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Ectopic *DICER-LIKE1* Expression in P1/HC-Pro *Arabidopsis* Rescues Phenotypic Anomalies but Not Defects in MicroRNA and Silencing Pathways

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Expression of the viral silencing suppressor P1/HC-Pro in plants causes severe developmental anomalies accompanied by defects in both short interfering RNA (siRNA) and microRNA (miRNA) pathways. P1/HC-Pro transgenic lines fail to accumulate the siRNAs that mediate RNA silencing and are impaired in both miRNA processing and function, accumulating abnormally high levels of miRNA/miRNA* processing intermediates as well as miRNA target messages. Both miRNA and RNA silencing pathways require participation of *DICER-LIKE* (*DCL*) ribonuclease III-like enzymes. Here, we investigate the effects of overexpressing *DCL1*, one of four Dicers in *Arabidopsis thaliana*, on P1/HC-Pro–induced defects in development and small RNA metabolism. Expression of a *DCL1* cDNA transgene (*35S:DCL1*) produced a mild gain-of-function phenotype and largely rescued *dcl1* mutant phenotypes. The *35S:DCL1* plants were competent for virus-induced RNA silencing but were impaired in transgene-induced RNA silencing and in the accumulation of some miRNAs. Ectopic *DCL1* largely alleviated developmental anomalies in P1/HC-Pro plants but did not correct the P1/HC-Pro–associated defects in small RNA pathways. The ability of P1/HC-Pro plants to suppress RNA silencing and the levels of miRNAs, miRNA*s, and miRNA target messages in these plants were essentially unaffected by ectopic *DCL1*. These data suggest that P1/HC-Pro defects in development do not result from general impairments in small RNA pathways and raise the possibility that *DCL1* participates in processes in addition to miRNA biogenesis.

INTRODUCTION

Small regulatory RNAs play crucial roles in a variety of genetic regulatory processes in a broad range of eukaryotes, including plants (recently reviewed in Hunter and Poethig, 2003; Baulcombe, 2004; Dugas and Bartel, 2004; Mallory and Vaucheret, 2004). One class of small regulatory RNAs, the short interfering RNAs (siRNAs), are produced in the RNA silencing pathway and serve as a defense against invading nucleic acids, such as viruses, transposons, and transgenes (Vance and Vaucheret, 2001). Such invaders trigger silencing by producing long fully double-stranded RNAs (dsRNA) that are cleaved into siRNAs by a ribonuclease III-like enzyme called Dicer. The siRNAs then act as guides in an endonuclease complex called RNA-induced silencing complex (RISC) that destroys any RNA having high homology to the dsRNA trigger. Consistent with the idea that RNA silencing is an antiviral mechanism, many plant viruses encode proteins that interfere with the process at one or more steps (reviewed in Roth et al., 2004; Silhavy and Burgyan, 2004).

Another class of small regulatory RNAs, the microRNAs (miRNAs), is produced from endogenous cellular genes and has been implicated as key developmental regulators. They arise from nonprotein encoding precursor RNAs at one locus and act in trans to negatively regulate the expression of a target mRNA from another locus. Similar to siRNAs, miRNAs are produced by the activity of a Dicer and then are incorporated into a RISC-like complex to act as guides to direct the complex to specific mRNA targets (reviewed in Ambros, 2004; Bartel, 2004; Dugas and Bartel, 2004; Mallory and Vaucheret, 2004). In contrast with the majority of animal miRNAs, most plant miRNAs direct the cleavage of target mRNAs (Llave et al., 2002; Kasschau et al., 2003; Palatnik et al., 2003; Xie et al., 2003), though at least some act primarily by inhibiting translation of the target mRNA (Aukerman and Sakai, 2003; Chen, 2004). Interestingly, miRNA-directed pathways in plants share a number of common biochemical features and genetic requirements with those directed by siRNAs. The integration of these two pathways can be seen in the biogenesis of a third class of small RNAs termed *trans*-acting siRNAs. These small RNAs arise after miRNA-directed cleavage

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of a precursor RNA that is subsequently copied into dsRNA by the activity of an RNA-dependent RNA polymerase that is also required for production of the siRNAs that mediate sense transgene–induced RNA silencing (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005; Williams et al., 2005). Perhaps reflecting the shared features of the small RNA pathways, many of the plant viral proteins that suppress RNA silencing also affect miRNA biogenesis and/or function and alter plant development (Mallory et al., 2002b; Kasschau et al., 2003; Chapman et al., 2004; Dunoyer et al., 2004; Roth et al., 2004; Silhavy and Burgyan, 2004).

The Dicers that mediate small RNA biogenesis are complex multifunctional proteins that contain an RNA helicase domain and usually a PAZ RNA binding domain in addition to the ribonuclease and dsRNA binding domains found in canonical RNase IIIs (Figure 3A; Schauer et al., 2002; Song et al., 2003). In addition to cleaving substrates to produce small RNAs, Dicers play a role in early steps in miRNA precursor processing (Kurihara and Watanabe, 2004) and are required for the assembly of small RNAs into the RISC complex (Lee et al., 2004; Pham et al., 2004). Many animals have a single Dicer that mediates all the required processing events for both siRNAs and miRNAs (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001), whereas *Drosophila melanogaster* has two Dicers that are functionally diverged, one required primarily for siRNA production and the other primarily for miRNA production (Lee et al., 2004; Pham et al., 2004; Tijsterman and Plasterk, 2004). The two Dicers in the fungi *Neurospora crassa* are redundant for transgene-induced RNA silencing (Catalanotto et al., 2004), whereas only one of the two Dicers in the fungus *Magnaporthe oryzae* is required for this process (Kadotani et al., 2004).

The *Arabidopsis thaliana* genome contains four putative *DICER-LIKE* genes (*DCL1-DCL4*; Schauer et al., 2002), and the exact roles of these enzymes are still being determined. Their function in small RNA biogenesis has been addressed via mutant analysis, using the weak loss-of-function *dcl1-7* and *dcl1-9* alleles and the null *dcl2-1*, *dcl3-1*, and *dcl4-1* insertion alleles (*dcl1* null alleles are embryo lethal) (Lang et al., 1994; Schwartz et al., 1994; Ray et al., 1996; Jacobsen et al., 1999; Golden et al., 2002; Xie et al., 2004, 2005; Gasciolli et al., 2005; Yoshikawa et al., 2005). The weak *dcl1* alleles are defective in at least two steps of miRNA precursor processing (Kurihara and Watanabe, 2004), accumulate reduced levels of most mature miRNAs (Park et al., 2002; Reinhart et al., 2002), and have severe developmental defects. By contrast, *dcl2-1*, *dcl3-1*, and *dcl4-1* null alleles have no reported defects in miRNA accumulation and exhibit mild or no developmental anomalies (Xie et al., 2004; Gasciolli et al., 2005). The roles of the other *Arabidopsis DCL* genes in RNA silencing are less well understood. The *dcl2-1* null allele causes a transient decrease in siRNA accumulation in response to infection with *Turnip crinkle virus* but not to either *Turnip mosaic virus* (TuMV) or *Cucumber mosaic virus* (Xie et al., 2004), suggesting that it plays a role in at least some types of virus-induced RNA silencing. *DCL3* has been implicated in production of a class of larger small RNAs that mediate chromatin modifications associated with transcriptional gene silencing (Chan et al., 2004; Xie et al., 2004). *DCL4* is required for the accumulation of *trans*-acting siRNAs (Gasciolli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005). However, the Dicer(s) that produces the siRNAs that direct transgene-induced RNA silencing has not yet been identified. While *dcl1-9* mutant plants were shown to be competent for silencing induced by a hairpin transgene (Finnegan et al., 2003), this finding does not eliminate a role for *DCL1* in transgene-induced RNA silencing since a null allele could not be used. The other *DCL* genes have not yet been tested to determine if they play a role in transgene-induced RNA silencing.

