

MicroRNAs prevent precocious gene expression and enable pattern formation during plant embryogenesis

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Arabidopsis embryos lacking *DICER-LIKE1* (*DCL1*), which is required for microRNA (miRNA) biogenesis, arrest early in development. To assess the functions of embryonic miRNAs, we determined the developmental and molecular consequences of *DCL1* loss. We found that *DCL1* is required for cell differentiation events as early as the eight-cell stage and soon thereafter for proper division of the hypophysis and subprotoderm cells. By the early globular (~32-cell) stage, *dcl1*-null mutant embryos overexpress ~50 miRNA targets. In *dcl1* eight-cell embryos, the two most up-regulated targets are those of miR156 and encode SPL10 and SPL11 transcription factors. SPL10 and SPL11 are derepressed >150-fold in *dcl1* embryos and are redundantly required for the *dcl1* early patterning defects. Moreover, as early as the eight-cell stage, miR156-mediated repression of zygotic SPL transcripts prevents premature accumulation of transcripts from genes normally induced during the embryonic maturation phase. Thus, the first perceptible molecular function of plant embryonic miRNAs is the opposite of that in vertebrates; in vertebrates, miRNAs sharpen the first developmental transition, whereas in plants, they forestall developmental transitions by repressing mRNAs that act later. We propose that, by preventing precocious expression of differentiation-promoting transcription factors, miRNAs enable proper embryonic patterning.

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MicroRNAs (miRNAs) are ~21-nucleotide (nt) RNAs that guide the post-transcriptional regulation of target genes during plant and animal development (Bartel 2004). Plant miRNAs recognize nearly perfect complementary binding sites in target mRNAs and mediate RNA cleavage (Llave et al. 2002; Rhoades et al. 2002; Tang et al. 2003). The high degree of complementarity between plant miRNAs and their binding sites in target mRNAs has allowed confident miRNA target predictions (Rhoades et al. 2002; Jones-Rhoades and Bartel 2004; Fahlgren and Carrington 2010). Plant miRNA targets tend to encode key developmental regulators, including many transcription factors (Rhoades et al. 2002; Jones-Rhoades et al. 2006). Accordingly, several studies have demonstrated that the miRNA-mediated repression of target transcripts is essential for correct cell differentiation and developmental timing during post-embryonic devel-

opment (Jones-Rhoades et al. 2006; Chen 2009). However, relatively little is known regarding the roles of miRNAs in embryonic cell differentiation, and miRNA functions during embryo developmental timing have not been characterized.

DICER-LIKE1 (*DCL1*) encodes an RNaseIII domain-containing protein that is nuclear-localized and required for processing primary miRNA transcripts into mature miRNAs (Park et al. 2002; Reinhart et al. 2002; Papp et al. 2003; Fang and Spector 2007). With this realization that *DCL1* is necessary for miRNA biogenesis, the recovery and analysis of *dcl1* mutant alleles from previous forward genetic screens performed using *Arabidopsis thaliana* (Schauer et al. 2002) provide insight into the roles of miRNAs during plant development. Null *dcl1* alleles (originally named *emb76*, then *sus1*) were recovered by Meinke's group (Errampalli et al. 1991; Castle et al. 1993) more than 15 years ago in screens for embryos with defective development. *dcl1* embryos are developmentally arrested at the globular stage of embryogenesis and exhibit abnormal divisions throughout the extraembryonic

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suspensor (Schwartz et al. 1994). These results suggest that *DCL1* and, by implication, miRNAs are required for embryo development and viability. Null mutations in other genes required for normal miRNA biogenesis or function also produce defects during embryogenesis (Lynn et al. 1999; Lobbes et al. 2006; Grigg et al. 2009). In addition, specific miRNA/target interactions are required for proper cotyledon formation during embryo development (Palatnik et al. 2003; Laufs et al. 2004; Mallory et al. 2004, 2005). Moreover, several plant miRNA targets are required for proper embryogenesis (Aida et al. 1997; Emery et al. 2003; Dharmasiri et al. 2005; Prigge et al. 2005). Collectively, these results strongly indicate that miRNAs have important functions during embryo development.

Morphogenesis is the phase of embryo development when the basic plant body plan is established. Embryonic cell types are specified in particular locations at precise developmental time points. Shoot meristem precursors and root meristem precursors are specified at the apical and basal poles of the early embryo, respectively (Mayer et al. 1991). The post-embryonic activity of these meristems generates the adult plant body. Patterning along the radial axis during early embryogenesis generates the outermost protoderm layer, the innermost vascular primordium, and a middle layer of ground tissue precursors (Laux et al. 2004). After morphogenesis, embryos transition to a maturation phase, where they accumulate storage proteins, undergo desiccation tolerance, and prepare to enter into a state of dormancy prior to germination (Gutierrez et al. 2007; Holdsworth et al. 2008). Although progress has been made (Weber et al. 2005; Braybrook and Harada 2008; Park and Harada 2008), the molecular basis of early embryonic patterning and the morphogenesis-to-maturation-phase transition are not completely understood.

To assess the regulatory functions of miRNAs during embryogenesis, we revisited the phenotypic characterization of *dcl1* embryos, with a focus on early morphogenesis and cell specification defects. *DCL1* was required for multiple embryonic cell differentiation events as early as the eight-cell stage. Genome-wide transcript profiling revealed that *DCL1* was required for the early embryonic repression of nearly 50 miRNA targets. Several of the miRNA targets up-regulated in eight-cell *dcl1* embryos encode transcription factors that promote differentiation during later stages of embryogenesis. Moreover, *dcl1* embryos prematurely accumulated transcripts from hundreds of genes typically expressed during the maturation phase of embryo development. The disruption of miR156-mediated regulation of two redundant *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL*) transcription factors was both responsible for the premature gene expression observed in *dcl1* embryos and partially required for the *dcl1* embryo patterning defects. Therefore, some of the earliest roles of miRNAs are to prevent the precocious expression of differentiation-promoting transcription factors during early embryogenesis and to enable proper pattern formation.

Results

DCL1 is required for early embryonic pattern formation

dcl1-null mutant embryos exhibit morphological defects and arrest at the globular stage of development (Schwartz et al. 1994). To identify the earliest *dcl1* morphological defects, we systematically analyzed *dcl1* embryos throughout early embryogenesis. Because *dcl1* embryos are not viable, embryos from self-pollinated plants heterozygous for *dcl1-5* and *dcl1-10*, two presumed null alleles (McElver et al. 2001; Schauer et al. 2002), were examined.

Embryos developing within a single silique are approximately at the same developmental stage, which enabled an estimate of *dcl1* embryonic stages based on the morphology of their wild-type siblings. It was reported previously that *sus1/dcl1* embryos exhibit abnormal cell divisions at the base of the embryo proper beginning at the globular stage and in the extraembryonic suspensor beginning at the heart stage (Schwartz et al. 1994). Our morphological analysis of *dcl1* embryos confirmed these findings and revealed previously unreported phenotypes (Fig. 1A). We identified initial morphological defects in the presumptive hypophysis cells (i.e., the suspensor cell most proximal to the embryo proper) of 19% (29 of 154) of dermatogen stage embryos from self-pollinated *dcl1-5/+* plants (Fig. 1A). By the early globular stage, morphological defects were observed in ~25% of embryos from selfed *dcl1-5/+* plants (Fig. 1A). These embryos with defects were presumably homozygous for *dcl1-5*-null alleles, and their defects included abnormal hypophysis cell divisions as well as the previously unreported loss of periclinal subprotoderm cell divisions in the embryo proper (Fig. 1A). In subsequent stages, these abnormal embryos did not produce cotyledons and were developmentally arrested and nonviable (Fig. 1A; Schwartz et al. 1994). Furthermore, *dcl1-5* homozygous seedlings could not be recovered from selfed *dcl1-5/+* plants, which suggested that the developmentally arrested embryos derived from selfed *dcl1-5/+* plants were indeed homozygous for *dcl1-5* (data not shown). Because embryos from selfed *dcl1-5/+* and selfed *dcl1-10/+* plants exhibited the same morphological defects at indistinguishable frequencies, subsequent analyses focused on embryos from selfed *dcl1-5/+* plants (Table 1; data not shown). *DCL1* transcripts were detected by quantitative RT-PCT (qRT-PCR) in both early globular embryos from wild-type plants and early globular embryos from selfed *dcl1-5/+* plants that developed normally, but were not detected in abnormal early globular embryos from selfed *dcl1-5/+* plants (Supplemental Fig. 1). These expression analyses further confirmed that abnormal embryos from selfed *dcl1/+* plants were homozygous for *dcl1*-null alleles.

