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Authors

Huo, Heqiang
Wei, Shouhui
Bradford, Kent J

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DELAY OF GERMINATION1 (*DOG1*) regulates both seed dormancy and flowering time through microRNA pathways

Heqiang Huo[a], Shouhui Wei[b], and Kent J. Bradford[a,1]

[a]Department of Plant Sciences, Seed Biotechnology Center, University of California, Davis, CA 95616; and [b]Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

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Seed germination and flowering, two critical developmental transitions in plant life cycles, are coordinately regulated by genetic and environmental factors to match plant establishment and reproduction to seasonal cues. The *DELAY OF GERMINATION1* (*DOG1*) gene is involved in regulating seed dormancy in response to temperature and has also been associated genetically with pleiotropic flowering phenotypes across diverse *Arabidopsis thaliana* accessions and locations. Here we show that *DOG1* can regulate seed dormancy and flowering times in lettuce (*Lactuca sativa, Ls*) and *Arabidopsis* through an influence on levels of microRNAs (miRNAs) miR156 and miR172. In lettuce, suppression of *LsDOG1* expression enabled seed germination at high temperature and promoted early flowering in association with reduced miR156 and increased miR172 levels. In *Arabidopsis*, higher miR156 levels resulting from overexpression of the *MIR156* gene enhanced seed dormancy and delayed flowering. These phenotypic effects, as well as conversion of *MIR156* transcripts to miR156, were compromised in *DOG1* loss-of-function mutant plants, especially in seeds. Overexpression of *MIR172* reduced seed dormancy and promoted early flowering in *Arabidopsis*, and the effect on flowering required functional *DOG1*. Transcript levels of several genes associated with miRNA processing were consistently lower in dry seeds of *Arabidopsis* and lettuce when *DOG1* was mutated or its expression was reduced; in contrast, transcript levels of these genes were elevated in a *DOG1* gain-of-function mutant. Our results reveal a previously unknown linkage between two critical developmental phase transitions in the plant life cycle through a DOG1–miR156–miR172 interaction.

seed dormancy | flowering | *DOG1* | miRNA | lettuce

The life cycles of flowering plants are characterized by distinct phase transitions such as from seed to seedling (germination) or from vegetative to reproductive development (flowering) (1). The timing of germination and flowering both require precise environmental sensing and integrated responses to multiple inputs so that developmental transitions can be accurately matched to seasonal conditions (1–3). Seeds use temperature as a signal of the seasonal and current environmental conditions to determine opportune times to germinate with respect to the potential for seedling survival (2, 4, 5). Similarly, in many plants the transition from vegetative to floral development occurs in response to environmental cues, particularly temperature and day length (6, 7). Ecological and evolutionary studies have found that seed germination and flowering traits within species are coadapted across habitat ranges (8–11). Seed dormancy and germination are regulated primarily by the antagonistic actions of the plant hormones gibberellin (GA; promotive) and abscisic acid (ABA; inhibitory), whose synthesis and action vary in response to environmental signals (12). Recent studies indicate that canonical genes regulating flowering, such as *FLOWERING LOCUS T* (*FT*) and *FLOWERING LOCUS C* (*FLC*), are also involved in the transition from seed dormancy to germination (13–16), suggesting that seed dormancy and flowering may be coordinately regulated through overlapping molecular pathways.

In *Arabidopsis*, expression of the *DELAY OF GERMINATION1* (*DOG1*) gene responds to seed maturation temperature and determines the depth of seed dormancy (2, 3, 5, 17–20). Deeper dormancy of freshly harvested seeds is associated with high *DOG1* transcript levels, which decrease in after-ripened (nondormant) and germinating seeds (18, 19). Functional analyses have shown that *DOG1* and ABA are essential for establishing primary seed dormancy (18, 19, 21), although *DOG1* can act independently of ABA to delay germination of less dormant seeds (20). Overexpression of *DOG1* also increases the sensitivity of seed germination to inhibition by warm temperatures (5, 20). In addition, genome-wide association and genetic linkage mapping studies revealed that the *DOG1* locus was associated with flowering phenotypes across diverse accessions and locations (22, 23). However, the mechanism of *DOG1* action on seed dormancy or flowering remains unknown.

Lettuce (*Lactuca sativa* L.) is a major leafy vegetable derived from a progenitor species (*Lactuca serriola* L.) having a winter annual lifecycle, normally flowering in spring after overwintering as a compact rosette. Lettuce seeds display little primary dormancy, but germination is strongly inhibited by warm temperatures during imbibition, a type of relative dormancy termed thermoinhibition (24, 25), resulting in reduced crop establishment during warm seasons (26). Previously, we genetically mapped and functionally characterized the role of *L. sativa 9-cis-EPOXYCAROTENOID DIOXYGENASE4* (*LsNCED4*), a gene encoding a key regulated enzyme in ABA biosynthesis, in lettuce seed thermoinhibition (24, 25, 27). Expression of this gene is induced in lettuce seeds by

Significance

Annual plants adapt to their environments by matching their life cycles, particularly seed germination and flowering, to the appropriate seasons. Although genetic evidence has suggested connections among genes regulating seed dormancy and flowering, specific mechanisms for such coordination are unknown. We report that a gene [*DELAY OF GERMINATION1* (*DOG1*)] involved in determining the depth of seed dormancy, and therefore the seasonal timing of germination, also influences the timing of flowering in *Arabidopsis* and lettuce. We further show that this gene acts through influencing the production of microRNAs that govern the progression of developmental phase transitions through the plant life cycle, providing a molecular genetic mechanism for the coordinate adaptation of seed dormancy and flowering phenotypes to environmental conditions.

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[1]To whom correspondence should be addressed. Email: kjbradford@ucdavis.edu.

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imbibition at high temperatures, maintaining high ABA content and preventing germination. A similar role was demonstrated for the homologous gene *Arabidopsis thaliana NCED9* (*AtNCED9*) (28). The mechanism by which these genes are regulated by high temperature remains unknown (29).

Here, we report that suppression of *DOG1* expression by RNAi in lettuce or mutation of *DOG1* in *Arabidopsis* markedly increased the maximum temperature for seed germination. In addition, *DOG1*-RNAi lettuce plants flowered much earlier than control plants. Further analyses in lettuce and *Arabidopsis* showed that these phenotypes are associated with reduced conversion of primary *MIR156* transcript into the microRNA (miRNA) miR156. Effects of overexpression of *MIR156* on flowering and seed dormancy were greatly compromised in loss-of-function *dog1-3* mutants of *Arabidopsis*. The expression of several miRNA processing genes was altered in seeds of *dog1-3* mutants or *DOG1*-RNAi lines, possibly associated with the reduced conversion of *MIR156* to miR156. In contrast, a gain-of-function *dog1-5* mutation that enhanced seed dormancy resulted in increased expression of miRNA processing genes. As have been described previously for flowering (30, 31) and seed and seedling development (32, 33), follow-on effects of modification of miR156 levels on miR172 abundance, or direct overexpression of *MIR172*, also affected seed dormancy. Our work provides a potential mechanism for DOG1 action and demonstrates a previously unknown linkage between seed dormancy and flowering phenotypes through a DOG1–miR156–miR172 interaction.