P1/HC-Pro (for proteinase1/helper component-proteinase) is a plant viral protein that blocks both virus-induced RNA silencing and transgene-induced RNA silencing (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998; Llave et al., 2000; Mallory et al., 2001; Dunoyer et al., 2004) but not transcriptional gene silencing (Marathe et al., 2000; Mette et al., 2001). In tobacco (*Nicotiana tabacum*), suppression of sense transgene–induced silencing by HC-Pro completely eliminates detectable levels of 21- to 24-nucleotide siRNAs (Mallory et al., 2001). The suppression of inverted repeat transgene silencing by HC-Pro also blocks the accumulation of the 21- to 24-nucleotide siRNA but allows accumulation of longer 25- 26-nucleotide small RNAs as well as long dsRNAs (Mallory et al., 2002b). Similarly, long dsRNA accumulates in transgenic *Arabidopsis* lines suppressed by HC-Pro for hairpin transgene-induced silencing (Dunoyer et al., 2004). The reduction in short siRNAs, which are the products of Dicer, accompanied by the simultaneous accumulation of long dsRNA, which is the substrate of Dicer, suggests that HC-Pro alters the activity of the enzyme that produces siRNAs from long dsRNA substrates.

Expression of P1/HC-Pro also impairs miRNA biogenesis and function. In contrast with siRNAs, miRNA levels are enhanced in P1/HC-Pro transgenic lines (Mallory et al., 2002b; Kasschau et al., 2003; Chapman et al., 2004; Dunoyer et al., 2004). Paradoxically, some miRNA target messages are also increased, suggesting that these miRNAs are not functional (Kasschau et al., 2003; Dunoyer et al., 2004). P1/HC-Pro lines also accumulate miRNA* species, small RNAs largely complementary to the mature miRNA sequence that are thought to arise as a labile intermediate in the processing of the miRNA precursor (Chapman et al., 2004; Dunoyer et al., 2004). These results suggest that P1/HC-Pro impairs strand separation of an intermediate miRNA/miRNA* duplex and/or the subsequent transfer of the miRNA strand into the RISC complex, thereby inhibiting miRNA-directed target cleavage. Because assembly of miRNA into a functional RISC requires Dicer activity in at least some organisms (Lee et al., 2004; Pham et al., 2004), these observations are consistent with the hypothesis that P1/HC-Pro interferes with miRNA-directed mRNA degradation at a Dicer-mediated step.

The developmental phenotypes of P1/HC-Pro plants have been ascribed to the inhibition of miRNA-directed target degradation (Kasschau et al., 2003; Xie et al., 2003; Chapman et al., 2004; Dunoyer et al., 2004). Supporting this view is the observation that mutations in genes involved in miRNA biogenesis (such as *DCL1*, *HUA ENHANCER1*, *HYPONASTIC LEAVES1*, and *ARGONAUTE1*) partially phenocopy P1/HC-Pro lines (Ray et al., 1996; Bohmert et al., 1998; Jacobsen et al., 1999; Lu and Fedoroff, 2000; Chen et al., 2002; Golden et al., 2002). However, because P1/HC-Pro transgenic *Arabidopsis* lines are additionally impaired in RNA silencing (Dunoyer et al., 2004; this article), it remains possible that RNA silencing also plays an important, yet unappreciated, role in

the regulation of developmental pathways. Alternatively, some of the effects of P1/HC-Pro on development may reflect an influence on factors that function independently of small RNA metabolism.

Here, we test the hypothesis that P1/HC-Pro interferes with small RNA metabolism and normal development by altering the activity of one or more Dicers. Ectopic overexpression of *DCL1* would be expected to overcome P1/HC-Pro–mediated defects that arise due to interference with DCL1 function and perhaps to compensate for P1/HC-Pro interference with the other Dicers as well. Indeed, we find that overexpression of *DCL1* is sufficient to relieve the developmental anomalies in P1/HC-Pro plants but surprisingly does not relieve the impairments in the biogenesis of small RNAs or those in small RNA-directed RNA degradation pathways in those plants. These results suggest that developmental defects in the P1/HC-Pro plants arise due to aberrant Dicer activity and raise the possibility that Dicers play an important role in development that is independent of target RNA degradation via small RNA pathways.

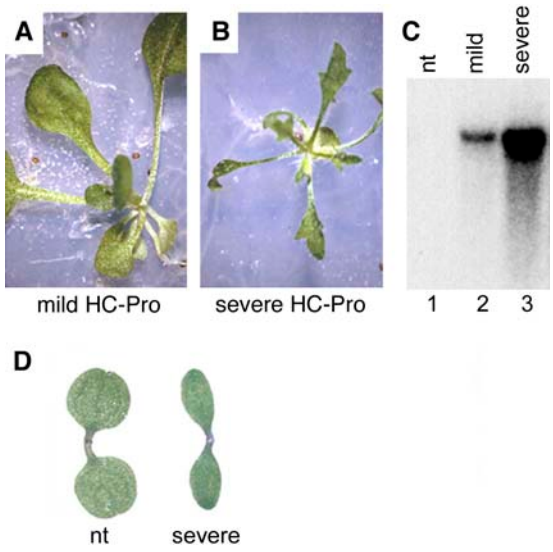

Figure 1. The Severity of P1/HC-Pro Phenotype in Primary Transformants Correlates with P1/HC-Pro mRNA Accumulation.

(A) The mild phenotype exhibited by a subset of P1/HC-Pro primary transformants in *Arabidopsis.*

(B) The severe phenotype exhibited by the majority of P1/HC-Pro primary transformants in *Arabidopsis.*

(C) RNA gel blot analysis showing the levels of P1/HC-Pro mRNA in total RNA from a pool of individual primary transformants. The level of P1/HC-Pro mRNA is higher in the pool of plants displaying a severe phenotype as shown in **(B)** (lane 3) than that in the pool of plants displaying a mild phenotype as shown in **(A)** (lane 2). Lane 1 shows the absence of P1/HC-Pro mRNA in vector only–transformed *Arabidopsis* plants.

(D) Severely phenotypic P1/HC-Pro *Arabidopsis* plants have narrow cotyledons as compared with those of nontransformed (nt) seedlings.

RESULTS

Effect of P1/HC-Pro Expression on Phenotype in *Arabidopsis*

Developmental abnormalities have been reported previously in both tobacco and *Arabidopsis* transgenic lines expressing P1/HC-Pro, and these have been attributed to defects in small RNA pathways (Anandalakshmi et al., 2000; Mallory et al., 2002a; Kasschau et al., 2003; Chapman et al., 2004; Dunoyer et al., 2004). To further investigate the nature of the defects caused by P1/HC-Pro, we generated *Arabidopsis* transgenic lines expressing the 5′ proximal region of the TuMV genome encoding the P1 and HC-Pro proteins. Analysis of TuMV P1/HC-Pro primary transformants indicated a range of severity in developmental phenotypes, which are first detectable after germination, with a positive correlation between the degree of phenotypic abnormality and the level of P1/HC-Pro expression (Figure 1). Thus, the primary transformants with a severe phenotype accumulated higher levels of P1/HC-Pro mRNA than transformants with a much weaker phenotype (Figures 1A to 1C). The most severely affected P1/HC-Pro transgenic lines had narrow cotyledons and thus could be distinguished from the wild type at germination (Figure 1D).

The *Arabidopsis* P1/HC-Pro line chosen for further experiments had severe developmental abnormalities in leaves and flowers similar to those previously reported by others (Kasschau et al., 2003; Dunoyer et al., 2004). Additional defects in branching pattern and vasculature were also noted in the P1/HC-Pro transgenic plants (Figures 2A to 2F). Reduced fertility was also noted in the P1/HC-Pro transgenic line and was associated with defects in both male and female floral organs (Figures 2G to 2J). The P1/HC-Pro transgenic line used in all our experiments was hemizygous for the transgene, as plants homozygous for the transgene were never recovered despite extensive efforts to do so (data not shown).