We next tested whether *DCL1* was required for embryonic cell differentiation by examining the expression of cell-specific markers in *dcl1-5* embryos. *WUSCHEL-related HOMEBOX2* (*WOX2*) transcripts are localized in the apical cell lineage of wild-type preglobular embryos (Haecker et al. 2004). After the two/four-cell stage, *WOX2* transcripts were not detected in about a quarter of

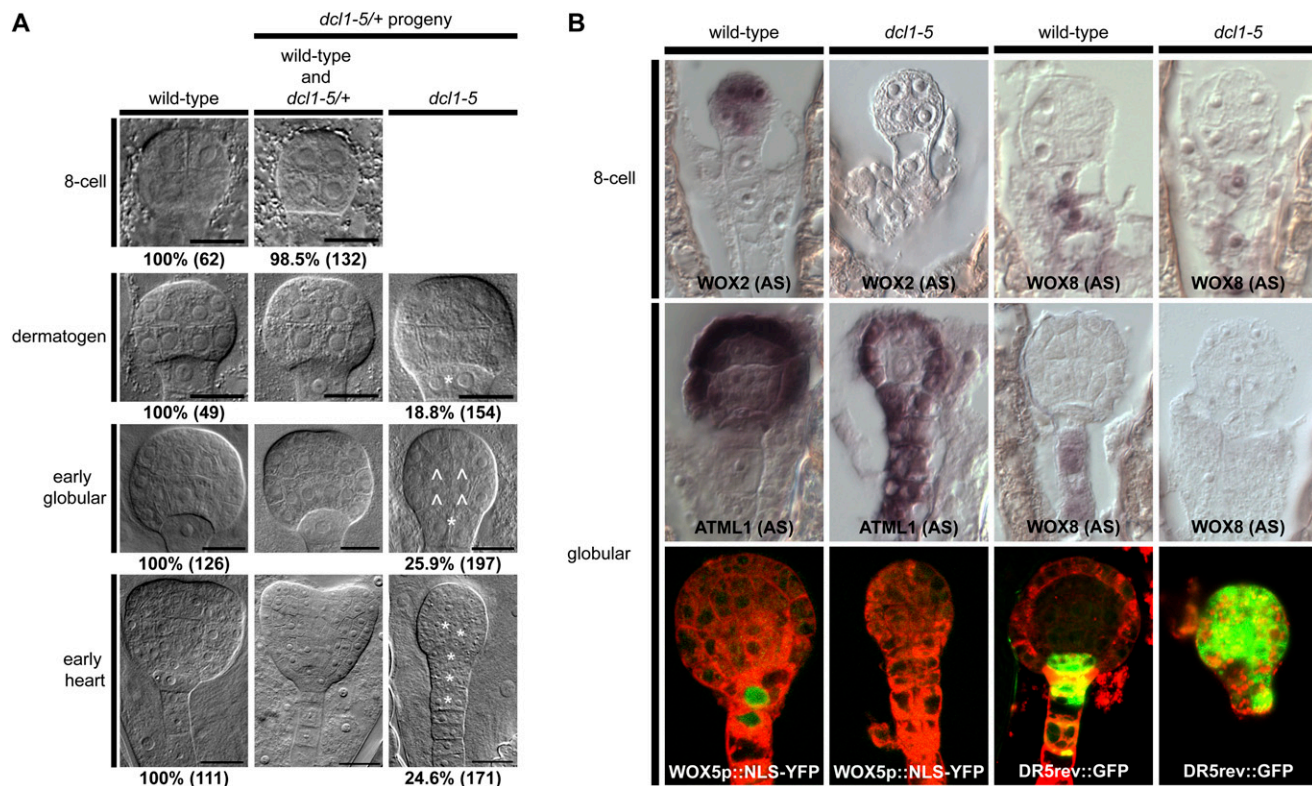


Figure 1. *DCL1* is required for pattern formation during early embryogenesis. (A) Representative Nomarski images of embryos from wild-type and *dcl1-5/+* self-pollinated plants. Phenotypic classes are denoted directly above each column, and developmental stages are indicated at the left of each row. The percent of embryos in each phenotypic class is noted below each image, and total numbers of embryos examined are in parentheses. Asterisks indicate products of abnormal divisions, and carets designate positions where cell divisions did not take place in the mutants. Bars, 20 μ m. (B) Representative micrographs of RNA in situ hybridizations performed with *WOX2*, *WOX8*, and *ATML1* antisense (AS) probes, and CSLM images with *WOX5p::NLS-YFP* and *DR5rev::GFP* reporters. Presumptive embryo genotypes are indicated above each column, and developmental stages are indicated to the left of each row. Probes/reporters are indicated at the bottom of each image. For the CSLM images, GFP and FM4-64 fluorescence are shown in green and red, respectively. See also Supplemental Figure 2.

eight-cell and dermatogen stage embryos (seven of 35 and 14 of 52, respectively) from self-pollinated *dcl1-5/+* plants (Fig. 1B), which implied that *dcl1-5* embryos had undetectable *WOX2* expression. Similar to *WOX2*, *PINHEAD*/

ZWILLE/*ARGONAUTE10* transcripts, hereafter referred to as *PNH*, are localized in the apical cell lineage of preglobular embryos (Lynn et al. 1999). Approximately 89% (72 of 81) of eight-cell and dermatogen stage embryos

Table 1. Frequencies of embryos with *Dcl1* phenotype

Parent genotypes	Frequency of <i>Dcl1</i> phenotype ^a (observed/total)		<i>P</i> -value for frequency \neq 25% ^b
Wild-type (Col-0)	0%	(0/237)	Not applicable
<i>dcl1-5/+</i> (self-pollinated)	24.6%	(42/171)	0.909
<i>dcl1-10/+</i> (self-pollinated)	24.5%	(25/102)	0.921
<i>spl10-1</i> (self-pollinated)	0%	(0/107)	Not applicable
<i>spl11-1</i> (self-pollinated)	0%	(0/113)	Not applicable
<i>dcl1-5/+ spl10-1</i> (self-pollinated)	25.4%	(45/177)	0.910
<i>dcl1-5/+ spl11-1</i> (self-pollinated)	16.8%	(114/680)	1.75E-05
<i>dcl1-10/+ spl11-1</i> (self-pollinated)	18.5%	(74/401)	0.009
<i>dcl1-5/+ spl10-1</i> (female) \times <i>dcl1-5/+ spl11-1</i> (male)	16.1%	(61/378)	0.001
<i>dcl1-5/+ spl11-1</i> (female) \times <i>dcl1-5/+ spl10-1</i> (male)	18.9%	(69/365)	0.020
<i>dcl1-5/+ spl11-1</i> (female) \times <i>dcl1-5/+</i> (male)	24.4%	(104/426)	0.809
<i>dcl1-5/+</i> (female) \times <i>dcl1-5/+ spl11-1</i> (male)	21.9%	(268/1222)	0.032
<i>dcl1-5/+ spl11-1 SPL10-RNAi</i> (self-pollinated)	1.5%	(3/203)	2.05E-11 ^c /1.04E-07 ^d

^aEmbryo phenotypes were determined when wild-type-like embryos were at the late globular/transition stages.

^bBased on χ^2 tests.

^c*P*-value for frequency \neq 25%.

^d*P*-value for frequency \neq frequency of *dcl1-5/+ spl11-1* (16.8%).

from selfed *dcl1-5/+* plants had detectable levels of *PNH* transcripts, suggesting that apical cell fate is not completely abolished in all preglobular *dcl1* embryos (Supplemental Fig. 2).

In contrast to *WOX2* and *PNH*, *WOX8* transcripts are localized to the basal cell lineage of preglobular wild-type embryos (Haecker et al. 2004). *WOX8* transcripts were detected in 90% (62 of 69) of eight-cell and dermatogen stage embryos from selfed *dcl1-5/+* plants, suggesting that basal cell lineage differentiation is not perturbed in all preglobular *dcl1* embryos (Fig. 1B). Thus, *dcl1* embryos had detectable defects in the differentiation of apical cell but not basal cell descendants as early as the eight-cell stage.

To test whether *DCL1* is required for radial patterning, we examined a series of markers that are differentially expressed along the radial axis. *A. thaliana* *MERISTEM LAYER1* (*ATML1*) and *PROTODERMAL FACTOR1* (*PDF1*) transcripts are detectable in the protoderm, but not the subprotoderm, of globular stage embryos (Lu et al. 1996; Abe et al. 1999). *ATML1* and *PDF1* transcripts were appropriately localized to the protoderm of globular stage *dcl1-5* embryos, but were ectopically localized in the suspensors of *dcl1-5* embryos whose wild-type siblings were at the early heart stage (Fig. 1B; Supplemental Fig. 2). This observation was consistent with the previously reported ectopic localization of embryo proper-specific starch grains and protein bodies in the suspensors of late stage *sus1/dcl1* embryos (Schwartz et al. 1994). Consistent with the reduction/loss of subprotodermal cell divisions in *dcl1* late globular/early heart stage embryos, we detected reduced levels of *RPS5A* transcripts in these cell types (Supplemental Fig. 2). Moreover, vascular primordium and ground tissue initial markers were absent from these cell types (Supplemental Fig. 2).

In addition to the ectopic localization of protoderm markers in the suspensor, globular *dcl1-5* suspensors had reduced levels of *WOX8* transcripts (Fig. 1B). Furthermore, hypophysis markers were not detectable in *dcl1* globular embryos (Fig. 1B; Supplemental Fig. 2). Considered together with the defective hypophysis cell divisions observed in *dcl1* embryos, the lack of hypophysis markers indicates that the hypophysis is misspecified in *dcl1* embryos.

