

# Partially Redundant Functions of *Arabidopsis* DICER-like Enzymes and a Role for DCL4 in Producing *trans*-Acting siRNAs

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## Summary

*Arabidopsis* encodes four DICER-like (DCL) proteins [1]. DCL1 produces miRNAs [2–4], DCL2 produces some virus-derived siRNAs, and DCL3 produces endogenous RDR2-dependent siRNAs [5], but the role of DCL4 is unknown. We show that DCL4 is the primary processor of endogenous RDR6-dependent *trans*-acting siRNAs (tasiRNAs). Molecular and phenotypic analyses of all *dcl* double mutants also revealed partially compensatory functions among DCL proteins. In the absence of DCL4, some RDR6-dependent siRNAs were produced by DCL2 and DCL3, and in the absence of DCL3, some RDR2-dependent siRNAs were produced by DCL2 and DCL4. Consistent with partial redundancies, *dcl2* and *dcl3* mutants developed normally, whereas *dcl4* and *dcl3 dcl4* mutants had weak and severe *rdr6* phenotypes, respectively, and increased tasiRNA target mRNA accumulation. After three generations, *dcl3 dcl4* and *dcl2 dcl3* mutants exhibited stochastic developmental phenotypes, some of which were lethal, likely owing to the accumulated loss of heterochromatic siRNA-directed marks. *dcl1 dcl3* and *dcl1 dcl4*, but not *dcl1 dcl2* mutants, had phenotypes more severe than *dcl1* mutants, consistent with DCL1, DCL3, and DCL4 acting as the primary processors of the three respective classes of endogenous silencing RNAs and DCL2 acting to produce viral-derived siRNAs and as an alternative DCL for endogenous siRNA production.

## Results and Discussion

Endogenous 21–24 nt small RNAs regulate gene expression transcriptionally or posttranscriptionally [6–8]. Dicer proteins are RNaseIII enzymes that process small RNAs from dsRNAs or hairpins. A single Dicer is present in mammals, worms, and fission yeast (*Schizosaccharomyces pombe*) [8]. *Neurospora crassa* has two functionally redundant Dicers [9], whereas some

other lineages have multiple Dicers with more specialized functions. Flies have two Dicers, Dcr-1 and Dcr-2, which process miRNAs and siRNAs, respectively [8]. *Arabidopsis* has four nuclear-localized DICER-like proteins, DCL1–DCL4 [1, 10, 11]. Analyses of single mutants have revealed functions for three of the four DCL proteins. DCL1 processes miRNAs from partially double-stranded stem-loop precursor RNAs transcribed from *MIR* genes [2–4]. DCL3 processes endogenous repeat and intergenic-region-derived siRNAs that depend on RNA-dependent RNA polymerase 2 (RDR2) [5]. DCL2 functions in the antiviral silencing response in turnip-crinkle-virus-infected plants but not in turnip-mosaic-virus- or cucumber-mosaic-virus-strain-Y-infected plants [5]. Thus far, no role in processing endogenous small RNAs has been assigned to DCL2, and the function of DCL4 has not been reported.

In addition to DCL1-dependent miRNAs and DCL3-RDR2-dependent siRNAs, plants also produce RDR6-dependent *trans*-acting siRNAs (tasiRNAs; [12, 13]). AGO1, DCL1, HEN1, and HYL1 are required for proper accumulation of both tasiRNAs and miRNAs, whereas RDR6 and SGS3 are required for proper accumulation of tasiRNAs but not miRNAs [12–14].

## tasiRNAs Are Produced by DCL4

We previously proposed that single-stranded tasiRNA precursor RNAs (*TAS*) were copied to dsRNA through an RDR6-SGS3-dependent pathway and subsequently processed to tasiRNAs through a mechanism similar to miRNA biogenesis, thus explaining the common requirements for AGO1, DCL1, HEN1, and HYL1 for both miRNAs and tasiRNAs [13]. However, an alternative explanation for the common requirement of these four proteins came with the recent discovery that certain miRNAs can be important for tasiRNA biogenesis [14]. Two miRNAs, miR173 and miR390, appear to set the phase for the production of tasiRNAs from *TAS1* and *TAS2*, and *TAS3*, respectively, a hypothesis supported by ectopic expression studies [14]. Overexpression of *TAS1* or *TAS2* and miR173 in the heterologous host *Nicotiana benthamiana* resulted in tasiRNA production, which could be abolished by mutations in the miRNA complementary sites of *TAS1* and *TAS2*. Further confirming that tasiRNA production relies on miR173, we found that an *Arabidopsis mir173* mutant, *mir173-1* (which has a T-DNA inserted at the *MIR173* locus upstream of the miRNA sequence), shows reduced miR173 accumulation and a corresponding reduction in *TAS1*- and *TAS2*-derived tasiRNA accumulation (Figure 1A).