Results

***DOG1* Influences Seed Thermoinhibition in Lettuce.** To explore whether lettuce homologs of *AtDOG1* are involved in regulation of seed thermoinhibition, cDNAs with homology to *DOG1* were isolated from four genotypes of lettuce having differing germination thermotolerance. Seeds of cv. Salinas (*L. sativa*; termed Sal) and PI261653 (*Lactuca saligna*; termed Saligna) were unable to germinate well at 32 °C, whereas seeds of two thermotolerant genotypes, accession US96UC23 (*L. serriola*) and PI251246 (*L. sativa*; termed PI), germinated fully at this temperature (*SI Appendix*, Fig. S1*A*). Sequences of *LsDOG1* cDNAs from the four genotypes were highly homologous and shared ∼50% overall protein amino acid sequence similarity with *Arabidopsis* DOG1 (54.7% similarity within DOG1 domain regions; *SI Appendix*, Fig. S2 and Table S1). *LsDOG1* is highly expressed in mature lettuce seeds, whereas its transcript level is much lower in leaves and roots (*SI Appendix*, Fig. S1*C*). Its expression in seeds decreased as seed maturation temperature increased (*SI Appendix*, Fig. S1*D*), as is also the case for *AtDOG1* expression in *Arabidopsis* seeds (17, 18).

Suppression of *LsDOG1* expression in Sal lettuce through RNAi caused 56% and 73% reductions in transcript abundance in two independent lines (Fig. 1*A*). Seeds of these *DOG1*-RNAi lines were able to germinate fully at 32 °C, whereas seeds of the segregated nontransgenic control line (i.e., Sal) were completely inhibited (Fig. 1 *B* and *C*). In contrast, ectopic expression of *LsDOG1* under the native Sal *DOG1* promoter (P_{DOG1}:*LsDOG1*) in the thermotolerant PI lettuce line resulted in increased *DOG1* transcripts in mature dry seeds and enhanced seed thermoinhibition at 30 °C (Fig. 1 *D*–*F*). A functional homolog of *DOG1* is therefore present in lettuce and is involved in regulating germination thermoinhibition.

***DOG1* Influences Seed Thermoinhibition in *Arabidopsis*.** Functional DOG1 action in *Arabidopsis* promotes seed dormancy and extends after-ripening periods required for dormancy alleviation (18, 19), whereas fresh seeds of loss-of-function *dog1* mutants can fully germinate without after-ripening or cold stratification (17, 19). To test whether *DOG1* might be involved in regulating germination thermoinhibition in *Arabidopsis*, we imbibed seeds of the Columbia (Col) *dog1-3* mutant (Salk_000867) at 32 °C. Mutant seeds germinated more than 80% whereas WT seeds germinated less than 20% at that temperature (*SI Appendix*, Fig. S1*B*). Molecular complementation showed that coding regions of *LsDOG1* genes from thermosensitive and thermotolerant lettuce genotypes were able to rescue the thermoinhibition phenotype when expressed in *Arabidopsis dog1-3* plants (*SI Appendix*, Fig. S1*B*), indicating that these homologs are all functional and that their role in regulation of seed thermoinhibition is conserved. The presence of functional *DOG1* alleles in US96UC23 and PI251246 is consistent with lack of association of this locus with quantitative trait loci for high-temperature germination in these genotypes (27, 34).

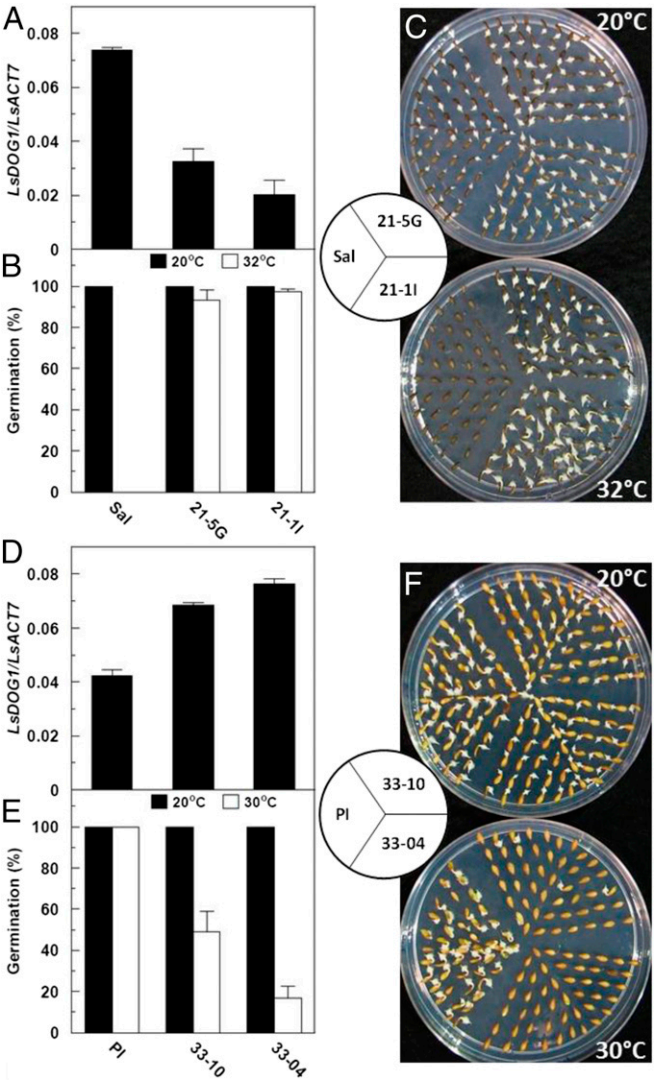

Fig. 1. Regulation by *LsDOG1* of lettuce seed thermoinhibition. (*A*) Relative *LsDOG1* mRNA levels in dry seeds of Sal lettuce and its *LsDOG1*-RNAi lines, 21–5G and 21–1I. (*B* and *C*) Seed germination of Sal, 21–5G, and 21–1I lines at 20 and 32 °C. (*D*) Relative *LsDOG1* mRNA levels in dry seeds of PI lettuce and its *LsDOG1* over-expression lines, 33–10 and 33–04. (*E* and *F*) Seed germination of PI, 33–10, and 33–04 lines at 20 and 30 °C. In *D* and *F*, seeds at 20 °C were imbibed for 16 h, whereas seeds at 32 and 30 °C were imbibed for 30 h. Error bars represent SE (n = 3).