The plant hormone auxin is transported from the embryo proper to the presumptive hypophysis and contributes to hypophysis specification (Friml et al. 2003; Aida et al. 2004; Weijers et al. 2006). Because the auxin signaling-responsive *DR5rev* promoter fused to GFP (*DR5rev::GFP*) is specifically active in the hypophysis cells of wild-type globular embryos (Friml et al. 2003), and hypophysis specification is perturbed in *dcl1* embryos, we examined the activity of *DR5rev::GFP* in *dcl1-5* embryos to test whether *DCL1* was required for appropriate auxin signaling. Although presumptive *dcl1-5* hypophysis cells often expressed *DR5rev::GFP*, cells located above the presumptive hypophysis ectopically expressed *DR5rev::GFP* (Fig. 1B). Therefore, *DCL1* is required for the hypophysis-specific increase in auxin signaling. Given the severe subprotodermal defects observed in *dcl1* embryos, the inappropriate auxin signaling observed in *dcl1* embryos was likely due to the incorrect expression of auxin transport and/or auxin

signaling factors. Therefore, the auxin response defects observed in *dcl1* embryos were probably secondary consequences of subprotoderm cell differentiation defects, which in turn were probably due to the apical cell lineage defects observed at the preglobular stages.

Many miRNA targets have increased transcript levels in dcl1 embryos

Because *DCL1* is required for miRNA biogenesis (Park et al. 2002; Reinhart et al. 2002), the patterning defects that we observed in *dcl1* embryos were likely due to the loss of specific miRNAs and the consequent up-regulation of their respective targets. To test which miRNA targets were up-regulated in *dcl1* embryos relative to wild-type embryos, we performed genome-wide transcript profiling of wild-type and *dcl1-5* early globular embryos using strand-specific mRNA-Seq. Of 205 annotated miRNA targets (see the Materials and Methods), 71 had at least 20 raw reads in *dcl1*, of which 68% (48 of 71), potentially targeted by 25 miRNA families, had at least twofold more reads in *dcl1* relative to wild type (Supplemental Fig. 3; Supplemental Table 1). To focus on the miRNA targets most likely to be responsible for the defects observed in *dcl1* embryos, predicted targets with at least 40 raw reads in *dcl1* and at least fivefold more reads in *dcl1* relative to wild type were selected for further analysis. Fifteen predicted targets of 10 miRNA families (miR156, miR159, miR160, miR166, miR168, miR319, miR393, miR400, miR778, and miR824) fulfilled these criteria (Fig. 2A). The most up-regulated target in *dcl1* relative to wild type was the miR156 target *SPL10*, which increased ~130-fold (Fig. 2A). Examination of *SPL10*-GFP translational fusions by confocal scanning laser microscopy (CSLM) confirmed that *SPL10* increased in early globular *dcl1-5* embryos (Fig. 2B). Signal corresponding to *SPL10*-GFP was mostly nuclear, which is consistent with its presumed role as a transcription factor.

Because the earliest differentiation defect detected in *dcl1* embryos occurred at the eight-cell stage (Fig. 1B), we hypothesized that the misregulation of one or more targets in *dcl1* eight-cell embryos led to the widespread patterning defects observed. To test which miRNA target transcripts were increased in *dcl1* eight-cell embryos, qRT-PCR was performed using RNA isolated from either wild-type eight-cell embryos or eight-cell embryos derived from selfed *dcl1-5/+* plants. Of the 15 predicted targets analyzed, nine had significantly increased transcript levels in embryos derived from selfed *dcl1-5/+* plants relative to wild type (Fig. 2C). All nine of the up-regulated targets encoded transcription factors that are predicted targets of either miR156, miR159, miR160, miR166, miR319, or miR824 (Fig. 2C). Therefore, multiple miRNAs appear to be repressing transcription factor production as early as the eight-cell stage of embryogenesis. Two known miR156 targets, *SPL10* and *SPL11* (Vazquez et al. 2004; Addo-Quaye et al. 2008; German et al. 2008), were the most up-regulated targets, with ≥150-fold increased transcript levels in progeny from self-fertilized *dcl1-5/+* plants (Fig. 2C). When considering that only one-quarter of the embryos from selfed *dcl1-5/+* plants were homozygous for *dcl1-5*, these

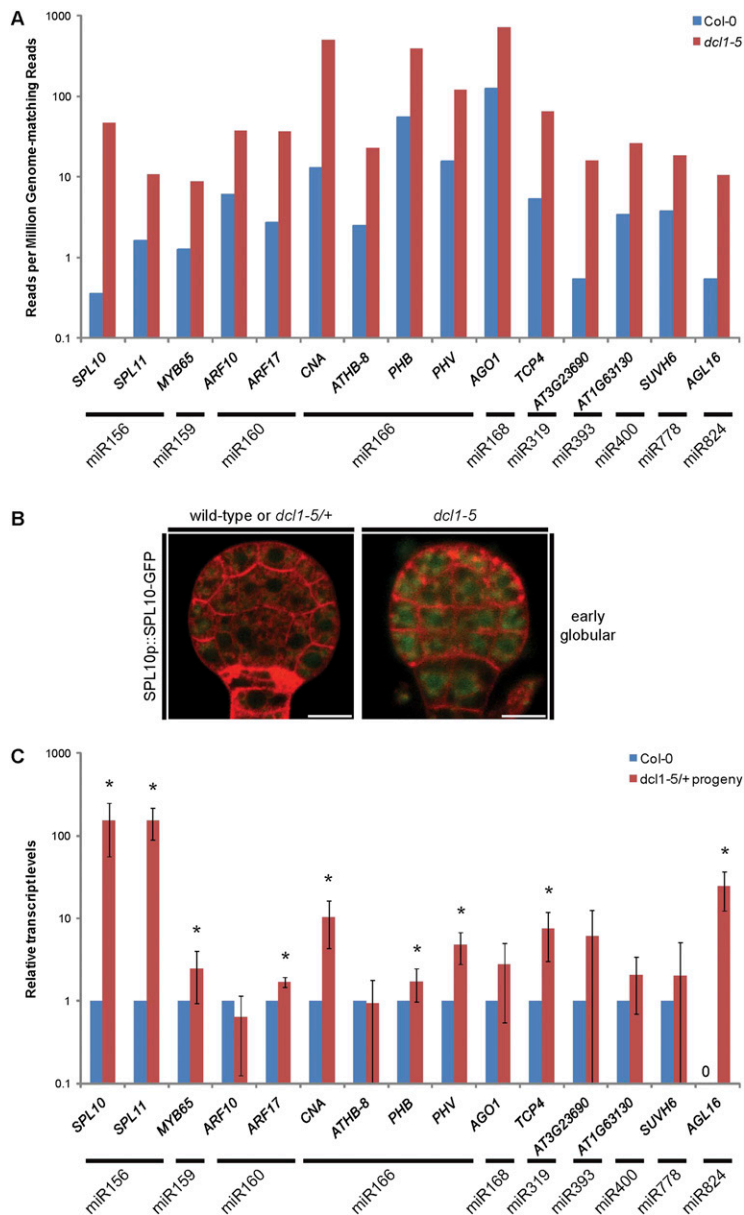


Figure 2. Many miRNA targets are overexpressed in *dcl1-5* embryos. (A) Number of reads matching miRNA targets in libraries prepared from Col-0 (wild-type) and *dcl1-5* early globular embryos. Only miRNA targets that had at least 40 raw reads in the *dcl1-5* library and were increased at least fivefold in *dcl1-5* embryos relative to Col-0 embryos are shown. Reads were normalized by dividing the number of reads that match each gene by the number of million genome-matching reads from each library. The corresponding miRNA is shown below each set of miRNA targets. See also Supplemental Figure 3. (B) CSLM images of early globular embryos expressing SPL10-GFP translational fusions under the control of upstream intergenic sequences (SPL10p::SPL10-GFP). Genotypes are indicated above images. GFP and FM4-64 fluorescence are shown in green and red, respectively. Bars, 20 μ m. (C) miRNA target transcript levels in eight-cell embryos from self-pollinated Col-0 and *dcl1-5/+* plants based on qRT-PCR and normalized to Col-0 levels. The corresponding miRNA is shown below each set of miRNA targets. Error bars represent one standard deviation of the sample mean. Transcripts that were not detected are indicated by a zero. Asterisks indicate target transcripts that were significantly increased in *dcl1-5/+* progeny compared with wild-type. Probabilities were calculated with heteroscedastic Student's *t*-tests using one-tailed distributions. Probabilities <0.05 were considered significant.

results suggest that *SPL10* and *SPL11* transcript levels were elevated as much as ~600-fold in *dcl1-5* eight-cell embryos. Such dramatic elevations spurred us to test whether derepression of *SPL10* or *SPL11* was responsible for the defects observed in *dcl1-5* embryos.

miR156 is localized and active throughout early embryogenesis

For the loss of miR156 to explain the observed up-regulation of *SPL10* and *SPL11* transcripts in *dcl1* embryos, miR156 must be present and active at the relevant embryonic stages. To test for miR156 presence, we performed RNA in situ hybridizations on wild-type and *dcl1-5* embryos using locked nucleic acid (LNA) probes antisense to miR156. Signal corresponding to miR156 was detected throughout two/four-cell and eight-cell embryos (Fig. 3A). Beginning at the globular stage, miR156 signal was increased in subpro-

dermal tissue layers, and this apparent differential accumulation of miR156 became more pronounced at the heart stage (Fig. 3A). Globular stage *dcl1-5* embryos had undetectable signal, confirming both the probe specificity and miR156 loss in *dcl1-5* embryos (Fig. 3A).

