Note

DICER-LIKE 1 and *DICER-LIKE 3* Redundantly Act to Promote Flowering via Repression of *FLOWERING LOCUS C* in *Arabidopsis thaliana*

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ABSTRACT

In *Arabidopsis thaliana*, *DICER-LIKE 1* and *DICER-LIKE 3* are involved in the generation of small RNAs. Double mutants between *dicer-like 1* and *dicer-like 3* exhibit a delay in flowering that is caused by increased expression of the floral repressor *FLOWERING LOCUS C*. This delayed-flowering phenotype is similar to that of autonomous-pathway mutants, and the flowering delay can be overcome by vernalization.

THE transition from vegetative to reproductive development is a highly regulated event in the plant life cycle. In Arabidopsis there are several pathways that influence time to flowering including the photoperiod, vernalization, autonomous, and *FRIGIDA* pathways. The photoperiod and vernalization pathways promote flowering in response to day length and the prolonged cold of winter, respectively. The vernalization pathway functions to epigenetically silence the strong floral repressor *FLOWERING LOCUS C* (*FLC*) (Bastow *et al.* 2004; Sung and Amasino 2004). The autonomous pathway acts to constitutively promote flowering by repressing *FLC* expression (for review see Simpson 2004), whereas *FRIGIDA* delays the floral transition by creating a vernalization requirement via upregulating *FLC* expression (Michaels and Amasino 1999; Sheldon *et al.* 1999). Because precise regulation of *FLC* is essential for proper timing of the floral transition, it is not surprising that *FLC* expression is controlled by multiple pathways.

Forward genetic screens have unveiled a large number of genes required for the floral transition (Sung and Amasino 2005). Components of the photoperiod pathway have been identified as mutants that flower at the same developmental stage regardless of the day length. Genes required for vernalization have been identified in screens for mutants that fail to flower rapidly after an extended exposure to cold temperatures. *FRIGIDA* pathway genes have been identified in screens for early-flowering mutants that block the ability of *FRIGIDA* to promote *FLC* expression. Finally, genes in the autonomous pathway have been identified as mutants that flower later than wild type in both inductive and non-inductive photoperiods.

In higher plants, it is common for members of gene families to be functionally redundant. For such gene families, forward genetic screens are less likely to reveal the role of a single gene in a particular developmental process. Recently, Gasciolli *et al.* (2005) used a reverse genetics approach to determine possible functional redundancy among the four member *DICER-LIKE* (*DCL*) gene family. DCLs are ribonucleases that generate small RNA species from double-stranded RNA (Bernstein *et al.* 2001; Hutvagner *et al.* 2001). Each of the DCL enzymes generates predominantly a particular class of small RNA species. *DCL1* is required for microRNA (miRNA) biogenesis (Park *et al.* 2002; Reinhart *et al.* 2002; Kurihara and Watanabe 2004), *DCL2* generates viral small interfering RNAs (siRNA) (Xie *et al.* 2004), *DCL3* forms heterochromatic siRNAs (Xie *et al.* 2004), and *DCL4* is required for transactivating siRNA (ta-siRNA) biogenesis (Dunoyer *et al.* 2005; Gasciolli *et al.* 2005; Xie *et al.* 2005; Yoshikawa *et al.* 2005). Although *DCL1–4* have predominant roles in generating specific small RNA species, these *DCLs* can also have compensating functions (Gasciolli *et al.* 2005; Blevins *et al.* 2006; Deleris *et al.* 2006). For example, *TAS1-3* mRNA levels in *dcl4* mutants are lower than those in Columbia (Col), whereas levels in *dcl2* or *dcl3* are indistinguishable from those in wild type. However, double mutants between *dcl4* and either *dcl2* or *dcl3* result in a further loss of *TAS1-3* mRNA expression, indicating that in the absence of *DCL4*, *DCL2* and *DCL3* process siRNAs that are otherwise primarily processed by *DCL4* (Gasciolli *et al.* 2005). Thus, double and triple *dcl* mutant combinations, created

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in the Col accession, result in phenotypes not observed in the single mutants (Gasciolli *et al.* 2005). For example, a double mutant between a weak *dcl1* allele (*dcl1* null alleles are lethal; Schauer *et al.* 2002) and *dcl3* results in defective floral structures, an extreme delay in flowering time, and sterility (Gasciolli *et al.* 2005).