Previous work in lettuce and *Arabidopsis* showed that *NCED* genes encoding a key enzyme in the ABA biosynthetic pathway are involved in thermoinhibition of germination (24, 28). We tested the interactive effects of *DOG1* and ABA on seed thermoinhibition by overexpressing *LsDOG1* in an *atnced6-1 atnced9-1* double mutant that displays strong germination thermotolerance

as a result of reduced ABA biosynthesis (24, 28). Overexpression of *LsDOG1* restored a thermosensitive phenotype to *atnced6-1atnced9-1* seeds at 32 °C (*SI Appendix*, Fig. S1*E*), indicating that *DOG1* and ABA can induce thermoinhibition via parallel pathways. We also generated a *dog1-3* × *nced9-1* double mutant to further examine the interactive effect of both genes on thermoinhibition. At a higher temperature (35 °C), seeds of the single mutants of *dog1-3* and *nced9-1* and of the double mutant *nced6-1 nced9-1* were fully inhibited from germinating, whereas *dog1-3 nced9-1* seeds germinated 95% (*SI Appendix*, Fig. S1*F*). This additional effect of *dog1-3* in the presence of ABA biosynthesis mutants is in contrast to reports that ABA biosynthesis and functional *DOG1* are required for induction of primary dormancy (18, 19). However, consistent with previous reports (17–20), overexpression of *LsDOG1* under the CaMV35S promoter in *Arabidopsis* Col plants resulted in deep seed dormancy that could not be alleviated by the ABA biosynthesis inhibitor fluridone, but could be alleviated by GA (*SI Appendix*, Fig. S3). Thus, *DOG1* and ABA can regulate seed primary dormancy and thermoinhibition, and may act in concert or independently depending on the conditions.

***DOG1* Influences Flowering Time in Lettuce Through Effects on miR156.** Suppression of *DOG1* expression in Sal lettuce caused early flowering (Fig. 2 *A*–*C*), as occurred also in wheat (35). Plants from seeds of seven independent transgenic *DOG1*-RNAi lines that germinated at 32 °C and from segregated control (nontransgenic) seeds were grown in the greenhouse under relatively noninductive day-length conditions for flowering in fall 2013. Salinas *DOG1*-RNAi lines flowered in ∼120–150 d, whereas control lines did not flower until >200 d (Fig. 2*B*). Similar results were observed in two *DOG1*-RNAi lines that were grown in spring 2014, although these plants all flowered earlier because of lengthening days (Fig. 2*C*).

To assess which flowering regulatory pathways were affected by suppression of *LsDOG1* expression, transcript levels of key genes in the different pathways were investigated (*SI Appendix*, Fig. S4). Consistent with early flowering, transcripts of the floral promoter *FT* were almost 25-fold higher in young *DOG1*-RNAi lettuce leaves than in similar leaves of the control line (*SI Appendix*, Fig. S4). Expression patterns of other flowering-related genes were not consistent with effects on the photoperiod-dependent, GA, vernalization, or autonomous flowering pathways (*SI Appendix*, *Supplementary Text* and Figs. S4 and S5). However, transcripts of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) *3*, *4*, and *9* were increased in leaves of the *DOG1*-RNAi line compared with its control line (*SI Appendix*, Fig. S4). Furthermore, the relative mRNA levels of *SPL3* and *4* were significantly elevated in apical meristems of *DOG1*-RNAi lines, although *SPL9* expression was not affected (Fig. 2*D*). *SPL* gene expression is inhibited by DELLA proteins (*SI Appendix*, Fig. S5), so a decrease in them could result in increased *SPL* expression (36). However, transcripts of genes encoding two major DELLA proteins (*RGL1* and *RGA*) were unchanged or elevated in *DOG1*-RNAi leaves (*SI Appendix*, Fig. S4), making it unlikely that this is the cause of increased *SPL* expression. *SPL* transcripts are targeted for degradation by miR156, a regulator of developmental phase transitions in plants, and SPL proteins can induce expression of *FT* and other genes promoting flowering (30). SPLs also promote production of miR172, a positive regulator of maturation (juvenile to adult) and floral phase transitions (30). Thus, if this mechanism is operative, we would expect early flowering and high *SPL* transcript levels to be associated with lower miR156 and higher miR172 levels. Consistent with this, miR156 abundance in apical meristems of *DOG1*-RNAi lines was only one third of that in the control line, whereas miR172 levels were approximately doubled (Fig. 2*D*). Thus, the early flowering in *DOG1*-RNAi lettuce plants may be a result of a reduction in miR156 levels, which would result in increased levels of the floral-promoting SPLs and of miR172 (*SI Appendix*, Fig. S5).

