

P1/HC-Pro, a Viral Suppressor of RNA Silencing, Interferes with *Arabidopsis* Development and miRNA Function

Kristin D. Kasschau,¹ Zhixin Xie,¹ Edwards Allen,¹ Cesar Llave, Elisabeth J. Chapman, Kate A. Krizan, and James C. Carrington*

Center for Gene Research and Biotechnology and Department of Botany and Plant Pathology Oregon State University Corvallis, Oregon 97331

Summary

The molecular basis for virus-induced disease in plants has been a long-standing mystery. Infection of *Arabidopsis* by *Turnip mosaic virus* (TuMV) induces a number of developmental defects in vegetative and reproductive organs. We found that these defects, many of which resemble those in miRNA-deficient *dicer-like1* (*dcl1*) mutants, were due to the TuMV-encoded RNA-silencing suppressor, P1/HC-Pro. Suppression of RNA silencing is a counterdefensive mechanism that enables systemic infection by TuMV. The suppressor interfered with the activity of miR171 (also known as miRNA39), which directs cleavage of several mRNAs coding for *Scarecrow-like* transcription factors, by inhibiting miR171-guided nucleolytic function. Out of ten other mRNAs that were validated as miRNA-guided cleavage targets, eight accumulated to elevated levels in the presence of P1/HC-Pro. The basis for TuMV- and other virus-induced disease in plants may be explained, at least partly, by interference with miRNA-controlled developmental pathways that share components with the antiviral RNA-silencing pathway.

Introduction

Systemic infection of plants by viruses requires replication at the single-cell level, cell to cell movement through plasmodesmata, systemic movement through the phloem, and invasion of distal tissues. A frequent consequence of systemic infection is disease, symptoms of which can range from mild discoloration to severe developmental defects and death (Hull, 2001). The fact that most symptoms occur in new tissues arising from growing points after initial infection suggests that disease involves disruption of normal growth and development.

In addition to factors that enable virus genome expression, replication, and movement, plant viruses generally require a suppressor of RNA silencing (Vance and Vaucheret, 2001). Virus mutants that lack a functional suppressor accumulate to low levels and are often restricted to inoculated cells or leaves (Kasschau and Carrington, 2001; Liu et al., 2002; Qiu et al., 2002; Voinnet et al., 1999, 2000; Yelina et al., 2002). Thus, RNA silencing functions as an antiviral defense response to limit the

extent of virus invasion. RNA silencing involves recognition and processing of double-stranded RNA by a DICER-like enzyme (Bernstein et al., 2001; Zamore et al., 2000), resulting in formation of small interfering RNAs (siRNAs) of ~21–25 nucleotides (nt) in length (Hamilton and Baulcombe, 1999). Viruses may form limited amounts of dsRNA during genome replication, or dsRNA may be produced through the activity of a silencing-associated RNA-dependent RNA polymerase (Dalmay et al., 2000; Mourrain et al., 2000). The siRNA assembles into an RNA-induced silencing complex (RISC) that acquires sequence-specific nucleolytic activity through the guide function of the siRNA (Hammond et al., 2000; Zamore et al., 2000). The RISC complexes then initiate sequence-specific degradation of viral genomic or messenger RNAs. RNA silencing, therefore, requires two nucleolytic steps—DICER-like cleavage of dsRNA and RISC-mediated degradation of viral RNA targets.

RNA silencing is part of a larger set of pathways involving small RNAs (Hannon, 2002). Developmental regulation in plants and animals requires microRNAs (miRNAs), which structurally resemble siRNAs except that they arise from structured precursor transcripts derived from miRNA genes (Pasquinelli and Ruvkun, 2002). Processing of miRNA precursors in animals requires DICER (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). In plants, the multidomain DICER-LIKE1 (DCL1), which was formerly known by other names, such as CARPEL FACTORY and SHORT INTEGUMENTS 1 (Schauer et al., 2002), catalyzes miRNA precursor processing (Reinhart et al., 2002), although its role in siRNA generation is not established. Biochemical data indicate that different subclasses of silencing-associated small RNAs require different DICER-like proteins in plants (Tang et al., 2003). *dcl1* mutants exhibit a range of embryo, vegetative, reproductive, and meristem defects (Golden et al., 2002; Jacobsen et al., 1999; Lang et al., 1994; Ray et al., 1996a, 1996b; Robinson-Beers et al., 1992; Schwartz et al., 1994), likely because they contain low levels of miRNAs.

miRNAs appear to negatively regulate genes required for stem/meristem cell identity, developmental timing, and other developmental processes by interacting with mRNAs encoding key regulatory factors (Ambros, 2001; Pasquinelli and Ruvkun, 2002). Interaction between an miRNA and an mRNA target can have at least two consequences. First, as shown for the small temporal RNAs of *C. elegans*, interaction through imperfect base pairing with the 3' nontranslated region of the target can lead to translation arrest (Olsen and Ambros, 1999; Wightman et al., 1993). And second, as shown for miR171 from *Arabidopsis*, perfect base pair interaction can trigger mRNA cleavage by a mechanism that resembles siRNA-guided cleavage (Llave et al., 2002b). A translation arrest-type miRNA can be converted to a target degradation-type miRNA by increasing the degree of complementarity between miRNA and target sequences (Hutvagner and Zamore, 2002). In addition to a set of mRNAs encoding *Scarecrow-like* transcription factors, which were validated as miRNA targets (Llave et al.,

*Correspondence: carrington@orst.edu

¹These authors contributed equally to this work.

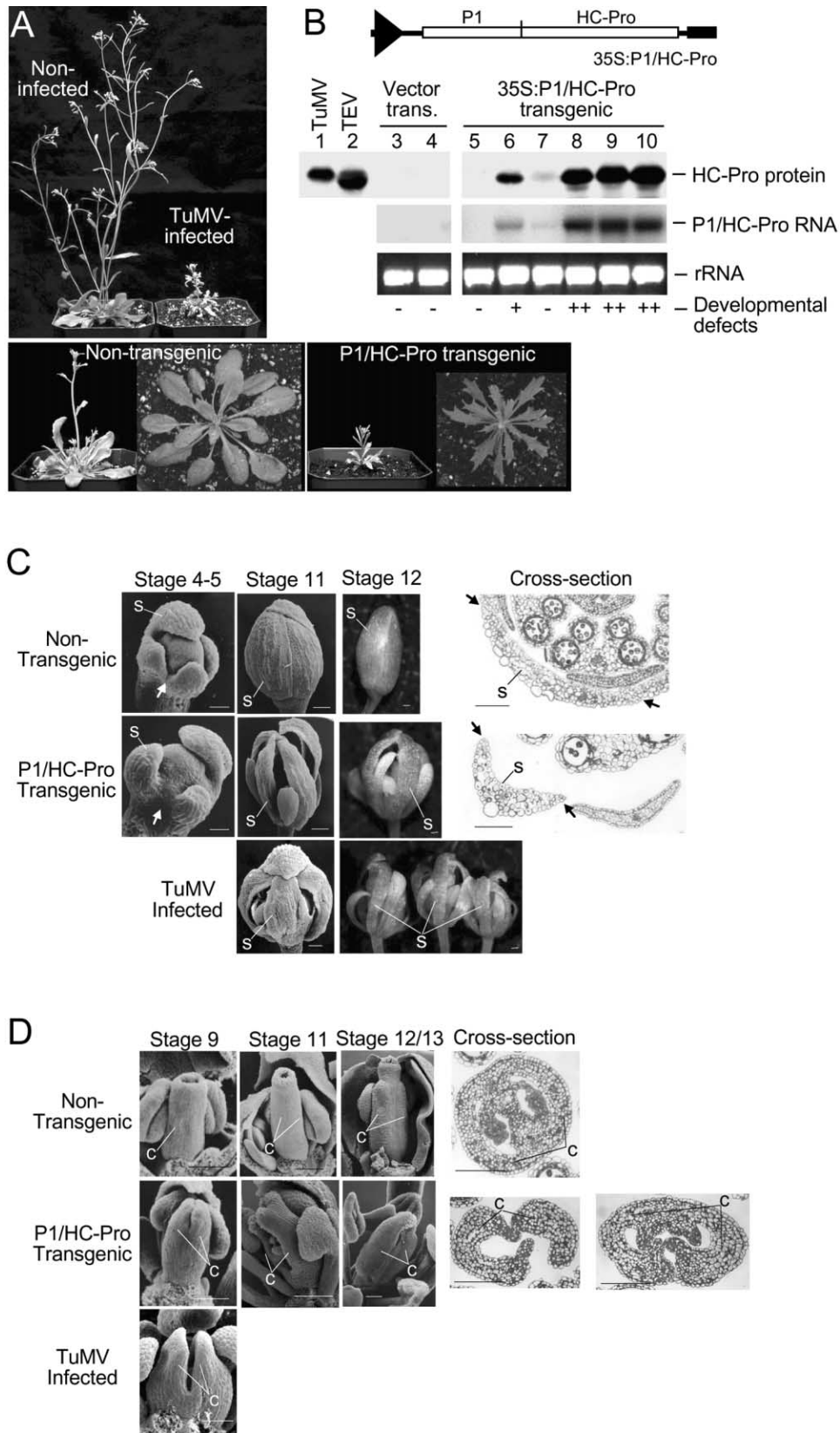


Figure 1. Developmental Defects Induced by TuMV Infection and TuMV P1/HC-Pro

(A) General vegetative effects of TuMV infection (top) and P1/HC-Pro transgene expression (bottom).