We explored the basis of the delayed-flowering phenotype of *dcl1;dcl3* double mutants in Col. *dcl1;dcl3* double mutants form many more rosette leaves than wild type from the primary shoot apical meristem in inductive photoperiods (Figure 1, A and B) as well as in noninductive photoperiods (Figure 1C). However, in a range of single mutants with lesions in genes required for small RNA production, there were no substantial effects on flowering behavior (Figure 1A). At a molecular level, *dcl1;dcl3* double mutants contain increased levels of *FLC* mRNA (Figure 1D). Therefore, *dcl1;dcl3* double mutants resemble mutants in the autonomous pathway. The delayed-flowering phenotype of autonomous-pathway mutants can be overcome by a vernalizing cold treatment or genetically by loss of *FLC* function (Michaels and Amasino 2001). To determine if the late-flowering phenotype of the *dcl1;dcl3* double mutant is responsive to vernalization, seedlings were exposed to 40 days of cold (4°) and transferred to long days for assessment of flowering time. *dcl1;dcl3* double mutants flowered rapidly after an extended exposure to cold (Figure 1A). In addition, the *dcl1;dcl3* delayed-flowering phenotype was suppressed by the *flc-3* mutation, as *dcl1;dcl3;flc-3* triple mutants flowered with the same number of rosette leaves as *flc-3* single mutants (Figure 1E). Therefore, *DCL1* and *DCL3* share a functionally redundant role in *FLC* repression and together could be considered a new component of the autonomous pathway.

In the Landsberg accession, mutations in *DCL1* alone result in a slight delay in flowering time (Ray *et al.* 1996; Liu *et al.* 2004). This is in contrast to Col, which displays no clear flowering delay caused by the *dcl1* single mutant under our growth conditions (Figure 1, A and C). The flowering delay in *dcl1* in Landsberg may be due to a failure to direct siRNA-mediated heterochromatin formation to a transposon located in the first intron of *FLC* (Liu *et al.* 2004); both the parental Landsberg accession and the derived *erecta* strain have a transposon insertion in *FLC*, but the Col allele of *FLC* does not have this transposon insertion (Michaels *et al.* 2003). Therefore, a measurable affect on flowering time of a *dcl1* single mutant may be unique to accessions in which *FLC* expression is attenuated by a transposon insertion. Despite the lack of a flowering phenotype in the Col *dcl1* single mutant, there appears to be a slight increase in *FLC* mRNA levels in *dcl1* and *dcl3* single mutants, although the increase is much less than that in the *dcl1;dcl3* double mutant (Figure 1D). Swiezewski *et al.* (2007) also noted an increase in *FLC* mRNA levels in the *dcl3* single mutant in Col, but did not report any change of flowering behavior in *dcl3 vs.* wild type.
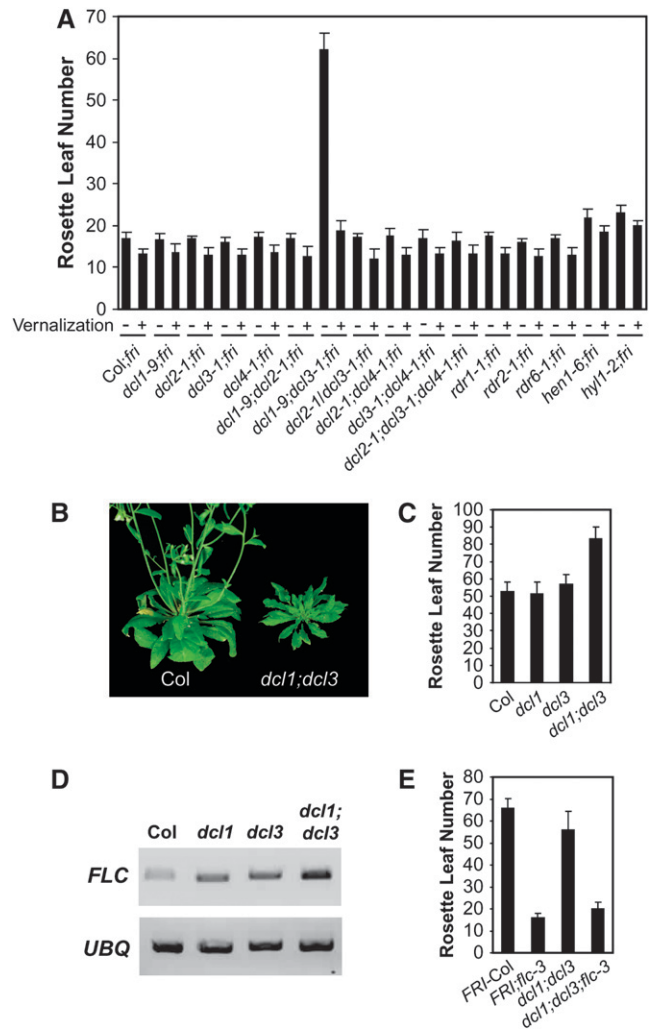