Ectopic Expression of *DCL1* cDNA

If the P1/HC-Pro–associated defects in development are due to defects in Dicer-mediated small RNA pathways, then overexpression of *DCL1* might suppress some or all aspects of the P1/HC-Pro phenotype. In order to test this hypothesis, we generated plant lines that ectopically overexpress *DCL1* by transformation of *Arabidopsis* with an expression cassette containing the full-length 6.2-kb *DCL1* cDNA under control of the constitutive cauliflower mosaic virus 35S promoter (*35S:DCL1*). Three of the twelve primary transformants that contained a single transgene locus (lines 2, 9, and 12) were found to overexpress the predicted 6.2-kb *DCL1* transcript in both seedling and adult stages (Figure 3B and 3C; data not shown). Interestingly, accumulation of the 6.2 kb *DCL1* mRNA was consistently higher in stems and flowers than in rosette leaves of adult plants in both lines (Figure 3C; data not shown). Two previously identified smaller *DCL1* transcripts were also detected in the *35S:DCL1* plants (Figure 3A): a 4.0-kb RNA containing 5′ *DCL1* sequences and a 2.5-kb RNA containing primarily 3′ *DCL1* sequences as well as variable amounts of intron 14 sequences (Jacobsen et al., 1999; Golden et al., 2002; Xie et al., 2003). It is thought that these smaller transcripts arise due to aberrant splicing of the 14th intron of *DCL1* (Xie et al., 2003). Interestingly, the 2.5-kb transcript was present at wild-type levels, as expected, but the 4.0-kb transcript accumulated in parallel with the 6.2-kb mRNA and was more heterogeneous than

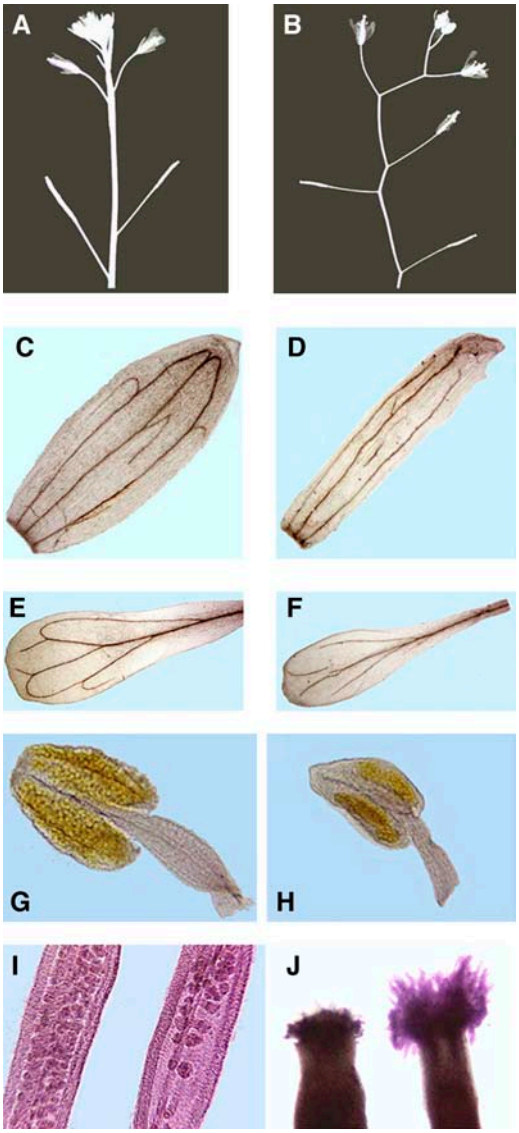

Figure 2. Developmental Phenotypes in P1/HC-Pro Transgenic *Arabidopsis* Plants.

(A) and **(B)** Branching abnormalities associated with the P1/HC-Pro transgene. The typical monopodial branching pattern of wild-type inflorescences **(A)**, unlike the primitive branching pattern in P1/HC-Pro inflorescences **(B)**.

(C) to **(F)** Vascular patterning abnormalities in P1/HC-Pro transgenic *Arabidopsis* flowers. A sepal from a nontransgenic flower **(C)**, as compared with a sepal from a P1/HC-Pro transgenic flower **(D)**, showing irregular edges and reduced interconnections in the vascular system. A petal from a nontransgenic flower **(E)**, as contrasted with a petal from a P1/HC-Pro transgenic flower **(F)**, showing reduced interconnections in the vascular system.

(G) to **(J)** Reduced fertility in P1/HC-Pro transgenic *Arabidopsis* is associated with defects in both male and female floral organs. The microsporangia (in yellow), the sites of pollen grain generation and maturation in the anther, of a nontransgenic stamen **(G)**. The microsporangia in the anther of a P1/HC-Pro transgenic stamen **(H)**, which are generally smaller and bound by more connective tissue in P1/HC-Pro transgenic plants than in nontransgenic plants. A pistil of a P1/HC-Pro transgenic flower (right), showing the reduced number of ovules compared with a nontransgenic pistil (left) **(I)**. The papillae on the pistil of a P1/HC-Pro transgenic flower (right) are longer and less dense than those of nontransgenic plants (left) **(J)**.

in the wild type (Figures 3B and 3C). These results suggest that the 4.0-kb RNA transcript in the *35S*:*DCL1* plant does not arise due to aberrant splicing but is a product of premature termination of the *35S*:*DCL1* transgene or the breakdown of *DCL1* mRNA. Expression of P1/HC-Pro increased the steady state level of the 6.2- and 4.0-kb *DCL1* transcripts by threefold to fivefold in both wild-type and *35S*:*DCL1* plants (Figure 3C).

Although *35S*:*DCL1* lines overexpressed the *DCL1* mRNA, it was possible that the lines would fail to accumulate DCL1 protein because the *DCL1* mRNA is targeted by miR162, which, in theory, could repress translation of the *DCL1* mRNA. To test if expression of the *35S*:*DCL1* transgene produced a protein with DCL1 activity, the *35S*:*DCL1* (line 12) transgene locus was crossed into the *dcl1-8* mutant background. Homozygous *dcl1-8* mutants show the same developmental abnormalities as the two other partial loss-of-function *dcl1* alleles (*dcl1-7* and *dcl1-9*), including delayed flowering, as seen by an increase in the number of rosette leaves at bolting (Table 1), adaxialized (curled under) rosette leaves (Figure 3D, Table 2), female sterility, and severe reductions in miRNA accumulation (Figure 3E) (Lang et al., 1994; Ray et al., 1996; Jacobsen et al., 1999; Golden et al., 2002; Park et al., 2002; Reinhart et al., 2002; Xie et al., 2004). The *dcl1-8* defects in leaf morphology and flowering time were largely complemented in homozygous *dcl1-8* plants carrying the *35S*:*DCL1* transgene (Figure 3D, Table 1); moreover, fertility was partially restored. Furthermore, the accumulation of each of three tested miRNAs was returned to wild-type or near wild-type level in these plants (Figure 3E). The ability of the *35S*:*DCL1* transgene to complement *dcl1-8* mutant phenotypes indicates that the transgenic locus produces functional protein capable of DCL1 activities.

To determine the effect of the *35S*:*DCL1* transgene in the wild-type *DCL1* background, developmental progression was examined in these lines. Ectopic *DCL1* in the wild-type *DCL1* background had only subtle effects on plant development. Plants homozygous for the *35S*:*DCL1* transgene had a slight acceleration of flowering time (Table 1) and an expansion of the abaxial fates of rosette leaves (Figure 3D, Table 2) and were fully fertile. No obvious developmental phenotypes were observed in plants hemizygous for the *35S*:*DCL1* transgene (data not shown). We can eliminate the possibility that the developmental phenotypes are due to transcriptional or RNA silencing of the *DCL1* gene or to feedback repression due to enhanced levels of miR162 because abundant full-length *DCL1* transcripts are detected in the *35S:DCL1* lines (Figures 3B and 3C) and because the *35S*:*DCL1* transgene is able to complement defects in the *dcl1-8* mutant (Figures 3D and 3E). As the observed phenotype of the *35S*:*DCL1* plants is the opposite of the loss-of-function *dcl1* alleles (early versus late flowering; abaxialized versus adaxialized leaves), we can conclude that the *35S*:*DCL1* transgene is functioning equivalently to a true gain-of-function allele of *DCL1*.