(B) Expression of constructs containing the TuMV P1/HC-Pro polyprotein coding sequence or empty vector in transgenic plants. The P1/HC-Pro polyprotein was accurately self-processed by P1 and HC-Pro proteinase activities (Carrington et al., 1990). The vector contained CaMV

2002b), mRNAs encoding several other transcription factors were predicted to be targets on the basis of near-perfect complementarity with miRNAs in *Arabidopsis* (Park et al., 2002; Rhoades et al., 2002).

In this study, infection of *Arabidopsis* by a virus (*Turnip mosaic virus* [TuMV]) or expression of an RNA-silencing suppressor (TuMV P1/HC-Pro) (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998) was shown to trigger developmental defects and to inhibit miRNA-guided cleavage of mRNA targets encoding members of several families of transcription factors. Interference with miRNA-guided functions by plant viruses may explain why certain viruses cause developmental abnormalities during infection.

Results and Discussion

Developmental Defects Induced by TuMV-Encoded RNA-Silencing Suppressor

Infection of young *Arabidopsis* plants (six- or eight-leaf stage) by TuMV resulted in severe developmental abnormalities of vegetative and reproductive organs, and these abnormalities were reproduced in transgenic *Arabidopsis* plants that expressed the TuMV-encoded RNA-silencing suppressor, P1/HC-Pro. Vegetative defects included stunting, reduced internodal distances, and pronounced lobing of leaves (Figure 1A). The severity of phenotypes in the transgenic plants correlated with accumulation levels of P1/HC-Pro mRNA and HC-Pro protein (Figure 1B).

Reproductive organs in TuMV-infected and P1/HC-Pro-expressing plants were analyzed by light and scanning electron (SEM) microscopy. Both TuMV infection and P1/HC-Pro expression induced narrow sepals that failed to encase developing flowers (Figure 1C). In P1/HC-Pro-expressing plants, the narrow sepals were due to reduced numbers of cells (51% of epidermal cells across the adaxial surface), rather than to decreased cell expansion (Figure 1C, cross-sections). Narrow sepals separated by gaps in the outer whorl were evident in stage 4–5 flowers (Figure 1C). TuMV-infected and P1/HC-Pro-expressing plants contained nonfused or partially fused carpels (Figure 1D). Unlike in control plants, these flowers frequently lacked a complete septum between the two carpels, exposing ovules to the space around the carpels (Figure 1D). Additionally, flowers from P1/HC-Pro transgenic plants contained fewer pollen sacs/anther (2.32, $n = 90$) than control plants (3.96, $n = 83$), failed to release pollen, and were sterile.

The flower developmental defects in P1/HC-Pro-expressing plants (Col-0 ecotype) were compared with those of *dcl1* mutants (La-*er* genetic background). Expression of P1/HC-Pro and each of two *dcl1* alleles (*dcl1-7* [Lang et al., 1994; Robinson-Beers et al., 1992] and *dcl1-9* [Jacobsen et al., 1999]) resulted in similar narrow sepal phenotypes (Figure 2A). P1/HC-Pro expression and the *dcl1-9* mutant both had nonfused or partially fused carpels (Figure 2B). P1/HC-Pro transgenic and *dcl1-9* mutant plants both had reduced numbers of pollen sacs/anther (Jacobsen et al., 1999). The *dcl1* mutants collectively possess other abnormalities, including ovule and embryo defects, but these tissues were not systematically analyzed in P1/HC-Pro-expressing plants. Thus, P1/HC-Pro expression and *dcl1* mutations induce a set of overlapping, although not necessarily identical, developmental phenotypes in flowers. It should also be noted that, although P1/HC-Pro and *dcl1* mutations induce vegetative phenotypes, these phenotypes are quite distinct (Figure 1A) (Lang et al., 1994; Robinson-Beers et al., 1992; Jacobsen et al., 1999).

P1/HC-Pro Suppresses miR171-Guided Cleavage of Scarecrow-like mRNAs

Given the overlap between TuMV P1/HC-Pro- and *dcl1*-associated phenotypes in *Arabidopsis* and the known role of P1/HC-Pro as an RNA-silencing suppressor, we hypothesized that P1/HC-Pro suppresses one or more reactions involved in miRNA production or activity. To test this hypothesis, we analyzed the effects of TuMV infection and P1/HC-Pro on miR171 accumulation and activity. miR171 is processed from a fold-back precursor derived from a locus in chromosome III (Llave et al., 2002a; Reinhart et al., 2002). It is complementary to an internal region of mRNAs coding for three members (SCL6-II, SCL6-III, and SCL6-IV) of the Scarecrow-like (GRAS domain) family of plant-specific transcription factors (Llave et al., 2002a; Reinhart et al., 2002). Members of this family control several developmental processes, including radial patterning in roots (DiLaurenzio et al., 1996; Helariutta et al., 2000) and hormone signaling (Silverstone et al., 1998). miR171 directs site-specific cleavage of complementary SCL mRNAs in inflorescence-associated tissues by a RISC-like mechanism (Figure 3A) (Llave et al., 2002b).

In inflorescence tissue of noninfected plants, *SCL6-III* and *SCL6-IV* RNAs each accumulated in two forms—full-length mRNA [(a) form] and a relatively stable, miR171-guided cleavage product derived from the

35S promoter (filled arrow) and terminator (filled rectangle) sequences. Six independent P1/HC-Pro transgenic plants were analyzed by immunoblot assay with anti-TEV HC-Pro serum and by RNA blot assay. Extracts from TuMV- and TEV-infected plants were positive immunoblot controls. The severity of developmental defects in P1/HC-Pro transgenic plants was rated as follows: –, no defects; +, mild defects in some, but not all, flowers, fertile, some stunting, and some lobed leaves; ++, narrow sepals in all flowers, infertile, deep lobing of leaves, slow growing, and stunted.

(C) Sepal phenotype in developing flowers of normal (top four panels), P1/HC-Pro transgenic (middle four panels), and TuMV-infected (bottom three panels) plants. The SEM micrographs were from flowers at stage 4–5 or 11, and the cross-section and macroscopic images were from stage 12 flowers. The arrows in the SEM images of stage 4–5 flowers indicate the junction or space between sepals. The arrows in the cross-section images indicate the boundaries of individual sepals. S, sepal. Bars are 20 μm in stage 4–5 images and 100 μm in stage 11 and cross-section images.

(D) Split or nonfused carpel phenotype in developing flowers of normal (top four panels), P1/HC-Pro transgenic (middle five panels), and TuMV-infected (bottom panel) plants. C, carpel. Bar is 100 μm .

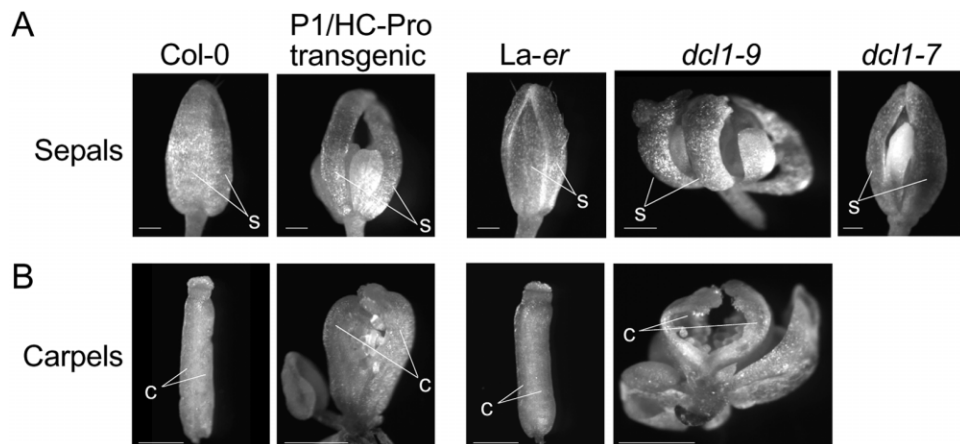


Figure 2. Similarity between *dcl1* Mutant and TuMV P1/HC-Pro-Induced Phenotypes in Developing Flowers

(A) Narrow sepal phenotype in P1/HC-Pro transgenic and *dcl1-9* and *dcl1-7* mutant plants. Macroscopic images of stage 11 or 12 flowers are shown. S, sepal. Bars are 300 μ M.
(B) Split or nonfused carpel phenotype in P1/HC-Pro transgenic and *dcl1-9* mutant plants. Macroscopic images of dissected stage 11 or 12 carpels are shown. C, carpel. Bars are 300 μ M.

mRNA 3' end [(b) form] (Figure 3B, lanes 1 and 2). In inflorescence tissue from TuMV-infected plants, accumulation of the cleavage product from both *SCL6-III* and *SCL6-IV* RNA was suppressed, as indicated by an increase in the (a)/(b) ratio (Figure 3B, lanes 3 and 4). To determine whether suppression of *SCL6-III* and *SCL6-IV* mRNA cleavage was due to P1/HC-Pro, we performed a similar analysis using P1/HC-Pro transgenic plants that displayed severe phenotypes. As in infected plants, miR171-guided cleavage of both mRNAs was significantly inhibited in inflorescence tissue of P1/HC-Pro transgenic plants (Figure 3B, lanes 5–8). The (a)/(b) ratio increased approximately 4- to 5-fold in P1/HC-Pro-expressing plants relative to the vector-transformed control plants. Although the (a)/(b) ratio was used as a relative measure of miR171-guided cleavage, it does not necessarily reflect the absolute amount of cleavage, as the differential stability of full-length mRNAs and cleavage products may affect the steady state levels that were measured.

