Running head: microRNAs and the timing of embryo maturation

Corresponding author: Pablo D. Jenik
Department of Biology, Franklin & Marshall College, Lancaster, PA 17604-3003, USA
(717) 358-4431
pjenik@fandm.edu

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microRNAs regulate the timing of embryo maturation in *Arabidopsis*

Matthew R. Willmann¹, Andrew J. Mehalick², Rachel L. Packer², Pablo D. Jenik² *

¹ Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA; ² Department of Biology, Franklin & Marshall College, Lancaster, PA 17604-3003, USA
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**Current addresses:** Andrew J. Mehalick: Philadelphia College of Osteopathic Medicine, Philadelphia, PA 19131; Rachel L. Packer: MCERT Secondary Masters of Education in Biology Program, University of Maryland, College Park, MD 20742.

**Author for correspondence:** Pablo D. Jenik, pjenik@fandm.edu
Abstract
The seed is a key evolutionary adaptation of land plants that facilitates dispersal and allows for germination when the environmental conditions are adequate. Mature seeds are dormant and desiccated with accumulated storage products that are to be used by the seedling after germination. These properties are imposed on the developing embryo by a maturation program, which operates during the later part of embryogenesis. A number of “master regulators” (the LEC genes) required for the induction of the maturation program have been described, but it is not known what prevents this program from being expressed during early embryogenesis. Here we report that Arabidopsis thaliana embryos mutant for strong alleles of DICER-LIKE1—the enzyme responsible for the biosynthesis of microRNAs (miRNAs)—mature earlier than their wild type counterparts. This heterochronic phenotype indicates that miRNAs are key regulators of the timing of the maturation program. We demonstrate that miRNAs operate in part by repressing the master regulators LEC2 and FUS3, and identify the trihelix transcription factors ASIL1 and ASIL2 and the histone deacetylase HDA6/SIL1 as components that act downstream of miRNAs to repress the maturation program early in embryogenesis. Both ASIL1 and HDA6/SIL1 are known to act to prevent the expression of embryonic maturation genes after germination, but this is the first time they have been shown to have a role during embryogenesis. Our data point to a common negative regulatory module of maturation during early embryogenesis and seedling development.
Introduction

One of the reasons for the evolutionary success of seed plants is their ability to generate a resistant structure, the seed, which facilitates dispersal and reinitiates development only in the appropriate environmental conditions. In *Arabidopsis thaliana*, wild-type embryos follow a predictable pattern of cell divisions, going through a series of stages named after the shape of the embryo: preglobular, globular, transition, heart, torpedo, bent green cotyledon and mature (Jürgens and Mayer, 1994). These stages encompass two major phases of development. The first part of embryogenesis, until the heart stage, is devoted to patterning, setting up the embryonic axes, meristems and tissue types (Jenik et al., 2007). The heart-to-late-heart stage transition marks the onset of embryonic maturation, first evidenced by the appearance of chlorophyll auto-fluorescence in the epidermis of the hypocotyl, signaling the beginning of proplastid maturation to chloroplasts (Mansfield and Briarty, 1991). The embryos turn green in color and start accumulating storage products at the early torpedo stage. The photosynthetic activity of the embryonic chloroplasts may contribute to the biosynthesis of storage lipids (Ruuska et al., 2004). In *Arabidopsis* the storage products include seed storage proteins and storage lipids (very long chain fatty acids (VLCFA), polyunsaturated fatty acids (PUFA) and triacylglycerol (TAG)) (Baud et al., 2008). Once the embryos fill the seed, they acquire desiccation tolerance, desiccate, and enter dormancy (Vicente-Carbajosa and Carbonero, 2005; Baud et al., 2008).

Because the maturation program directs seed dormancy, it needs to be carefully timed, ensuring it starts mid-embryogenesis and is repressed after germination. A complex network is involved in timing maturation during embryo development, including positive regulators of maturation during mid-embryogenesis and negative regulators after germination. The existence of repressors of maturation in early embryogenesis has been postulated, but none have been identified so far (reviewed by (Baud et al., 2008; Santos-Mendoza et al., 2008).