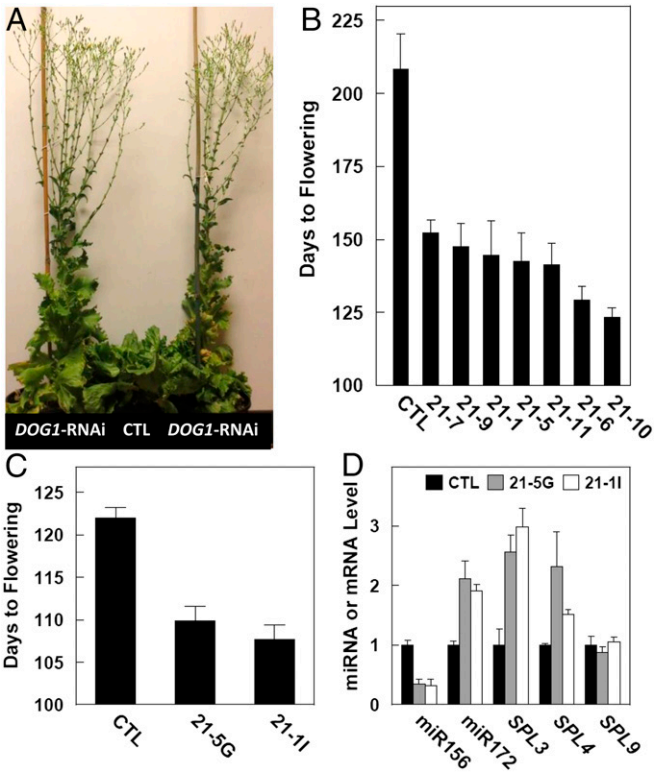

Fig. 2. Regulation of flowering time in lettuce by *DOG1*. (*A*) Two independent early-flowering lettuce lines (*DOG1*-RNAi) and the control Sal line (CTL) are shown at 120 d after seeding. (*B*) Times to flowering of F2 plants of seven independent transgenic lines and a segregated control line (CTL). Plants were sown in September 2013 and were genotyped for the presence of the transgene ($n \geq 20$ plants per line). (*C*) Times to flowering of two homozygous *DOG1*-RNAi lines and a control line that were sown in late February 2014 ($n \geq 20$ plants per line). (*D*) Relative levels of miR156 and miR172 and mRNA levels of *SPL3*, *SPL4*, and *SPL9* in apical meristems of 6-wk-old control (CTL) and *DOG1*-RNAi (21-5G, 21–1I) lettuce plants; levels were first normalized by *LsUBQ10* and are shown relative to those in control for each gene or miRNA. Error bars represent SE ($n = 3$).

***DOG1* Can Influence Flowering in *Arabidopsis*.** In contrast to early flowering when *LsDOG1* expression was suppressed in lettuce, no effects on flowering times were observed in *Arabidopsis dog1-3* mutant plants under long-day or short-day conditions (Table 1 and *SI Appendix*, Table S2). However, when miR156 levels were elevated by overexpression of the gene encoding it, *MIR156* (Col-*35S*:*LsMIR156*), flowering times were significantly delayed (Fig. 3*A*), with a majority of plants beginning to flower more than 50 d after germination (DAG) and having more than 36 rosette leaves; in contrast, flowering of homozygous *dog1-3* plants also expressing *35S*:*LsMIR156* was only slightly delayed compared with Col and *dog1-3* plants (Table 1 and *SI Appendix*, Table S2). Further evaluation of 48 *dog1-3*–*35S*:*LsMIR156* and 72 Col-*35S*:*LsMIR156* independent transgenic lines showed that 87% of the former had flowered within 50 DAG and 95% had less than 30 leaves at flowering, whereas 72% of the latter required more than 50 d to flower and 83% of flowering lines had more than 30 leaves (Table 1 and *SI Appendix*, Table S2). Although it is possible that sequence homology between the CaMV35S promoter and the T-DNA insertion in *dog1-3* could silence the transgene (37), this result was also reproduced in Landsberg *erecta* (L*er*) and its γ-irradiation–induced *dog1-1* mutant (19), although the

Table 1. *DOG1* effect on flowering times (days to flowering) of *Arabidopsis* genotypes

Experiment	Hm/Ht	PN	Interval and incidence of flowering plants							
			Experiment 1 (LD, 22 °C, 135 μmol·m^{-2}·s^{-1})							
Days to flowering			21–25	26–30	31–35	36–40	41–50	51–60	61–70	>71
			Flowering plants, %							
Col	Hm	72	6.9	91.7	1.4	—	—	—	—	—
Col-*35S:LsMIR156-G*	Hm	65	—	—	—	—	1.5	32.3	27.7	38.5
Col-*35S:LsMIR156-C*	Hm	53	—	—	—	—	28.3	64.1	1.9	5.7
Col-*35S:AtMIR156*	Hm	70	—	—	—	—	—	7.1	15.7	77.2
dog1-3	Hm	61	3.3	55.7	41.0		—	—	—	—
dog1-3–35S:LsMIR156-I	Hm	69	—	27.5	58.0	14.5	—	—	—	—
dog1-3–35S:LsMIR156-G	Hm	68	—	11.8	83.8	4.4	—	—	—	—
dog1-3 × *35S:AtMIR156-A*	Hm	63	—	9.5	41.3	49.2	—	—	—	—
dog1-3 × *35S:AtMIR156-G*	Hm	65	—	6.2	76.9	16.9	—	—	—	—
nced9-1	Hm	48	—	77.1	22.9	—	—	—	—	—
nced9-1×*AtMIR156#7*	Hm	40	—	—	—	—	2.5	5	17.5	75
			Experiment 2 (LD, 21 °C, 100 μmol·m^{-2}·s^{-1})							
Days to flowering			21–25	26–30	31–35	36–40	41–50	51–60	61–70	>71
			Flowering plants, %							
Col	Hm	48	—	66.7	33.3	—	—	—	—	—
Col-*35S:LsMIR156*	Ht	72	—	—	—	1.4	26.4	8.3	5.6	58.3
dog1-3	Hm	36	—	41.7	58.3	—	—	—	—	—
dog1-3–35S:LsMIR156	Ht	48	—	—	8.4	33.3	45.8	12.5	—	—
dog1-5	Hm	20	—	55	45	—	—	—	—	—
dog1-5–35S:LsMIR156	Ht	28	—	—	—	—	7.1	7.1	21.4	64.4
nced9-1	Hm	47	—	56.3	43.7	—	—	—	—	—
nced9-1–35S:LsMIR156	Ht	37	—	—	—	5.4	16.2	8.1	16.2	54.1
			Experiment 3 (LD, 22 °C, 135 μmol·m^{-2}·s^{-1})							
Days to flowering			21–25	26–30	31–35	36–40	41–50	51–60	61–70	>71
			Flowering plants, %							
Ler	Hm	48	20.8	79.2	—	—	—	—	—	—
Ler-*35S:LsMIR156*	Ht	63	—	4.8	14.3	6.3	55.5	14.3	4.8	—
dog1-1	Hm	57	17.5	82.5	—	—	—	—	—	—
dog1-1–35S:LsMIR156	Ht	52	—	40.4	50	9.6	—	—	—	—
			Experiment 4 (SD, 22 °C, 135 μmol·m^{-2}·s^{-1})							
Days to flowering			61–70		71–80	81–90		91–100	>100	
			Flowering plants, %							
Col	Hm	37	—		—	10.8		32.4	56.8	
Col-*35S:LsMIR172*	Ht	77	10.4		10.4	18.2		44.2	16.8	
dog1-3	Hm	64	—		—	9.5		29.7	60.8	
dog1-3–35S:LsMIR172	Ht	100	—		1.0	8.0		42.0	49.0	

Hm, homozygous; Ht, heterozygous; LD, long day; PN, number of plants when homozygous or number of independent transgenic individuals when heterozygous; SD, short day. Dashes indicate absence of plants in that category.