The *SCL6-IV* mRNA forms were also analyzed in miRNA-deficient *dcl1* mutant plants (Figure 3C). This was done in the *La-er* genetic background, which has relatively low steady-state levels of *SCL6-IV*-associated RNAs, as *La-er* is the ecotype in which *dcl1-7* and *dcl1-9* mutations were characterized. As expected, miR171-guided cleavage was suppressed in both mutants, as indicated by an \sim 4-fold increase in the (a)/(b) ratio (Figure 3C).

The levels of miR171 in control, TuMV-infected, P1/HC-Pro-expressing, and *dcl1* mutant plants were measured with extracts from inflorescence tissue. In both TuMV-infected and P1/HC-Pro-expressing plants, miR171 accumulated to levels approximately 2-fold higher than those in control plants (Figure 3D, lanes 1–8). These results are similar to those reported for tobacco miR171 (Mallory et al., 2002) and indicate that suppression of *SCL* mRNA cleavage is not due to lack of miR171. In

contrast, miR171 in *dcl1-7* and *dcl1-9* plants accumulated to less than 10% of normal levels (Figure 3D, lanes 9–11), as expected from previous studies (Reinhart et al., 2002). These results suggest that suppression of miR171-guided cleavage in *dcl1* plants is due to low levels of miR171, whereas suppression by P1/HC-Pro is due to interference with a step downstream from miR171 biosynthesis.

To confirm that P1/HC-Pro interferes with miR171-guided cleavage, but not with miR171 production, we used the *Agrobacterium*-mediated injection (Agro injection) assay in *Nicotiana benthamiana* to coexpress *SCL6-IV* mRNA, miR171, and wild-type and mutant forms of *Tobacco etch virus* (TEV) P1/HC-Pro. The construct *35S:IGR-miR171* contained the entire 1.8 kb intergenic region (IGR) with the *miR171* gene adjacent to a 35S promoter (Figure 4A). Transcription of the *35S:IGR-miR171* gene yields a synthetic precursor RNA that is processed by DCL1 to generate miR171 in Agro-injected *N. benthamiana* leaves. Expression of this construct, but not a construct containing the same IGR sequence in the opposite orientation relative to the 35S promoter (*35S:AS-IGR-miR171*), results in high levels of miR171 expression (Llave et al., 2002b). Expression of the *35S:SCL6-IV* construct alone resulted in accumulation of predominantly full-length mRNA [(a) form], although a low proportion of cleavage product [(b) form] accumulated because of the activity of endogenous *N. benthamiana* miR171 (Llave et al., 2002b) (Figure 4B, lanes 5 and 6). Coinjection of *35S:IGR-miR171* and *35S:SCL6-IV* constructs resulted in processing of the full-length mRNA and elevated accumulation of the 3' cleavage product (Figure 4B, lanes 7 and 8). As in *Arabidopsis*, the full-length mRNA and 3' product should not necessarily accumulate in stoichiometric amounts because they likely have different stabilities. miR171-guided cleavage of *SCL6-IV* RNA was effectively suppressed in tissues that were co-Agro injected with mixtures containing

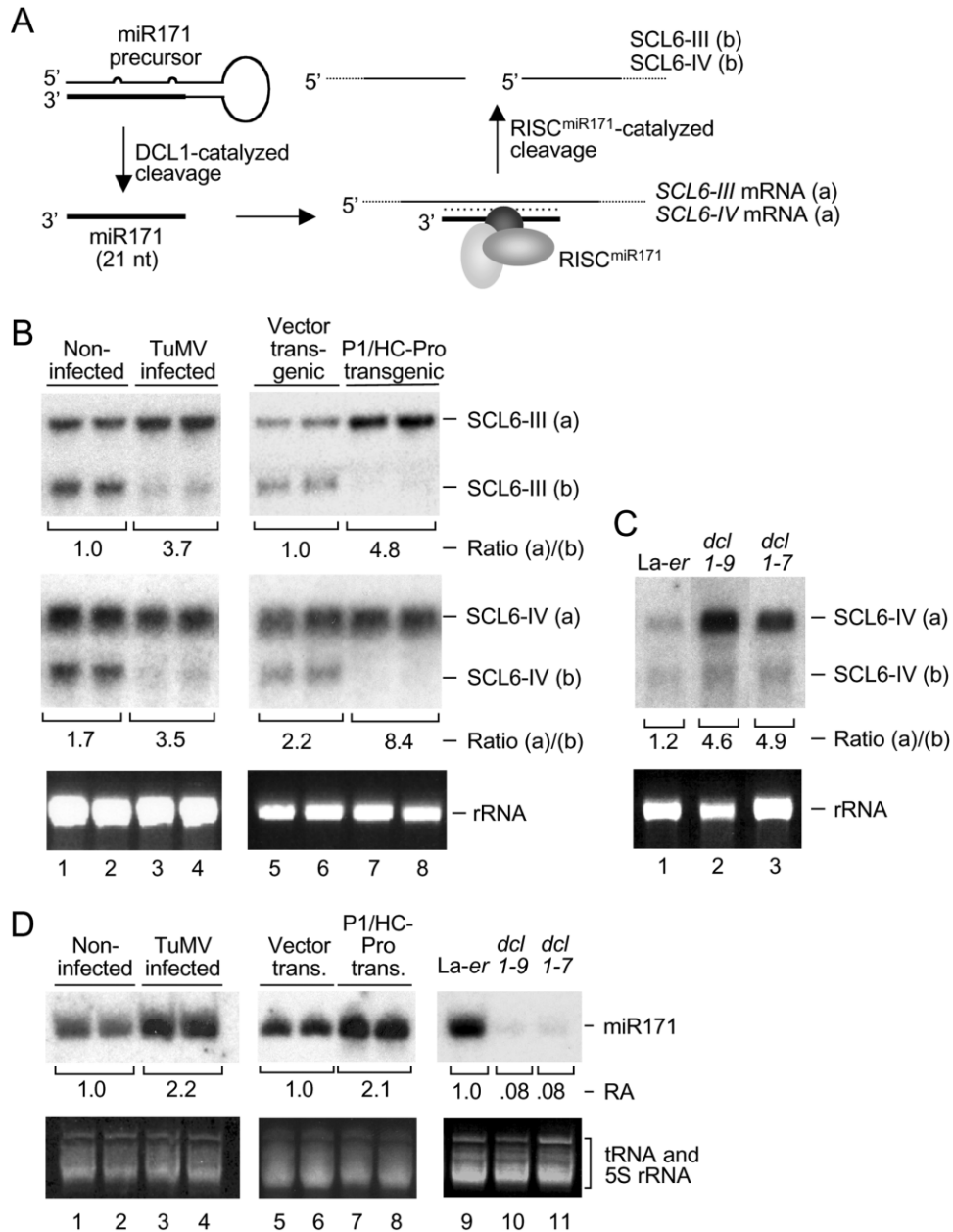


Figure 3. Interference with miR171 Activity by TuMV Infection, P1/HC-Pro, and *dcl1* Mutations

(A) Model for miR171 generation and activity. miR171 arises by DCL1-catalyzed processing of a fold-back precursor transcript, assembles into a RISC-like complex (RISC^{miR171}), and interacts with several *SCL* mRNAs. The complex catalyzes mRNA cleavage in the middle of the base-paired sequence. Full-length *SCL6-III* and *SCL6-IV* mRNAs are indicated with an "(a)" suffix, and the 3' cleavage products are indicated with a "(b)" suffix.

(B) Effect of TuMV infection and P1/HC-Pro on *SCL6-III* and *SCL6-IV* mRNA cleavage in inflorescence tissue (Col-0). Tissue from two pools of ten noninfected control (lanes 1 and 2), TuMV-infected (lanes 3 and 4), vector transgenic (lanes 5 and 6), and P1/HC-Pro transgenic (lanes 7 and 8) plants were analyzed by RNA blot assay with a probe corresponding to the 3' end of the *SCL6-III* or *SCL6-IV* genes.

(C) Effect of *dcl1-9* and *dcl1-7* mutations on *SCL6-IV* mRNA cleavage in inflorescence tissue (La-er genetic background). Samples were analyzed as in panel (B).

(D) Effect of TuMV infection, P1/HC-Pro, and *dcl1* mutations on miR171 accumulation in inflorescence tissue. Small RNA preparations from two pools of ten noninfected (lanes 1 and 2), TuMV-infected (lanes 3 and 4), vector transgenic (lanes 5 and 6), and P1/HC-Pro transgenic (lanes 7 and 8) plants and from single pools of La-er control, *dcl1-9*, and *dcl1-7* plants (lanes 9–11) were analyzed by RNA blot assay with an miR171-specific probe. The mean relative accumulation (RA) level for each set of samples is indicated (level in tissue from noninfected, nontransgenic, or control plants was arbitrarily designated as 1.0 in each set).