To address the question of which DCL processes *TAS* dsRNA into tasiRNAs, we monitored *TAS1*, *TAS2*, and *TAS3* siRNA accumulation with a panel of *dcl* mutants that included *dcl1-9* (a viable hypomorph with a disrupted dsRNA binding domain; [1]) and *dcl3-1* [5] and the newly identified *dcl2-4* and *dcl4-1* (three presumed null mutants). As reported previously, an essential role

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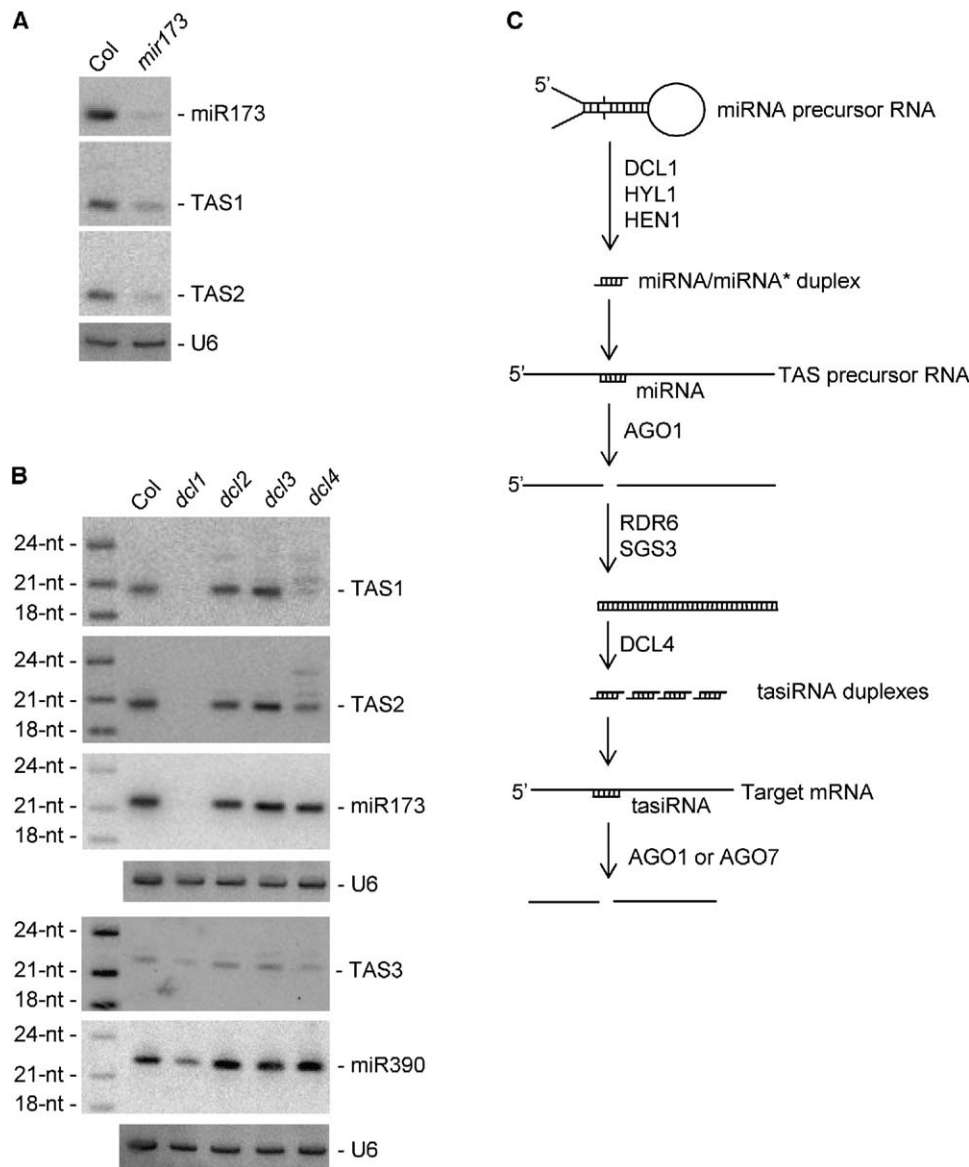


Figure 1. DCL4 Processes tasiRNAs

(A) RNA gel blot analysis of 10  $\mu$ g total RNA prepared from floral inflorescence of wild-type Col and the *mir173-1* mutant. The blot was successively probed with DNAs complementary to miR173, *TAS1*-derived tasiRNA siR480(+) [13], also named ASRP255 [12, 14], *TAS2*-derived tasiRNA F [3], also named ASRP1511 [14], and the U6 snRNA, which served as a loading control.

(B) RNA gel blot analysis of 10  $\mu$ g total RNA prepared from floral inflorescence of wild-type Col and *dcl1-9*, *dcl2-4*, *dcl3-1*, and *dcl4-1* mutants. The blots were probed with DNAs complementary to miR173, *TAS1*-derived tasiRNA siR480(+), *TAS2*-derived tasiRNA F, miR390, *TAS3*-derived tasiRNAs, and U6, which served as a loading control.