delay in flowering time caused by *35S:LsMIR156* in L*er* was not as strong as in Col (Table 1 and *SI Appendix*, Table S2). As in *Arabidopsis*, overexpression of *LsMIR156* also greatly delayed flowering in Sal lettuce (*SI Appendix*, Fig. S6). To test whether this phenomenon is specific to lettuce *MIR156*, we crossed *dog1-3* with a Col-*35S:AtMIR156* line that exhibits delayed flowering (38). Effects on flowering were essentially identical to those for *LsMIR156* (Fig. 3*A*, Table 1, and *SI Appendix*, Table S2). The lack of response of *dog1-3* plants to overexpression of *LsMIR156* or *AtMIR156* was evidently caused by reduced conversion of *pri-MIR156* transcripts to miR156, as *LsMIR156* (or *AtMIR156*) transcripts were present at equal or greater levels in *dog1-3* meristems (Fig. 3*B* and *SI Appendix*, Fig. S7*F*) or leaves (*SI Appendix*, Fig. S7*A*) as in Col tissues also expressing *LsMIR156* or *AtMIR156*. The levels of miR156 in *dog1-3–35S:LsMIR156* meristems were only 28% or 13% of those in 25-d-old apical meristems or leaves, respectively, of the Col-*35S:AtMIR156* plants (Fig. 3*B* and *SI Appendix*, Fig. S7*B*). This was associated with an increase in miR172 and *SPL3*, *4*, *5*, and *9* transcripts in *dog1-3–35S:LsMIR156* meristems and leaves (Fig. 3*B* and *SI Appendix*, Fig. S7 *C–E*). Similar differences in miR156 and miR172 levels were also observed in Col and *dog1-3* meristems in response to overexpression of *AtMIR156* (*SI Appendix*, Fig. S7*F*).

It was recently reported that *dog1-5* (SALK_022748) is a gain-of-function mutant in which the level of DOG1 protein is greatly increased and seed dormancy is enhanced compared with Col WT (39), as we have confirmed here (*SI Appendix*, Fig. S8). In this case, the mutation in *DOG1* did not negate the effect of *MIR156* overexpression on delaying flowering, but rather somewhat enhanced it; 93% of independent *dog1-5-LsMIR156* transgenic lines required more than 50 DAG to flower compared with 72% of Col-*LsMIR156* plants, with similar data for numbers of leaves at flowering (Table 1 and *SI Appendix*, Table S2). Interestingly, as for *dog1-3*, we did not observe a significant difference in flowering time for *dog1-5* plants in the absence of *MIR156* overexpression (Table 1 and *SI Appendix*, Table S1). Thus, in contrast to lettuce, DOG1 regulation of flowering time in *Arabidopsis* is evident only when *MIR156* expression is enhanced.

ABA has also been reported to be involved in floral transitions (40), raising the question of whether the early flowering observed in lettuce *DOG1*-RNAi plants and *dog1-3* plants overexpressing *MIR156* could be caused by the alteration of ABA contents, as

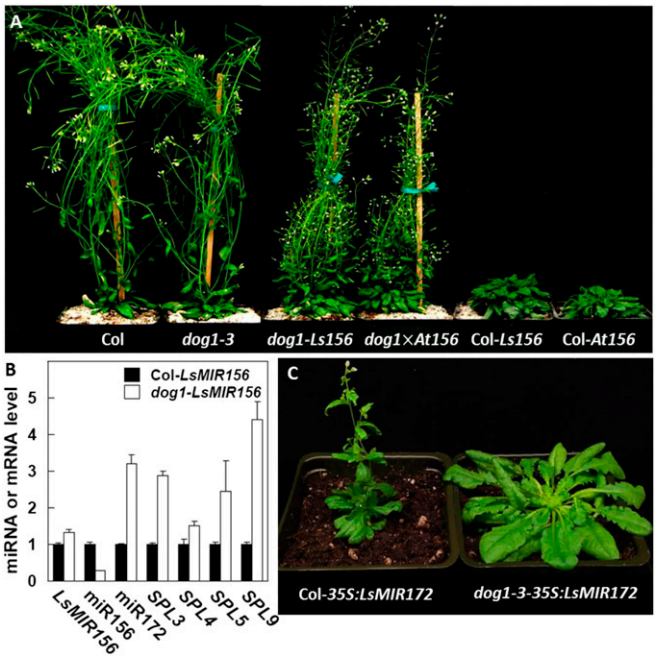

Fig. 3. Regulation of flowering time in *Arabidopsis* by DOG1. (*A*) Seven-week-old Col, *dog1-3*, *dog1-3–35S:LsMIR156* (*dog1-Ls156*), *dog1-3* × *Col-35S:AtMIR156* (*dog1×At156*), Col-*35S:LsMIR156* (Col-*Ls156*), and Col-*35S:AtMIR156* (Col-*At156*) plants grown in long days. (*B*) Relative levels of miR156 and miR172 and mRNA levels of *LsMIR156*, *SPL3*, *SPL4*, *SPL5*, and *SPL9* in apical meristems of Col and *dog1-3* plants expressing *LsMIR156* at 25 DAG; levels were first normalized by *AtACT2*, and values relative to those in the Col-*LsMIR156* meristems for each gene or miRNA are shown. Error bars represent SE ($n = 3$). (*C*) Col-*35S:LsMIR172* and *dog1-3–35S:LsMIR172* plants grown for 65 d in short days.

mutants in ABA biosynthetic genes have similar seed germination phenotypes as *dog1-3* mutant seeds (*SI Appendix*, Fig. S1 *B* and *E*). However, the delayed flowering time phenotypes conferred by *35S:AtMIR156* and *35S:LsMIR156* in Col plants also occurred in an *nced9-1* mutant background (Table 1 and *SI Appendix*, Table S2). Thus, although phenotypic effects of *MIR156* overexpression on flowering depend upon functional *DOG1*, expression of *NCED9*, which is primarily seed-expressed and required for thermoinhibition (28), is not necessary.