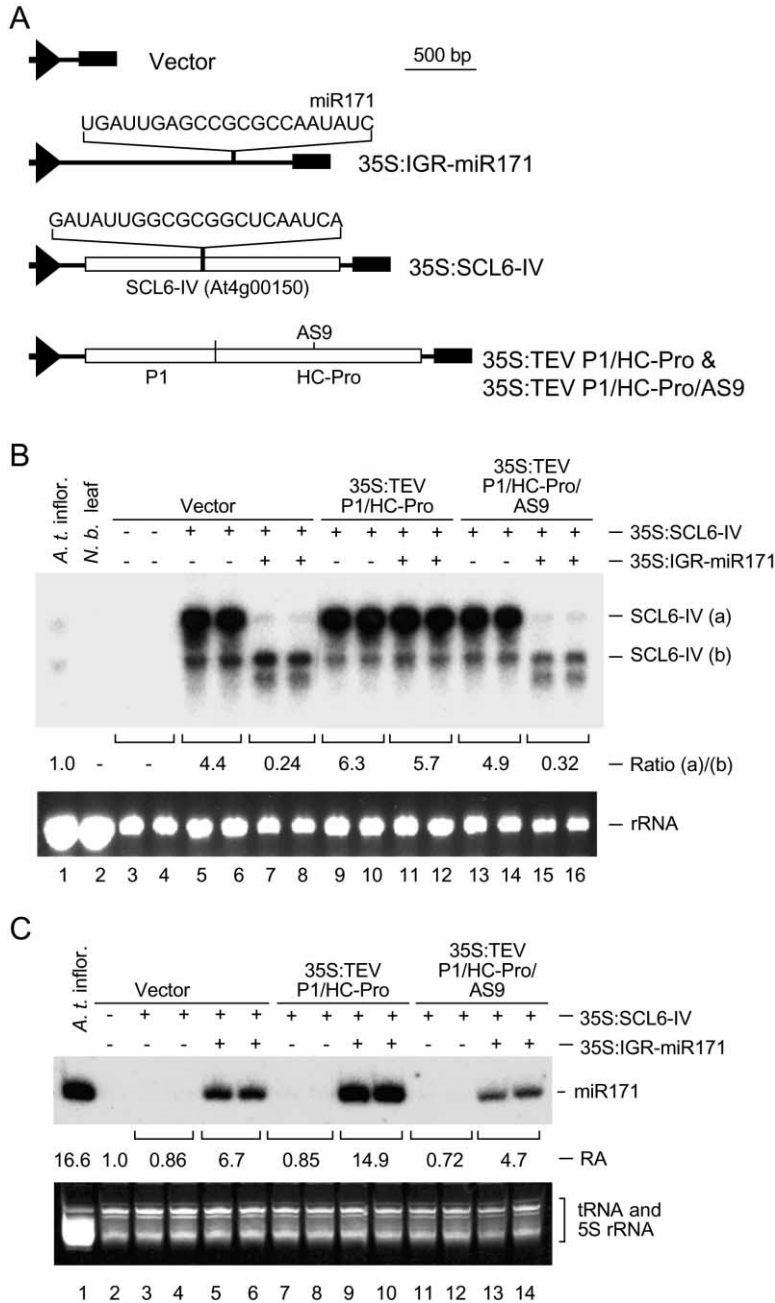


Figure 4. Interference with miR171 Activity by TEV P1/HC-Pro in the Agro Injection Assay in *N. benthamiana*

(A) Constructs for expression of miR171 (35S:IGR-miR171), SCL6-IV mRNA (35S:SCL6-IV), and wild-type (35S:TEV P1/HC-Pro) or mutant (35S:TEV P1/HC-Pro/AS9) forms of TEV P1/HC-Pro each contained a 35S promoter (arrow) and 35S terminator (filled rectangle). The miR171 sequence from the 35S:IGR-miR171 construct and the miR171 complementary sequence in the 35S:SCL6-IV construct are indicated.

(B) Effect of wild-type and mutant TEV P1/HC-Pro on miR171-guided cleavage of SCL6-IV mRNA. *N. benthamiana* leaves were co-Agro injected with constructs as indicated, and SCL6-IV mRNA (a) and 3' cleavage product (b) were detected in two independent tissue pools by RNA blot assay. The non-Agro-injected *Arabidopsis* (A.t.) and *N. benthamiana* (N.b.) samples contained four times as much RNA as the Agro-injected samples. The precise nature of the minor species below the SCL6-IV (b) band in lanes 7, 8, 15, and 16 was not determined. The mean (a)/(b) ratio was calculated for each set of samples.

(C) Effect of TEV P1/HC-Pro on miR171 accumulation in the Agro injection assay. miR171 was detected in the same Agro injection samples that were shown in (B).

35S:IGR-miR171, 35S:SCL6-IV and wild-type 35S:TEV P1/HC-Pro (Figure 4B, lanes 9–12). A silencing suppressor-defective form of the P1/HC-Pro construct (encoded by 35S:TEV P1/HC-Pro/AS9) (Kasschau and Carrington, 2001) failed to inhibit miR171-guided cleavage in the Agro injection assay (Figure 4B, lanes 13–16). As in the virus-infected and P1/HC-Pro transgenic plants, miR171 accumulated to higher levels in tissue expressing active P1/HC-Pro (Figure 4C).

These results collectively lead to three key conclusions. First, TuMV infection interferes with miR171 activity. Second, functional P1/HC-Pro is necessary and sufficient for this interference. And third, P1/HC-Pro suppresses one or more steps downstream from DCL1-dependent

processing and maturation of miR171. It seems reasonable to propose that P1/HC-Pro interferes with formation or activity of miR171-containing RISC complexes. This is consistent with results of RNA-silencing experiments in the Agro injection system with inducers containing extensive dsRNA structure, in which P1/HC-Pro was shown to suppress target degradation, but not siRNA formation (Johansen and Carrington, 2001).

P1/HC-Pro Suppresses miRNA-Guided Cleavage of Multiple Target mRNAs

Computational approaches yielded an extensive list of potential mRNA targets for several *Arabidopsis* miRNAs

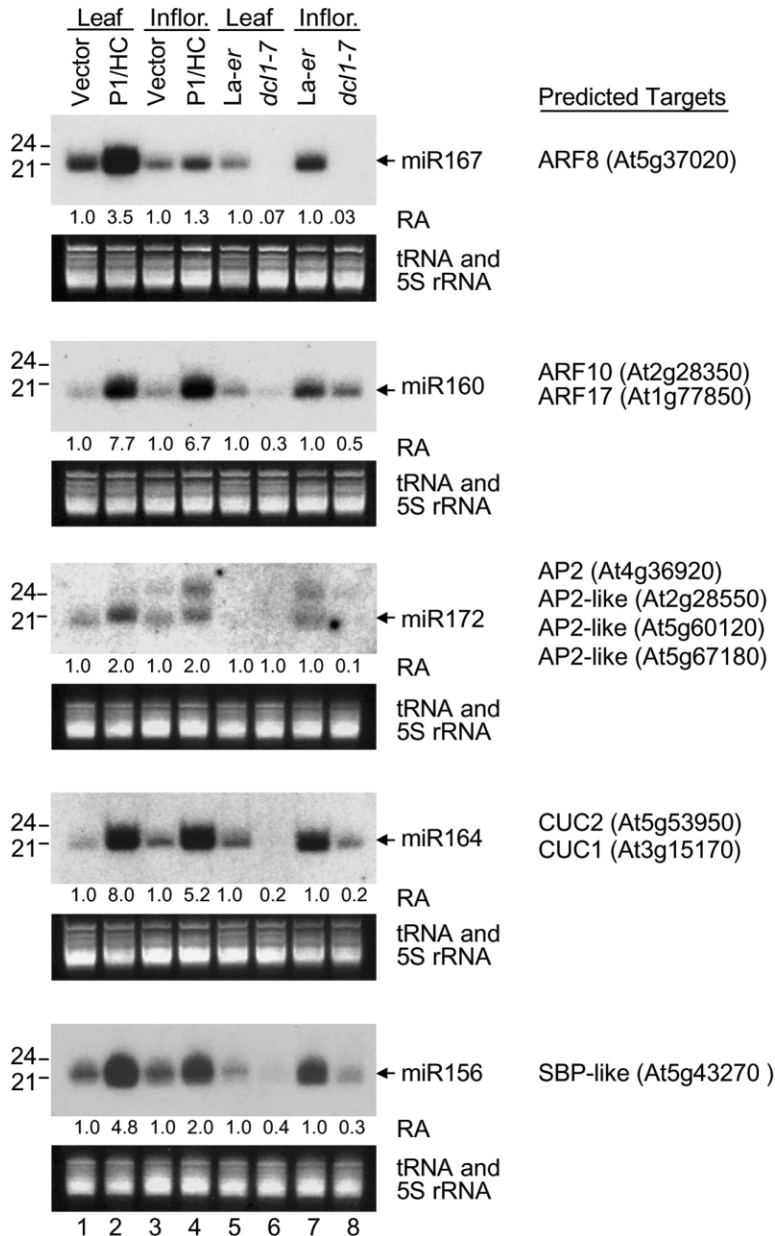


Figure 5. Analysis of miRNA Accumulation in P1/HC-Pro Transgenic and *dcl1* Mutant Plants

Small RNA preparations from leaf and inflorescence (Inflor.) tissue of vector transgenic Col-0 (lanes 1 and 3), P1/HC-Pro transgenic (lanes 2 and 4), *La-er* (lanes 5 and 7), and *dcl1-7* (lanes 6 and 8) plants were analyzed by RNA blot assay with oligonucleotide probes that were complementary to miR167, miR160, miR172, miR164, and miR156. The positions of the miRNA (arrow) and oligonucleotide standards are indicated. The mean relative accumulation (RA) level for each set of samples is indicated (levels in tissue from vector transformed and *La-er* plants were arbitrarily designated as 1.0). Predicted mRNA targets for each miRNA are listed to the right.