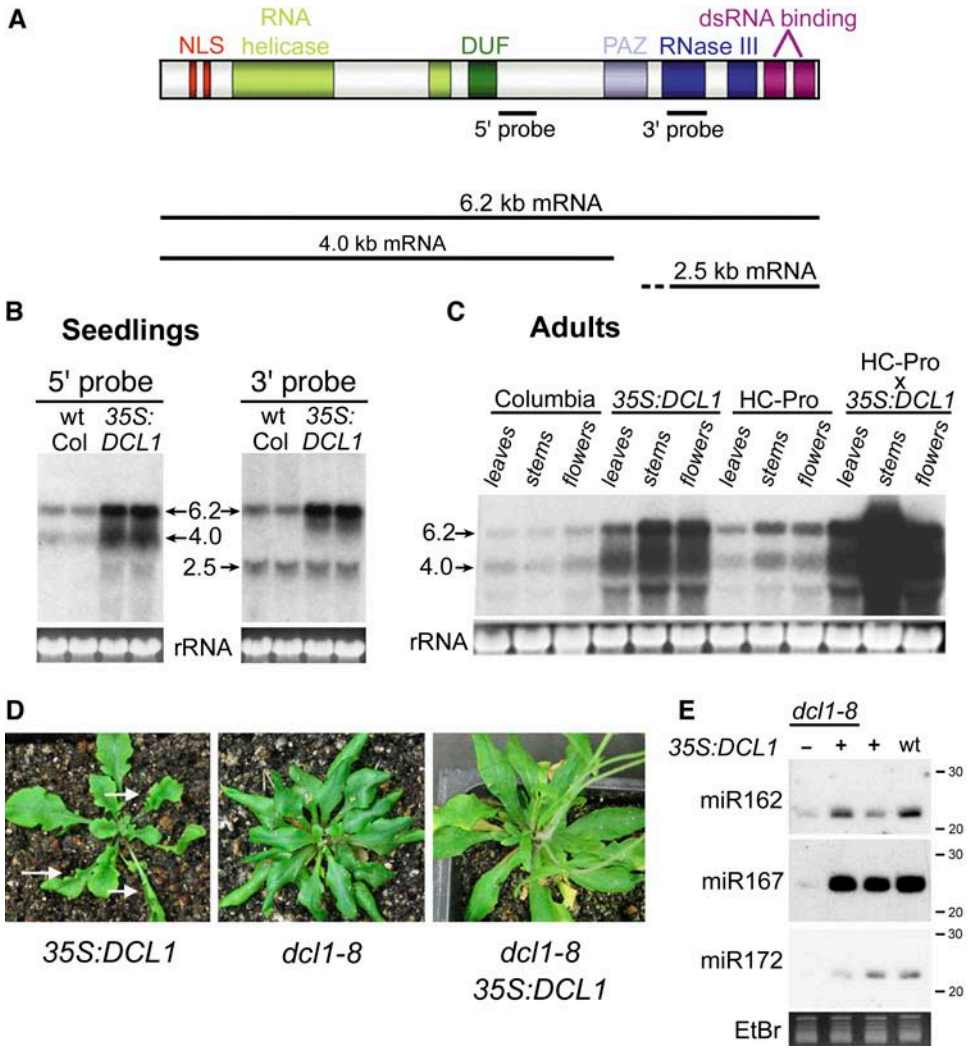

Figure 3. Overexpression of *DCL1* mRNA Largely Complements the *dcl1-8* Mutation.

(A) The predicted domains of the DCL1 protein from Schauer et al. (2002). NLS is the nuclear localization signal, and DUF refers to domain of unknown function 283. The location of 5′ and 3′ probes used in **(B)** and **(C)** and the sequence content of the major *DCL1* transcripts are indicated. The dashed line at the beginning of the 2.5-kb RNA represents the known heterogeneity in the 5′ end of this transcript containing intron 14 sequences.

(B) *35S:DCL1* seedlings accumulate high levels of *DCL1* mRNA. RNA gel blot analysis of RNA isolated from 10-d seedlings of wild-type plants or plants homozygous for *35S:DCL1* transgene (line 12), as indicated, using the 5′ or 3′ hybridization probes indicated in **(A)**. Arrows indicate the location of the 6.2-kb full-length *DCL1* transcript as well as the 4.0- and 2.5-kb smaller *DCL1* transcripts. Ethidium bromide staining of 25S rRNA is shown as a loading control.

(C) Adult *35S:DCL1* plants accumulate high levels of *DCL1* mRNA. RNA gel blot analysis of RNA from rosette leaves, stems, or flowers of adult wild-type plants, *35S:DCL1* transgenic plants, P1/HC-Pro transgenic plants, and P1/HC-Pro × *35S:DCL1* plants, as indicated, using the 5′ hybridization probe indicated in **(A)**. The location of the 6.2-kb full-length and the 4.0-kb *DCL1* transcripts is indicated. Ethidium bromide staining of 25S rRNA is shown as a loading control.

(D) Rosette leaf morphology is affected by altered levels of DCL1 activity. *35S:DCL1 Arabidopsis* plants sometimes show abaxialized (curled up) rosette leaves as shown in the left picture, in contrast with *dcl1-8* mutant rosette leaves, which are adaxialized (curled under) as shown in the middle picture. The *dcl1-8* adaxialized rosette leaf phenotype is largely complemented in plants expressing the *35S:DCL1* transgene (right picture). Arrows indicate adaxialized leaves.

(E) RNA gel blot showing the levels of the indicated miRNAs in rosette leaves of young *dcl1-8* rosette leaves in the absence (–) or presence (+) of the *35S:DCL1* transgene as compared with those in the equivalent tissues of *35S:DCL1* plants or wild-type plants. The location of molecular weight RNA markers of 20 and 30 nucleotides are indicated to the right of the figure. Ethidium bromide (EtBr) staining of the predominant RNA species in the low molecular weight fraction is shown as a loading control.

Table 1. Effects of *35S:DCL1* on Flowering Time and Leaf Number at Flowering

Strain	Transgene	N[a]	Light[b]	Flowering time (Days)[c]	Leaf Number[c]
Co-*gl*[d]	–	68	18L, 6D	25.3 ± 1.8	7.6 ± 0.7
Line 2	*35S:DCL1*	72	18L, 6D	25.7 ± 2.1[e]	6.4 ± 1.0[f]
Line 9	*35S:DCL1*	72	18L, 6D	24.2 ± 1.7[f]	4.8 ± 0.7[f]
Line 12	*35S:DCL1*	72	18L, 6D	23.3 ± 1.8[f]	5.3 ± 0.6[f]
Co[d]	–	19	24L	ND	12.5 ± 1.7[g]
Dcl1$^+$	–	21	24L	ND	12.8 ± 2.3[e,g]
Dcl1$^+$	*35S:DCL1*	31	24L	ND	13.4 ± 1.8[e,g]
dcl1-8	*35S:DCL1*	6	24L	ND	16.3 ± 1.9[f,g]
dcl1-8	–	5	24L	ND	30.2 ± 3.2[f]

[a] Number of plants scored per strain.
[b] 18L, 6D, growth in 18 h light/6 h dark; 24L, growth in constant light.
[c] Mean ± SD; ND, not determined.
[d] Co-*gl*, Columbia *glabrous1*; Co, Columbia.
[e] Not significantly different from wild-type plants using Fisher-Behrens procedure (for means with unequal population variances; Campbell, 1989).
[f] *d* value significant at the 99% level compared with wild-type plants using Fisher-Behrens.
[g] *d* value significant at the 99% level compared with homozygous *dcl1-8* plants using Fisher-Behrens.

35S:DCL1 Suppresses the Developmental Phenotype of P1/HC-Pro Plants

To investigate the possibility that P1/HC-Pro phenotypes are due to altered Dicer activity, we examined the effect of ectopic overexpression of *DCL1* on the morphology of plants expressing P1/HC-Pro in the F1 offspring of a cross between the homozygous *35S:DCL1* and the dominant hemizygous P1/HC-Pro plants. Surprisingly, plants that were hemizygous for both transgenes had morphologically normal fertile flowers, unlike the nearly sterile flowers produced by plants hemizygous for P1/HC-Pro (Figure 4A). Additionally, the rosette leaves of the double hemizygous plants displayed only a modest lobing as compared with the severely serrated leaves of the P1/HC-Pro line (Figure 4A). The weakening of the P1/HC-Pro phenotype by *35S:DCL1* is not due to either transcriptional or RNA silencing of the P1/HC-Pro transgene because the level of P1/HC-Pro mRNA is similar in P1/HC-Pro and P1/HC-Pro × *35S:DCL1* plants (Figure 4B). Thus, ectopic expression of *DCL1* suppresses the majority of the morphological defects observed in P1/HC-Pro plants, supplying further evidence for the model that P1/HC-Pro affects normal plant development by altering the activity of a Dicer.