To test for miR156 activity, a sensor was constructed that contained a miR156 target site in the 5' untranslated region (UTR) of a ubiquitously expressed nuclear-localized GFP (Fig. 3B). An identical construct without the miR156 target site served as a control. Transgenic plants expressing miR156 and control sensors were generated to determine the embryonic spatiotemporal activation domains of miR156. Compared with embryos with the control sensor, those with the miR156 sensor had reduced GFP signal throughout the eight-cell, dermatogen, and globular stages (Fig. 3C). Furthermore, GFP signal corresponding to the miR156 sensor was derepressed in *dcl1-5*

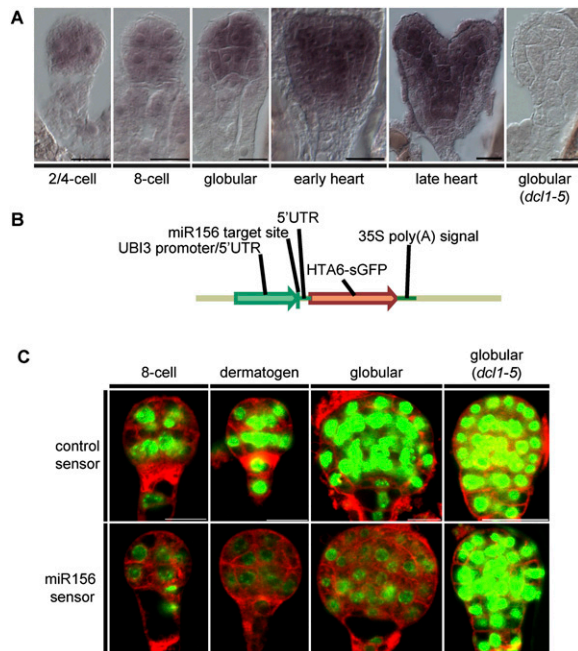


Figure 3. miR156 is localized and active throughout early embryogenesis. (A) Representative micrographs of RNA in situ hybridizations performed with LNA probes antisense to miR156. All embryos are wild type except for the indicated *dcl1-5* embryo. The stage of embryo development is listed below each image. (B) miR156 sensor construct used to detect miR156 activity. The UBI3 promoter/5'UTR, miR156 target site, translational fusion between *Arabidopsis* HISTONE 2A (HTA6) and sGFP, and Cauliflower Mosaic Virus 35S poly(A) site are indicated. (C) CSLM images of embryos expressing sensor constructs with either no site (control sensor; top) or miR156 target site (miR156 sensor; bottom). Embryo developmental stages are indicated above each column of images. All embryos are wild type except for the indicated *dcl1-5* embryos. GFP and FM4-64 fluorescence are shown in green and red, respectively. Bars, 20 μ m.

embryos. Together, these results demonstrate that miR156 is localized and active throughout early embryogenesis, and that miR156 activity is lost in *dcl1-5* embryos.

SPL10 and SPL11 are partially responsible for dcl1 mutant phenotypes

The results presented so far indicate that miR156 is lost in *dcl1* embryos and, as a result, *SPL10* and *SPL11* transcripts increase during early embryogenesis. To determine whether the up-regulation of *SPL10* or *SPL11* in *dcl1* embryos was responsible for the Dcl1 phenotype, we tested whether *spl10* or *spl11* mutants suppressed *dcl1* embryonic defects. Plant lines carrying T-DNA insertions in either *SPL10* (*spl10-1*) or *SPL11* (*spl11-1*) were obtained from the *Arabidopsis thaliana* Resource Center for Genomics at the French National Institute for Agricultural Research (Samson et al. 2002). Although *SPL10* transcripts were detected in *spl10-1* plants, *SPL10* function was likely at least partially disrupted in *spl10-1* plants due to a T-DNA insertion in the first coding sequence exon, which deletes 51 base pairs (bp) of adjacent genomic sequence (Supplemental Fig. 4; data

not shown). Full-length transcripts corresponding to *SPL11* could not be detected from *spl11-1* plants (Supplemental Fig. 4). Embryos homozygous for *spl10-1* or *spl11-1* did not exhibit detectable morphological phenotypes.

Late globular and early heart stage embryos from self-pollinated *dcl1-5/+ spl10-1*, *dcl1-5/+ spl11-1*, and *dcl1-10/+ spl11-1* plants were examined to test for a decrease in the frequency or severity of the Dcl1 phenotype. Selfed *dcl1-5/+ spl10-1* plants produced ~25% Dcl1 embryos, suggesting that *SPL10* alone is not required for the phenotypes observed in *dcl1* embryos (Table 1). In contrast, selfed *dcl1-5/+ spl11-1* and selfed *dcl1-10/+ spl11-1* plants yielded only ~17% and ~19% Dcl1 embryos, respectively (Table 1). Thus, *spl11-1* suppressed the penetrance of the early embryonic Dcl1 phenotype.

Because *SPL10* and *SPL11* transcripts were both increased in *dcl1-5* embryos and encode proteins that are ~76% identical, we then tested whether *SPL10* and *SPL11* were redundantly required for the Dcl1 phenotype. *dcl1/+ spl10 spl11* mutants could not be generated due to the tight linkage of the *SPL10* and *SPL11* loci (Supplemental Fig. 4). However, F1 embryos derived from crosses between *dcl1-5/+ spl10-1* and *dcl1-5/+ spl11-1* plants exhibited the Dcl1 phenotype at frequencies that were significantly less than those observed in embryos from self-pollinated *dcl1-5/+ spl10-1* plants or crosses between *dcl1-5/+* and *dcl1-5/+ spl11-1* plants (Table 1). Therefore, simultaneously reducing the *SPL10* and *SPL11* wild-type alleles by one-half suppressed the penetrance of the Dcl1 phenotype. To further test whether *SPL10* and *SPL11* were redundantly required for the Dcl1 phenotype, we generated *SPL10*-RNAi constructs under the control of a ubiquitously expressed embryonic promoter, and transformed these *SPL10*-RNAi constructs into *dcl1-5/+ spl11-1* plants. A strong knockdown of *SPL10* transcripts was observed in embryos from *dcl1-5/+ spl11-1 SPL10*-RNAi plants relative to embryos from *dcl1-5/+ spl11-1* plants, which demonstrated the functionality of this RNAi construct (Supplemental Fig. 5). Only a small percentage (1.5%; three of 203) of late globular and early heart embryos from selfed *dcl1-5/+ spl11-1 SPL10*-RNAi plants exhibited the Dcl1 phenotype. However, 8% (17 of 203) of embryos from these selfed plants exhibited a partial Dcl1 phenotype, characterized by defects in hypophysis but not subprotoderm cell division (Fig. 4A). With regard to later stages in development, ~25% of late heart and torpedo stage embryos from selfed *dcl1-5/+ spl11-1* plants (23.4%; 32 of 137) and selfed *dcl1-5/+ spl11-1 SPL10*-RNAi plants (22.1%; 35 of 159) resembled *dcl1* embryos. More specifically, they failed to form cotyledons, exhibited aberrant cell divisions throughout the embryo and suspensor, and were arrested in development. Furthermore, seedlings homozygous for *dcl1-5* were never recovered from the progeny of these plants. These results suggest that either the *SPL10*-RNAi construct is not effective at later stages of embryo development, or knockdown of other miRNA targets is also required for a full rescue of *dcl1* embryos. Nevertheless, in globular and early heart stage *dcl1* embryos, *SPL10* and *SPL11* are redundantly required for the observed morphological defects.

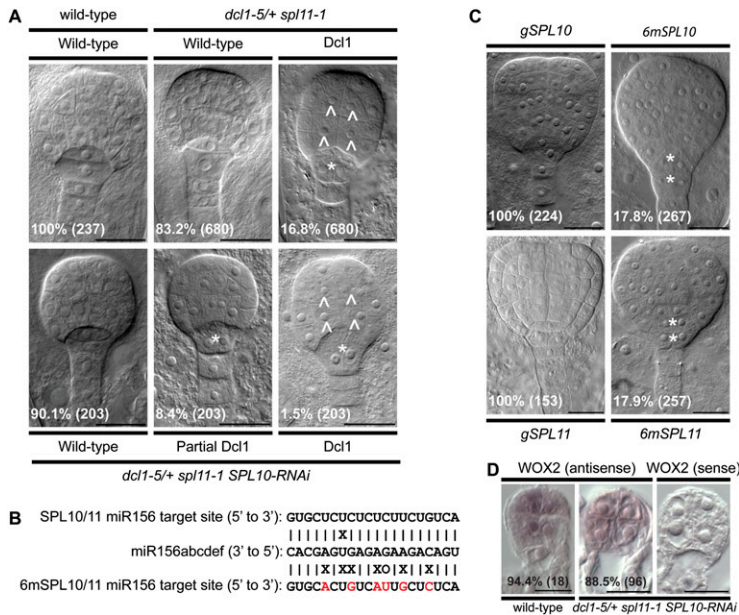


Figure 4. *SPL10* and *SPL11* are redundantly required for *dcl1* mutant phenotypes. (A) Representative Nomarski images of late globular embryos from self-pollinated wild-type, *dcl1-5/+ spl11-1*, and *dcl1-5/+ spl11-1 SPL10-RNAi* plants. Phenotypic classes are indicated directly above or below images and parental plant genotypes are listed directly above or below phenotypic classes. Phenotypic class frequencies for the given parental genotype and the total number of embryos examined are shown in the bottom left corner of each image. Asterisks indicate abnormal products of cell divisions, and carets designate positions where cell divisions did not take place in the mutants. (B) Schematic of base-pairing interactions between *SPL10* and *SPL11* (*SPL10/11*) and *miR156*, and between *6mSPL10/11* and *miR156*. Mutations introduced by site-directed mutagenesis are shown in red. Watson-Crick base-pairing (I), non-base-pairing (X), and G:U wobbles (O) for each pair are indicated. (C) Representative Nomarski images of late globular/transition stage embryos from self-pollinated *gSPL10*, *6mSPL10*, *gSPL11*, and *6mSPL11* plants. Parental genotypes are listed directly above or below each image. Phenotypic frequencies and total number of embryos examined are indicated in the bottom left