Figure 1.—*dcl1;dcl3* double mutants result in phenotypes similar to autonomous-pathway mutants. (A) Mutations in genes encoding components required for small RNA biogenesis do not result in a strong effect on flowering time except for the *dcl1;dcl3* double mutant. The *dcl1;dcl3* double mutant has a late-flowering phenotype that responds to vernalization. (B) The *dcl1;dcl3* double mutant phenotype includes late flowering, altered leaf shape, and a smaller rosette size. Data and images in A and B are from plants grown in inductive long-day photoperiods (16 hr light/8 hr dark). (C) The *dcl1;dcl3* double mutant flowers later than Col wild type in noninductive photoperiods (8 hr light/16 hr dark) indicating that there is not a defect in detecting a noninductive photoperiod. (D) Steady-state levels of *FLC* mRNA are increased in *dcl1;dcl3* double mutants. RNA was isolated from the shoot apices of plants after they had formed a total of six visible leaves. (E) The late-flowering phenotype of the *dcl1;dcl3* double mutant is completely suppressed by the *flc-3* mutation.

dcl1;dcl3 double mutants exhibit altered developmental timing. For example, abaxial trichomes (often used as a marker of the juvenile to adult transition; Willmann and Poethig 2005) appear on the second to third true leaves of *dcl1;dcl3* double mutants, whereas abaxial trichomes do not appear until the fifth and sixth leaves of wild-type Col. In addition, the *dcl1;dcl3* double mutants develop at a slower rate than wild type (for example,

when leaf 13 becomes visible in wild type, leaf 7 is emerging in the *dcl1;dcl3* double mutant). These results are consistent with previous reports of the involvement of miRNAs in regulating phase change in Arabidopsis (WILLMANN and POETHIG 2005).

Given the role of small RNAs in repression of gene activity, we further explored the possibility of their involvement in epigenetic silencing of *FLC* by vernalization. We genetically introduced a range of mutants affected in either miRNA or siRNA biogenesis into the vernalization-requiring *FRI*–Col genetic background (MICHAELS and AMASINO 1999) and assayed their vernalization response. All mutants tested, including the *dcl1;dcl3* double mutant, responded like wild type to a vernalizing cold treatment (Figure 2A). In addition, we monitored *FLC* expression during a vernalization time course in *dcl1;dcl3* using a reporter line. GUS expression was detected throughout the shoot and root apex as well as the vasculature prior to vernalization, but expression was repressed in seedlings immediately after 40 days of cold. This repression was maintained as plants grew in warm temperatures following cold exposure (Figure 2B). Thus, we found no evidence that known genes involved in small RNA metabolism (listed in Figure 2) play a role in initiation or maintenance of the vernalized state.

Recently it was reported that *DCL3, RDR2,* and *NRPD2* are involved in directing small RNA-mediated heterochromatin formation to a target site downstream of the region corresponding to the mature *FLC* transcript; mutations in these genes prevent production of a 24-nucleotide small RNA that is complementary to this 3′ site (SWIEZEWSKI *et al.* 2007). Additionally, a T-DNA insertion that disrupts this small RNA binding site and prevents production of this small RNA causes a slight delay in flowering (SWIEZEWSKI *et al.* 2007). Although *dcl3, rdr2,* and *nrpd2* also prevent production of this small RNA, the flowering time of these mutants was not reported in SWIEZEWSKI *et al.* (2007). As noted above, we do not observe any change in the flowering behavior of the *dcl3* or *rdr2* mutant *vs.* wild type, although we do observe a slight increase in *FLC* mRNA in the *dcl3* single mutant. It is possible that this increase in *FLC* mRNA may be below a threshold required to cause a change in flowering time or that this increase results from an expansion in the spatial expression of *FLC* to cells that do not contribute to the timing of flowering.