35S:DCL1 Does Not Alter the Accumulation of miRNA or miRNA*s in P1/HC-Pro Plants

The developmental abnormalities of P1/HC-Pro plants have previously been ascribed to impairments in miRNA biogenesis and function (Kasschau et al., 2003; Chapman et al., 2004). This is because both miRNAs and miRNA* species accumulate to high levels in P1/HC-Pro plants, suggesting that HC-Pro interferes with the assembly of the mature miRNA strand into the RISC complex. If the P1/HC-Pro effects on miRNA biogenesis are mediated through a DCL protein, then ectopic DCL1 might be expected to correct the anomalous accumulation of miRNA* and/or miRNA in the P1/HC-Pro line. We therefore examined the accumulation of four miRNAs and two miRNA*s in leaves, stems, and flowers of *35S:DCL1*, P1/HC-Pro, and P1/HC-Pro × *35S:DCL1* plants. Surprisingly, the accumulation of a subset of miRNAs was impaired in *35S:DCL1* plants in a tissue-specific and developmental manner. The accumulation of two of the four miRNAs was decreased ~10-fold in both stem and flower tissues (Figure 5). In addition, levels of three of the four miRNAs examined were dramatically reduced in the rosette leaves of plants in the late stages of flowering (Figure 5), though little or no reduction was noted in the rosette leaves of plants immediately after bolting (Figure 3E). By contrast, levels of all four miRNAs were increased severalfold in the P1/HC-Pro plants as compared with wild-type plants (Figure 5). Furthermore, the accumulation of miRNA* species, which are nearly undetectable in wild-type and *35S:DCL1* plants, was increased to an even greater extent, consistent with previously reported results (Figure 5; Chapman et al., 2004; Dunoyer et al., 2004). Interestingly, the levels of both miRNAs and miRNA* species in the P1/HC-Pro × *35S:DCL1* plants were almost indistinguishable from those in the P1/HC-Pro plants (Figure 5). Thus, even though the severe developmental anomalies in the P1/HC-Pro line were alleviated in the *35S:DCL1* background, the anomalies in accumulation of miRNAs and miRNA*s were not. These results suggest that the alleviation of P1/HC-Pro phenotype by ectopic *DCL1* is not

Table 2. Effects of the Level of *DCL1* Gene Activity on the Patterning of Lateral Organs[a]

Strain	Phenotypic Class	N[b]	% Fused	% Adaxialized	% Abaxialized
Co-*gl*[c]	Wild type	373	0.0	0.0	0.0
F2 of *dcl1-7*/+	Dcl1$^+$	312	0.0	0.0	0.0
(Co-*gl*)	Dcl1$^-$	188	0.0	29.3[d]	0.0
F2 of *dcl1-8*/+	Dcl1$^+$	307	0.0	0.0	0.0
(Co-*gl*)	Dcl1$^-$	280	0.0	23.8[d]	0.0
F2 of *dcl1-7*/+	Dcl1$^+$	316	1.0	0.0	0.0
(L*er*)[c]	Dcl1$^-$	219	0.5	14.6	0.0
F2 of *dcl1-8*/+	Dcl1$^+$	312	0.0	0.0	0.0
(L*er*)	Dcl1$^-$	201	1.4	14.1	0.0
F2 of *dcl1-9*/+	Dcl1$^+$	212	0.0	0.5	0.0
(L*er*)	Dcl1$^-$	105	0.0	30.5[d]	0.0
Co-*gl*	Wild type	520	0.0	0.0	0.6
Line 2 (Co)[c]	*35S:DCL1*	458	0.2	2.0	1.1
Line 9 (Co)	*35S:DCL1*	345	0.3	0.0	30.4[d]
Line 12 (Co)	*35S:DCL1*	382	0.0	0.0	10.7[e]
Null hypothesis[f]		4311	0.2	3.6	4.8

[a] Data were analyzed using the $p \times q$ table and a one-sided significance test with 45° of freedom (Campbell, 1989).
[b] Number of leaves scored per strain.
[c] Co-*gl*, Columbia *glabrous1*; L*er*, Landsberg *erecta*; Co, Columbia.
[d] χ^2 value significant at the 99.9% level.
[e] χ^2 value significant at the 95% level.
[f] Based on the frequency of each phenotypic class in the total population (Campbell, 1989).

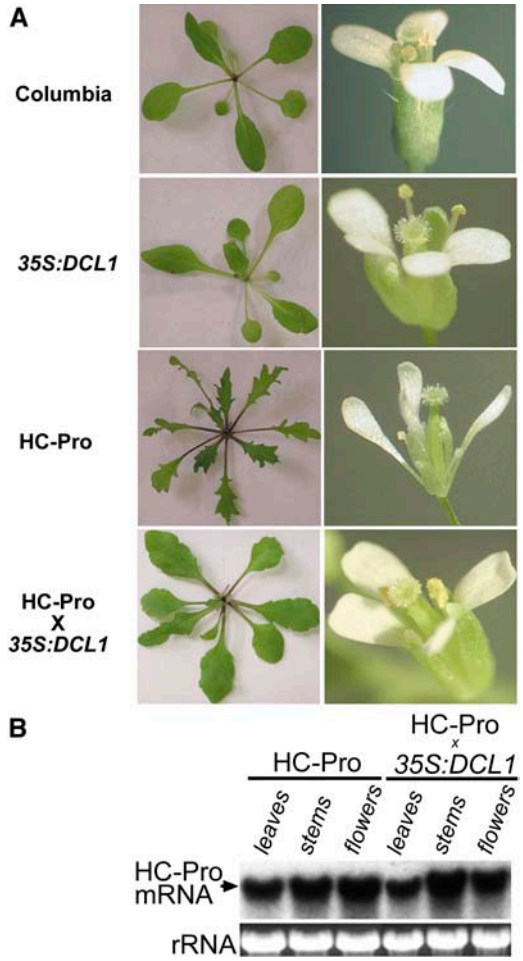

Figure 4. The *35S*:*DCL1* Locus Largely Rescues Morphological Features of P1/HC-Pro–Expressing *Arabidopsis* Plants.

(A) Ectopic *DCL1* rescues developmental defects in P1/HC-Pro transgenic plants. The morphology of the rosette leaves and flowers is shown for wild-type (Columbia), homozygous *35S*:*DCL1*, hemizygous P1/HC-Pro, and doubly hemizygous P1/HC-Pro × *35S*:*DCL1* lines. The heavily serrated leaves and sterile flowers of the P1/HC-Pro plants are almost completely suppressed in the P1/HC-Pro × *35S*:*DCL1* lines.
(B) P1/HC-Pro mRNA is expressed in P1/HC-Pro × *35S*:*DCL1*offspring. RNA gel blot analysis showing levels of P1/HC-Pro mRNA in the leaves, stems, and flowers of P1/HC-Pro transgenic plants and the equivalent tissues from P1/HC-Pro × *35S*:*DCL1* plants, as indicated. Ethidium bromide staining of 25S rRNA is shown as a loading control.

mediated by a general correction of the abnormal accumulation of miRNAs and miRNA*s in these plants.

35S:DCL1 Does Not Alter the Accumulation of miRNA Targets in P1/HC-Pro Plants

Despite the increased miRNA accumulation in P1/HC-Pro plants, the levels of some, but not all, miRNA target mRNAs are elevated in these plants, suggesting a P1/HC-Pro–mediated impairment in miRNA function (Kasschau et al., 2003; Dunoyer et al., 2004). Thus, even though *35S*:*DCL1* does not alter the accumulation of miRNAs or miRNA* species in P1/HC-Pro plants, it is still possible that the *35S*:*DCL1* transgene could suppress the P1/HC-Pro phenotype by restoring miRNA-directed target degradation. To test this hypothesis, the accumulation of selected miRNA target mRNAs was examined in P1/HC-Pro plants, with and without *35S*:*DCL1*. Two of the three target mRNAs examined, *AUXIN RESPONSE FACTOR8* (*ARF8*; At5g37020; miR167 target) and *AP2-LIKE* (At2g28550; miR172 target), were previously shown to undergo miRNA-directed cleavage that was inhibited by P1/HC-Pro in some tissues (Kasschau et al., 2003). The third, encoding a *NAC* domain protein (At5g61430), is a target of miR164 as shown by the detection of cleavage products (Mallory et al., 2004). Consistent with earlier results, the levels of some target messages were higher in plants expressing P1/HC-Pro than in the equivalent tissues of wild-type controls (for example, the *ARF8* and *NAC* domain protein mRNAs; Figure 6). However, we did not observe an increase in accumulation of *AP2-LIKE* mRNA in P1/HC-Pro plants as previously reported (Kasschau et al., 2003), possibly because of differences in the particular tissues and developmental stages examined. The effect of *35S*:*DCL1* alone on miRNA target accumulation was inconclusive because the observed changes were small and