The results reported here show that loss of DOG1 function negatively affects processing of *MIR156* to miR156 in lettuce and *Arabidopsis* and elevates miR172 abundance. As miR172 is known to promote early flowering in *Arabidopsis* (30), we tested the effect of overexpression of *LsMIR172* on flowering in Col WT and *dog1-3* mutant plants. To maximize phenotypic differences, we grew 100 (for Col-*35S:LsMIR172*) and 77 (for *dog1-3–35S:LsMIR172*) independent transgenic lines under short-day conditions. Under short days, WT Col plants developed more than 40 leaves before flowering and 90% of plants did not flower before 90 d, whereas 85% of Col-*35S:MIR172* plants had less than 40 leaves at flowering and 39% started flowering in less than 90 DAG (Fig. 3*C*, Table 1, and *SI Appendix*, Table S2). This advancement of flowering as a result of overexpression of *MIR172* was much reduced in *dog1-3* plants: more than 60% of *dog1-3–35S:LsMIR172* plants had more than 40 leaves at flowering, as did the *dog1-3* plants, and only 9% of *dog1-3:35S:LsMIR172* plants flowered in less than 90 DAG (Fig. 3*C*, Table 1, and *SI Appendix*, Table S2). Thus, functional DOG1 plays a role in the processing or action of *MIR172* as well as of *MIR156* (*SI Appendix*, Fig. S5).

miR156 and miR172 Affect Seed Germination. Suppression of *DOG1* expression in lettuce, which improved germination at warm temperatures (Fig. 1 *B* and *C*), also resulted in large reductions in miR156 content in dry seeds, with levels only 5–7% of that in control seeds (Fig. 4*A*). In mature *dog1-3* mutant seeds, the miR156 level was slightly lower than that in mature WT Col seeds, but this difference was more significant when *LsMIR156* was overexpressed (Fig.4*B*). We therefore tested whether expression of *MIR156* and *MIR172* would affect primary dormancy and thermoinhibition of *Arabidopsis* seed germination in WT and *dog1-3* mutant plants. Expression of *35S:LsMIR156* or *35S:*

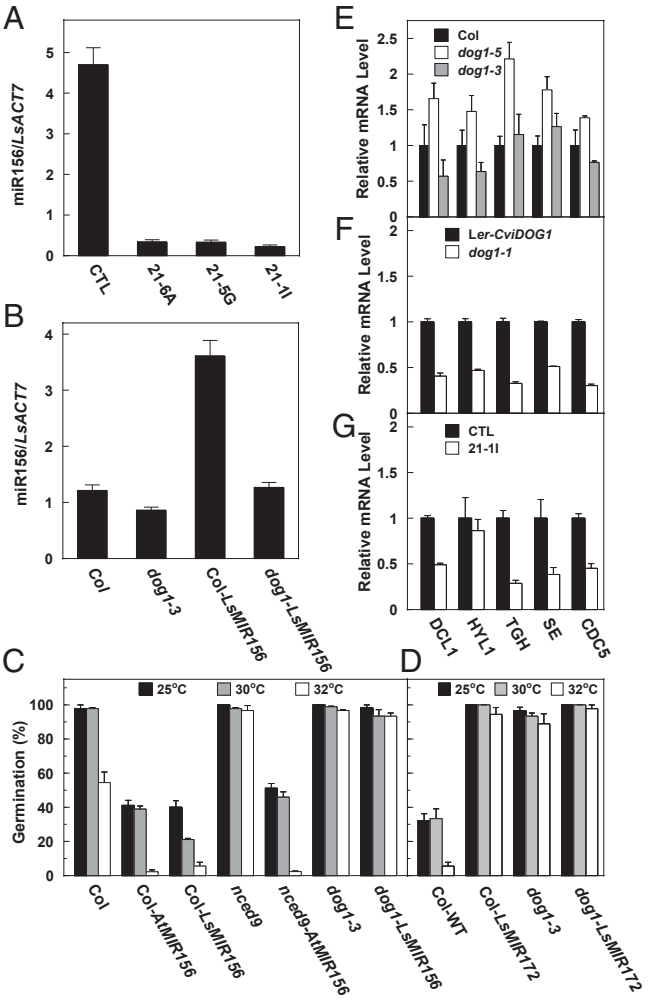

Fig. 4. Lettuce seed content of miR156, effects of modification of miR156 or miR172 expression on germination, and effects of *dog1* mutations on expression of DICER-related genes. (*A*) Levels of miR156 in dry seeds of control and three homozygous *DOG1*-RNAi lettuce lines, 21–6A, 21–5G, and 21–1I. (*B*) miR156 levels in dry seeds of Col, *dog1-3* mutant, Col expressing *LsMIR156*, and *dog1-3* mutants expressing *LsMIR156*. (*C*) Effect of expression of *35S:AtMIR156* and *35S:LsMIR156* on germination of seeds of Col, *nced9-1*, and *dog1-3* genotypes of *Arabidopsis*. All seeds were tested at 3 wk after harvesting. (*D*) Germination at 25, 30, or 32 °C of Col WT or *dog1-3* mutant seeds and of seeds of these genotypes that had been transformed to express *35S:LsMIR172*. Seeds were tested 5 d after harvesting when primary dormancy was still present in Col-WT seeds to demonstrate alleviation of dormancy by *LsMIR172* overexpression. (*E–G*) Transcripts of genes associated with miRNA processing or transcription (*DCL1*, *HYL1*, *TGH*, *SE*, and *CDC5*) in dry seeds of Col and its T-DNA *dog1-3* (loss-of-function) and *dog1-5* (gain-of-function) mutants (*E*), of *Ler-CviDOG1* introgression line and its irradiation-induced mutant *dog1-1* (*F*), and of Salinas lettuce (CTL) and its *DOG1*-RNAi silencing line (21–1I) (*G*). mRNA levels were first normalized by *AtACT2*; values relative to those in Col (*E*), in *Ler-CviDOG1* (*F*), and in Sal (*G*) are shown. Error bars represent SE ($n = 3$).

AtMIR156 in Col plants reduced germination at 25 °C, likely reflecting increased primary dormancy, and strongly enhanced seed thermoinhibition at warmer temperatures (Fig. 4*C*). In contrast, overexpressing *LsMIR156* in *dog1-3* plants did not affect germination at 25, 30, or 32 °C (Fig. 4*C*). Although seeds of the *nced9-1* mutant, like *dog1-3* seeds, were thermotolerant, expression of *35S:AtMIR156* in *nced9-1* plants had similar effects in suppressing germination as in Col plants (Fig. 4*C*). This demonstrates that elevation of miR156 can inhibit germination independently of seed ABA biosynthesis and that the effect of *MIR156* expression on seed germination inhibition is dependent on functional *DOG1*. In contrast, overexpression of *LsMIR172* promoted germination of fresh (primary dormant) Col seeds, even at elevated temperatures (Fig. 4*D*). As *dog1-3* seeds did not exhibit primary dormancy and germinated fully at all temperatures tested, an additional effect of *LsMIR172* overexpression on germination of *dog1-3* seeds could not be detected (Fig. 4*D*). Thus, miR156 and miR172 can influence seed thermoinhibition as well as flowering and other developmental phase transitions (*SI Appendix*, Fig. S5).