(C) Model for tasiRNA biogenesis and action. miR173 and miR390, processed from partially double-stranded precursor RNAs in a DCL1-, HYL1-, and HEN1-dependent manner, direct the AGO1-dependent cleavage of partially complementary *TAS* precursor RNAs. The *TAS* cleavage products generated by miRNA-directed cleavage are copied to dsRNA in an RDR6- and SGS3-dependent manner and subsequently diced to tasiRNAs by DCL4. tasiRNAs direct the cleavage of partially complementary target mRNAs in an AGO1-dependent manner, but AGO7 also may play a role in tasiRNA-directed target cleavage because mRNA targets of *TAS3*-derived tasiRNAs (ARF3 and ARF4) overaccumulate in *ago7/zip*, *rdr6*, and *sgs3* mutants [12, 14].

of DCL2 and DCL3 in tasiRNA production could be ruled out because mutations in *DCL2* and *DCL3* do not affect tasiRNA accumulation [13, 14]. In contrast, the mutation in *DCL4* led to both misprocessing and reduced accumulation of *TAS1*- and *TAS2*-derived

tasiRNAs and reduced accumulation of *TAS3*-derived tasiRNAs, without affecting miR173 or miR390 accumulation (Figure 1B). These results suggest a model in which DCL4 is primarily responsible for processing RDR6-generated dsRNA into tasiRNAs (Figure 1C).

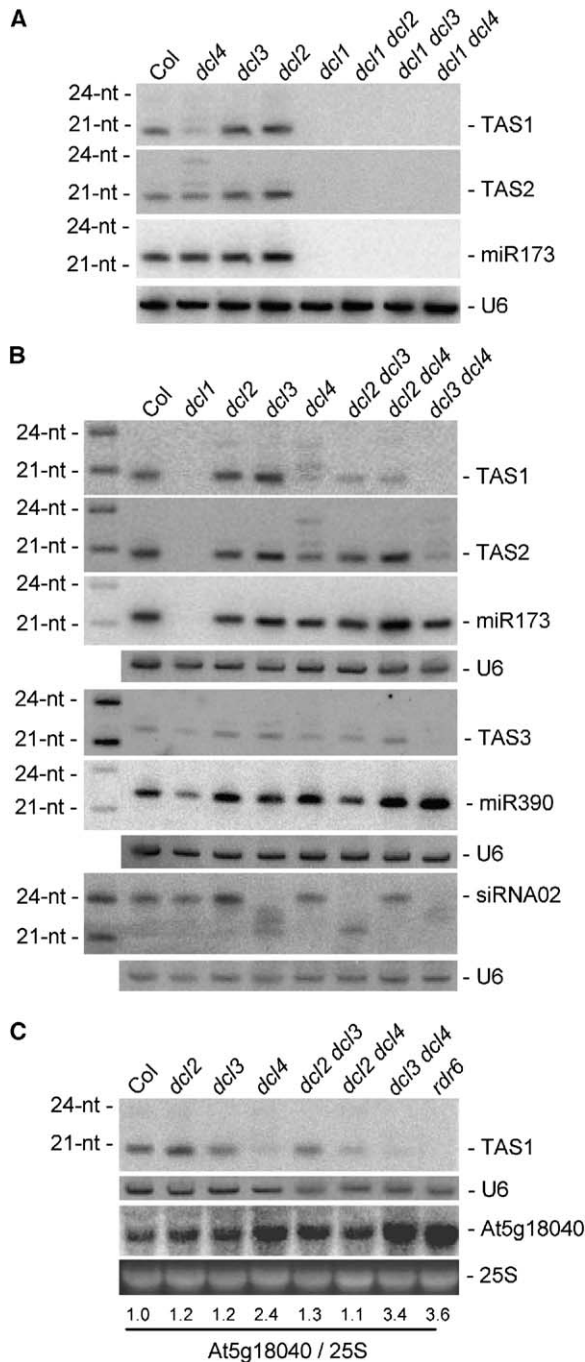


Figure 2. Analysis of *dcl* Double Mutants Reveals Partially Complementary Functions

(A) RNA gel blot analysis of 10 µg total RNA prepared from floral inflorescence of wild-type Col, *dcl1-9*, *dcl2-4*, *dcl3-1*, and *dcl4-1* single mutants and the three *dcl1* double mutants. The blot was successively probed with DNAs complementary to *TAS1*-derived tasiRNA siR480(+), *TAS2*-derived tasiRNA F, miR173, and U6, which served as a loading control.