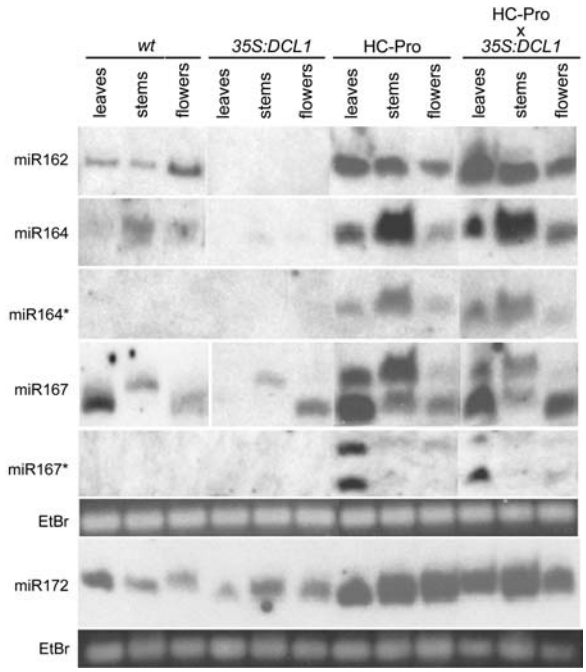

Figure 5. The Accumulation of miRNAs in *35S*:*DCL1*, P1/HC-Pro, and P1/HC-Pro × *35S*:*DCL1* Plants.

The accumulation of the indicated miRNAs and miRNA*s was determined from RNA gel blot analysis of low molecular weight RNA. RNA was isolated from the indicated tissues of adult plants at a late stage of flowering. The same RNA samples were fractionated on two gels. Probes to miR162, miR164, miR164*, miR167, and miR167* were hybridized to a blot derived from one gel, whereas the probe to miR172 was hybridized to a blot derived from the other gel. Ethidium bromide (EtBr) staining of the predominant RNA species in the low molecular weight fraction is shown as a loading control.

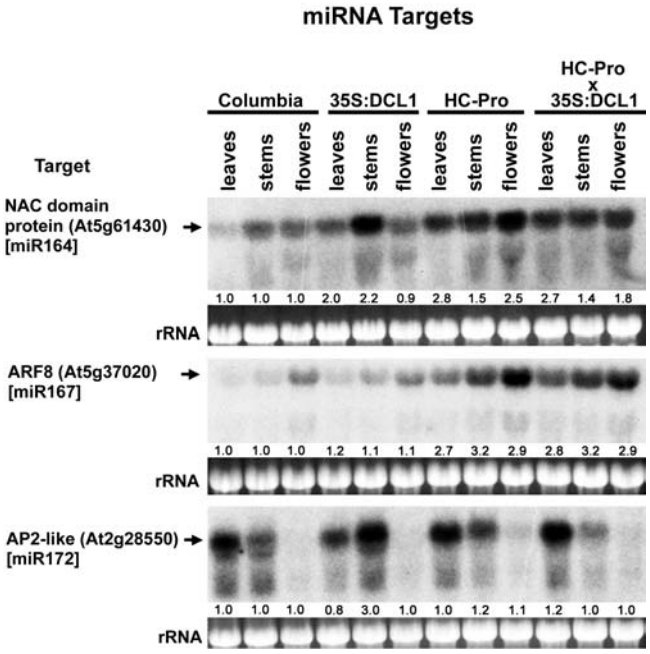

Figure 6. The Accumulation of miRNA Target mRNAs in *35S:DCL1*, P1/HC-Pro, and P1/HC-Pro × *35S:DCL1* Plants.

The accumulation of the indicated mRNA targets was determined from RNA gel blot analysis of high molecular weight RNA isolated from the indicated plants. Ethidium bromide staining of 25S rRNA is shown as a loading control.

inconsistent; although an inverse correlation between the level of a particular miRNA and its corresponding target could sometimes be noted (for example, a decrease in miR164 in stems is correlated with an increase in the *NAC* domain protein target mRNA in stems; Figures 5 and 6). By contrast, the effect of ectopic *DCL1* on miRNA target messages in the P1/HC-Pro background was clear in every case. The levels of all three miRNA targets in the P1/HC-Pro × *35S:DCL1* plants were the same as in the P1/HC-Pro plants (Figure 6). It seems likely, therefore, that the developmental abnormalities of P1/HC-Pro plants and the phenotypic rescue by ectopic *DCL1* are not mediated by a general miRNA-directed effect on target mRNA stability or at least not by effects mediated by the specific miRNAs examined here.

35S:DCL1 Does Not Rescue either Sense Transgene–Induced Silencing or Virus-Induced RNA Silencing in P1/HC-Pro Plants

To investigate if *35S:DCL1* could prevent P1/HC-Pro–mediated suppression of RNA silencing in addition to correcting P1/HC-Pro–mediated developmental anomalies, we looked at the effect of *35S:DCL1* on P1/HC-Pro suppression of both sense transgene–induced silencing and virus-induced RNA silencing (VIGS). For the sense transgene–induced silencing experiments, P1/HC-Pro, *35S:DCL1*, and P1/HC-Pro × *35S:DCL1* plants were crossed to the well-characterized *L1* line, which contains a silenced sense transgene encoding β-glucuronidase (GUS). *L1* plants initiate silencing at ~2 to 3 weeks after germination as evidenced by a dramatic decrease in *GUS* mRNA and the simultaneous appearance of high levels of *GUS* siRNAs (Figure 7A, lanes 5 and 6). By contrast, *L1* plants expressing P1/HC-Pro fail to silence and thus had high levels of full-length *GUS* mRNA and undetectable levels of *GUS* siRNAs at the same developmental stage (Figure 7A, lane 1). Overexpression of *DCL1* did not rescue *L1* silencing in the P1/HC-Pro line: plants with all three loci had high levels of *GUS* mRNA and undetectable levels of *GUS* siRNAs (Figure 7A, lanes 3 and 4). Unexpectedly, the *35S:DCL1* plants were also partially suppressed for silencing of the *L1* transgene in the absence of a P1/HC-Pro transgene (Figure 7A, lane 2). Analysis of F2 offspring indicated that suppression of *L1* silencing occurred whether the plants were homozygous or hemizygous for either the *L1* or the *35S:DCL1* locus (data not shown). These results raise the possibility that DCL1 plays a role in at least some kinds of RNA silencing and strongly suggest that the alleviation of the P1/HC-Pro developmental phenotype by *35S:DCL1* does not reflect a rescue of sense transgene–induced RNA silencing.

To determine if ectopic *DCL1* prevents P1/HC-Pro suppression of VIGS, we bombarded wild-type, P1/HC-Pro, *35S:DCL1*, and P1/HC-Pro × *35S:DCL1* plants with a *Cabbage leaf curl geminivirus* (CbLCV) vector carrying a portion of the endogenous gene *CHLORATA42* (*CH42*), which encodes a component of the magnesium chelatase complex (Turnage et al., 2002). Silencing of this gene causes the inability to accumulate chlorophyll and results in pronounced yellowing or chlorosis of leaf tissue. Bombardment with the *CH42* vector DNA resulted in viral infection in all inoculated plants as shown by the presence of symptoms and viral DNA (data not shown). When infected with the *CH42* vector, both wild-type and *35S:DCL1* plants developed the severe chlorosis characteristic of VIGS of *CH42*, had nearly undetectable levels of *CH42* mRNA, and accumulated *CH42* siRNAs (Figure 7B, lanes 5 and 6). By contrast, both P1/HC-Pro and P1/HC-Pro×*35S:DCL1* plants infected with the *CH42* vector had levels of *CH42* mRNA equivalent to those in uninfected controls and showed no signs of chlorosis, indicating that P1/HC-Pro blocked VIGS even when *DCL1* was overexpressed (Figure 7B, lanes 7 and 8). Interestingly, the siRNAs that accumulate in response to VIGS in this system were primarily of the larger variety previously associated with chromatin modifications rather than with RNA degradation (Mallory et al., 2002b; Chan et al., 2004; Xie et al., 2004). Moreover, the level of these larger small RNAs was not reduced in either P1/HC-Pro plants or in the P1/HC-Pro× *35S:DCL1* plants even though VIGS was suppressed as assayed by accumulation of the targeted *CH42* mRNA. Overall, these results suggest that it is unlikely that the alleviation of P1/HC-Pro developmental phenotypes caused by ectopic *35S:DCL1* is due to rescue of an endogenous pathway related to either VIGS or sense transgene–induced RNA silencing.