(Rhoades et al., 2002). Unlike miR171-SCL mRNA interaction, most of the predicted interactions involve base pair mismatches at one or a few positions. How many of these candidates are targeted for miRNA-guided cleavage and degradation? For those miRNA-mRNA interactions that involve target cleavage, how many are suppressed by P1/HC-Pro?

From the known *Arabidopsis* miRNAs, other than miR171, five (miR156, miR160, miR164, miR167 [also known as miRNA5], and miR172) were selected for analysis by small RNA blot assay in leaf and inflorescence tissues from control plants, P1/HC-Pro transgenic plants, and *dcl1* mutant plants (*dcl1-7* allele). These miRNAs were collectively represented in three small RNA libraries from shoot, seedling, and inflorescence tissues (Llave et al., 2002a; Park et al., 2002; Reinhart et al., 2002). These

were predicted to interact with one or more mRNA targets encoding members of the auxin response factor (ARF) family, apetela 2 (AP2) domain family, NAC domain family, and squamosa promoter binding protein (SBP) family (Park et al., 2002; Rhoades et al., 2002) (Figure 5). Members of these families control a range of developmental processes, including hormone response and signaling, shoot and floral meristem identity, and organ identity and separation (Aida et al., 1997; Cardon et al., 1999; Liscum and Reed, 2002; Reichmann and Meyero-witz, 1998).

Each miRNA was detected in leaf and inflorescence tissues from empty vector-transformed Col-0 plants, although the relative abundance varied between the two tissues (Figure 5, lanes 1 and 3). Each miRNA increased in abundance in the presence of P1/HC-Pro (Figure 5,

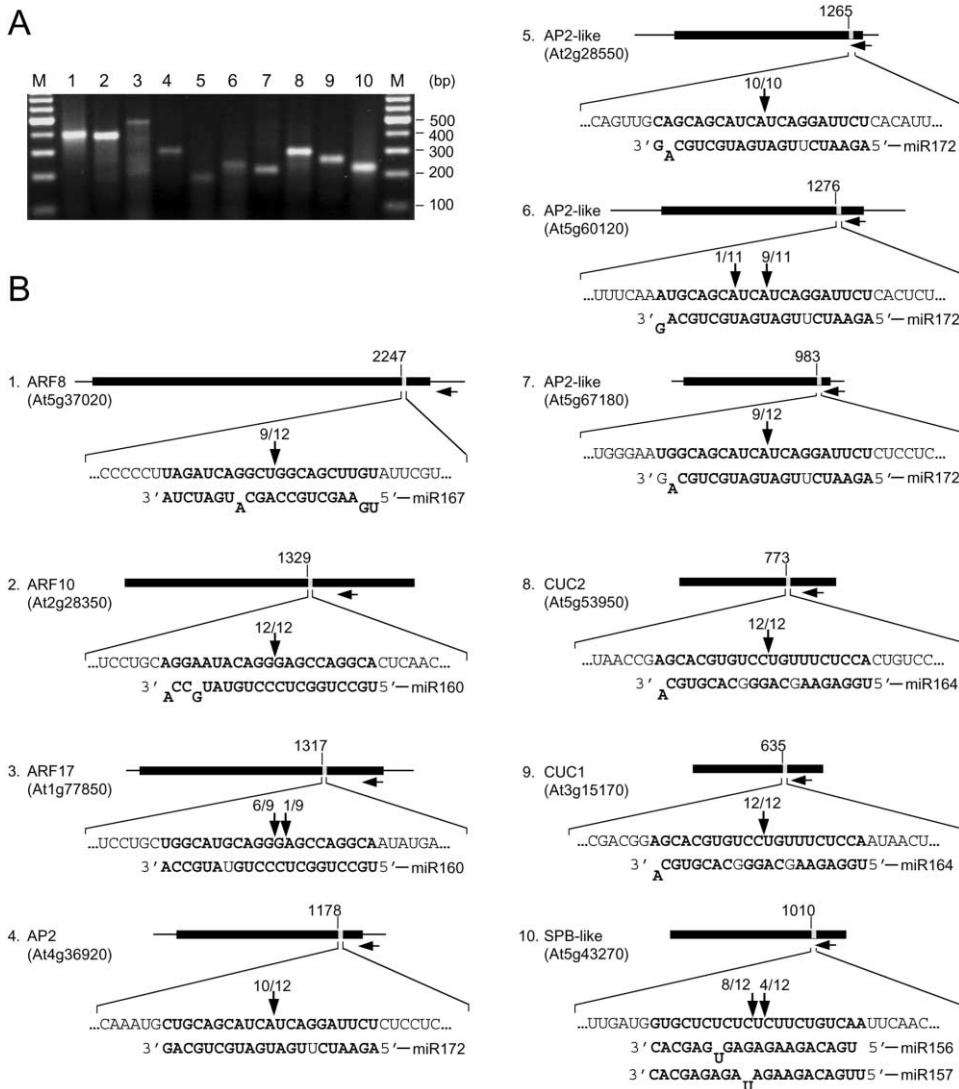


Figure 6. Mapping of mRNA Cleavage Sites by RNA Ligase-Mediated 5' RACE

(A) Ethidium bromide-stained agarose gel showing the 5' RACE products for ten miRNA targets. Numbers on top of each lane correspond to 5' RACE reactions specific for each of the ten genes listed in (B). Lane M, 100 bp DNA ladder.

(B) Diagrammatic representation of target mRNA cleavage sites. Thick black lines represent open reading frames (ORFs), while the flanking thin lines represent nontranslated regions (if known). The putative miRNA interaction site is shown as a gray box, with the nucleotide position within the ORF indicated. The miRNA sequence and partial sequence of the target mRNA are shown in the expanded regions. All predicted G-U base pairing is shown (not in bold text). The positions inferred as 5' ends of miRNA-guided cleavage products, as revealed by 5' RACE, and the number of sequenced 5' RACE clones corresponding to each site are indicated by vertical arrowheads. Horizontal arrowheads indicate gene-specific primer sites used for 5' RACE.

lanes 2 and 4), indicating that this is a general feature of miRNAs in *Arabidopsis*. In each *La-er* control sample, all five miRNAs were detected, with the exception of miR172 in leaf tissue (Figure 5, lanes 5 and 7). In contrast, the abundance of each miRNA in both tissues was lower in the *dcl1* mutant plants than the *La-er* controls (Figure 5, lanes 6 and 8), although the extent of diminution varied among the different miRNAs. For example, while miR167 was nearly eliminated by the *dcl1-7* mutation in inflorescence tissue, accumulation of miR160 was decreased by only 50% in the mutant. The basis for different effects of the *dcl1* mutation was not clear, although it is possible

that residual *dcl1* protein has differential affinity for, or catalytic function on, various miRNA precursors. It is also possible that one of the other multidomain DICER-LIKE proteins (DCL2, DCL3, or DCL4 [Schauer et al., 2002]) can compensate for the loss of processing activity on certain miRNA precursors.

Ten mRNAs that were predicted to interact with at least one of these miRNAs were subjected to target cleavage analysis. As shown with *SCL* mRNA targets (Llave et al., 2002b), miRNA-guided cleavage events have two diagnostic features. First, cleavage occurs near the middle of the region of complementarity with

the miRNA, just as siRNAs direct cleavage of silencing or RNAi targets (Elbashir et al., 2001). And second, the 5' end of the 3'-derived cleavage product can be ligated directly to an RNA adaptor with T4 RNA ligase. Thus, miRNA-guided cleavage events can be detected by RNA ligase-mediated 5' RACE with untreated RNA extracts followed by sequence analysis of the cloned PCR products. Gene-specific 5' RACE primers that were predicted to yield products of between ~200 and 500 bp, if miRNA-guided cleavage occurred in vivo, were designed and used. With each primer, a major PCR product of the size predicted to be generated from a template resulting from an miRNA-guided cleavage event was detected (Figure 6A). These products were cloned, and the sequence of at least nine inserts was determined for each reaction. In all cases, at least 75% of the 5' ends of inserts terminated at a position corresponding to the middle of the region of complementarity with the respective miRNA (Figure 6B). This feature is characteristic of a RISC-like processing event (Elbashir et al., 2001) and provides strong evidence that at least part of each predicted target pool is cleaved by a process dependent on the indicated miRNAs. It should be emphasized that each of these predicted miRNA-mRNA interactions contains G-U base pairs or mismatched positions. This indicates that perfect base pairing throughout the entire length of the miRNA-mRNA interaction is not a strict requirement to guide cleavage of target RNAs. Tang et al. (2003) reached a similar conclusion after showing that miR165/miR166-containing RISC complexes in wheat germ extracts functioned catalytically to cleave an imperfectly base-paired mRNA target from the *Phavoluta* gene.

To determine whether the ten miRNA-sensitive targets were affected by TuMV P1/HC-Pro, we performed RNA blot assays with gene-specific probes using extracts from leaf and inflorescence tissues from empty vector-transformed and P1/HC-Pro-expressing transgenic plants (Col-0). Samples from *La-er* control and *dcl1* mutant plants were also analyzed. As controls, blots were analyzed with probes for mRNAs encoding tyrosine aminotransferase (TyrAT) and β -tubulin, which were predicted to be unaffected by miRNAs. Hybridization signals were quantitated, and relative accumulation (RA) levels in both tissue types were calculated by comparison of the P1/HC-Pro-expressing or *dcl1* mutant values with the vector-transformed or wild-type values (each arbitrarily set at 1.0), respectively. Relative levels were calculated independently for each tissue type.

