predicted miR156/mRNA duplex, which contains one mismatch at the second nucleotide from the 3′ terminus of miR156 in addition to a G:U wobble (Rhoades et al., 2002) (Figure 6C). For this purpose, cDNA corresponding to the predicted 3′ fragment
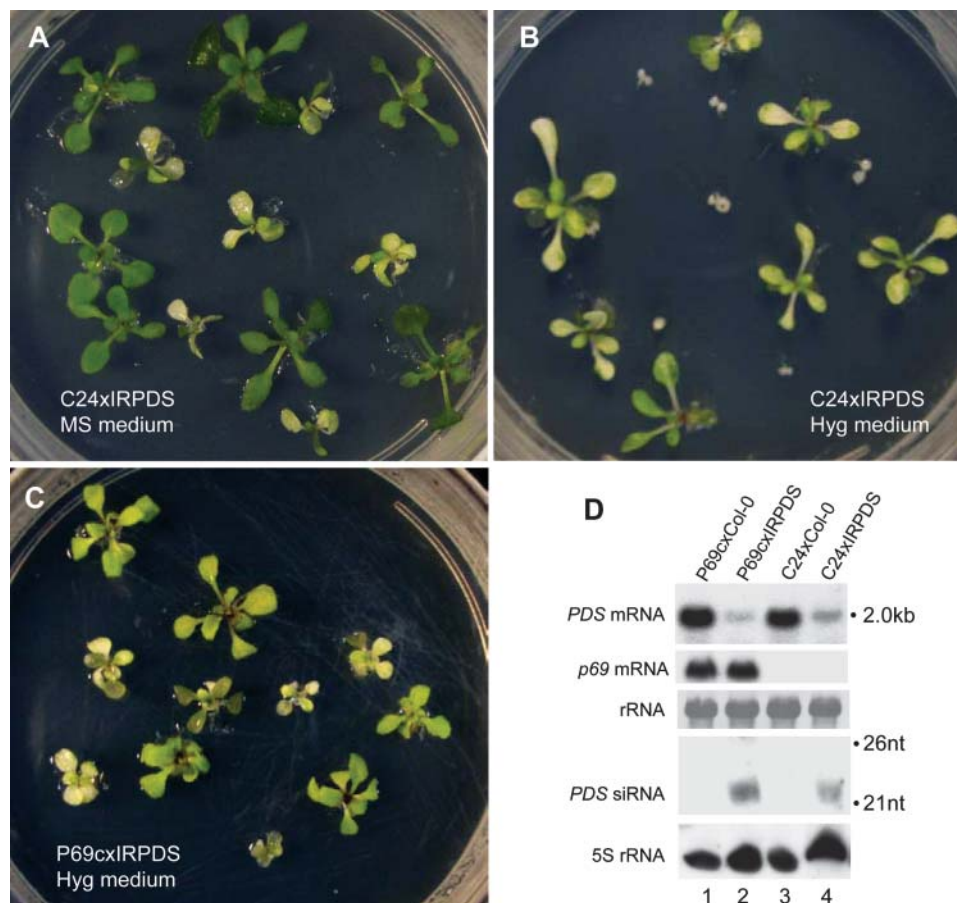
Figure 5. p69 Does Not Suppress the IR-RNA–Induced PTGS Targeting of *PDS*.

(A) to **(C)** Genetic segregation of the photobleaching RNA interference phenotype of the *IRPDS* transgene after crossing an *IRPDS* heterozygote (*IRPDS*/−, in Col-0 background) with either C-24 or a P69c homozygote (in the C-24 background). A representative plate is shown for each except for P69cxIRPDS, which did not show difference with or without hygromycin selection. P69c plants appear yellow in contrast with the photobleached plants **(C)**.

(D) Detection of *P69* mRNA and *PDS* mRNA/siRNAs. For comparison to the level of PDS mRNA in the C24xCol-0 genetic background with and without *P69*, RNA samples from the progeny of C24xCol-0 (lane 3) and P69cxCol-0 (lane 1) were included as controls. Equal loading was monitored for mRNA by rRNA staining and for small RNAs by reprobing for 5S rRNA.