corner of each image. Asterisks indicate abnormal cell divisions. (D) Representative micrographs of RNA in situ hybridizations performed with either *WOX2* antisense or sense probes on eight-cell embryos from self-pollinated wild-type and *dcl1-5/+ spl11-1 SPL10-RNAi* plants. Probes and parental genotypes are indicated directly above and below each image, respectively. Frequencies of embryos with detectable *WOX2* transcripts and total number of embryos examined are shown at the bottom of each image. Bars, 20 μ m.

We then tested whether the misregulation of *SPL10* or *SPL11* was sufficient to cause the *Dcl1* phenotype. The *miR156* target sites of *SPL10* and *SPL11* genes were disrupted with synonymous codon mutations within transformation constructs (*6mSPL10* and *6mSPL11*) (Fig. 4B). Upstream and downstream intergenic regions were included in these constructs to preserve other potential *cis*-regulatory elements that control *SPL10* and *SPL11* expression (Supplemental Fig. 4). Transgenic plants carrying nonmutated *SPL10* and *SPL11* loci (*gSPL10* and *gSPL11*) were also generated as controls. Plants carrying either the *6mSPL10* or *6mSPL11* transgenes exhibited traits characteristic of precocious juvenile-to-adult vegetative-phase transitions (Supplemental Fig. 6), as reported for plants expressing *miR156*-resistant *SPL10* or *SPL11* transgenes (Wu et al. 2009). This, together with the observation that *6mSPL10* and *6mSPL11* embryos had significantly increased levels of *SPL10* and *SPL11* transcripts relative to wild type (Supplemental Fig. 5), indicated that the *6mSPL10* and *6mSPL11* transgenes used in our study were expressed and functional. Although they did not phenocopy *dcl1* embryos, *6mSPL10* and *6mSPL11* embryos resembled *dcl1* embryos in exhibiting division defects in cells at the position of the hypophysis derivatives (cf. Figs. 4C and 1A). Embryos expressing *gSPL10* or *gSPL11*, the wild-type versions of these transgenes, resembled wild-type embryos, which suggested that disruption of *miR156*-mediated regulation of *SPL10* or *SPL11* was responsible for the observed phenotypes (Fig. 4C).

6mSPL10 and *6mSPL11* embryos had decreased levels of *SPL10* and *SPL11* transcripts relative to *dcl1-5* embryos (Supplemental Fig. 5), which suggested the possibility that they did not phenocopy the *dcl1* embryos because

the *6mSPL10* and *6mSPL11* transgenic lines that expressed *SPL10* and *SPL11* at the levels observed in *dcl1-5* embryos were not viable and could not be recovered because they did indeed phenocopy the *dcl1* embryos. Alternatively, expression of *SPL10* and *SPL11* at the levels observed in *dcl1-5* embryos still might not have been sufficient to cause the defects observed in *dcl1* embryos. To help distinguish between these two possibilities, we examined embryos from self-pollinated *6mSPL10/6mSPL11* plants, some of which had increased dosage of the *miR156*-resistant mRNAs because they were homozygous at both alleles. Approximately 16% (21 of 128) resembled the abnormal embryos observed in *6mSPL10* and *6mSPL11* embryos, but none phenocopied *dcl1* embryos. Thus, disrupting *miR156*-mediated regulation of *SPL10* and *SPL11* caused defects during embryo morphogenesis but appears insufficient to phenocopy *dcl1* embryos.

To test whether *SPL10* and *SPL11* were required for the loss of the apical cell lineage-specific transcript *WOX2* in *dcl1-5* preglobular embryos (Fig. 1B), we examined *WOX2* transcripts in eight-cell and dermatogen stage embryos from selfed *dcl1-5/+ spl11-1 SPL10-RNAi* plants using RNA in situ hybridizations. The frequency of eight-cell and dermatogen stage embryos with detectable *WOX2* transcripts significantly increased in the progeny of selfed *dcl1-5/+ spl11-1 SPL10-RNAi* plants relative to selfed *dcl1-5/+* plants [88.5% [85 of 96] vs. 75.9% [66 of 87], respectively; $P < 0.05$] (Fig. 4D). Therefore, *SPL10* and/or *SPL11* appeared to be required for the reduction of *WOX2* transcripts in *dcl1-5* preglobular embryos. To test whether misregulated *SPL10* or *SPL11* were sufficient for *WOX2* transcript reduction, we examined *WOX2* transcript levels in eight-cell

embryos from *6mSPL10* and *6mSPL11* plants. There was not a significant difference in transcript levels between wild-type, *6mSPL10*, and *6mSPL11* eight-cell embryos (Supplemental Fig. 7). Collectively, our results indicate that derepression of *SPL10* and *SPL11* is required for a substantial part of the *dcl1* patterning defects, but disrupting miR156-mediated regulation of these genes is not sufficient to fully recapitulate the *dcl1* patterning defects.

SPL10 and SPL11 zygotic transcripts are repressed by miR156 during early embryogenesis

Because *SPL10* and *SPL11* transcripts were dramatically repressed by miR156 in eight-cell embryos (Fig. 2C; Supplemental Fig. 5), we tested whether miR156 clears maternal or paternal *SPL10/SPL11* transcripts from early embryos. We did not detect significant parent-of-origin effects of the *spl11-1* allele or the *6mSPL10/6mSPL11* transgenes on zygotic *SPL10/SPL11* transcript levels (Supplemental Fig. 8).

To assess the spatiotemporal expression patterns of *SPL10* and *SPL11* during embryogenesis, we examined the activity of *SPL10* and *SPL11* promoters transcriptionally fused to a nuclear-localized GFP (*SPL10p::NLS-GFP* and *SPL11p::NLS-GFP*). Weak signal corresponding to *SPL10p::NLS-GFP* was detected in the subprotoderm and suspensors of dermatogen stage embryos and became more intense throughout the embryo during the globular stages (Fig. 5). Low levels of *SPL11p::NLS-GFP* signal were detected throughout eight-cell embryos and, like *SPL10p::NLS-GFP* signals, became progressively stronger during the globular stages (Fig. 5). Signal corresponding to *SPL10p::NLS-GFP* and *SPL11p::NLS-GFP* transgenes was more intense in the basal half of the embryo proper at the late globular stage, had peak levels at the heart stage, and were detected in torpedo stage embryos with increased levels in the shoot and root meristem precursors and vascular primordium (Fig. 5). These results were consistent with the Harada-Goldberg *Arabidopsis* seed development laser capture microdissection microarray data set

(National Center for Biotechnology Gene Expression Omnibus [NCBI GEO]: GSE12404). We conclude that *SPL10p::NLS-GFP* and *SPL11p::NLS-GFP* are therefore expressed in overlapping patterns at the stages and in the cell types that exhibited patterning defects in *dcl1*, *6mSPL10*, and *6mSPL11* embryos. Furthermore, they are expressed in the embryonic spatiotemporal domains where miR156 is active (cf. Figs. 3C and 5). Taken together, our results indicate that miR156 represses the zygotic expression of both *SPL10* and *SPL11* during early embryogenesis.

Hundreds of genes are expressed prematurely in dcl1 embryos

In addition to the many miRNA targets up-regulated in *dcl1-5* early globular embryos, we found many other genes that were also up-regulated. More specifically, 37.1% (4331 of 11,570) of genes with at least 20 raw reads in the *dcl1-5* early globular library had at least twofold significantly more reads in *dcl1-5* relative to wild type. Four of the 10 most up-regulated genes in *dcl1-5* early globular embryos encoded OLEOSINS (Fig. 6A). Because OLEOSINS are found in the oil bodies of mature embryos (Frandsen et al. 2001), this suggested that *OLEOSIN* genes are expressed prematurely in *dcl1-5* early globular embryos, and that the same might be true for other genes. Indeed, using the Harada-Goldberg data set (NCBI GEO: GSE12404), we found that nine out of the 10 most up-regulated genes in *dcl1-5* early globular embryos had increased transcript levels in mature embryos relative to globular embryos (Fig. 6B). Moreover, significantly elevated transcript levels were detected for six out of nine of these genes in eight-cell embryos derived from selfed *dcl1-5/+* plants relative to wild-type eight-cell embryos (Fig. 6C). In fact, transcripts corresponding to only one of the nine genes were detected in wild-type eight-cell embryos, consistent with their expression being mostly restricted to the maturation phase of embryogenesis. In summary, we observed that the most up-regulated genes in *dcl1-5* early globular embryos are typically expressed at post-globular stages of embryo development, and are expressed prematurely as early as the eight-cell stage in *dcl1-5* embryos.