We assessed whether a range of genes required for small RNA production were required for the correct spatial expression of *FLC*. For these experiments we crossed mutants defective in either siRNA or miRNA biogenesis [*dcl1, dcl2, dcl3, dcl1;dcl3, rdr1, rdr2, rdr6 (RNA DEPENDENT RNA POLYMERASE),* hyl1 (*HYPONASTIC LEAVES 1*)] to a line containing an *FLC:GUS* reporter that includes the 3′ target site for the 24-nucleotide small RNA mentioned above. In segregating F$_2$ populations, for the mutants listed above, no altered *FLC:GUS* expression patterns were detected (data shown only for
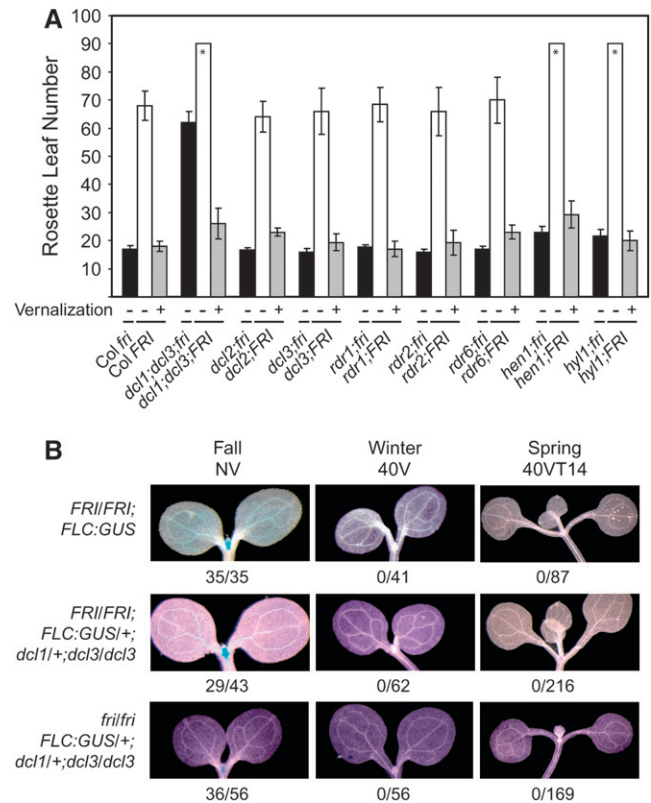
FIGURE 2.—The *dcl1;dcl3* double mutant does not block vernalization in a winter-annual genetic background. (A) Eight mutants with lesions in components required for small RNA biogenesis were crossed into the vernalization-requiring Col *FRI* genetic background (MICHAELS and AMASINO 1999) and examined for their flowering phenotypes before and after vernalization. All mutants flowered much more rapidly after exposure to 40 days of cold. Data representing the flowering-time phenotypes in Col (which lacks *FRI* activity) are identical to the data presented in Figure 1A and are presented for comparison. The asterisk indicates that plants produced >90 rosette leaves. Accurate leaf counts for these genotypes were difficult to obtain due to developmental defects in combination with the late-flowering phenotype. Solid bars represent genotypes in Col without exposure to cold, open bars represent genotypes in Col *FRI* without vernalization, and shaded bars represent genotypes in Col *FRI* with vernalization. (B) An *FLC:GUS* reporter line was used to monitor the expression of *FLC* during a vernalization time course. The *FLC:GUS* construct consists of a 15-kb genomic region around *FLC* that rescues the *flc-3* mutant. The *GUS* coding region was inserted into exon 4 of *FLC* within this construct (MICHAELS and AMASINO 2000). GUS expression was examined in seedlings exposed to no cold (NV, Fall), 40 days of (4°) cold (40V, Winter), and 40 days of cold followed by 14 days of warm (40VT14, Spring). The parental genotypes of populations screened for GUS activity are listed on the left. The number of seedlings with GUS activity relative to the total examined are noted below each panel (seedlings were not selected for the GUS transgene).

the *dcl1;dcl3* double mutant, Figure 2B). Considering the substantial increase in *FLC* mRNA observed in the *dcl1;dcl3* double mutant, this would be the most likely genotype in which there might be such an expansion in spatial expression. In summary, we found no evidence

for a role for these genes in controlling spatial expression of *FLC* using this reporter gene assay.

Except for the 24-nucleotide RNA reported in SWIEZEWSKI *et al.* (2007), small RNA species at the *FLC* locus have not yet been observed on RNA blots or identified in deep-sequencing projects (GUSTAFSON *et al.* 2005; LU *et al.* 2005, 2006). Nevertheless, *DCL1* and *DCL3* may be required to generate additional small RNAs that target *FLC*. Indeed, the lack of a strong flowering phenotype in lines that lack the 24-nucleotide RNA (SWIEZEWSKI *et al.* 2007) compared to the strong flowering phenotype of the *dcl1;dcl3* double mutant indicates that, if a meaningful level of *FLC* repression is due to small RNAs targeted to *FLC*, this repression may be due to the collective effects of multiple small RNA species. Perhaps *DCL1* and *DCL3* are involved in the formation of different *FLC*-targeted small RNAs. Alternatively, *DCL1* and *DCL3* may be involved in down regulating an activator of *FLC*.

Regardless, the data presented here show that multiple DICERs are required for proper control of *FLC* mRNA levels. The specific role of *DCL1* and *DCL3* in flowering time control awaits the identification of the specific small RNA(s) that are altered in the *dcl1;dcl3* double mutant.

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Communicating editor: B. BARTEL