of *SPL3* mRNA was amplified using the RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) essentially as described (Llave et al., 2002). Total mRNAs isolated from P69c plants were ligated to a 45-nucleotide RACE adapter RNA and reverse transcribed using a downstream *SPL3*-specific primer. Two rounds of nested PCR using the cDNAs obtained as templates and two sets of RACE and gene-specific primers yielded a discrete band that migrated within the predicted size range (Figure 6C, top panel). Sequencing and alignment of the cloned PCR products derived from the *SPL3* mRNA identified two cleavage sites in the center of the predicted miR156/mRNA duplex (Figure 6C, bottom panel). Therefore, cleavage at the predicted duplex of miR156 and *SPL3* mRNA indeed occurred in the P69c plants.

p69 Expression Increases the Accumulation Level of Seven miRNAs and *DCL1* mRNA

The enhanced cleavage of miRNA targets observed in p69-expressing plants might be because of a higher accumulation level of miRNAs. To investigate this possibility, total RNA was extracted from newly bolted inflorescence and analyzed for the accumulation of seven *A. thaliana* miRNAs, miR156, miR157, miR158, miR162, miR164, miR167, and miR171. We found that each miRNA increased in abundance in the presence of the *P69* transgene in P69c plants (Figures 7A and 7B). Similar increases were observed in the P69d plants but not in line G for miR156 and miR171, and we detected no difference in the accumulation of miR156, miR157, miR158, miR167, or miR171 between L1 and
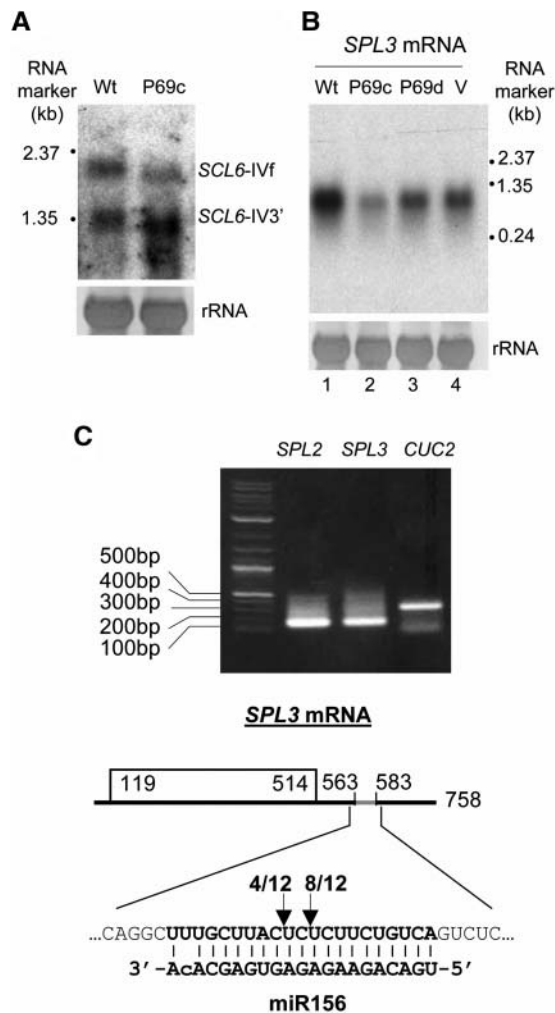
Figure 6. Reduced Accumulation of Two miRNA-Targeted mRNAs in p69-Expressing Plants.

(A) Detection of full-length *SCL6-IV* mRNA and its 3′ cleavage product by miR171 in wild-type C-24 and P69c plants.
(B) Accumulation of *SPL3* mRNA in P69c and P69d plants as well as in C-24 plants before (Wt) and after systemic TYMV infection (V).
(C) Determination of the miR156 cleavage sites within *SPL3* mRNA. The 5′-terminal portion of the predicted 3′ cleavage product for *SPL3* mRNA as well as *SPL2* and *CUC2* mRNAs (Kasschau et al., 2003; used here as controls) was amplified by 5′RACE (top panel), and the dominant products were recovered, cloned, and sequenced. The arrows indicate the positions of two cleavage sites inferred (bottom panel). The number of the 5′RACE clones that correspond to each site is indicated.

the *sgs2*(L1) mutant (see Supplemental Figure 3 online), suggesting that SDE1 may not be involved in miRNA biogenesis.

As the first step to investigate the mechanism of p69-mediated enhancement of miRNA silencing, we examined expression of two genes in the RNA silencing pathway using RNA gel blotting (Figure 7C) and real-time PCR (Figure 7D). No significant differences were detected in the accumulation of either *AGO1* or *β-TUBULIN-1* mRNA between P69c and wild-type plants.