To extend the analysis to the thousands of other genes up-regulated in *dcl1-5* relative to wild-type early globular embryos, we tested whether these genes also tended to be expressed preferentially in mature embryos compared with globular embryos. Compared with control gene sets, genes with at least twofold significantly increased transcript levels in *dcl1-5* early globular embryos were significantly enriched for genes with increased transcript levels in mature embryos relative to globular embryos, and were significantly depleted of genes with decreased transcript levels in mature embryos relative to globular embryos (Fig. 6D). Conversely, genes with transcript levels significantly decreased at least twofold in *dcl1-5* early globular embryos were significantly enriched for genes that have decreased transcript levels in mature embryos relative to globular embryos, and were significantly depleted of genes that had increased transcript levels in mature embryos (Fig. 6D). Therefore, many

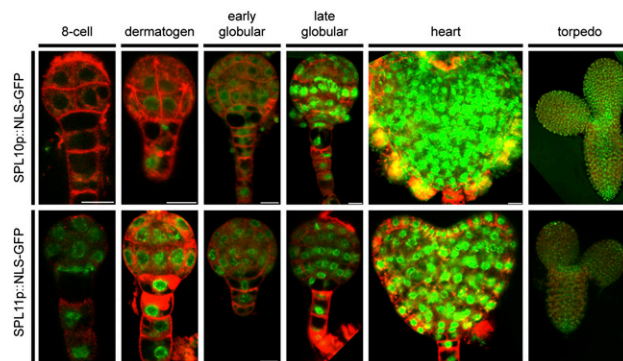


Figure 5. Spatiotemporal activation domains of *SPL10* and *SPL11* promoters. Representative CSLM images of embryos from plants carrying *SPL10p::NLS-GFP* or *SPL11p::NLS-GFP* transgenes. Parental genotypes are indicated to the left of each row of images, and embryonic stages are designated above each column of images. The green signal corresponds to GFP, and the red signal corresponds to FM4-64. Bars, 20 μ m.

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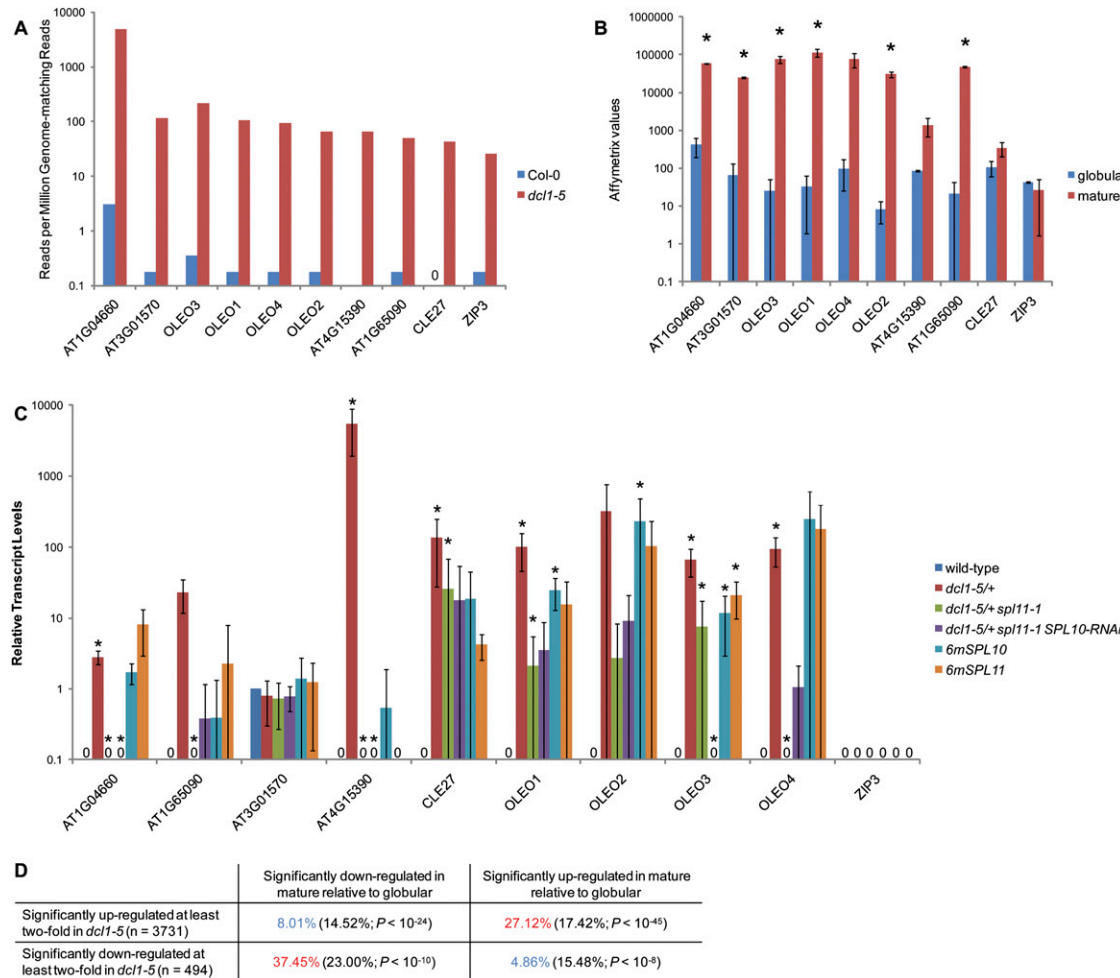


Figure 6. miR156 regulation of *SPL10* and *SPL11* prevents premature gene expression during early embryogenesis. (A) Normalized read numbers of the top 10 up-regulated genes in *dcl1-5* early globular embryos relative to wild-type early globular embryos based on mRNA-Seq. Transcripts that were not detected are indicated by a zero. (B) Transcript levels of the top 10 up-regulated genes in *dcl1* early globular embryos at globular and mature stages of embryo development based on the Harada-Goldberg data sets (NCBI GEO: GSE12404). Error bars represent one standard deviation of the sample. (C) Relative transcript levels of the top 10 up-regulated genes in eight-cell embryos from self-pollinated Col-0, *dcl1-5/+*, *dcl1-5/+ spl11-1*, *dcl1-5/+ spl11-1 SPL10-RNAi*, *6mSPL10*, and *6mSPL11* plants based on qRT-PCR and normalized to Col-0 levels. Transcripts that were not detected are indicated by a zero. Error bars represent one standard deviation of the sample mean. Asterisks indicate either significantly increased transcript levels compared with wild-type (*dcl1-5/+*, *6mSPL10* and *6mSPL11* samples) or significantly decreased transcript levels compared with *dcl1-5/+* (*dcl1-5/+ spl11-1* and *dcl1-5/+ spl11-1 SPL10-RNAi* samples). Probabilities were calculated with heteroscedastic Student's *t*-tests. Probabilities < 0.05 were considered significant. (D) Frequency of genes that are either significantly up-regulated or down-regulated in *dcl1-5* early globular embryos (left column) that also have significantly different transcript levels in mature embryos relative to globular embryos (top row) based on publicly available Affymetrix microarray data sets (NCBI GEO: GSE12404). The total number of genes that are either significantly up-regulated or down-regulated in *dcl1-5* and that also have corresponding probes on the Affymetrix arrays are represented by *n*. Frequencies are color-coded in red or blue to indicate enrichment or depletion, respectively. Frequencies in parentheses are what is expected based on randomly selected control sets corrected for gene expression levels (see the Materials and Methods for more details). Probabilities (*P*) that differences between observed frequencies and expected frequencies (based on random control sets) are different due to chance are shown and were calculated using χ^2 distributions.

genes are prematurely up-regulated or down-regulated in *dcl1* early globular embryos relative to wild type.

Loss of miR156-mediated regulation of SPL transcripts is responsible for premature accumulation of transcripts in eight-cell embryos

The premature expression of many genes in *dcl1-5* embryos could be a consequence of the up-regulation of

miRNA targets (Fig. 2; Supplemental Table 1). Because *SPL10* and *SPL11* transcripts were dramatically up-regulated in eight-cell *dcl1-5* embryos (Fig. 2C), we tested whether *SPL10* and *SPL11* function upstream of prematurely expressed genes. The levels of transcripts corresponding to the 10 most up-regulated genes in *dcl1-5* early globular embryos were examined in eight-cell embryos from self-pollinated *dcl1-5/+ spl11-1* plants to test whether *spl11-1* suppressed the premature accumulation

of transcripts observed in *dcl1* embryos. Among the genes examined by qRT-PCR, transcript levels for all six that were significantly increased in *dcl1-5* relative to wild-type eight-cell embryos were suppressed in eight-cell embryos from *dcl1-5/+ spl11-1* plants (Fig. 6C). Transcript levels for these genes were also suppressed in eight-cell embryos from *dcl1-5/+ spl11-1 SPL10-RNAi* plants (Fig. 6C). These results indicate that *SPL11* is necessary for the accumulation of transcripts corresponding to at least some of the genes expressed prematurely in *dcl1-5* embryos.