(B) RNA gel blot analysis of 10 µg total RNA prepared from floral inflorescence of wild-type Col, *dcl1-9*, *dcl2-4*, *dcl3-1*, and *dcl4-1* single mutants and *dcl2-4 dcl3-1*, *dcl2-4 dcl4-1*, and *dcl3-1 dcl4-1* double mutants. The blots were probed with DNAs complementary to *TAS1*-derived tasiRNA siR480(+), *TAS2*-derived tasiRNA F, miR173, *TAS3*-derived tasiRNAs, miR390, and siRNA02. Each of

### DCL2 and DCL3 Contribute to RDR6-Dependent siRNA Production in the Absence of DCL4

Because *dcl4-1* carries a T-DNA in intron 10, upstream of the RNaseIII domain, and *DCL4* mRNA is not detected by RT-PCR with primers complementary to exons 10 and 11 (data not shown), it is likely that *dcl4-1* is a null allele and that the remaining 21 nt tasiRNAs, as well as the misprocessed 22–24 nt siRNAs, are produced by another DCL(s) that can process RDR6-generated dsRNAs in the absence of DCL4. To test this hypothesis, we generated the six possible double *dcl* mutants. Because *dcl1* single mutants are impaired in miR173 production (Figures 1B and 2), *dcl1 dcl2*, *dcl1 dcl3*, and *dcl1 dcl4* double mutants did not accumulate detectable levels of *TAS1*- and *TAS2*-derived tasiRNAs (Figure 2A). A *dcl2 dcl3* double mutant accumulated *TAS*-derived siRNAs, miR173, and miR390 (Figure 2B), consistent with DCL2 and DCL3 not being the primary producers of tasiRNAs and miRNAs (Figures 1B and 2B). In contrast, *dcl2 dcl4* and *dcl3 dcl4* double mutants showed modified patterns of tasiRNA accumulation. The *dcl3 dcl4* double mutant showed a more dramatic reduction in 21 nt *TAS*-derived siRNA levels than the *dcl4* mutant, suggesting that DCL3 is able to produce 21 nt *TAS*-derived siRNAs in the absence of DCL4. Because 21 nt tasiRNA accumulation was not completely abolished in *dcl2 dcl4* and *dcl3 dcl4* plants (Figure 2B), it is possible that DCL1 can also produce 21 nt RDR6-dependent tasiRNAs. Accumulation of the 22–24 nt *TAS*-derived siRNAs detected in the *dcl4* single mutant was reduced in *dcl2 dcl4* and *dcl3 dcl4* double mutants (Figure 2B), indicating that DCL2 and DCL3 are able to produce 22–24 nt *TAS*-derived siRNAs in the absence of DCL4.

In contrast to the *dcl3 dcl4* plants, the *dcl2 dcl4* plants had an increase in the *TAS1*-, *TAS2*-, and *TAS3*-derived 21 nt tasiRNAs, as well as an increase in both miR173 and miR390, when compared to the *dcl4* plants (Figure 2B). That the disruption of a second DCL led to an increase in 21 nt silencing RNAs was unexpected, and it suggests that DCL2 might antagonize the function of DCL1 and DCL3. Another possibility is that DCL2 might antagonize the function of DCL1 but not that of DCL3, and the increased miR173 and miR390 in the *dcl2 dcl4* plants results in increased tasiRNA production. Perhaps DCL2 competes for a protein factor(s) needed for the function of DCL1 and DCL3. One such

the three blots was stripped and reprobated with an oligonucleotide complementary to U6 as a loading control. Note that the wild-type and single mutant lanes probed for *TAS1*, *TAS2*, miR173, *TAS3*, and miR390 are the same as those shown in Figure 1B and are reiterated here for clarity and side-by-side comparison to the double mutants.

(C) RNA gel blot analysis of 10 µg total RNA prepared from 17-day-old seedlings of wild-type Col and the indicated single and double mutants. The blots were probed with DNAs complementary to *TAS1*-derived tasiRNA siR480(+) and At5g18040, a target of *TAS1*-derived tasiRNA siR480(+) [12, 13]. The *TAS1* blot was stripped and reprobated with an oligonucleotide complementary to U6 as a loading control, and 25S rRNA is shown as a loading control for the mRNA blot. Normalized values of At5g18040 mRNA to 25S rRNA (with Col levels set at 1.0) are indicated.

factor might be HYL1 or another member of this family of double-stranded RNA binding (DRB) proteins that collaborate with the DICER proteins [11].