The central positive regulators of the seed maturation program are the “LEC genes” (the B3 domain transcription factors *LEAFY-COTYLEDON2* (*LEC2*) and *FUSCA3* (*FUS3*), and the B subunits of the NF-Y family of trimeric transcription factors
LEC1 (also called NF-YB9) and LEC1-LIKE (LIL, NF-YB6)) and the B3 domain transcription factor ABA INSENSITIVE3 (ABI3). Single loss-of-function mutations in all of these genes result in deficiencies in the accumulation of storage products, desiccation intolerance and/or a heterochronic change of cotyledons into true leaves. These factors regulate each other at the transcriptional level in embryos, and their interactions vary depending on tissue type. The hormones abscisic acid (ABA) and gibberellic acid (GA) interact with this network of transcription factors. High ratios of ABA to GA promote maturation via ABI3 and ABI5 (Santos-Mendoza et al., 2008). In vitro studies have suggested that LEC1 and L1L interact with NY-FC1, 2 and 6 to upregulate the induction of storage product genes by ABA (Yamamoto et al., 2009). Several lines of evidence have identified other positive regulators, acting either at the same level or downstream of the LEC genes, by binding to the promoters of genes encoding storage proteins and other genes involved in seed maturation: bZIP transcription factors that cooperate with the NF-Y complexes (ABI5, bZIP10, bZIP25, bZIP53 and bZIP67) (Bensmihen et al., 2002; Alonso et al., 2009; Yamamoto et al., 2009), MYB transcription factors (AtMYB115 and AtMYB118) (Zhang et al., 2008; Wang et al., 2009), and MADS-box transcription factors (AGAMOUS-LIKE15 (AGL15) and AGL18) (Zheng et al., 2009).

A number of factors are known to prevent the expression of embryonic traits after seed germination (reviewed by (Zhang and Ogas, 2009). This negative regulation involves transcriptional mechanisms, via the B3-domain proteins VAL1/HSI2 (VP1/ABI3-LIKE or HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE) and VAL2/HSL1 and the trihelix protein ASIL1 (ARABIDOPSIS 6B-INTERACTING PROTEIN 1-LIKE1). Epigenetic factors also repress the maturation program in seedlings, including histone deacetylases (HDA6/SIL1 and HDA19), Polycomb-group proteins (SWINGER, CURLY LEAF and MEDEA), and chromatin remodelers (BRAHMA, AtSWI3c, BSH and PICKLE). These transcriptional and epigenetic regulators appear to act both directly and indirectly (by modulating the actions of GA) to prevent the expression of the LEC genes and of the genes encoding storage products. It is not known whether any of these regulators or other factors are responsible for the repression of the maturation early in embryogenesis.
miRNAs are 21-nucleotide single-stranded RNA molecules that act by binding complementary target mRNAs to promote their cleavage or interfere with translation (reviewed by (Voinnet, 2009). miRNAs are generated by cleavage of a precursor miRNA (pri-miRNA) by a complex that includes the RNase III DICER-LIKE1 (DCL1), the double-stranded RNA-binding protein HYponastic LEAVES1 (HYL1) and the C2H2-zinc finger protein SERRATE (SE). The resulting miRNAs are then methylated by HUA-ENHANCER1 (HEN1) and incorporated into a RISC complex, which leads the miRNA to its target and effects either cleavage or repression of translation.

ARGONAUTE proteins such as AGO1 and AGO10/ZWILLE/PINHEAD are central components of miRNA RISC complexes. Null alleles of dcl1 are embryonic lethal, but embryos mutant for other elements of the miRNA pathway have either non-overlapping phenotypes, or no observable phenotype (Lynn et al., 1999; Lu and Fedoroff, 2000; Chen et al., 2002; Grigg et al., 2005; Ronemus et al., 2006; Kurihara et al., 2009). Null alleles of dcl1 do accumulate storage products (Schwartz et al., 1994) but, other than interactions of miR159 with the ABA pathway during germination (Reyes and Chua, 2007), no connections have been reported between the miRNA pathway and seed maturation.

Here we report that miRNAs are key repressors of the maturation program during embryogenesis. We demonstrate that embryos mutant for strong dcl1 alleles are heterochronic, maturing much earlier than normal. This heterochronic phenotype requires the action of the LEC genes LEC2 and FUS3 downstream of DCL1. We also present the first evidence for repression of maturation during early embryogenesis by ASIL1, ASIL2 and HDA6/SIL1. These genes also act downstream of the miRNA pathway. Our data suggest that one or more miRNA targets sit at the top of the regulatory cascade controlling both activators and repressors of the embryonic maturation program.