However, both analyses showed that mRNA of *DCL1* was at least threefold higher in P69c plants than in wild-type plants.

DISCUSSION

In this work, we identified TYMV p69 as a unique viral virulence protein that suppresses the host RNA silencing antiviral defense but promotes RNA silencing of host genes mediated by miRNAs.
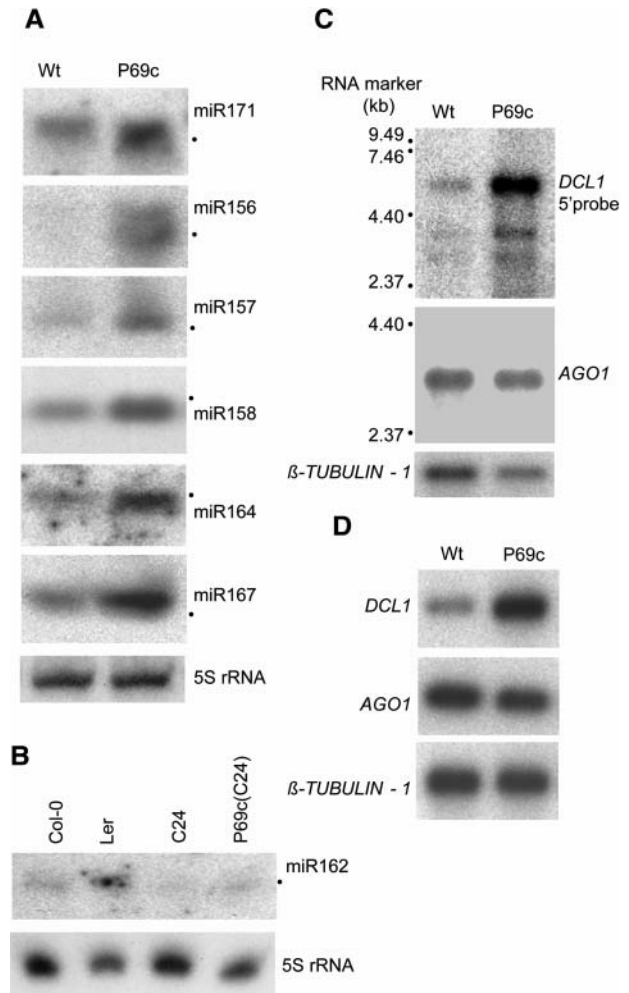

Figure 7. Enhanced Accumulation of Seven miRNAs and mRNAs for *DCL1* and *SDE1/SGS2* in p69-Expressing Plants.

(A) and **(B)** Detection of miRNAs in P69c and wild-type C-24 plants as described in Methods. We noted that the level of miR162 was much lower in wild-type ecotype C-24 than in either Landsberg *erecta* (Ler) or Col-0.
(C) Detection of *DCL1* and *AGO1* mRNAs by RNA gel blot hybridizations. Two micrograms of poly(A)$^+$ mRNAs from P69c and C-24 plants were loaded in each lane, and the filter was probed sequentially for the mRNA of *AGO1*, *DCL1*, and *β-TUBULIN-1* (as a loading control). The full-length *DCL1* and *AGO1* mRNAs are 6.2 and 3.5 kb, respectively, as indicated.
(D) Real-time PCR analyses of the mRNA accumulation for *AGO1*, *DCL1*, and *β-TUBULIN-1* in P69c and C-24 plants.

Here, we discuss the mode of action of p69 and the potential role of p69 in viral pathogenesis by miRNAs.

p69 Suppresses RNA Silencing Upstream of dsRNA

Several lines of evidence indicate that among the viral suppressors characterized to date, p69 targets a unique step in the RNA silencing pathway. The fact that p69 suppressed PTGS induced by sense RNA transgenes but not by IR-RNA transgenes indicates that p69 inhibits a cellular function leading to dsRNA production. This conclusion is consistent with the observation that p69 had similar effects on RNA silencing to those of genetic mutations in cellular genes involved in dsRNA production such as *SGS2/SDE1*. For example, both *P69* expression and the *sgs2/sde1* mutation eliminated the siRNAs production (Figure 3B) and DNA methylation (Figures 3C) associated with RNA silencing induced by the sense RNA *GUS* transgene at the L1 locus (Mourrain et al., 2000). In the amplicon-based two-component silencing system (Figure 4), both *P69* expression and the *sgs2/sde1* mutation suppressed RNA silencing and DNA methylation of the *35S-GFP* transgene, but both increased the accumulation of virus-specific siRNAs (Dalmay et al., 2000b). The increase in the production of viral siRNAs is likely from Dicer processing of the more abundant viral RdRP products after p69 suppression of silencing. However, these siRNAs may not be fully functional because of lack of both amplicon silencing and DNA methylation in the amplicon plants either expressing *P69* or defective for *SDE1* (Dalmay et al., 2000b; Mette et al., 2000).