#### DCL2 and DCL4 Produce RDR2-Dependent siRNAs in the Absence of DCL3

DCL2, DCL3, and perhaps DCL1 were able to produce RDR6-dependent siRNAs in the absence of DCL4, suggesting partially compensatory functions among some DCL proteins. To address the issue of redundancy for RDR2-dependent siRNA production, we examined, in the different mutant backgrounds, the accumulation of the siRNA02, an RDR2-dependent heterochromatic siRNA [5]. Analogous to the size shifts observed for TAS-derived siRNAs in a *dcl4* mutant (Figures 1 and 2), a *dcl3* mutant accumulated 21–23 nt RDR2-dependent siRNAs (Figure 2B) instead of the 24 nt RDR2-dependent siRNA02 observed in wild-type plants ([5]; Figure 2B). In *dcl2* and *dcl4* single mutants and the *dcl2 dcl4* double mutant, RDR2-dependent siRNAs accumulated at wild-type levels (Figure 2B). Only the 21 nt siRNAs were detected in *dcl2 dcl3* mutants (Figure 2B), whereas only the 22 and 23 nt siRNAs were detected in *dcl3 dcl4* mutants (Figure 2B), indicating that in the absence of DCL3, DCL4 and DCL2 are able to produce 21 nt and 22–23 nt RDR2-dependent siRNAs, respectively.

#### A *dcl3 dcl4* Double Mutant Phenocopies *rdm6* and *sgs3* Mutants

*dcl1* mutants have a dramatic phenotype [1], whereas *dcl2* and *dcl3* mutants do not exhibit obvious developmental defects ([5]; Figure 3A). *dcl4* leaves are curled slightly downward at the margins (Table 1; Figure 3A), consistent with the reduction in tasiRNA accumulation and a 2-fold increase in tasiRNA target mRNA accumulation (Figures 2B and 2C). The *dcl3 dcl4* double mutant had more severely downward-curved leaf margins (Table 1; Figure 3A), which correlated with a stronger reduction in tasiRNA levels (Figures 2B and 2C) and a 3-fold increase in tasiRNA target mRNA accumulation (Figure 2C), similar to the increase seen in an *rdm6* null allele (Figures 2C and 3A). In contrast, the *dcl3* mutant did not exhibit the *rdm6* phenotype (Table 1; Figure 3A), consistent with wild-type levels of tasiRNA target accumulation (Figure 2C).

#### *dcl2 dcl3* and *dcl3 dcl4* Double Mutants Exhibit Stochastic Developmental Defects after Three Generations

After three generations, additional phenotypes were visible, in a stochastic manner, in *dcl2 dcl3* and *dcl3 dcl4* mutants but not in *dcl2*, *dcl3*, *dcl4*, and *dcl2 dcl4* mutants (Figure 3B; data not shown). The appearance of phenotypes over generations suggested that they resulted from accumulated epigenetic lesions that occur at the chromatin level, similar to the appearance of phenotypes in *ddm1* after three to five generations [15, 16]. On the basis of these cumulative findings, we speculate that stochastic phenotypes that appear over generations will be characteristic of mutations in genes that have important, nonredundant functions during epigenetic control. Although DCL3 appears to be the primary

DCL producing RDR2-dependent siRNAs, which direct transcriptional gene silencing and heterochromatin modification [17, 5], the absence of phenotypes in the *dcl3* mutant, even after several generations, indicated that both DCL2 and DCL4 are functionally compensatory in this regard.

#### *dcl1 dcl3* and *dcl1 dcl4* but Not *dcl1 dcl2* Exhibit Phenotypes More Severe than *dcl1*

MicroRNA accumulation is reduced in *dcl1-7* and *dcl1-9* mutants, which have a point mutation in the RNA helicase domain and a truncation of the second dsRNA binding domain, respectively [1–3]. Because miRNA accumulation is substantially reduced in partial-loss-of-function *dcl1-7* and *dcl1-9* mutants, DCL1 was proposed to be the primary DCL processing miRNA precursors [2–4]. The residual accumulation of some miRNAs in *dcl1-7* and *dcl1-9* could result from partial activity of the mutant proteins because *dcl1* null alleles are embryo lethal [1]. *MIR163* transcripts are misprocessed in *dcl1-7* and *dcl1-9* [4], which, in light of our results showing partially compensatory functions among DCLs, could result from the aberrant processing of miRNA precursors by DCL2, DCL3, or DCL4. To test this hypothesis, we examined the miRNA accumulation and phenotypes of double mutants between the partial-loss-of-function *dcl1-9* mutant and null *dcl2*, *dcl3*, and *dcl4* alleles. *dcl1 dcl2* mutants resembled *dcl1* mutants, whereas *dcl1 dcl3* and *dcl1 dcl4* mutants had phenotypes more severe than did *dcl1* (Figure 4A). Both *dcl1 dcl3* and *dcl1 dcl4* mutants flowered later and showed more dramatic floral defects than did *dcl1*. *dcl1 dcl3* consistently produced more rosette leaves, which is often characteristic of late-flowering plants. In contrast, *dcl1 dcl4* had increased anthocyanin accumulation, indicative of stress, and approximately 90% of the plants died before flowering. MicroRNAs that remained detectable in the partial-loss-of-function *dcl1-9* mutant also accumulated in the *dcl1 dcl2*, *dcl1 dcl3*, and *dcl1 dcl4* double mutants (Figure 4B), suggesting that none of the three other DCLs was responsible for the residual accumulation of these miRNAs in *dcl1-9*. However, because the residual miRNA accumulation might be from the compensatory function of more than one of the other DCLs, the analysis of triple and quadruple mutants will be useful to determine whether any of the three other DCL proteins is able to process miRNA precursors. In the double mutant contexts, if there is no substantive decrease in miRNA processing than that observed in *dcl1-9*, then the stronger phenotypes in the *dcl1 dcl3* and *dcl1 dcl4* mutants might result from the combined diminution of both miRNAs and heterochromatic siRNAs or miRNAs and tasiRNAs, respectively.