Eight of the ten target mRNAs accumulated to elevated levels (mean RA \geq 2.0) in leaf and/or inflorescence tissue in the presence of P1/HC-Pro (Figure 7). The ARF8 and ARF10 mRNAs accumulated to substantially higher levels in P1/HC-Pro-expressing leaf tissue but were essentially unchanged in inflorescence tissue. The ARF17 mRNA accumulated to the same levels in the presence and absence of P1/HC-Pro (data not shown). Three of the AP2-like mRNAs, but not AP2 mRNA, were detected at higher levels in P1/HC-Pro-expressing leaves, but not inflorescence tissues. The pattern of expression of the SBP-like mRNA was similar to the AP2-like mRNAs, with enhanced accumulation specifically in P1/HC-Pro-expressing leaves. The two NAC domain protein mRNAs (CUC1 and CUC2) accumulated to much higher levels

in P1/HC-Pro-expressing inflorescence tissues but were not detected in leaves from the same plants. In contrast to most of the miRNA targets, the accumulation levels of TyrAT and β -tubulin mRNAs were essentially the same in the presence and absence of P1/HC-Pro (Figure 7).

Except for the AP2 and ARF17 mRNA results, these data are consistent with the hypothesis that P1/HC-Pro suppresses RISC-like cleavage activity guided by multiple miRNAs. Interestingly, of the eight P1/HC-Pro-responsive targets, all but the NAC domain mRNAs (CUC1 and CUC2) were detected or relatively abundant in leaves only in the presence of the suppressor (Figure 7). As P1/HC-Pro has no direct effect on transcription (Kasschau and Carrington, 1998, 2001), this suggests that these genes are transcribed in leaves but that the mRNAs are normally held to low steady-state levels by miRNA-guided degradation. The two NAC domain mRNAs were stimulated specifically in inflorescence tissue but were undetectable in leaves, suggesting that these mRNAs are either not transcribed in leaf tissue or are subject to additional negative posttranscriptional regulation by non-miRNA-directed mechanisms. Likewise, the AP2-like (At2g28550) gene in inflorescence tissue is either not expressed or negatively regulated by non-miRNA mechanisms, as no expression was detected in the presence or absence of P1/HC-Pro. Several mRNAs (e.g., ARF8 and SBP-like) that were P1/HC-Pro responsive in leaves exhibited no response in inflorescence tissue, even though the corresponding miRNAs accumulated in these tissues. It is possible that these mRNAs are actually targeted for cleavage, as shown in the 5' RACE analysis, but only in relatively few cells in inflorescence tissues, which include shoot and floral meristems, developing flowers, and other tissues. It is also possible that RISC-like complexes containing some miRNAs do not form, or are inactive, in the cell types and tissues analyzed.

The miRNA targets were predicted to accumulate to higher levels in *dcl1* mutant plants. However, higher steady-state levels (RA \geq 2.0) were measured in leaf and/or inflorescence tissue for only five of the ten targets (ARF8, ARF10, AP2-like [At2g28550], AP2-like [At5g60120], and CUC2; Figure 7). The higher level of *dcl1*-responsive mRNAs was often associated with a decreased level of faster-migrating RNA species (see AP2-like [At2g28550]; Figure 7) that were presumed to be cleavage products similar to those detected from *SCL* mRNA targets (Figures 3 and 4). When mRNA accumulation was elevated in *dcl1* plants, the extent of the increase was generally less than that detected in the presence of P1/HC-Pro. Because the *dcl1-7* mutation does not completely eliminate miRNAs (Figure 5), it is reasonable to assume that these plants contain residual miRNA-guided degradation activity. Attempts to identify or generate complete *dcl1* knockouts or null alleles by insertion mutagenesis or RNA silencing have been unsuccessful (our unpublished data), suggesting that some miRNA biosynthetic capacity is required for *Arabidopsis* viability.

Why do miRNAs accumulate to elevated levels in the presence of P1/HC-Pro? If the suppressor inhibits assembly or activity of RISC complexes and miRNA turnover is coupled to RISC activity, then miRNAs may build

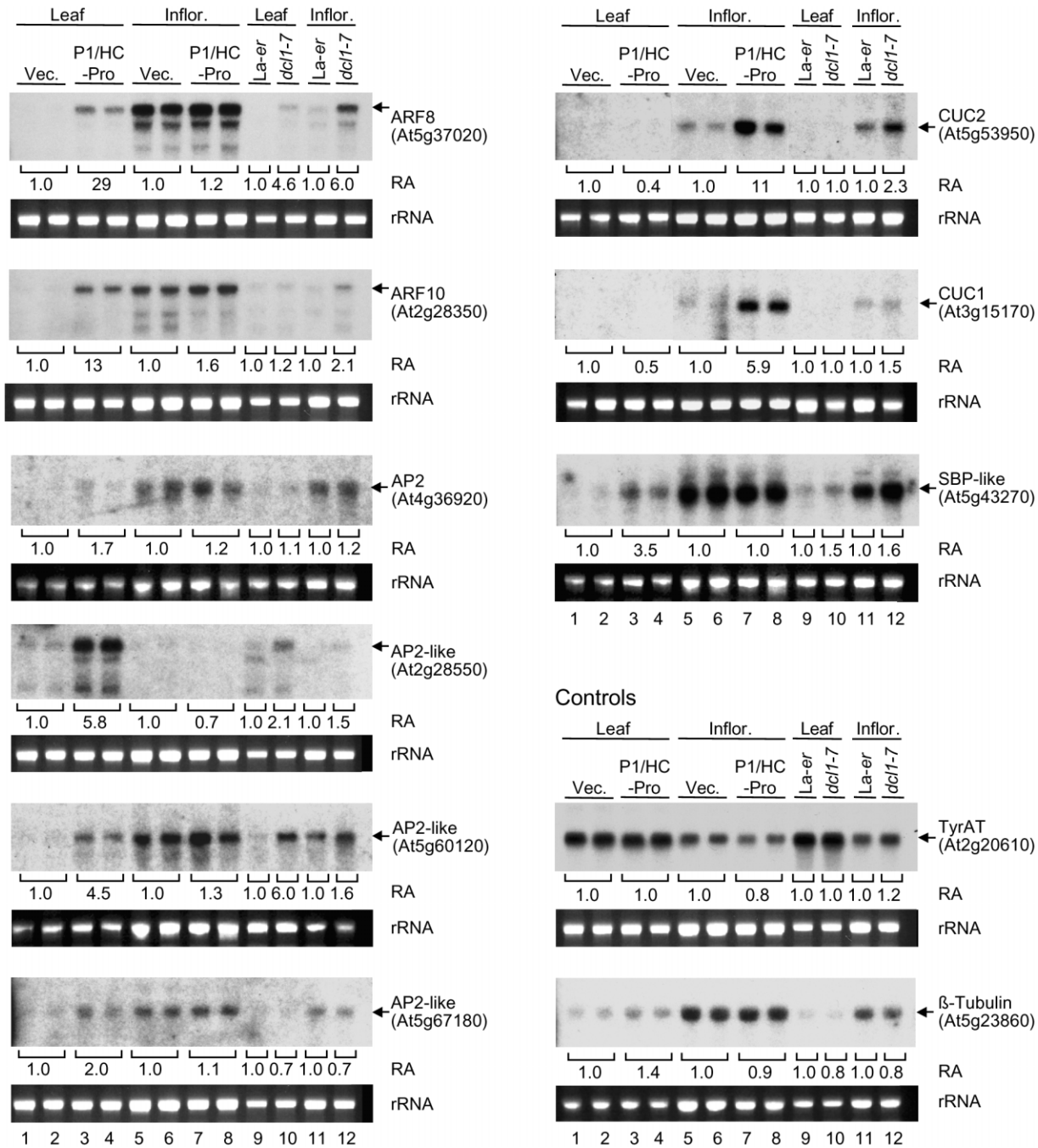


Figure 7. Analysis of Target mRNAs in P1/HC-Pro-Expressing and *dcl1* Mutant Plants

Pooled samples from leaf and inflorescence (Inflor.) tissue of vector transgenic (lanes 1, 2, 5, and 6) and P1/HC-Pro transgenic (lanes 3, 4, 7, and 8) plants (Col-0) and *La-er* (lanes 9 and 11) and *dcl1-7* (lanes 10 and 12) plants were analyzed by blot assay with probes for nine miRNA target genes and two control genes. The arrow in each panel indicates the full-length mRNA. The mean relative accumulation (RA) level for each set of samples is indicated (levels in tissue from vector-transformed Col-0 and *La-er* control plants were designated as 1.0). Ethidium bromide-stained rRNA for each blot is shown. Three of the blots were each analyzed sequentially with two probes (including a membrane stripping procedure); in each of these cases, the same ethidium bromide-stained gel is shown (ARF8 and AP2-like [At2g28550], ARF10 and TyrAT, and AP2 and SBP-like).

up as stable intermediates to relatively high levels. Alternatively, P1/HC-Pro may stimulate, either directly or indirectly, a limiting factor required for miRNA biosynthesis. For example, if miRNA formation requires a factor

that is itself encoded by an miRNA-targeted mRNA, suppression of RISC activity by P1/HC-Pro may increase expression of the limiting factor and stimulate production of miRNA. In other words, P1/HC-Pro may impede

a negative-feedback loop that normally limits expression of miRNAs.