PVX p25 also suppresses the host RdRP-dependent branch of RNA silencing (Voinnet et al., 2000). Interestingly, both p25 and p69 are essential for virus cell-to-cell movement as compared with a dispensable role for other viral suppressors in the cell-to-cell movement. Unlike p69, however, p25 is not active in the suppression of either established silencing targeted against sense RNA transgenes (Brigneti et al., 1998) or silencing targeted its own amplicon transgene (Angell and Baulcombe, 1997; Dalmay et al., 2000a). Furthermore, it appears that p25 expression also does not prevent DNA methylation of the *35S-GUS* transgene in the tobacco host (Mallory et al., 2003). A recent work suggests that p25 acts downstream of dsRNA synthesis by a specific inhibition of the production of the longer siRNA species of 24 to 26 nucleotides (Hamilton et al., 2002). However, the recently described NSs protein of tomato spotted wilt virus (Takeda et al., 2002) may be mechanistically similar to p69.

p69 Suppression May Trigger a Negative Feedback Regulation

Our analysis revealed that *P69* expression was associated with enhanced accumulation of all of the seven miRNAs examined and a correspondingly decreased accumulation of two mRNA targets of miRNAs. Increased accumulation was also observed for the mRNA of *DCL1* (Figure 7C) and *SDE1* (shown by real-time PCR analysis; our unpublished data) but not for the mRNA of *AGO1* (Figure 7C). It is not known if p69 expression leads to increased expression of additional RNA silencing pathway genes, such as the homologs of *DCL1* and *SGS2/SDE1* (Finnegan et al., 2003; Yu et al., 2003) or if any of the p69-

induced changes are mechanistically related. Given the known role of *DCL1* in the production of miRNAs (Park et al., 2002; Reinhart et al., 2002), however, we propose that the RNA silencing pathway in *A. thaliana* is under a negative feedback regulation that can be triggered by p69 suppression and that *DCL1* and *SDE1* represent those genes in the pathway that are responsive to this feedback regulation at the level of transcription. It is possible that an upregulated *DCL1* expression will lead to elevated levels of miRNAs by a more efficient processing of miRNA precursors, which may in turn cause enhanced cleavages of those host mRNAs targeted by miRNAs. In this regard, it will be interesting to determine if p69 expression also inhibits host gene expression by miRNA-guided translational repression (Aukerman and Sakai, 2003; Chen, 2003).

Recent work indicates that the *DCL1* mRNA is targeted for cleavage in *A. thaliana* plants by miR162 (Xie et al., 2003). miR162 also accumulated to a higher level in p69-expressing plants than in wild-type plants (Figure 7B), which intriguingly, did not lead to a correspondingly lower level of the *DCL1* mRNA as expected, in contrast with miR156 and miR171. It is not clear if this is because of an overall lower accumulation level of miR162 in C24 than in Col-0 and Landsberg *erecta* (Figure 7B; Xie et al., 2003). Alternatively, miRNA cleavage of mRNAs may be a rate-limiting process so that it becomes ineffective to control mRNA accumulation of those genes that are under transcriptional induction. It is less likely, however, that the increased accumulation of *DCL1* mRNA found in p69-expressing plants is a result of p69-mediated inhibition of miRNA cleavage because mRNA cleavages by miR156 and miR171 were not inhibited (Figure 6) and consistent HC-Pro inhibition of miRNA cleavages was observed for 10 target mRNAs (Kasschau et al., 2003; Xie et al., 2003). It should be pointed out that a putative role for *DCL1* in elevated levels of miRNAs is based on an assumption that p69 does not inhibit miRNA-mediated translational arrest, which remains to be determined.

Viral Pathogenesis by miRNAs?