DISCUSSION

The plant viral protein P1/HC-Pro has dramatic affects on RNA silencing and miRNA pathways and normal progression through

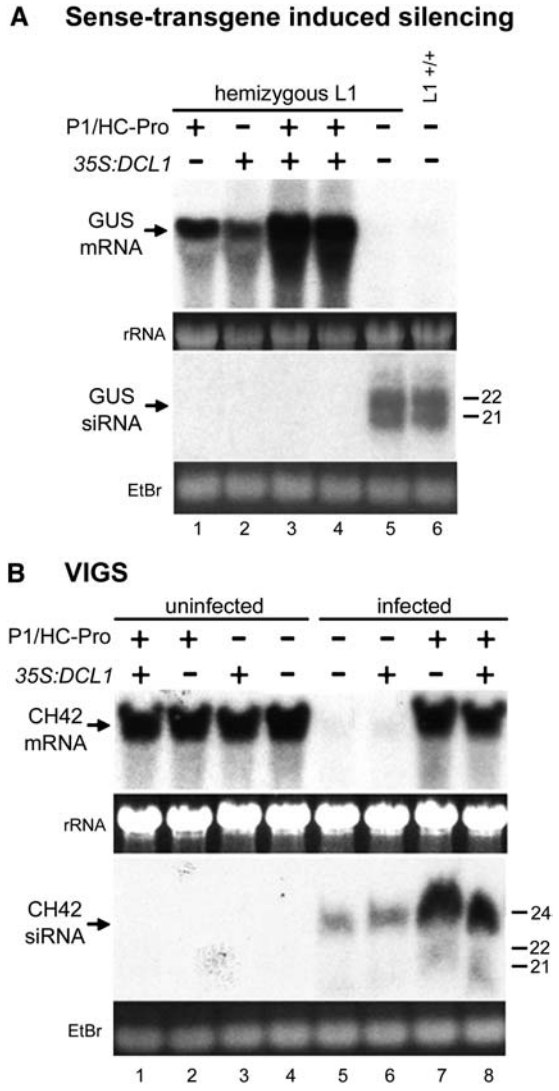

Figure 7. *35S:DCL1* Does Not Rescue P1/HC-Pro Suppression of either Sense Transgene–Induced or Virus-Induced RNA Silencing.

(A) Sense transgene–induced RNA silencing was monitored by RNA gel blot analysis of high molecular weight RNA to detect *GUS* mRNA (top blot) and of low molecular weight RNA to detect *GUS* siRNAs (bottom blot). The RNAs in lanes 1 to 5 are derived from plants hemizygous for the *L1 GUS*-silenced locus. The presence or absence of the P1/HC-Pro and *35S:DCL1* transgenes is indicated above the blot. The RNA in lane 2 is from a single plant hemizygous for both *35S:DCL1* and *L1* transgenes and is representative of four separate individuals that were assayed. The RNAs in lanes 3 and 4 are from two different individual plants hemizygous for all three transgenes. Lanes 5 and 6 show RNA from control plants lacking both P1/HC-Pro and *35S:DCL1* transgenes and either hemizygous (lane 5) or homozygous (lane 6) for the *L1* locus.

(B) VIGS was assayed by RNA gel blot analysis of high molecular weight RNA to detect *CH42* mRNA (top blot) and of low molecular weight RNA to detect *CH42* siRNA (bottom blot). RNA was isolated from tissue pooled from 10 plants at 21 d after bombardment with the *CH42* silencing vector. The presence or absence of the P1/HC-Pro and *35S:DCL1* transgenes is indicated above the blot. Ethidium bromide (EtBr) staining of 25S rRNA and the predominant RNA species in the low molecular weight fraction are shown as loading controls in **(A)** and **(B)**. The molecular weight of the indicated small RNA species in **(A)** and **(B)** was inferred from the migration of DNA oligonucleotides that were subsequently standardized to RNA markers.

development. The effect of P1/HC-Pro on the miRNA pathway is manifest as an increase in the levels of miRNA and miRNA*s and an increase in the levels of some miRNA target mRNAs in some plant tissues (Mallory et al., 2002b; Kasschau et al., 2003; Chapman et al., 2004; Dunoyer et al., 2004). Suppression of sense transgene–induced RNA silencing by P1/HC-Pro is associated with the elimination of siRNAs and the accumulation of target mRNA (Llave et al., 2000; Mallory et al., 2001). Both defects in small RNA metabolism are consistent with the hypothesis that P1/HC-Pro alters the activity of DCL enzymes, which produce small RNAs and are essential for their assembly into RISC. If P1/HC-Pro interferes with one or more DCL proteins, then additional copies of a Dicer might restore Dicer activity and allow the plant to overcome the effects of P1/HC-Pro on both development and small RNA metabolism. To test this hypothesis, we ectopically expressed *Arabidopsis DCL1*, a Dicer required for miRNA biogenesis, under the control of a constitutive promoter to see if it could rescue any aspect of the P1/HC-Pro phenotype.

Ectopic expression of *DCL1* largely alleviated the severe developmental anomalies that characterize P1/HC-Pro transgenic *Arabidopsis*, resulting in plants with a near wild-type phenotype. To determine if the rescue of developmental anomalies by *35S:DCL1* correlated with the alleviation of any of the known defects in small RNA processes induced by P1/HC-Pro, we examined RNA silencing and miRNA pathways in P1/HC-Pro plants with and without the *35S:DCL1* transgene. Surprisingly, we found no significant differences with respect to small RNA metabolism in these experiments: the VIGS and sense transgene silencing phenotypes for both P1/HC-Pro plants and P1/HC-Pro × *35S:DCL1* plants were essentially identical. Furthermore, the levels of miRNAs, miRNA*s, and miRNA target messages in P1/HC-Pro × *35S:DCL1* plants were similar to those in P1/HC-Pro controls. Thus, the rescue of P1/HC-Pro developmental anomalies by ectopic *DCL1* was not coupled to a general correction of P1/HC-Pro–mediated defects in either RNA silencing or miRNA pathways.

The simplest interpretation of these results is that P1/HC-Pro–mediated developmental defects, but not the defects in small RNA metabolism, are caused by interference with a DCL1 activity that can be rescued by increased expression of the gene. Alternatively, the mechanism of P1/HC-Pro action might be via interference with one or more of the other *Arabidopsis* DCL enzymes (DCL2, 3, or 4), and ectopic DCL1 can compensate for the effect of these enzymes on development but not for the other effects on small RNA-directed RNA degradation pathways. A further possibility is that P1/HC-Pro may not directly interfere with any aspect of Dicer function but rather with some other activity for which overexpression of DCL1 can compensate, perhaps by enhancing the efficiency of a downstream step in the pathways controlling development.

Current models attribute the developmental anomalies in P1/HC-Pro plants to an impairment in miRNA precursor processing

that allows accumulation of a normally labile miRNA/miRNA* intermediate, thereby preventing assembly of a functional RISC and blocking miRNA-directed target degradation (Chapman et al., 2004). These models would predict that correction of defects in development would be accompanied by a correction of the aberrant accumulation of miRNA, miRNA*s, and miRNA target mRNAs. However, our results clearly show that the P1/HC-Pro developmental anomalies can be rescued without a concomitant rescue of these miRNA pathway defects. How then might *35S:DCL1* relieve the effects of P1/HC-Pro on development? Since our studies included a limited number of miRNAs, it remains possible that the observed alleviation of P1/HC-Pro phenotype by ectopic *DCL1* is due to an effect on a subset of miRNAs rather than a general correction of the biogenesis and function of all miRNAs. An alternative hypothesis is that DCL1 and perhaps one or more of the other *Arabidopsis* DCL enzymes have a function in addition to their known roles in small RNA metabolism. In general, RNase IIIs cleave highly base-paired or fully dsRNAs and are important in the processing of rRNAs (Venema and Tollervey, 1999) and small nucleolar RNAs (Tycowski and Steitz, 2001) and in regulating the level of some pre-mRNAs (Danin-Kreiselman et al., 2003). Based on these known roles of canonical RNase IIIs, one possibility is that Dicers are involved in RNA processing events that directly regulate the accumulation of pre-mRNAs, small nuclear RNAs, or small nucleolar RNAs that play important roles in development.