To test whether disruption of miR156-mediated regulation of *SPL10* or *SPL11* is sufficient for the premature accumulation of transcripts in eight-cell embryos, we examined premature transcript levels in embryos expressing miR156-resistant *6mSPL10* or *6mSPL11* transgenes. Eight of the top 10 most up-regulated genes in *dcl1-5* early globular embryos had both increased transcript levels in mature embryos relative to globular embryos and increased transcript levels in eight-cell embryos from selfed *dcl1-5/+* plants (Fig. 6B,C). Seven of these eight genes also had at least twofold increased transcript levels in embryos expressing either the *6mSPL10* or *6mSPL11* transgene (Fig. 6C). These results suggest that, upon the disruption of miR156-mediated regulation, *SPL10* and *SPL11* are sufficient to promote the precocious accumulation of maturation-phase transcripts in eight-cell embryos.

Discussion

We found that *DCL1* and, by implication, miRNAs were required for multiple embryonic patterning events beginning as early as the eight-cell stage. In fact, the only cell type that appeared to differentiate correctly in *dcl1* embryos was the outermost protoderm layer. Furthermore, protoderm markers that are typically restricted to the embryo proper were ectopically expressed in *dcl1* suspensors at the late globular and early heart stages (Fig. 1B; Supplemental Fig. 2), which helps explain a previously reported function of *DCL1/SUS1* in maintaining the extraembryonic cell fate of the suspensor (Schwartz et al. 1994).

The requirement of miRNAs for proper embryonic patterning and cell differentiation might have suggested that embryonic miRNAs act to trigger developmental transitions, as was first reported for metazoan miRNAs (Lee et al. 1993; Wightman et al. 1993; Moss et al. 1997; Reinhart et al. 2000), where induced miRNAs act to down-regulate genes that contribute to the differentiation state of the precursor cells. Or they might act to sharpen developmental transitions, as observed in fish, frog, and, perhaps, mammalian embryos, where miR-430 or its orthologs clears maternally expressed messages to sharpen the transition from maternal to zygotic expression programs (Farh et al. 2005; Giraldez et al. 2006; Lund et al. 2009). Indeed, an analogous function in sharpening developmental transitions was proposed and has been observed for plant miRNAs at later developmental stages (Rhoades et al. 2002; Aukerman and Sakai 2003; Lauter et al. 2005; Wu et al. 2009). However, our molecular characterization of the *Dcl1* phenotype shows that the earliest known developmental roles of miRNAs in plants is not to trigger or sharpen developmental transitions by repressing genes with developmental functions in precursor cell types, but instead is the opposite (Fig. 7). In eight-cell embryos, miRNAs repress genes that function in daughter cell types later in embryo development. Instead of attenuating pre-existing function, they prevent precocious function. Dicer and other miRNA biogenesis proteins are required for formation of the mammalian mesoderm and differentiation of murine embryonic stem cells (Bernstein et al. 2003; Kanellopoulou et al. 2005; O'Rourke et al. 2007; Wang et al. 2007). Perhaps as the roles of individual vertebrate miRNAs are examined in more detail, analogous functions in preventing precocious embryonic expression will help explain the requirement of miRNAs for mammalian embryonic development. Indeed, recent studies show that the extraembryonic lineage of mouse embryos uses miRNAs to prevent premature differentiation and maintain extraembryonic stem cell multipotency (Spruce et al. 2010).

One of the earliest perceptible roles of plant miRNAs is to repress transcription factors (Fig. 2C). We propose that, by

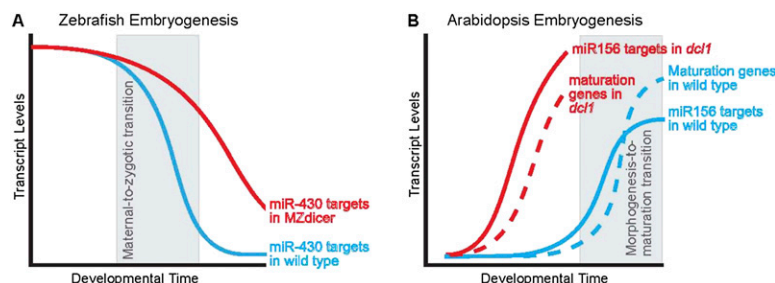


Figure 7. Opposite functions of animal and plant miRNAs during early embryogenesis. (A) In zebrafish embryos, miR-430 sharpens the maternal-to-zygotic transition by directing the destabilization of maternally derived target transcripts, which function earlier in development (Giraldez et al. 2006). MZdicer (maternal-zygotic dicer) embryos lack Dicer and miR-430 and exhibit delayed reduction of maternal miR-430 targets. (B) In *Arabidopsis* embryos, miR156 delays the production of maturation transcripts by directing the repression of *SPL10/11*. Early *dcl1* mutant embryos lack miR156

and exhibit premature expression of miR156 targets *SPL10* and *SPL11*, which in turn induces precocious expression of genes normally induced during the maturation phase of embryogenesis. We propose that additional plant miRNAs—including miR160, miR166, and miR319—also forestall expression of differentiation-promoting transcription factors such as *ARF17*, *CNA*, *PHB*, *PHV*, and *TCP4*. A link between delayed reduction of maternal transcripts and the morphogenesis phenotypes observed in MZdicer embryos awaits experimental confirmation. The same is true for the precocious expression of maturation transcripts and the patterning phenotypes observed in *dcl1* embryos, although *SPL10* and *SPL11* have been experimentally linked to defects in both patterning and maturation gene expression.

repressing differentiation-promoting transcription factors (including *SPL10*, *SPL11*, *ARF17*, *CNA*, *PHB*, *PHV*, and *TCP4*), miRNAs maintain the potential of preglobular cells to generate diverse cell types at the subsequent globular stages. Since the reduction of *WOX2* transcripts in *dcl1* eight-cell embryos was the first detectable differentiation defect, and homeobox genes related to *WOX2* have been implicated in maintaining stem cells during post-embryonic development (Laux et al. 1996; Mayer et al. 1998; Wu et al. 2005), we speculate that premature expression of miRNA targets leads to the reduction of *WOX2* transcripts in *dcl1* embryos and ultimately contributes to precocious differentiation and subsequent loss of developmental potential in preglobular cell types.

The two transcription factor genes that were most derepressed in eight-cell *dcl1* embryos were miR156 targets *SPL10* and *SPL11*, for which transcripts were up-regulated ≥ 150 -fold (≥ 600 -fold if only the homozygous mutant embryos contributed to the increase). These two genes were redundantly required for the embryonic patterning defects observed in *dcl1* embryos (Table 1; Fig. 4A,D). The ability to suppress such a pleiotropic phenotype by knocking out/down only two miRNA targets was surprising when considering that dozens of miRNA targets were derepressed in *dcl1* embryos. However, miR156-resistant *SPL10* and *SPL11* transgenes did not phenocopy *dcl1* embryos, which suggests that the misregulation of additional targets contributes to the patterning defects observed. Consistent with this idea, at least seven miRNA target transcripts in addition to *SPL10* and *SPL11* have increased levels as early as the eight-cell stage, and all of these encode transcription factors.

Of these seven misregulated targets, five (*ARF17*, *CNA*, *PHB*, *PHV*, and *TCP4*) have reported embryonic functions after the preglobular stage (Palatnik et al. 2003; Mallory et al. 2005; Prigge et al. 2005). miR166-mediated regulation of *PHB* and *PHV* has also been reported to be important during early embryonic patterning (Grigg et al. 2009). However, *phb phv* double mutants do not suppress *dcl1*-null embryonic phenotypes (Grigg et al. 2009), suggesting that the misregulation of other miRNA targets is required for the *dcl1* embryonic phenotypes. Perhaps the loss of both miR156-mediated regulation of SPL transcripts and miR166-mediated regulation of HD-ZIPIII transcripts in *dcl1* embryos is responsible for the embryonic patterning phenotypes observed. Future characterization of how multiple miRNA/target interactions facilitate embryonic cell differentiation will contribute to a better understanding of how the basic plant body plan is established during embryo morphogenesis.

We also report previously uncharacterized roles of miR156-mediated *SPL* gene repression during embryogenesis. After morphogenesis, seed plant embryos transition to a maturation phase, when they accumulate storage proteins, undergo desiccation tolerance, and prepare to enter into a state of dormancy prior to germination. We found that miR156-mediated regulation of *SPL10* and *SPL11* prevents the transcription factor products of these genes from prematurely inducing seed maturation genes before the embryo has formed. To the extent that this

regulation forestalls the morphogenesis-to-maturation-phase transition, this newly identified role for miR156-mediated repression of *SPL* genes resembles that observed for vegetative-, reproductive-, and meristem identity-phase transitions during post-embryonic development (Wu and Poethig 2006; Gandikota et al. 2007; Schwarz et al. 2008; Wang et al. 2009; Wu et al. 2009; Yamaguchi et al. 2009). This role for *SPL10* and *SPL11* repression might be tied to the requirement of these targets for the patterning defects observed in *dcl1* embryos. Perhaps the premature induction of maturation-phase genes arrests morphogenesis before it is complete.