Recent work has established a regulatory role for miRNAs in the development of plants and animals by targeting mRNAs for either cleavage or translational repression (Carrington and Ambros, 2003; Bartel, 2004). Although p69 could play a role in viral pathogenesis by miRNA-independent mechanisms, an attractive hypothesis is that the disease symptoms/developmental defects associated with *P69* expression in *A. thaliana* plants represent a consequence of enhanced miRNA-guided inhibition of host gene expression regardless of whether one or both mechanisms are used. For example, the late flowering phenotype of the P69 plants may be in part attributable to the p69-stimulated miR156 knockdown of *SPL3* mRNA because it is known that constitutive overexpression of *SPL3* results in early flowering (Cardon et al., 1997). Interestingly, the level of *P69* expression is highest in the young leaves systemically infected with TYMV but decline as infected leaves expand and mature (Bozarth et al., 1992), possibly because of selective proteolysis by the proteasome (Drugeon and Jupin, 2002). Presumably, young tissues are most sensitive to developmental cues such as miRNAs, a suggestion consistent with the observation that systemic viral symptoms

always appear first in young leaves. Thus, miRNAs may play a novel pathogenic role in the induction of viral diseases.

HC-Pro expression in transgenic plants is known to cause developmental defects (Anandalakshmi et al., 2000). Recent studies show that even though miRNAs accumulate to elevated levels in the presence of HC-Pro, miRNA cleavage of target mRNAs is inhibited in HC-Pro–expressing plants, as found previously for PTGS induced by either sense- or IR-RNA transgenes (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998; Mallory et al., 2002, 2003; Kasschau et al., 2003; Xie et al., 2003). Accordingly, it has been proposed that the role of HC-Pro in disease development is to inhibit host developmental pathways that depend on negative regulation by miRNAs (Kasschau et al., 2003). Therefore, our work on p69 of TYMV not only establishes a distinct mechanism in viral suppression of the RNA silencing antiviral defense, but also suggests an alternative model for the induction of viral diseases by upregulating the role of miRNAs in the inhibition of host gene expression.

METHODS

Plant Materials and Growth Conditions

L1 carries a silenced *GUS* transgene in the *Arabidopsis thaliana* ecotype Col-0 background, and *sgs2* is a loss-of-function mutant of L1 at the *SGS2/SDE1* locus (Mourrain et al., 2000). Lines G, A, and GxA (in C-24 ecotype background) correspond to GFP142, Amp243, and GFP142xAmp243, respectively (Dalmay et al., 2000a). *sde1-3* was derived from GxA (Dalmay et al., 2000b).

The *P69* coding sequence of TYMV-Blue Lake (BL) (Skotnicki et al., 1992) was cloned into pCambia1301 by replacing the GUS sequence between the 35S promoter and nopaline synthase terminator to give 35S-P69. The start codon (ATG) of the viral RdRP, which is four nucleotides downstream of the start codon for p69, was mutated to ACG in 35S-P69. As a result, the RdRP open reading frame (ORF) is likely not to be translated from 35S-P69 because the next Met codon in the RdRP ORF is 95 codons downstream. A single G-to-T substitution converting the 46th codon of ORF P69 into a stop codon (TAA) was introduced to create 35S-ΔP69. 35S-P69 and 35S-ΔP69 were transferred into *A. thaliana* ecotype C24 as described (Clough and Bent, 1998). The IR-RNA transgene targeting PDS contained an inverted repeat corresponding to nucleotides 128 to 532 of the PDS mRNA. The IR-RNA targeting GFP corresponded to nucleotides 360 to 716 of the GFP mRNA. Inserted between the repeats of either PDS or GFP was the third intron of *A. thaliana* actin gene 11 (nucleotides 1957 to 2111; GenBank accession number ATU27981). The PDS and GFP IR-RNA cassettes were cloned into pCambia1300 between 35S promoter and nopaline synthase terminator and transformed into wild-type Col-0 and line G (Dalmay et al., 2000a), respectively. Transformants were selected by hygromycin at 20 mg/L. Lines with a single copy transgene were selected by a 3:1 segregation of HygR and HygS among selfed progeny. Homozygous lines for *35S-P69* or *35S-ΔP69* were used in crosses. For plant inoculation, purified viral particles derived from an infectious plasmid containing the full-length TYMV-BL cDNA (Skotnicki et al., 1992) under the 35S promoter (A. Mackenzie, unpublished data) in the pCass vector as described (Ding et al., 1995) were used. TYMV amplicon plants, which were kindly supplied by N.H. Chua, carried the minimal 35S promoter (−46 to +1)–controlled TYMV-BL cDNA with a 1.6-kb insertion of nonviral sequence between the viral RdRP and CP genes (Aoyama and Chua, 1997). These plants became symptomatic

without dexamethasone induction, possibly because of a leaky transcription.