Altered *DOG1* Expression or Function Affects Expression of Genes Encoding miRNA Processing Proteins. As the conversion of primary *MIR156* to miR156 was greatly compromised in seeds of lettuce *DOG1*-RNAi suppression lines and of *Arabidopsis dog1-3* mutants (Fig. 4 *A* and *B*), we tested whether transcripts of genes encoding proteins associated with miRNA transcription or processing were altered. After preliminary screening, we focused on five genes: *DICER-LIKE 1* (*DCL1*), *HYPONASTIC LEAVES1* (*HYL1*), the G-patch domain protein *TOUGH* (*TGH*), the zinc finger protein *SERRATE* (*SE*), and *CELL DIVISION CYCLE 5* (*CDC5*) that binds RNA polymerase II and *MIR* promoters to positively regulate transcription of *MIR*-encoding genes (41, 42). Although the specific effects varied somewhat among the *Arabidopsis* and lettuce genotypes and mutants tested, transcript levels of all of these genes differed from their respective WTs in at least some cases (Fig. 4 *E*–*G*). For example, the transcript levels of *DCL1*, *HYL1*, and *CDC5* in dry *dog1-3* mutant seeds were 56%, 55%, and 76%, respectively, of those in dry Col WT seeds, whereas expression of these genes (and of *TGH* and *SE*) was elevated compared with Col-WT in seeds of the gain-of-function *dog1-5* mutant (Fig. 4*E*). Consistent reduction in expression of these genes was also observed in dry seeds of the *dog1-1* mutant compared with those in L*er*-Cvi*DOG1* (Fig. 4*F*), which contains a strong Cvi*DOG1* allele (19), and transcripts of *DCL1*, *SE*, *TGH*, and *CDC5* were also lower in dry *DOG1*-RNAi lettuce seeds (Fig. 4*G*). These results indicate that DOG1 could affect miR156 (and possibly miR172) processing in seeds through altering expression of genes encoding miRNA-processing proteins (*SI Appendix*, Fig. S5).

Discussion

Seed germination and flowering are two critical developmental-phase transitions in the plant life cycle. Plants sense environmental cues such as temperature to coordinate endogenous genetic and molecular mechanisms that determine the timing of both phase transitions (1, 6, 7, 12, 43, 44). Although its mechanism of action remains unknown, DOG1 has been demonstrated to be a positive regulator for the establishment and maintenance of primary seed dormancy in several species (19, 20, 35). DOG1 is involved in transducing maternal environmental conditions during seed development into effects on seed dormancy and in the loss of dormancy through dry after-ripening or moist chilling (2, 5, 17, 45, 46). ABA is also a strong positive regulator of seed dormancy (12) and promoted *DOG1* expression in imbibed *Lepidium sativum* seeds (47). *Arabidopsis* seeds may require ABA and functional *DOG1* to establish primary dormancy, as reduced dormancy in *Arabidopsis* seeds was still observed in ABA-deficient *aba1* mutants, even when the strong Cvi-*DOG1* allele was present (18, 19), whereas seeds of a *dog1-2 cyp707a2-1* double mutant that have higher levels of ABA nonetheless exhibited reduced seed dormancy as a result of the loss of *DOG1* function (18). Here, we demonstrated that *DOG1* is involved in regulation of thermoinhibition of seed germination in lettuce and *Arabidopsis*, but, unlike the dependence of primary dormancy on ABA and DOG1, DOG1 can act in the absence of enhanced ABA synthesis to impose thermoinhibition, consistent with the relative ineffectiveness of an ABA biosynthesis inhibitor to alleviate effects of overexpression of *DOG1* on thermoinhibition (*SI Appendix*, Fig. S3) (20). Thus, ABA and DOG1 may regulate seed thermoinhibition through parallel but mutually reinforcing pathways that may not be identical to those by which they act on primary dormancy (29). On the contrary, GA can alleviate the effects of overexpression of *DOG1* on thermoinhibition (*SI Appendix*, Fig. S3) (20), as might be expected from the suppression of expression of GA biosynthetic enzymes by high temperatures in *Arabidopsis* and lettuce (25, 28, 34).

Ecological genetics studies have shown that, under natural seasonal variation, *DOG1* can influence seed germination and flowering times (48). Although, initially, the effects of *DOG1* on flowering were suggested to be an indirect response to the seasonal timing of germination, further study showed that the alleviation of seed dormancy resulted in an acceleration of flowering that was independent of germination timing (49). Genome-wide association and genetic linkage mapping analyses have implicated the *DOG1* locus in the regulation of flowering phenotypes (22, 23), but these results were attributed to the possibility of being caused by closely linked genes, as *DOG1* was assumed to be active only in seeds. However, our results demonstrate that *DOG1* itself can have effects on seed dormancy and flowering, suggesting a more direct mechanism for the evolutionary coadaptation of life-cycle transitions to match seasonal environmental conditions (16, 44, 49, 50). In addition, previous studies have shown that genes related to flowering are expressed in association with seed development and germination. For example, a genome-wide expression correlation network in *Arabidopsis* showed that maturity-phase transition regulators such as *SE*, *EMBRYONIC FLOWER1*, and *EARLY BOLTING IN SHORT DAYS* are coexpressed with germination-associated genes and affect seed germination (14). *Arabidopsis HISTONE MONOUBIQUITINATION1* and *2* (*HUB1* and *HUB2*) genes regulate flowering and seed germination; seeds of *hub1* and *hub2* mutants displayed reduced seed dormancy (51), and *hub1* seeds also exhibit strong thermotolerance to high temperature at germination (52). The increased germination of *hub1* and *hub2* seeds could be attributed to the altered expression of several seed dormancy-related genes, including *DOG1* and *NCED9* (51). Loss of function of *HUB1* and *HUB2* also caused early flowering phenotypes through repression of *FLC* by chromatin modification (53). In addition to repressing flowering, *FLC* also promotes seed germination at low temperature (10 °C) via changing expression of ABA-catabolism (*CYP707A2*) and GA-biosynthesis (*GA20ox1*) genes (15). Consistent with these observations, the flowering-promotive protein *FT* maternally controls seed dormancy through alteration in seed coat tannin content in *Arabidopsis* (13). Thus, although there have been previous indications that seed dormancy and flowering might share common genetic and molecular mechanisms, our understanding of how such mechanisms might be connected to each other and to environmental cues has been limited.