Results

Precocious maturation in dcl1 embryos

During an EMS screen for embryo defective mutants (Jurkuta et al., 2009), we isolated a mutant with two conspicuous phenotypes: very abnormal patterning (Fig. 1A, B) and early chlorophyll accumulation (Fig. 1D-H). Due to the latter phenotype, we
named it *green too early* (*gt*)). Siliques from heterozygous plants segregated ~25% (458/1864; $\chi^2$ test *p < 0.01) defective embryos, indicating that *gt* was a single recessive nuclear mutation. *gt* was mapped to the tip of chromosome I, close to marker *nga59* and in the region of the known embryo defective mutant *dcl1* (*At1g01040*). *gt* failed to complement the null alleles *dcl1-3* and *dcl1-5* and the weak allele *dcl1-9* (Schauer et al., 2002). Sequencing of the *gt* allele from genomic DNA uncovered a G-to-A transition at position 7096 (counting from the ATG; chromosomal position 30241). This mutation is predicted to change a conserved glycine to glutamic acid in one of two active sites of the RNase IIIb domain (position 1692), presumably affecting catalysis (Du et al., 2008) (Fig. S1). We therefore renamed *gt* as *dcl1-15*.

Because the morphology of the mutant embryos was altered, we staged them by referring to the wild-type embryos in the same silique. *dcl1-15* embryos developed normally until the early globular stage. At this point two distinct phenotypes were observed. The patterns of cell division became very abnormal, starting with misdvisions of the hypophysis, leading to mature embryos that had very disturbed tissue patterns (Fig. 1A, B). The defects were not as severe as those of the null allele *dcl1-5* (Fig. 1C and (Schwartz et al., 1994)), suggesting that *dcl1-15* is not a null allele. The patterning defects will be discussed in detail in a separate paper. At the early globular stage we also observed chlorophyll auto-fluorescence throughout the mutant embryo proper and suspensor (Fig. 1D). The fluorescence became more intense by the early heart stage, when *dcl1-15* embryos turned green (Fig. 1G, H, compare with Fig. 1E, F). This “early fluorescence” phenotype was also observed in embryos mutant for the null allele *dcl1-5* (Fig. 1M), but not in mutants for the weak allele *dcl1-9*. The early fluorescence phenotype was not affected by crossing *dcl1-15* into other genetic backgrounds (*Ler*, *Ws*, or *Col*) for the purpose of generating double mutants or analyzing reporter genes (data not shown). *dcl1-15* embryos aborted late in embryogenesis. Bent-cotyledon stage *dcl1-15* embryos could not be rescued by culturing them on MS-agar plates (data not shown), suggesting that embryo abortion was not due to desiccation intolerance (Meinke et al., 1994).

The early accumulation of chlorophyll suggested early maturation of chloroplasts. We collected wild-type embryos at the heart stage (when they do not yet show
chlorophyll auto-fluorescence) and dcl1-15 embryos from the same siliques (recognizable for their abnormal cell division patterns) and studied the morphology of the chloroplasts using TEM (598 wild-type vs. 310 dcl1-15 chloroplasts). We found that chloroplasts in dcl1-15 embryos were more developed than those in wild-type embryos (Fig. 2A, B). They contained more stacks of grana per chloroplast (2.48 ± 0.08 vs. 0.75 ± 0.04; ANOVA p < 0.001), and each stack had more thylakoids (2.67 ± 0.06 vs. 1.53 ± 0.04; ANOVA p < 0.001). There were also more starch grains in chloroplasts per cell (0.62 ± 0.08 vs. 0.04 ± 0.01; ANOVA p < 0.01). By the time they reached the bent cotyledon stage, however, the chloroplasts in dcl1-15 and wild-type embryos were indistinguishable (Fig. 2C, D), although the mutant embryos contained significantly lower amounts of total chlorophyll (0.21 ± 0.03 µg/30 embryos vs. 0.83 ± 0.04 µg/30 embryos; t-test p < 0.001).

Our TEM analysis revealed that heart stage dcl1-15 embryos not only contained developmentally more advanced chloroplasts, but they also had a significant number of structures that looked like lipid bodies, which are not found in wild-type embryos until the torpedo stage (3.7 ± 0.37 per cell vs. 0.22 ± 0.04 per cell; ANOVA p < 0.001) (Fig. 2E, F). To see if the entire embryo maturation program was switched on earlier in dcl1-15, we studied the expression of the gene for the embryonic storage protein At2S3, using the reporter gene pAt2S3:GFP (Kroj et al., 2003). In wild-type embryos pAt2S3:GFP was not expressed until the late heart stage, and GFP did not accumulate significantly until the late torpedo stage (Fig. 1I, J). dcl1-15 embryos, in contrast, showed GFP expression starting at the early heart stage (Fig. 1K), and by the late torpedo stage they had much higher levels than wild-type embryos (Fig. 1L). These data indicate that the whole embryonic maturation program (chloroplast development and synthesis of storage products) was induced precociously in dcl1-15, making it a heterochronic mutant. These maturation events were induced in a normal sequence in dcl1-15, with chloroplast maturation occurring ahead of the accumulation of storage proteins and lipids.