Later in development, the miR156-mediated repression of *SPL10* and *SPL11* must presumably be overcome in order for the embryo to undergo the morphogenesis-to-maturation transition. Although miR156 levels do not appear to decrease at later stages of morphogenesis (Fig. 3A), *SPL10* and *SPL11* promoter activities do increase at the same stages that the morphogenesis-to-maturation-phase transition occurs (Fig. 5). Therefore, at later stages of morphogenesis, miR156 may establish a threshold that *SPL10* and *SPL11* transcript levels must surpass in order to accumulate and promote maturation-phase gene expression programs. Despite the characterization of hormonal, metabolic, and genetic factors involved in embryo maturation (Nambara and Marion-Poll 2003; Weber et al. 2005; Braybrook and Harada 2008), the regulatory mechanisms that control the morphogenesis-to-maturation-phase transition are still not well understood. Multiple factors may influence *SPL10* and *SPL11* transcript levels and ultimately regulate the timing of the morphogenesis-to-maturation-phase transition. Further characterization of the embryonic functions of *SPL* transcription factors may provide a handle to acquire a better molecular understanding of the morphogenesis-to-maturation-phase transition.

Materials and methods

Growth conditions and genetic analyses

The *dcl1-5* and *dcl1-10* alleles were generated by McElver et al. (2001) and obtained from the *Arabidopsis* Biological Resource Center (ABRC). *DR5rev::GFP* lines were obtained from the Nottingham *Arabidopsis* Stock Center (Scholl et al. 2000). Plants were grown at 20°C–24°C in a growth room with a 16-h light/8-h dark cycle.

Generation of transgenic lines

SPL10p::SPL10-GFP constructs were generated by cloning genomic fragments, including 1.7 kb upstream of and including the *SPL10* coding sequence (CDS), into pENTR/D-TOPO (Invitrogen), and then recombining these plasmids with Gateway-compatible pBIB-KAN-GWR-GFP plasmids using LR clonase (Invitrogen). The primers used in this study are listed in Supplemental Table 2.

Control sensor constructs were created by cloning the potato *UBI3* promoter into pENTR/D-TOPO (Invitrogen). The resulting plasmids were then recombined with pCGTAG. A similar procedure was used to create miR156 sensor constructs, except that miR156 target sites were introduced into the construct by PCR.

SPL10p::NLS-GFP and *SPL11p::NLS-GFP* constructs were generated by PCR amplification of 1.9-kb and 2.2-kb genomic

DNA, respectively. The resulting amplicons were then cloned into pCR8/GW-TOPO (Invitrogen) and recombined with pCGTAG.

6mSPL10 and 6mSPL11 constructs were created by cloning 4.9-kb (including 1.8 kb upstream of CDS, CDS, and 1.6 kb downstream from CDS) and 5.5-kb (including 2.7 kb upstream of CDS, CDS, and 1.3 kb downstream from CDS) genomic fragments in pENTR/D-TOPO (Invitrogen), respectively. The QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene) was used on the resulting plasmids to disrupt miR156-binding sites, and both nonmutagenized and mutagenized plasmids were recombined with pBIB-KAN-GW using LR clonase (Invitrogen).

SPL10-RNAi constructs were generated by amplifying 0.6 kb of the *SPL10* CDS from cDNA prepared from inflorescence tissue total RNA isolated using TRIzol reagent (Invitrogen) and reverse-transcribed with oligo(dT) primers using SuperScript III Reverse Transcriptase (Invitrogen). The resulting amplicons were then cloned into pCR8/GW-TOPO (Invitrogen) and then recombined with UBI3p::pFSPGW-HYGv02 to create an inverted repeat of the *SPL10* cDNA fragment under the control of the *UBI3* promoter. The UBI3p::pFSPGW-HYGv02 vector was created by first adding AatII and XhoI sites on the 5' and 3' ends of the *UBI3* promoter by PCR, cloning the amplicons into pENTR/D-TOPO (Invitrogen) to create UBI3F2R2::pENTR, double-digesting UBI3F2R2::pENTR and pFSPGW with AatII and XhoI, and ligating together the appropriate digestion fragments to create UBI3p::pFSPGW. A hygromycin resistance cassette was then amplified from pCGTAG with primers that introduced Bsu36I and Acc65I sites on the 5' and 3' ends of the amplicons, respectively, and these were cloned into pCR8/GW-TOPO to create HYGf3R3::pCR8. HYGf3R3::pCR8 and UBI3p::pFSPGW were then double-digested with Bsu36I and Acc65I and ligated together to create UBI3p::pFSPGW-HYGv02.

All constructs were transformed into Col-0 and *dcl1-5/+* plants via *Agrobacterium*-mediated transformation (Clough and Bent 1998). More than 20 embryos from at least five independent transgenic lines were examined for each construct.

Microscopy

Ovules were fixed and cleared as described previously (Ohad et al. 1996), and embryos were examined using Nomarski optics on a Nikon E800 upright microscope for morphological analysis. Images were collected with a Hamamatsu C4742-95 CCD camera and viewed with Openlab acquisition software (PerkinElmer).

For CSLM analysis, embryos were dissected in pH 7.2 potassium phosphate buffer, stained with 1 μ g/mL FM4-64, and mounted in 5% glycerol. A 488-nm laser on a Zeiss LSM 510 confocal microscope was used to excite GFP, YFP, and FM4-64, and images were collected at 505–530 nm, 500–550 nm, and 644–719 nm, respectively.

RNA in situ hybridizations

WOX2, WOX8, RPS5A, eIF4A1, and PDF1 probes were generated by introducing T7 promoters with PCR and using the procedure outlined by Hejatko et al. (2006). miR156 LNA oligonucleotides were ordered directly from Integrated DNA Technologies. ATML1, PNH, and SCR probe generation; fixing, embedding, and sectioning of siliques; hybridization; washes; and immunological detection were performed as described previously (Nordine et al. 2007) except for miR156 LNA in situ, where 10 pmol of probe was used in each hybridization reaction.

mRNA sequencing and analysis

For RNA isolation, pools of 30 Col-0 (wild-type) and *dcl1-5* early globular embryos were hand-dissected in water, immediately transferred to 30 μ L of RNeasy lysis buffer (Qiagen), incubated at 60°C

with 500 μ L of TRIzol reagent (Invitrogen) for 30 min, and then purified according to the TRIzol reagent protocol for RNA isolation from small quantities of tissue (Invitrogen). Two rounds of linear amplification of poly(A) RNA were performed using the Arcturus RiboAmp HS Plus kit (Molecular Devices). Strand-specific mRNA-Seq libraries were generated as described previously (Guo et al. 2010). After removing adapter sequences, sequences were mapped to the *A. thaliana* genome (TAIR9 assembly) using the Bowtie short-read aligner (Langmead et al. 2009), allowing up to two mismatches within a 25-nt “seed” sequence and retaining reads mapping uniquely to the genome. Wild-type and *dcl1* libraries yielded 5.6 million and 4.5 million reads that uniquely matched the *Arabidopsis* genome, respectively. Since the Arcturus RiboAmp HS Plus kit generates amplified RNAs in the antisense orientation, reads were counted as matching TAIR9 annotated genes if they overlapped the antisense strand. Approximately 90% of genome-matching reads (5,061,561 for wild-type and 3,973,657 for *dcl1-5*) matched annotated genes of the TAIR9 genome release (Swarbreck et al. 2008). To normalize for differences in library size, the number of reads matching each gene was divided by the total number of million genome-matching reads for each library. We then considered the number of normalized reads that matched miRNA targets listed in the *Arabidopsis* Small RNA Project (ASRP) database (Gustafson et al. 2005), comparing the read numbers in wild-type and *dcl1-5* early globular embryo libraries (Supplemental Fig. 3). For comparisons between the set of genes up-regulated in *dcl1-5* relative to wild-type embryos and the set of genes up-regulated in mature relative to globular embryos, we generated a control gene set. The control gene set was generated by first placing the genes significantly up-regulated at least twofold in *dcl1-5* early globular embryos into 10 approximately equally sized bins based on expression levels. Genes expressed in Col-0 early globular embryos were then sorted into the established bins, and were randomly chosen uniformly from these bins to correct for associations between gene expression levels and the tendency for a gene to be up-regulated in either *dcl1-5* early globular embryos or wild-type mature embryos. The mRNA-Seq data sets generated in this study have been deposited in NCBI GEO.

qRT-PCR analysis

Total RNA from pools of at least 20 embryos was isolated and linearly amplified as described above, and 100 ng of amplified RNA was used in reverse transcription reactions using Random Primers (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen). Gene-specific and *eIF4A1* (endogenous control) primers were used in the real-time PCR using a 7900HT Fast Real-Time PCR system from Applied Biosystems. $\Delta\Delta$ Ct values were calculated for each gene, and relative transcript levels were derived from these values. For transcripts that were not detectable in wild type, test samples (from selfed *dcl1-5/+* plants) were serially diluted with wild-type samples in order to determine the maximum cycle number within the exponential phase of the PCR for which fluorescence could be detected. The corresponding Δ Ct value was then used to calculate the relative transcript levels in the test samples.

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