#### Concluding Remarks

*Arabidopsis* has complex RNAi machinery, including four DCL, six RDR, and ten AGO proteins, half of which have unknown functions. Although molecular and phenotypic characteristics often are associated with single mutations, evidence exists for partial redundancies. For example, AGO1 and AGO10 (PINHEAD) have overlapping functions in the meristem [18], and RDR2 and RDR6 both affect PTGS induced by an amplicon trans-

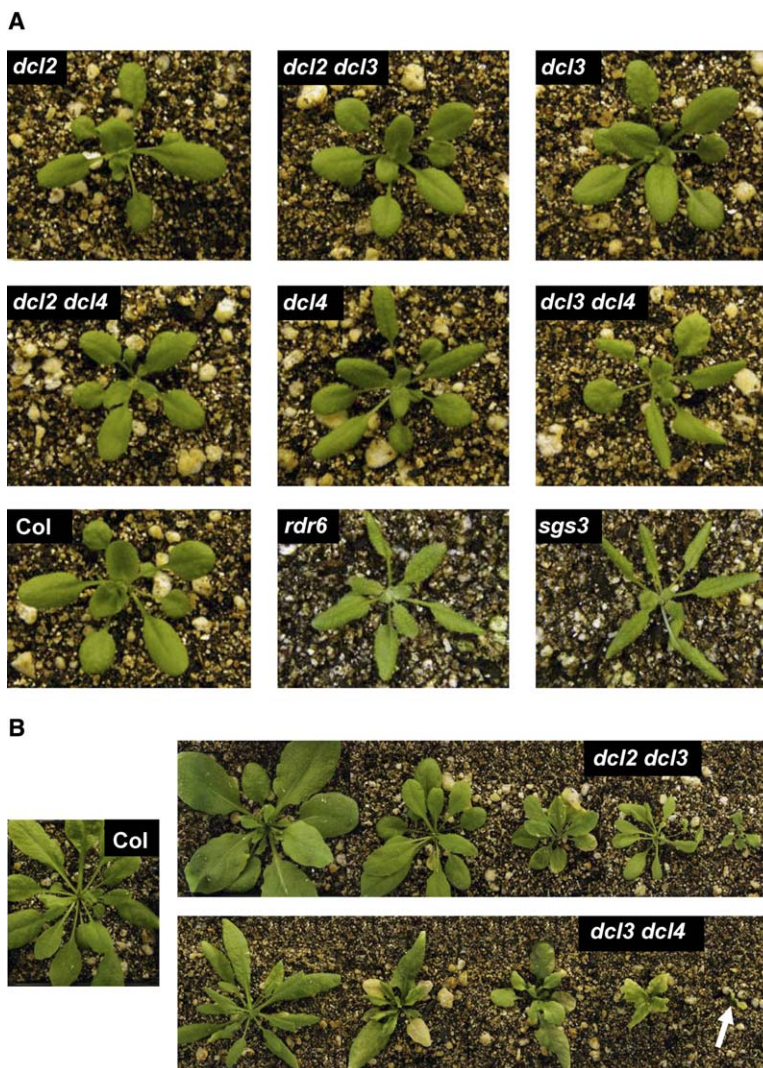


Figure 3. A *dcl3 dcl4* Double Mutant Phenocopies *rdr6* and *sgs3* Mutants

(A) One-month-old wild-type Col, *dcl2-4*, *dcl3-1*, and *dcl4-1* single mutants, the three corresponding double mutants (second generation), *rdr6 (sgs2-2)*, and *sgs3-1* mutant plants. *dcl2-4*, *dcl3-1*, *dcl2-4 dcl3-1*, and *dcl2-4 dcl4-1* mutants do not display obvious vegetative defects, whereas *dcl4-1*, *rdr6 (sgs2-2)*, *sgs3-1*, and *dcl3-1 dcl4-1* mutants have downward-curved leaf margins.