**Early maturation in other mutants of the miRNA pathway**

The strong phenotype of dcl1-15 embryos and the location of the mutation in the active site of the enzyme suggested that the embryonic defects were due to a significant reduction in the levels of miRNAs, since miRNA synthesis appears to be the primary role
of DCL1 (Laubinger et al., 2010). However, DCL1 also participates in the biosynthetic pathway for other sRNAs, including 21-22 nt ta-siRNAs (Peragine et al., 2004) (trans-acting siRNAs; due to the need for miRNAs to generate ta-siRNAs) and nat-siRNAs (natural antisense siRNAs) (Borsani et al., 2005). Unlike miRNA biogenesis, these other pathways also require RDR6. We distinguished between those options genetically by testing a variety of mutants in the miRNA pathway, using the early fluorescence phenotype as a proxy for early maturation. Mutations in most of the other members of the miRNA biosynthetic and utilization pathways either have no obvious embryonic defects (hyl1-2, hen1-1) (Chen et al., 2002; Vazquez et al., 2004), or have phenotypes that are distinct from those of dcl1-15 (se-3, ago1-8, pnh-2) (Lynn et al., 1999; Grigg et al., 2005). This is most likely due to redundancy, since there are several homologs of HYL1, HEN1 and AGO1 in Arabidopsis (Hiraguri et al., 2005; Vaucheret, 2008), and the levels of miRNAs are reduced but not eliminated in the single mutants (Vazquez et al., 2004; Grigg et al., 2005). Not surprisingly, hyl1-2, hen1-1, se-3, ago1-8 and pnh-2 single mutant embryos did not show early chlorophyll fluorescence. However, pnh-2 ago1-8 double mutant embryos did, although not as early as dcl1-15 or dcl1-5 embryos (late globular stage instead of early globular stage) (Fig. 1N). Interestingly, the morphology of pnh-2 ago1-8 embryos is similar to that of mutants for strong dcl1 alleles (Lynn et al., 1999; Mallory et al., 2009). This result is consistent with other studies that indicate that AGO1 and PNH redundantly mediate the action of miRNAs (Lynn et al., 1999; Mallory et al., 2009). In addition, embryos of a functionally null RDR6 mutant (rdr6-11) (Peragine et al., 2004) do not show early chlorophyll fluorescence. These data support the hypothesis that the dcl1-15 early maturation phenotype is most likely due to the misregulation of one or more miRNA targets.

Transcript profiling of dcl1 confirms the heterochronic phenotype

The mutation in dcl1-15 is predicted to significantly reduce the levels of all miRNAs present in embryos. There are more than 500 validated or predicted miRNA targets, and a significant proportion of these are transcription factors. Therefore, it seemed likely that many pathways would be altered in dcl1-15 embryos. In order to gain deeper insight into the observed phenotypes, we conducted a global transcriptional
profiling experiment of wild-type and \textit{dcl1-15} torpedo stage embryos in triplicate using Affymetrix ATH1 microarrays. The torpedo stage was chosen because it was the earliest time point when wild-type and mutant embryos could be easily differentiated and isolated from the seed coat and endosperm in sufficient numbers.

Differential expression analysis by LIMMA found 2960 increasing and 3428 decreasing genes at a Benjamini-Hochberg multiple test correction (MTC) \( \leq 0.05 \) (Tables S2 and S3). As expected from the phenotypes described above, most of the genes involved in the synthesis of storage products were significantly upregulated in \textit{dcl1-15} embryos (Table I), including all eight seed storage protein genes (most of them more than 12-fold), all 10 genes encoding the oil body proteome (\textit{CLO1} and \textit{HSD1} more than 140-fold), and the genes encoding enzymes involved in the synthesis of PUFA (\textit{FAD3} and \textit{FAD2}), TAG (\textit{DAGAT} and \textit{ECR}) and VLCFA (\textit{FAE}) storage lipids (Baud et al, 2008). GO term and SP-PIR keyword enrichment analysis of the differentially expressed genes confirmed that genes involved in storage protein, storage lipid, and carbohydrate biosynthesis and localization are overrepresented in the genes upregulated in \textit{dcl1-15}, and genes involved in cell cycle, basic cellular processes, and development are downregulated (Table S4).