Our results demonstrate that DOG1 regulates seed germination and flowering time at least in part through an influence on generation and/or action of miR156 and miR172. miRNAs have been implicated previously as being involved separately in seed germination and flowering time (30, 32). Mutants that are defective in DCL1, HYL1, and SE, all of which are required for miRNA biogenesis (41), display abnormal flowering and alteration in ABA sensitivity of seed germination (54, 55). Interestingly, the delayed-flowering phenotype of *dcl1-7* mutant could be rescued by

expression of a DCL4-dependent *MIR839* in which the mature sequence was replaced by the one encoding *MIR172*, suggesting that the delayed flowering in *dcl1-7* plants is a result of the reduction in miR172 (56). Earlier transition from the juvenile to the adult phase was also observed in loss-of-function mutants of *SE* and *HYL1*, in association with substantial reduction in miR156, and could be rescued by overexpression of *MIR156* (57, 58). Another miR156 target, *SPL13*, plays an important role in the regulation of the postgerminative switch from the cotyledon stage to the vegetative-leaf stage during seedling growth (59). Mutants in *dcl1* exhibit early seed maturation programming, probably caused by increased levels of *SPL10* and *SPL11* as a consequence of reduced miR156 (60, 61). Our data indicate that DOG1 may affect the conversion of primary *MIR156* to miR156 or primary *MIR172* to miR172 through an effect on expression of genes involved in miRNA processing (*SI Appendix*, Fig. S5). Loss of function or gain of function of *DOG1* decreases or increases, respectively, expression of DICER-related genes (Fig. 4 *E*–*G*), and previous studies found that miR156 and miR172 levels are altered in mutants of *DCL1*, *HYL1*, *SE*, *TGH*, and *CDC5* (42, 56, 58, 62, 63). However, multiple feedback loops are associated with transcription and processing of miRNAs (41), so further study is needed to clarify the specific mechanism by which DOG1 affects miRNA levels. In addition, the strong effects of *DOG1* overexpression in enhancing seed dormancy even in the absence of *MIR156* overexpression (*SI Appendix*, Fig. S3) (18, 19) indicate that DOG1 may also act on seed germination through additional mechanisms (*SI Appendix*, Fig. S5).

miR156 accumulates to high levels during seed development (32, 64) and is abundant in young seedlings and decreases as plants age, in association with the transition to adult phase and flowering (30, 38, 65). This pattern indicates that high miR156 levels are reset during embryogenesis and seed development, as occurs for *FLC*, a strong floral repressor (7). Winter annual flowering plants require prolonged chilling to epigenetically silence *FLC* to flower the next spring; to ensure the requirement for vernalization in every generation, it is essential to reactivate *FLC* gene expression during seed development (66). Consistent with this, expression and maintenance of *FLC* during late embryogenesis are required for late flowering phenotypes (67, 68). Similarly, DOG1 may play a role in resetting high miR156 levels during seed development (Fig. 4*A*), and defects in DOG1 function could result in altered postgerminative plant development and floral transition as well as reduced seed dormancy (*SI Appendix*, Fig. S5) (32, 41, 64, 65). We also note that a primary phenotype of DOG1 action is an extension of dry after-ripening times required for dormancy alleviation (19). A role for miRNAs produced during seed development in maintaining seed dormancy provides a mechanism for the after-ripening effect, in which seeds lose dormancy during dry storage when metabolism is prevented (12). miR156 accumulation during seed development, and its subsequent loss during storage as a result of oxidation, as shown for stored mRNAs (69), would provide a timing mechanism for after-ripening via loss of the inhibitory effect of stored miR156 on germination.

Our results support the conclusion that the transition from dormancy to germination is a life-cycle phase transition comparable to vegetative maturation or flowering, and apparently is influenced by the same miRNA-dependent systems (*SI Appendix*, Fig. S5) (32). Effects on these systems are also consistent with the hypothesis that DOG1 primarily affects the underlying environmental sensitivity of processes it influences, rather than directly determining final phenotypes (5). As the miR156/SPL/miR172 pathway is involved in modulating the inputs from a diverse array of environmental signals and transducing them into developmental responses (6, 7, 32, 59), interaction of DOG1 with this system would enable it to play an integrating role by matching the environmental sensitivities of seed dormancy and flowering to current or anticipated conditions. A dual role in sensing environmental signals (e.g., temperature) and coordinating developmental-phase transitions in the plant life cycle would explain the repeated identification of *DOG1* as a significant locus in ecological genetics studies of flowering phenotypes (22, 23, 48). It would also make evolutionary sense, as seed dormancy and germination timing influence the environment in which subsequent flowering and reproduction occur, and vice versa (16, 44, 50). An integrated mechanism for coordinating these two major life cycle transitions having significant impacts on fitness and survival would be subject to coselection to optimize (or bet-hedge) both (9, 11, 43, 49, 50). Much remains to be done to fully understand these complex interactions between plant life cycles and the environment, but our results demonstrate that DOG1 functions as an important molecular integrator that exerts its effects on developmental phase changes at least in part through miRNA-regulated pathways.

Materials and Methods

Seeds of lettuce (*L. sativa* L.) were germinated at room temperature and then transferred to an automatically controlled greenhouse at 23 ± 2 °C (day) and 20 ± 2 °C (night) in long days (14 h light/10 h dark) for seed production unless stated otherwise. Seeds of *A. thaliana* were stratified at 4 °C for 2–4 d before being transferred to a growth chamber at 21–22 °C under long days (16 h light/8 h dark, 100 or 135 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity) or short days (8 h light/16 h dark, 135 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity) for seed production. Seeds of the γ-irradiation–induced *dog1-1* mutant were provided by Leonie Bentsink, Wageningen University, Wageningen, The Netherlands (19), seeds of the T-DNA mutant *nced9-1* by Eiji Nambara, University of Toronto, Toronto, and seeds of the T-DNA double-mutant *nced6-1 nced9-1* by Annie Marion-Poll, Institut Jean-Pierre Bourgin, Versailles, France. Seeds of *Arabidopsis* WT (Col and Ler), T-DNA *dog1-3* mutant (Salk_000867) (19), T-DNA *dog1-5* mutant (SALK_022748) (39), and *AtMIR156*-overexpression line (CS67849) (30) were purchased from the *Arabidopsis* Biological Resource Center. The *dog1-3* mutant line was selfed and genotyped for three generations before this study. The *Arabidopsis* T-DNA mutants were genotyped using the primers listed in *SI Appendix*, Table S3. Details of experimental procedures including isolation of *LsDOG1* and vector construction, generation of *dog1-3* × *35S:AtMIR156* and *nced9–1* × *35S:LsMIR156* lines, seed germination assays, measurements of flowering times, identification of *LsMIR156* and *LsMIR172*, vector construction for overexpression of *LsMIR156* and *LsMIR172*, plant transformation, and mRNA and miRNA analyses are described in *SI Appendix, Supplementary Methods*. Primers used in this study are listed in *SI Appendix*, Table S3.

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