(B) Eight-week-old wild-type Col plant (left), third-generation *dcl2-4 dcl3-1* plants, and third-generation *dcl3-1 dcl4-1* plants exhibiting stochastic phenotypes increasing in severity from left to right. Inflorescence stems were removed to visualize vegetative structures. In the most severe case, *dcl3-1 dcl4-1* plants died before flowering (arrow).

gene, although to different extents [19]. Our systematic analysis of single and double *dcl* mutants also reveals redundancies among DCLs for the production of *trans*-acting siRNAs and heterochromatic siRNAs but not miRNAs. Because the endogenous tasiRNA pathway

and the transgene-induced PTGS pathways function similarly with regard to their requirement for RDR6 and SGS3 [20, 12, 13], these compensatory functions among DCL proteins may explain why single *dcl* mutants were not isolated in forward genetic screens for reactivation of posttranscriptionally silenced transgenes.

When combined with previous studies, our work suggests the following specificities and redundancies of *Arabidopsis* DCL proteins: MicroRNAs do not require the action of any RDR and seem to be produced by DCL1 only ([2–4]; Figure 4B). RDR2-dependent siRNAs are produced primarily by DCL3 [5]. However, DCL4 and DCL2 produce RDR2-dependent siRNAs in the absence of DCL3 (Figure 2B), and some of these siRNAs are functional in that both *dcl2 dcl3* and *dcl3 dcl4* double mutants but not the *dcl3* single mutant exhibit stochastic phenotypes reminiscent of the heterochromatin changes seen in *dmm1* (Figure 3B). RDR6-dependent tasiRNAs are produced primarily by DCL4 (Figure 1C). However, DCL3, DCL2, and perhaps DCL1 produce RDR6-dependent siRNAs in the absence of DCL4 (Figure 2B). Some of these siRNAs probably are functional

Table 1. Rosette Leaf Phenotype of *dcl*, *rdr*, and *sgs* Mutants

Plant	Rosette Leaves 1–2 Length/Width <sup>a</sup>	Rosette Leaf 4 Length/Width <sup>b</sup>
Col	1.27 ± 0.10	1.58 ± 0.08
<i>dcl3-1</i>	1.26 ± 0.11	1.65 ± 0.11
<i>dcl4-1</i>	1.62 ± 0.21	1.84 ± 0.13
<i>dcl3-1 dcl4-1</i>	1.79 ± 0.20	1.94 ± 0.11
<i>sgs3-1</i>	1.88 ± 0.22	1.97 ± 0.11
<i>rdr6 (sgs2-2)</i>	1.60 ± 0.23	1.98 ± 0.10

The average length and width of the first and second rosette leaves of 17-day-old plants<sup>a</sup> and of the fourth rosette leaf of 24-day-old plants<sup>b</sup> were measured, and the average length divided by width and standard deviations are reported. Measurements were taken at the widest and longest point of each leaf. Twelve individual plants were measured for each class.