The phenotypes of \textit{dcl1-15} mutants suggest that they are maturing faster than normal embryos. We tested this idea further using a recently generated microarray dataset of laser capture microdissected preglobular, globular, heart, torpedo, and green cotyledon stage embryos from the Goldberg and Harada laboratories (http://www.seedgenenetwork.net/arabidopsis). Hierarchical clustering of these arrays and our arrays using the 6388 genes differentially expressed in \textit{dcl1-15} found that, for the best clustering pattern (as determined using average silhouette widths; see Figure S3), wild-type samples clustered with the torpedo arrays from the public dataset, consistent with the timing of their development. The \textit{dcl1-15} samples, however, clustered with the more mature green cotyledon arrays, further suggesting that \textit{dcl1-15} is a heterochronic mutation (Fig. 3A). We then identified temporally expressed genes in embryos —genes whose expression levels vary at different developmental stages—using the Goldberg-Harada dataset, and compared these genes to those differentially expressed in \textit{dcl1-15} torpedo embryos. 6139 probesets that fell into nine clusters of expression patterns were
temporally expressed in the Goldberg-Harada dataset (Fig. 3B; Table S5). We found that 32.4% of the genes upregulated in dcl1-15 were genes more highly expressed in developmentally older embryos (temporally increasing genes) and 33.9% of the genes downregulated in dcl1-15 were genes more highly expressed in developmentally younger embryos (temporally decreasing genes) (Fig. 3C and D; Tables S6 and S7). This supports the conclusion that the expression profile of dcl1-15 torpedo stage embryos resembles more the expression profile of developmentally older wild type embryos.

Differential regulation of miRNA targets in the seed

To look at the effect of dcl1-15 on miRNA target expression, we compiled a list of validated or predicted miRNA targets using public lists from the Arabidopsis thaliana Small RNA Project (ASRP) at Oregon State University (http://asrp.cgrb.oregonstate.edu/) and the Arabidopsis Next-Gen Sequence Databases at the University of Delaware (http://mpss.udel.edu/), the latter of which includes the results of the PARE (parallel analysis of RNA ends) sequencing data from (German et al., 2008). The ATH1 array has 499 probesets that recognize miRNA targets on this list, with 148 of these being temporally expressed in embryos (Tables S8, 9). Of the 499 probesets on the array, 409 passed the MAS5.0 filter in our arrays, with 67 upregulated (targeted by 35 miRNAs; including 18 temporally-expressed genes), 80 downregulated (targeted by 34 miRNAs; including 48 temporally-expressed genes), and 262 not changing in dcl1-15 (Tables S10, 11). Interestingly, in many cases different targets of the same miRNA are upregulated while others are downregulated, further suggesting that the regulation of the steady state levels of miRNA targets is complex.

Known regulators of the seed maturation program are regulated by DCL1

The observed heterochronic phenotype and gene expression patterns of dcl1-15 suggest that one or more DCL1-generated miRNAs and their target genes regulate the timing of maturation, possibly by altering the expression of the master regulators of maturation. Consistent with this hypothesis, LIL and FUS3 transcripts were significantly higher in the microarray (1.8 and 2.6-fold, respectively) and LEC2 transcripts were higher, though just below the statistical cutoff (1.7-fold, p = 0.079). WRI1, encoding a
transcription factor that induces the expression of the genes involved in the synthesis of storage lipids, was also significantly upregulated in the mutant (1.57-fold) (Table 1).

To strengthen the inferences suggested by the microarray analysis, we decided to study the expression of FUS3 and LEC2 using reporter genes. This method allowed us to check the spatial expression of the genes at different stages of embryogenesis. In wild-type embryos, both pFUS3:GUS and pLEC2:GUS are expressed from early embryogenesis in the suspensor and the hypophysis (Fig. 4A, C). At the late globular/early heart stage, expression spreads to encompass the whole embryo (although pLEC2:GUS is weaker in the upper half of the embryo) (Kroj et al., 2003). In dcl1-15 early to mid-globular stage embryos, both reporter genes were expressed throughout the embryo proper (also weaker in the upper tier for pLEC2:GUS), in a fashion similar to later stage wild-type embryos (Fig. 4B, D). After the early heart stage, both wild-type and dcl1-15 embryos stained similarly. We conclude that DCL1 represses FUS3 and LEC2 in the embryo proper during early embryogenesis, and that the loss of miRNAs leads to their ectopic expression.