An unexpected finding of our studies is that the *35S:DCL1* plants are not uniformly enhanced for known DCL1 functions. While the *35S:DCL1* transgene can largely complement a *dcl1* mutation (indicating that the transgene produces a functional DCL1 protein), its phenotype in wild-type plants suggests that some DCL1 activities are enhanced, whereas others are impaired. The developmental phenotype in the homozygous *DCL1* overexpressing plants is opposite those of *dcl1* partial loss-of-function mutants with respect to flowering time and adaxialized/abaxialized patterning of leaves, suggesting enhanced DCL1 function. However, the accumulation of at least some miRNAs is decreased in the *35S:DCL1* plants in the wild-type *DCL1* background, suggesting decreased DCL1 activity with regard to miRNA biogenesis. It is not unreasonable to think that overexpressing a Dicer might result in either increased or decreased activity depending upon the process being examined. For example, if a Dicer function was dependent on assembly with two or more other factors, then overexpression might titrate out these other factors, resulting in the formation of inactive complexes that contain only a subset of the required components. Similarly, if such factors are shared with other Dicers, then overexpression might also interfere with the activities of these other Dicers. By contrast, overexpression ought to enhance DCL1 activities that are not dependent on limiting amounts of cofactors by simply providing more enzyme. Using this rationale, the finding that both miRNA accumulation and sense transgene silencing are impaired in the *35S:DCL1* plants suggests that both processes depend on a Dicer complex that is not properly assembled when DCL1 is overexpressed. By contrast, an increase in a DCL1 activity that does not require cofactors may account for the rescue of P1/HC-Pro–induced developmental anomalies. Overall, the relatively mild developmental phenotypes of both the *35S:DCL1* and the P1/HC-Pro × *35S:DCL1* plants suggest that some degree of impairment in both RNA silencing and miRNA pathways can be tolerated in *Arabidopsis* without severe developmental consequences.

METHODS

Plant Lines and Transformation

35S:DCL1 lines were generated by transforming *Arabidopsis thaliana* (Columbia ecotype) with the 6.2-kb *DCL1* sequence containing the 5′ and 3′ untranslated regions in binary vector pAL144 (Lloyd et al., 1992; kind gift of A. Lloyd). Plants expressing TuMV P1/HC-Pro were made by transforming *Arabidopsis* (Columbia ecotype) with binary vector PCX305 containing the P1/HC-Pro coding region from TuMV isolate 1 (kind gift of T. Pirone). A translation termination signal was inserted to produce the authentic C terminus of HC-Pro, which is generated by the autocatalytic activity of HC-Pro when the polyprotein is expressed from the virus. Both the *DCL1* and the P1/HC-Pro cDNAs were cloned under control of the cauliflower mosaic virus 35S promoter. Line *L1* (*23b1*) was the kind gift of H. Vaucheret and has been previously described (Elmayan et al., 1998).

Genotyping to Identify Homozygous *dcl1-8* Plants Containing the *35S:DCL1* Transgene

The genotype of F2 plants segregating for both the *dcl1-8* mutation and the *35S:DCL1* transgene was determined by a PCR-based assay using *DCL1*-specific primers (5′-CACGCTGTCAAGAAACATCCATAC-3′ and 5′-GCAGCTTTGTCATACTCGACGAC-3′). Using these primers, a PCR product of 340 bp is amplified from the wild-type *DCL1* gene and the *dcl1-8* mutant gene; however, the product amplified from the *35S:DCL1* transgene is only 262 bp because it lacks the introns that are present in the endogenous gene. The single nucleotide change causing the *dcl1-8* mutation fortuitously also disrupts the recognition sequence for the restriction endonuclease *Sna*BI, and this polymorphism was used to distinguish the *dcl1-8* mutant from the wild-type *DCL1* gene (Golden et al., 2002; S. Schauer, D. Merchant, and A. Ray, unpublished data). DNA amplified from a wild-type plant produces 229- and 111-bp bands when digested with *Sna*BI, while DNA amplified from a homozygous *dcl1-8* mutant is not cut by *Sna*BI, producing the original 340-bp PCR fragment. The presence of all three DNA fragments indicates that the sampled plant was heterozygous for the *dcl1-8* mutation. The presence of the *35S:DCL1* transgene in the sampled plant DNA is indicated by the production of 151- and 111-bp fragments upon cleavage of the 262-bp transgene-specific PCR fragment with *Sna*BI.

VIGS Assay

For the VIGS assay, *Arabidopsis* plants were bombarded at the four- to six-leaf stage using the CbLCV-based vector system for silencing of the *CH42* gene encoding magnesium cheletase that was kindly provided by D. Robertson and has been previously described (Turnage et al., 2002). Each cartridge contained 0.125 mg of 0.6-μm gold particles coated with a mixture of 1 μg each of the CbLCV A and B component vectors as described (Turnage et al., 2002). Plants were bombarded twice/cartridge using a Bio-Rad PDS 100 Helium system at a pressure of 80 p.s.i.

RNA Isolation and Hybridization

Plant material was frozen in liquid nitrogen, ground to a powder, and immediately vortexed in a preheated (80°C) mixture of ~5 volumes/g tissue of extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, and 1% sodium dodecyl sulfate) and an equal volume of phenol. Two

volumes of chloroform/volume of phenol were added, the solution was mixed intermittently for 5 min, and phases were separated by centrifugation. An equal volume of 4 M LiCl and 10 mM EDTA, pH 7.0, was added to the aqueous phase and incubated overnight at 4°C. High molecular weight RNA was recovered by centrifugation at 12,000*g* for 15 min. The supernatant, containing DNA and low molecular weight RNAs, was made 10% in polyethylene glycol 8000 and 0.5 M NaCl and incubated for 30 min on ice to precipitate the DNA. After centrifugation to remove DNA, low molecular weight RNA was concentrated by ethanol precipitation.

RNA gel blot analysis of high and low molecular weight RNA were performed exactly as described (Vance, 1991; Mallory et al., 2002b) using Ambion ULTRAhyb hybridization solution at 45°C for DNA probes and 68°C for RNA probes. The nucleotide coordinates of hybridization probes (nucleotide numbers derived from mRNA sequences) were as follows: *DCL1* 5′ probe (At1g01040), nucleotides 3171 to 3410; *DCL1* 3′ probe (At1g01040), nucleotides 4577 to 4829; *CH42* (At4g18480), nucleotides 1241 to 1557; *NAC* domain protein (At5g614330), nucleotides 870 to 1284; *ARF8* (At5g37020), nucleotides 1988 to 2395; *AP2-LIKE* (At2g28550), nucleotides 11 to 452. *GUS* probes were derived from the 3′ 700 nucleotides of the GUS coding sequence. The P1/HC-Pro probe was derived from the TuMV P1/HC-Pro coding region. Probes were generated using PCR and either labeled directly using Ambion DECAprime II kit or transcribed with T7 RNA polymerase using Ambion MAXIscript after addition of a T7 promoter with an Ambion Lig'n Scribe kit. Specific miRNA probes were prepared by end-labeling antisense oligonucleotides with T4 polynucleotide kinase (New England Biolabs). Probes for miRNA* species 164 and 167 were an equal mix of end-labeled antisense oligonucleotides to the predicted miRNA* sequence of the two precursor genes (miR164a and b; miR167a and b) (Reinhart et al., 2002).

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers At1g01040 (*DCL1*), At4g18480 (*CH42*), At5g614330 (*NAC* domain protein), At5g37020 (*ARF8*), and At2g28550 (*AP2-LIKE*).

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