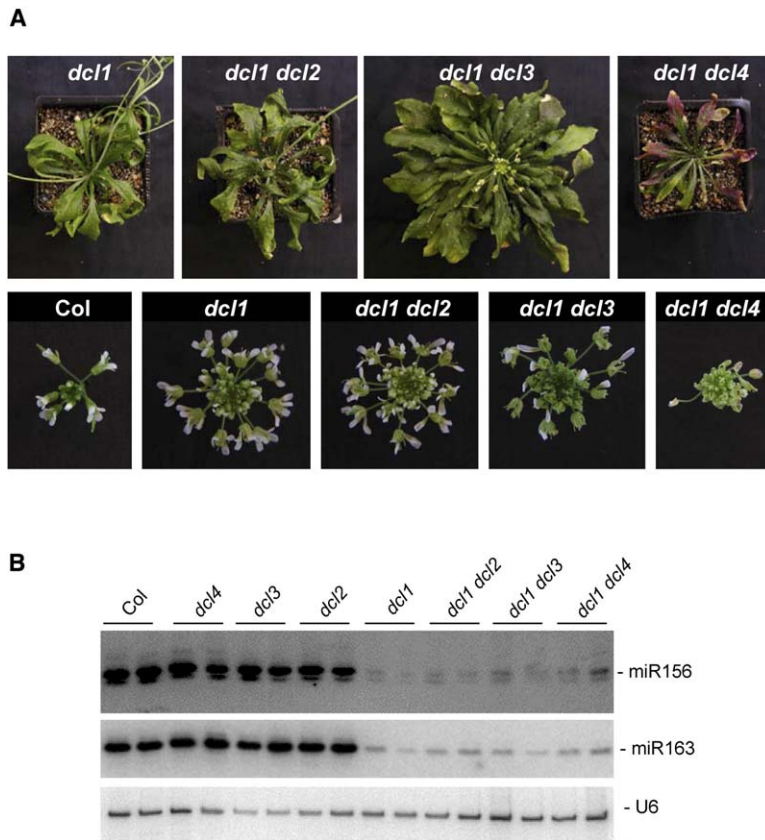


Figure 4. *dcl1 dcl3* and *dcl1 dcl4* Exhibit Phenotypes More Severe than *dcl1*

(A) Vegetative and floral phenotypes of *dcl1-9*, *dcl1-9 dcl2-4*, *dcl1-9 dcl3-1*, and *dcl1-9 dcl4-1* plants. *dcl1-9* and *dcl1-9 dcl2-4* plants flower later than wild-type Col plants, and *dcl1-9 dcl3-1* and *dcl1-9 dcl4-1* flower later than *dcl1-9* and *dcl1-9 dcl2-1* plants. Col plants of the equivalent age had completed a life cycle. *dcl1-9 dcl3-1* plants produced many more leaves than wild-type Col plants, indicative of late flowering, and *dcl1-9 dcl4-1* plants were small and accumulated anthocyanins, indicative of stress. The floral phenotypes of *dcl1-9* and *dcl1-9 dcl2-4* were indistinguishable, whereas *dcl1-9 dcl3-1* and *dcl1-9 dcl4-1* mutants had more severe floral defects, including decreased petal number. An example of a wild-type Col inflorescence at a similar stage is shown at the left.

(B) RNA gel blot analysis of 10 μg total RNA prepared from rosette leaves of two independent plants of the indicated genotype. The blot was probed with DNAs complementary to miR156 and miR163, two miRNAs that are detectable in *dcl1-9*, then reprobated with an oligonucleotide complementary to U6 as a loading control.

because the *dcl3 dcl4* double mutant exhibits molecular and phenotypic similarities to *rdp6* and *sgs3* null mutants impaired in the production of tasiRNAs, whereas the *dcl4* single mutant exhibits a milder phenotype (Table 1; Figures 2C and 3A). Whether DCL3 produces a consequential amount of 21 nt tasiRNAs in plants that have a functional DCL4 remains to be determined. It is possible that by abrogating the function of DCL4, TAS transcripts become available for processing by other DCLs that normally have little access to these RNAs.

It is tempting to assign size classes to the products of each DCL, with DCL1 making the predominantly 21 nt miRNAs [3], DCL2 making the 22–23 nt siRNAs, DCL3 making 24 nt siRNAs [5], and DCL4 making 21 nt tasiRNAs. However, this picture becomes more complicated when we consider some of the *dcl* double mutants. For example, 21 nt tasiRNAs decreased in the *dcl3 dcl4* mutant when compared to the *dcl4* single mutant, suggesting that in some circumstances, DCL3 can make 21 nt siRNAs. Perhaps the product size is determined not only by the DCL but also by the partners of DCL proteins, particularly HYL1 and related DRB proteins [11]. In this scenario, loss of DCL4 would free its partner, DRB4, to interact with DCL3, thereby imparting a shorter length specificity to DCL3. Analysis of small RNA length and accumulation in *drb* mutants and *dcl drb* double mutants will help to shed light on this possibility.

#### Experimental Procedures

*rdp6* (*sgs2-2*), *sgs3-1*, and *dcl3-1* are in the Col ecotype and have been previously described [20, 13, 5]. *dcl1-9* has been backcrossed

five times to Col [13]. *dcl2-4* and *dcl4-1*, identified in the INRA T-DNA collection (FLAG\_451B03 and FLAG\_330A04) [21], are in the Ws ecotype and have been backcrossed two times to Col before crossing to the other *dcl* mutants to generate double mutants. *mir173-1* was identified in the SALK T-DNA collection (SALK\_013393) [22]. Molecular analyses were performed as described before [13], and mRNA hybridization signals were quantified with a Fuji phosphorimager. <sup>32</sup>P end-labeled oligonucleotide probes were:

siRNA02: 5'-GTTGACCAGTCCGCCAGCCGAT-3'  
miR156: 5'-GTGCTCACTCTCTCTGTCA-3'  
miR163: 5'-ATCGAAGTTCCAAGTCCCTCTTCAA-3'  
miR173: 5'-GTGATTCTCTCTGCAAGCGAA-3'  
miR390: 5'-GGCGCTATCCCTCCTGAGCTT-3'  
TAS1-siR480(+): 5'-TACGCTATGTTGGACTTAGAA-3'  
TAS2-siR-F: 5'-AAGTATCATCATTGCTTGA-3'

Pooled <sup>32</sup>P end-labeled oligonucleotide probes for TAS3 were: siR1769 5'-GGGATAG ACAAGGTAGGAGAA-3' and siR1778 5'-GGT CAAGAAAAGGCCTTACAA-3' [14], and 5'-CATCTAGATAAAAACAA TAA-3', 5'-TCATTATTCTCTTTTCTTGA-3', 5'-CCATCTCTTTCTAAAC GTTTT-3', and 5'-ACCTCTAATTCTGTTTCGAGTCA-3' (R. Rajagopalan and D.P.B., unpublished data). Random-primed At5g18040 DNA probe template was PCR-amplified with oligonucleotides At5g18040 forward 5'-GCGTCTCAGCTCAGTATTGGTG-3' and At5g18040 reverse 5'-GAACACATGGAGTCTTGCACCC-3'.

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