The microarray also suggested that LEC1 expression is reduced in dcl1-15. pLEC1:GUS expression in wild-type embryos is first detected at the early heart stage, increasing until the late torpedo stage and then decreasing (Lotan et al., 1998) (Fig. 4E). Consistent with the microarray results, we were not able to detect pLEC1:GUS expression in dcl1-15 embryos at any stage (Fig. 4F).

**FUS3, LEC2 and AGL23 are required for the early maturation phenotype**

The analysis of reporter genes indicated that both FUS3 and LEC2 are negatively regulated by miRNA pathways during early embryogenesis. We wondered whether the early maturation seen in dcl1-15 embryos required the presence of FUS3 and LEC2 proteins, or whether miRNA target genes triggered the program independently of these regulators. Previous studies have shown that the timing of embryo maturation in fus3-3 and lec2-1 embryos is normal, based on the stages of appearance of chlorophyll, storage proteins and the expression of pAt2S3:GFP. In both mutants, the levels of chlorophyll and pAt2S3:GFP could be significantly reduced, however, with pAt2S3 expression more severely affected in fus3-3 (Meinke et al., 1994; To et al., 2006). To distinguish between
these hypotheses, we generated plants heterozygous for dcl1-15 and homozygous for pAt2S3:GFP and fus3-3 or lec2-1. We evaluated the presence of chlorophyll auto-fluorescence and the expression of pAt2S3:GFP in fus3-3 dcl1-15 and lec2-1 dcl1-15 double mutant embryos. Preliminary observations suggest that lec2-1 does not rescue the morphological defects of dcl1-15 embryos, while fus3-3 enhances them (data not shown).

lec2-1 dcl1-15 embryos did not show chlorophyll auto-fluorescence or pAt2S3:GFP expression until the heart stage (n = 29 and 13, respectively), a bit earlier than wild type, but later than dcl1-15 (Fig. 5A). After the heart stage the double mutant embryos showed intense chlorophyll auto-fluorescence and pAt2S3:GFP expression, although the reporter was concentrated on the basal part of the embryo (n = 52 and 14, respectively) (Fig. 5B, C). A functional LEC2 protein is, therefore, required for dcl1-15 embryos to mature before the heart stage, but becomes dispensable afterwards.

In contrast, fus3-3 dcl1-15 embryos were very variable in their early maturation phenotypes. The proportion of fus3-3 dcl1-15 embryos that had chlorophyll auto-fluorescence before the heart stage varied from silique to silique, even in the same plant, from 0% to 100%. On average, 68% of the double mutant embryos in a silique fluoresced (n= 122 embryos, 8 siliques) or expressed pAt2S3:GFP (n=29) earlier than normal. The proportion of fluorescing (n=74) or pAt2S3:GFP-expressing (n=9) fus3-3 dcl1-15 embryos increased to 88% by the heart stage. In some of these embryos there were patches (towards the apical end) that had no chlorophyll fluorescence and were not green (Fig 5D, E). pAt2S3:GFP was also expressed in a patchy way, mostly in the basal part of the embryo (Fig 5F). These data indicate that FUS3, like LEC2, is necessary for the early maturation phenotypes of dcl1-15, but not required after the heart stage. The variable and patchy nature of the phenotype suggests redundancy with other factors, as has been proposed previously (To et al., 2006).

We also analyzed whether AGL23 was required for the dcl1-15 phenotypes. AGL23 is one of the few known transcription factors that specifically regulates chloroplast maturation during embryogenesis. agl23-2 embryos are white or very pale green with underdeveloped chloroplasts (Colombo et al., 2008), but accumulate storage proteins normally (Fig. S2). Homozygotes are seedling lethal (Colombo et al., 2008). In our microarrays AGL23 was not significantly expressed, consistent with the observation
that its expression decreases after the early torpedo stage (Colombo et al., 2008). We wanted to test whether AGL23 was required for establishing the early chloroplast maturation we saw in dcl1-15. We looked at the progeny of DCL1/dcl1-15 AGL23/agl23-2 self-pollinated plants. We found that 4% of dcl1-15 heart stage embryos (n = 162) were not green, nor had chlorophyll fluorescence (Fig. 5G). A similar percentage of pale dcl1-15 embryos was observed at the bent cotyledon stage (n = 511). These percentages are not significantly different from those expected for the dcl1-15/dcl1-15 agl23-2/agl23-2 class (chi-square test, p = 0.2 and 0.46, respectively for heart and bent cotyledon stage, assuming 60% female transmission of agl23-2 as described by (Colombo et al., 2008)). Our conclusion is that agl23-2 is epistatic to dcl1-15, and, therefore, a functional AGL23 protein is required for the heterochronic maturation of chloroplasts.