Conclusions

These results lead to several conclusions and propositions about the basis for virus-induced disease in plants. We propose that many, or most, of the developmental defects triggered by TuMV in *Arabidopsis* are due to interference with pathways that depend on negative regulation by miRNAs and that P1/HC-Pro is the virus-encoded factor that mediates this interference. The suppression of miRNA-directed function and RNA silencing by P1/HC-Pro is likely due to interference with a common reaction, probably involving assembly or activity of RISC-like complexes. The consequence of virus infection is ectopic expression of some mRNAs that are normally negatively regulated by miRNA-guided cleavage. Infected plants, therefore, display a range of developmental abnormalities because the aberrantly expressed target mRNAs encode proteins belonging to families that control meristem identity (NAC domain and SBP-like proteins), organ identity and separation (AP2 domain and NAC domain proteins), radial patterning (SCL-like proteins), and hormone signaling (ARF proteins). Interference with leaf and flower formation and developmental timing, ectopic induction of cell division in nonmeristematic tissues, and disruption of hormone production, signaling, and response are some of the well-characterized effects of different viruses in certain susceptible host plants (Hull, 2001). Given that many of the miRNA target genes are expressed or repressed in specific cell-types in meristematic and organ primordium zones, we further propose that viruses triggering the most severe developmental defects are those that (1) invade meristematic and dividing cells and (2) encode potent RNA silencing suppressors. Indeed, although many viruses are known to be excluded from meristematic zones, *in situ* analysis revealed that meristems and organ primordia are effectively invaded by TuMV in *Arabidopsis* (data not shown).

Experimental Procedures

Virus, Genes, and Constructs

Turnip mosaic virus was derived from an infectious clone (Lellis et al., 2002). All plant expression vectors were constructed with the base vector pRTL2 (Restrepo et al., 1990). The expression cassette from each pRTL2-based clone was transferred to the plant transformation vector pSLJ75515 (Jones et al., 1992), and the resulting constructs were introduced into *A. tumefaciens* strains GV3101 and GV2260. The 35S:P1/HC-Pro and 35S:TEV P1/HC-Pro constructs contained the coding sequence for the P1/HC-Pro polyprotein from TuMV and TEV, respectively (Kasschau and Carrington, 2001). The 35S:TEV P1/HC-Pro/AS9 construct was similar to 35S:TEV P1/HC-Pro, except that it contained alanine-scanning mutations affecting HC-Pro codons 240–242 and was defective in silencing suppressor activity (Kasschau and Carrington, 2001). The 35S:SCL6-IV construct contained the coding sequence of SCL6-IV (locus At4g00150) (Llave et al., 2002b). The 35S:I_{GR}-miR171 construct, also known as 35S:I_{GR}-mi39 (Llave et al., 2002b), contained the 1841 bp sequence annotated as the intergenic region following *Arabidopsis* locus At3g51370. The construct contained the miR171 precursor gene in the sense orientation relative to the 35S promoter and yielded a synthetic miR171 precursor transcript that was processed accurately in *Agro* injection assays (Llave et al., 2002b). The 35S:AS-I_{GR}-miR171 construct (Llave et al., 2002b) contained the 1841 bp

I_{GR} in the antisense orientation relative to the 35S promoter. Plasmids containing sequences corresponding to the 3' proximal regions of the SCL6-III (At3g60630) and SCL6-IV genes were used as hybridization probes.

Transgenic and Mutant Plants

Transgenic *Arabidopsis* plants were produced by the vacuum infiltration method (Clough and Bent, 1998) with the 35S:P1/HC-Pro and empty vector (pSLJ75515) constructs. Seed from primary transformants was grown under selection in soil in a standard greenhouse. *Arabidopsis* plants (La-er background) with *dcl1-7* and *dcl1-9* mutant alleles were obtained from the *Arabidopsis* Biological Resource Center (stock numbers CS3089 and CS3828, respectively).

Protein and RNA Blot Analysis

Protein extracts were prepared and analyzed by immunoblot assay after SDS-PAGE with anti-TEV HC-Pro serum (Kasschau and Carrington, 2001). Total RNA was extracted from leaves or inflorescence tissue (containing stage 1–10 flowers) of TuMV-infected, transgenic, or mutant plants with Trizol reagent (Johansen and Carrington, 2001). High- and low-molecular weight RNA was isolated with the RNeasy Plant Mini Kit and the RNA/DNA Midi Kit (Qiagen), respectively. Blot hybridization of normalized high-molecular weight (10 μg/lane, except where noted) and low-molecular weight (5 μg/lane) RNA was done as described (Llave et al., 2002b). Ethidium bromide staining of gels prior to and after blot transfer was used to detect ribosomal RNA. Hybridization intensity was measured by phosphorimage analysis.

Radiolabeled probes from cloned sequences were made by random priming reactions with [³²P]dATP (Feinberg and Vogelstein, 1983). Oligonucleotides complementary to miR171 (also known as miRNA39), miR167, miR160, miR172, miR164, and miR156 were end-labeled and used as probes to detect their respective miRNAs. Probes to detect miRNA target or control mRNAs were designed from exons greater than 200 nt that had little or no sequence similarity to other *Arabidopsis* genes as revealed by BLAST. Gene-specific sequences were amplified by PCR from genomic DNA (Col-0) and cloned in pGEM-T Easy (Promega). Plasmids were sequenced to confirm the identity of each insert. The cloned probes contained the following sequences, with nucleotide numbers starting at the beginning of the known or predicted open reading frame: ARF8 (At5g37020), nt 1164–1880; ARF10 (At2g28350), nt 1089–1878; ARF17 (At1g77850), nt 1069–1753; AP2 (At4g36920), nt 1077–1296; AP2-like (At2g28550), nt 4–510; AP2-like (At5g60120), nt 1086–1368; AP2-like (At5g67180), nt 1–290; CUC2 (At5g53950), nt 1–1198; CUC1 (At3g15170), nt 568–933; SBP-like (At5g43270), nt 161–620; TyrAT (At2g20610), nt 892–1366; β-tubulin (At5g23860), nt 1–417.

5' RACE

A modified procedure for RNA ligase-mediated rapid amplification of cDNA ends (5' RACE) was followed with the GeneRacer Kit (Invitrogen, CA) as described previously (Llave et al., 2002b). Total RNA was isolated from 4-week-old flowering *Arabidopsis* (Col-0) plants. poly(A)⁺ mRNA was prepared by two rounds of purification with an Oligotex mRNA Midi Kit (Qiagen, CA) and directly ligated to the GeneRacer RNA Oligo adaptor without further modification. The GeneRacer Oligo dT primer was used to prime cDNA synthesis with reverse transcriptase. This cDNA was subjected to an amplification procedure with the GeneRacer 5' Primer (5'-CGACTGGAGCAC GAGGACACTGA-3') and the GeneRacer 3' Primer (5'-GCTGTCAAC GATACGCTACGTAACG-3') to generate a pool of non-gene-specific 5' RACE products. The conditions used for this amplification step were the same as those for gene-specific RACE recommended by the manufacturer, with the exception that an extension time of 2.5 min was used. Gene-specific 5' RACE reactions were done with the GeneRacer 5' Nested Primer and gene-specific primers as follows: ARF8 (At5g37020)-2608R (5'CGCTAAAGTTTCAGGACCAT ACT3'), ARF10 (At2g28350)-1675R (5'CCGCTCCGCCTCTTCTTCC AAAA3'), ARF17 (At1g77850)-1750R (5'GGGAGCTAGAACCTGCGT TGCTGTT3'), AP2 (At4g36920)-1441R (5'CGCCTAAGTTAACAA GAGGAT3'), AP2-like (At2g28550)-1412R (5'GGGACTAGAGTGT GAGAGAGAGA3'), AP2-like (At5g60120)-1471R (5'GCGGAGGAAG

GAGGAAGCTAT3'), AP2-like (At5g67180)-1163R (5'GAGCCC AAAATAGCTCGAGAAT3'), CUC2 (At5g53950)-1032R (5'GACGCC TCCGCCGCTTGGAAAGAA3'), CUC1 (At3g15170)-856R (5'CCGT GGGAGGCAGAGAAGTAGATT3'), and SPB-like (At5g43270)-1198R (5'GCAGAGCGGTCGGGCTTCAA3'). The 5' RACE products were gel purified, cloned, and sequenced.

Light and SEM Microscopy

Developing flowers from noninfected, TuMV-infected (14 days post-inoculation), vector transgenic, and P1/HC-Pro transgenic plants were fixed overnight in 2% glutaraldehyde/2% paraformaldehyde/0.05 M PIPES (pH 7.2). Samples for light microscopy were washed three times in 0.05 M PIPES (pH 7.2) for 10 min each, treated with 1% osmium tetroxide overnight, and rinsed in 0.05 M PIPES (pH 7.2) twice for 10 min each. Samples were dehydrated through a series of acetone treatments (30%, 50%, 70%, 95%, and 100%) for 10 min at each step. The 100% acetone treatment was repeated two more times. Dehydrated samples were embedded in SPURRS resin. Sections were cut (100 μ m) and stained with 1% toluidine blue in 1% sodium borate. Samples for SEM were fixed and dehydrated as described above, but without osmium tetroxide treatment. In some cases, samples were dissected with tiny glass shards affixed to sewing needles. Dehydrated samples were put in the critical point dryer, mounted on SEM stubs, and gold coated. Samples were viewed with an acceleration voltage of 15 kV.