Seeds were either imbibed on wet filter paper at 4°C for 7 d before planting on the Florobella potting compost/sand mix (3:1) or surface sterilized for growth on MS medium with/without antibiotics, but both were maintained in a growth room (16 h light/8 h darkness, 20 to 23°C).

DNA and RNA Analyses

The analysis of DNA methylation of either the *GFP* coding sequence (Dalmay et al., 2000a) or the *GUS* coding sequence (Mourrain et al., 2000) was performed as described using ^{32}P-labeled DNA probes corresponding to the full coding sequences. As a control, the GUS filter was rehybridized with a *SPL3*-specific probe because there is only one *Hpa*II/*Msp*I recognition sequence (CCGG) within the *SPL3* genomic DNA.

Total plant RNA extraction and RNA gel blotting analysis were performed as described previously (Li et al., 1999). Hybridization probes were labeled with ^{32}P using the Amersham Megaprime DNA labeling kit (Piscataway, NJ). *SPL3*, *SCL6-IV*, and *PDS* probes corresponded to nucleotides 293 to 532, 774 to 1485, and 128 to 532 of the *SPL3*, *SCL6-IV*, and *PDS* mRNAs, respectively. Total mRNAs were extracted from whole plants including leaves, stems, flowers, and siliques but not roots using the Qiagen Oligotex mRNA Midi kit (Valencia, CA). The probes for *DCL1* (Jacobsen et al., 1999), *AGO1* (Fagard et al., 2000), and *β-TUBULIN-1* corresponded to nucleotides 475 to 2283, 484 to 748, and 500 to 992 of their respective mRNAs.

siRNA detection was as described (Guo and Ding, 2002). These siRNA filters were reprobed for the 5S rRNA to show equal loading. For miRNA detection, total RNA was isolated from newly bolted inflorescence, and 50 μg was loaded in each lane as described (Reinhart et al., 2002) and probed by end-labeled DNA oligonucleotides complementary to miRNA. All experiments on the DNA and RNA analyses were repeated at least twice.

Real-Time RT-PCR Analysis

Total cDNA was first synthesized using the oligo(dT)$_{15}$ primer from total RNAs and used as templates for the real-time PCR with gene-specific primers. The amplified regions corresponded to nucleotides 721 to 949, 628 to 1057, 484 to 748, and 500 to 992 of the *SDE1/SGS2* (*RdRP*), *DCL1*, *AGO1*, and *β-TUBULIN-1* mRNAs, respectively. The LightCycler-FastStartDNA Master SYBR Green kit and LightCycler Instrument real-time PCR machine (Roche Applied Science, Penzberg, Germany) were used. The reactions were terminated when PCR was in the log-linear phase but before the later plateau phase, which was 27 cycles for *RdRP* and 29 for *DCL1*, *AGO1*, and *β-TUBULIN-1*. After confirming the identity by direct sequencing, PCR products were blotted and detected by hybridizations with gene-specific probes.

RLM-RACE

The FirstChoice RLM-RACE kit (Ambion, Austin, TX) was used for RLM-RACE essentially following the manufacturer's instructions. Briefly, poly(A)$^{+}$ mRNA was obtained from flowering plants using Oligotex mRNA Midi kit (Qiagen) and directly ligated to RLM-RACE 5′RACE RNA oligo adaptor (45 nucleotides) before cDNA synthesis using oligo(dT)$_{15}$ primer. The first round of nested PCR was performed using 5′RACE outer primer included in the kit and a gene-specific outer primer that was complementary to nucleotides 690 to 710 of *SPL3* (At2g33810 complete cDNA), 1166 to 1185 of *SPL2* (At5g43270), or 1075 to 1094 of *CUC2* (At5g53950). For the second round, 5′RACE inner primer was used with the second set of gene-specific inner primers that were respectively complementary to nucleotides 665 to 685 of *SPL3*, 1134 to 1152 of *SPL2*,

and 1057 to 1078 of *CUC2*. In each case, a unique gene-specific DNA fragment was amplified (Figure 6C). The PCR products were gel purified and cloned into pGEM-T Easy vector (Promega, Madison, WI) for DNA sequencing.

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