**ASIL1, ASIL2 and HDA6/SIL1 repress the maturation program during early embryogenesis**

Our transcript profiling data also revealed that a handful of the genes that have been shown to repress the expression of the embryonic program after germination were down-regulated in torpedo-stage dcl1-15 mutants (Table I). These were CLF, HDT1, HDA6/SIL1 and ASIL1. We also saw down-regulation of several other histone deacetylases (HDA5, 9 and 10) (none of which are known to affect embryogenesis,(Hollender and Liu, 2008)) and of the closest homolog of ASIL1, At3g14180 (ASIL2 hereafter).

We hypothesized that since these genes are involved in repressing the maturation program post-germination, they might also be involved in repressing it during early embryogenesis, downstream of one or more miRNA targets. To test this idea, we looked at asil1-1 (Gao et al., 2009), asil2-1 (line SAIL_258_F06 from ABRC) and sil1-1 (Furner et al., 1998) embryos, in both single and double mutant combinations. The results are summarized in Table 2. We observed no obvious morphological defects in the single or double mutant embryos. All the homozygous single mutant plants were viable and fertile (data not shown). Of the three single mutants, only a fraction (9.2%) of asil2-1 embryos showed early chlorophyll fluorescence during the globular stages, in a similar pattern to
that seen in dcl1-15 embryos. By the early heart stage that fraction had increased to 27.6%. At this stage, 43.5% of asil1-1 embryos also start to show chlorophyll fluorescence. In both these cases, the fluorescence is localized throughout the embryo (Fig. 5H), which is distinct from the protodermal localization seen in wild-type heart stage embryos (Fig. 1F). sill-1 embryos do not mature early, at least by this measure.

The phenotypes of double mutant combinations strongly suggest that the timing of maturation is controlled at least in part by the concerted action of ASIL1, ASIL2 and HDA6/SIL1. Double mutant embryos of all combinations phenocopy the early maturation phenotype of dcl1-15. The fraction of embryos that show chlorophyll fluorescence at the globular stage indicates that these three factors regulate the timing of maturation in a dose dependent manner, dominantly enhancing each other, because many embryos that are homozygous for one mutation and heterozygous for the other show the phenotype (Table II).

We were also able to analyze the expression of pAt2S3:GFP in asil1-1 and asil1-1 asil2-1 embryos. In both cases we observed expression as early as the early heart stage, phenocopying dcl1-15 embryos, although the levels and extent of expression were lower than in dcl1-15 embryos (Fig. 5I). These data support the conclusion that these mutants are also heterochronic.

Discussion

miRNAs regulate embryonic maturation

The maturation program was a key innovation of seed plants (Vicente-Carbajosa and Carbonero, 2005). Like any other important developmental process, this program is tightly regulated. Several of the mechanisms involved in inducing maturation during embryogenesis and repressing it after germination have been worked out (Baud et al., 2008; Santos-Mendoza et al., 2008; Zhang and Ogas, 2009). However, other pieces of the puzzle are missing, in particular the genes that repress maturation during early embryogenesis. The data presented here strongly suggest that miRNAs are these key negative regulators of the maturation program. In dcl1-15 embryos, we observed early chloroplast maturation, and early accumulation of starch grains, lipid bodies and storage proteins. Our conclusion is reinforced by the transcriptional profile of dcl1-15 torpedo
stage embryos, which resemble that of older embryos. Similarly, Nodine and Bartel (2010) described the early accumulation of OLEOSIN transcripts and a heterochronic change in the transcriptional profile in dcl1-5 embryos. Interestingly, even though the maturation program starts too early, the timing of the different components of the program seem to be unaffected. In particular, we still see accumulation of chlorophyll preceding the expression of the seed storage protein At2S3, as in wild-type embryos. It is tempting to speculate that miRNAs regulate the initiation, but not the progression, of maturation.