Agro Injection Assays in *N. benthamiana*

Details of miR171 production from a synthetic precursor gene (35S:*IGR-miR171*) and cleavage of *SCL6-IV* mRNA (transcribed from 35S:*SCL6-IV*) in *N. benthamiana* leaves after Agro injection were described elsewhere (Llave et al., 2002b). Cultures were injected in combinations at the following concentrations: 35S:*IGR-miR171* or 35S:*AS-IGR-miR171*, OD₆₀₀ = 0.75; 35S:*SCL6-IV*, OD₆₀₀ = 0.1; 35S:*TEV P1/HC-Pro* or 35S:*TEV P1/HC-Pro/AS9*, OD₆₀₀ = 0.15; empty vector, OD₆₀₀ = variable. The concentration of bacteria in all experiments was normalized to OD₆₀₀ = 1.0 by varying the concentration of cells containing empty vector. Infiltration zones were harvested 3 days postinjection for RNA analysis.

Acknowledgments

We thank Valerie Lynch-Holm and the Washington State University Electron Microscopy Center for SEM analysis, Brenda Shaffer for assistance with data analysis, and Adam Gustafson for the small RNA database. This work was supported by grants from the National Science Foundation (MCB-0209836), National Institutes of Health (AI43288), and the U.S. Department of Agriculture (NRI 2002-35319-11560). C.L. received a fellowship from the Ministerio de Educación y Cultura (Spain).

Received: December 2, 2002

Revised: January 17, 2003

References

Aida, M., Ishida, T., Fukaki, H., Fujisawa, H., and Tasaka, M. (1997). Genes involved in organ separation in *Arabidopsis*: an analysis of the cup-shaped cotyledon mutant. *Plant Cell* 9, 841–857.

Ambros, V. (2001). microRNAs: tiny regulators with great potential. *Cell* 107, 823–826.

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Smith, T.H., and Vance, V.B. (1998). A viral suppressor of gene silencing in plants. *Proc. Natl. Acad. Sci. USA* 95, 13079–13084.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366.

Brigneti, G., Voinnet, O., Wan-Xiang, L., Ding, S.W., and Baulcombe, D.C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing. *EMBO J.* 17, 6739–6746.

Cardon, G., Hohmann, S., Klein, J., Nettesheim, K., Saedler, H., and Huijser, P. (1999). Molecular characterization of the *Arabidopsis* SBP-box genes. *Gene* 237, 91–104.

Carrington, J.C., Freed, D.D., and Oh, C.-S. (1990). Expression of potyviral polyproteins in transgenic plants reveals three proteolytic activities required for complete processing. *EMBO J.* 9, 1347–1353.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D.C. (2000). An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101, 543–553.

DiLaurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M.G., Feldmann, K.A., and Benfey, P.N. (1996). The *SCARECROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsis* root. *Cell* 86, 423–433.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15, 188–200.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 257, 8569–8572.

Golden, T.A., Schauer, S.E., Lang, J.D., Pien, S., Mushegian, A.R., Grossniklaus, U., Meinke, D.W., and Ray, A. (2002). Short integuments1/suspensor1/carpel factory, a Dicer homolog, is a maternal effect gene required for embryo development in *Arabidopsis*. *Plant Physiol.* 130, 808–822.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Bailly, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* 106, 23–34.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small anti-sense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950–952.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404, 293–296.

Hannon, G.J. (2002). RNA interference. *Nature* 418, 244–251.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M.T., and Benfey, P.N. (2000). The *SHORT-ROOT* gene controls radial patterning of the *Arabidopsis* root through radial signaling. *Cell* 101, 555–567.

Hull, R. (2001). *Matthews' Plant Virology*, Fourth Edition (San Diego: Academic Press).

Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Bálint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293, 834–838.

Hutvagner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* 297, 2056–2059.

Jacobsen, S.E., Running, M.P., and Meyerowitz, E.M. (1999). Disruption of an RNA helicase/RNase III gene in *Arabidopsis* causes unregulated cell division in floral meristems. *Development* 126, 5231–5243.

Johansen, L.K., and Carrington, J.C. (2001). Silencing on the spot: induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiol.* 126, 930–938.

Jones, J.D., Shlumukov, L., Carland, F., English, J., Scofield, S.R., Bishop, G.J., and Harrison, K. (1992). Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. *Transgenic Res.* 1, 285–297.

Kasschau, K.D., and Carrington, J.C. (1998). A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell* 95, 461–470.

Kasschau, K.D., and Carrington, J.C. (2001). Long-distance movement and replication maintenance functions correlate with silencing suppression activity of potyviral HC-Pro. *Virology* 285, 71–81.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. (2001). Dicer functions in RNA interference and

- in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* **15**, 2654–2659.
- Lang, J.D., Ray, S., and Ray, A. (1994). *sin1*, a mutation affecting female fertility in *Arabidopsis*, interacts with *mod1*, its recessive modifier. *Genetics* **137**, 1101–1110.
- Lellis, A.D., Kasschau, K.D., Whitham, S.A., and Carrington, J.C. (2002). Loss-of-susceptibility mutants of *Arabidopsis thaliana* reveal an essential role for eIF(iso)4E during potyvirus infection. *Curr. Biol.* **12**, 1046–1051.
- Liscum, E., and Reed, J.W. (2002). Genetics of AUX/IAA and ARF action in plant growth and development. *Plant Mol. Biol.* **49**, 387–400.
- Liu, H., Reavy, B., Swanson, M., and MacFarlane, S.A. (2002). Functional replacement of the tobacco rattle virus cysteine-rich protein by pathogenicity proteins from unrelated plant viruses. *Virology* **298**, 232–239.
- Llave, C., Kasschau, K.D., Rector, M.A., and Carrington, J.C. (2002a). Endogenous and silencing-associated small RNAs in plants. *Plant Cell* **14**, 1605–1619.
- Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. (2002b). Cleavage of *Scarecrow-like* mRNA targets is directed by a class of *Arabidopsis* miRNA. *Science* **297**, 2053–2056.
- Mallory, A.C., Reinhart, B.J., Bartel, D., Vance, V.B., and Bowman, L.H. (2002). A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. *Proc. Natl. Acad. Sci. USA* **99**, 15228–15233.
- Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., et al. (2000). *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**, 533–542.
- Olsen, P.H., and Ambros, V. (1999). The *lin-4* regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216**, 671–680.
- Park, W., Li, J., Song, R., Messing, J., and Chen, X. (2002). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr. Biol.* **12**, 1484–1495.
- Pasquinelli, A.E., and Ruvkun, G. (2002). Control of developmental timing by microRNAs and their targets. *Annu. Rev. Cell Dev. Biol.* **18**, 495–513.
- Qiu, W., Park, J.W., and Scholthof, H.B. (2002). Tombusvirus P19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. *Mol. Plant Microbe Interact.* **15**, 269–280.
- Ray, A., Lang, J.D., Golden, T., and Ray, S. (1996a). *SHORT INTEGUMENTS1 (SIN1)*, a gene required for ovule development in *Arabidopsis*, also controls flowering time. *Development* **122**, 2631–2638.
- Ray, S., Golden, T., and Ray, A. (1996b). Maternal effects of the short integuments1 mutation on embryo development. *Dev. Biol.* **180**, 365–369.
- Reichmann, J.L., and Meyerowitz, E.M. (1998). The AP2/EREBP family of plant transcription factors. *Biol. Chem.* **379**, 633–646.
- Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. (2002). MicroRNAs in plants. *Genes Dev.* **16**, 1616–1626.
- Restrepo, M.A., Freed, D.D., and Carrington, J.C. (1990). Nuclear transport of plant potyviral proteins. *Plant Cell* **2**, 987–998.
- Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. (2002). Prediction of plant microRNA targets. *Cell* **110**, 513–520.
- Robinson-Beers, K., Pruitt, R.E., and Gasser, C.S. (1992). Ovule development in wild-type *Arabidopsis* and two female sterile mutants. *Plant Cell* **4**, 1237–1250.
- Schauer, S.E., Jacobsen, S.E., Meinke, D.W., and Ray, A. (2002). *DICER-LIKE1*: blind men and elephants in *Arabidopsis* development. *Trends Plant Sci.* **7**, 487–491.
- Schwartz, B.W., Yeung, E.C., and Meinke, D.W. (1994). Disruption of morphogenesis and transformation of the suspensor in abnormal *suspensor* mutants of *Arabidopsis*. *Dev. Biol.* **120**, 3235–3245.
- Silverstone, A.L., Ciampaglio, C.N., and Sun, T. (1998). The *Arabidopsis* RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *Plant Cell* **10**, 155–169.
- Tang, G., Reinhart, B.J., Bartel, D.P., and Zamore, P.D. (2003). A biochemical framework for RNA silencing in plants. *Genes Dev.* **17**, 49–63.
- Vance, V., and Vaucheret, H. (2001). RNA silencing in plants—defense and counterdefense. *Science* **292**, 2277–2280.
- Voinnet, O., Lederer, C., and Baulcombe, D.C. (2000). A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**, 157–167.
- Voinnet, O., Pinto, V.M., and Baulcombe, D.C. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. USA* **96**, 14147–14152.
- Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862.
- Yelina, N.E., Savenkov, E.I., Solov'yev, A.G., Morozov, S.Y., and Valkonen, J.P. (2002). Long-distance movement, virulence, and RNA silencing suppression controlled by a single protein in Hordei- and Potyviruses: Complementary functions between virus families. *J. Virol.* **76**, 12981–12991.
- Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33.