**Positive regulators of maturation are downstream of miRNAs**

The key positive regulators of the maturation program are the LEC genes, probably assisted by several bZIP and MYB transcription factors (Baud et al., 2008). All of these bind to the promoters of seed storage protein genes and genes involved in the synthesis of storage lipids (e.g. (Lara et al., 2003; Kagaya et al., 2005; Braybrook et al., 2006; Santos-Mendoza et al., 2008). We found that several of these genes were upregulated in the dcl1-15 mutant: FUS3, LEC2, L1L, and several MYBs and bZIPs (Table 1). In contrast, ABI3 was unaffected, and LEC1 was downregulated.

We observed that in the wild type the FUS3 and LEC2 transcriptional reporter genes are excluded from the embryo proper until the early heart stage, just before the beginning of maturation. In dcl1-15 they are expressed in the embryo proper at least by the early globular stage, coinciding with the onset of chlorophyll accumulation in the mutant. Our analysis of lec2-1 dcl1-15 and fus3-3 dcl1-15 double mutants confirms that both proteins are required for the early maturation phenotype, further supporting their position downstream of miRNAs. The requirement for FUS3 was less pronounced, and it has been suggested previously that it acts redundantly with LEC2 (To et al., 2006). This redundancy, and the fact that FUS3 positively regulates itself, likely explain the patchy appearance (in terms of chlorophyll and pAt2S3:GFP expression) of fus3-3 dcl1-15 embryos. We propose, therefore, that miRNA targets are responsible, directly or indirectly, for repressing FUS3, LEC2 and possibly other inducers of maturation in the embryo proper until they are required. The ectopic expression of these factors is sufficient to induce maturation even in the absence of LEC1. Interestingly, after the heart
stage, fus3-3 dcl1-15 and lec2-1 dcl1-15 embryos have high levels of chlorophyll and pAt2S3:GFP, something not seen in either fus3-3 or lec2-1 embryos (Meinke et al., 1994; Kroj et al., 2003; To et al., 2006), suggesting that after that stage other miRNA-regulated factors can compensate for the absence of LEC2 or FUS3.

Identification of repressors of the maturation program during embryogenesis

One lingering mystery has been the identity of the genes directly repressing the maturation program early in development. Our microarray analysis indicated that ASIL1, HDA6/SIL1 and CLF were downregulated in dcl1-15. These genes have previously been shown to repress the transcription of the LEC genes and other maturation-related genes in seedlings. Interestingly, several other histone deacetylases were downregulated (HDA5, 9, 10 and HDT1), as well as the closest homolog to ASIL1 (ASIL2). It has already been shown that ASIL1 binds to a GT-box, which is present in both the promoters of the LEC genes and of the genes encoding storage products, regulating the program at least at two levels (Gao et al., 2009). We analyzed single and double mutant combinations of asil1-1, asil2-1 and sill-1 and found that these genes redundantly repress the maturation program during early embryogenesis, downstream of miRNA targets. Thus, our studies demonstrate for the first time that the same genetic mechanism negatively regulates seed storage genes during early embryogenesis and seedling development.

miRNA targets as key regulators of maturation

Based on our findings, we suggest that during early embryogenesis specific miRNAs downregulate one or more miRNA targets to both promote the repressors and repress the inducers of maturation. Later in embryonic development a reduction in those miRNAs leads to the induction of maturation. We cannot deduce from our data whether this is a linear pathway (i.e. miRNA targets repress the repressors which in turn silence the activators) or whether miRNA targets act at more than one level.

In a report published while this manuscript was under preparation, Nodine and Bartel (Nodine and Bartel, 2010) demonstrated that globular stage dcl1-5 embryos show heterochronic gene expression. Their genetic data also suggest that the increased expression of the miR156 targets SQUAMOSA PROMOTER BINDING PROTEIN-LIKE
10 (SPL10) and SPL11 are partly responsible for the early onset of the maturation program in the mutant, despite the fact that the RNA levels of these genes do not change significantly during embryogenesis, according to the Goldberg-Harada dataset. Another SPL gene—SPL5—does significantly increase in expression during embryogenesis in this dataset, however. Thus, it might be possible that multiple miR156-targetted SPL genes redundantly regulate the same targets, with the developmental increase in SPL5 expression allowing for the threshold for maturation to be crossed. In such a model, removing the functions of both SPL10 and SPL11 may have lowered the overall SPL activity below the threshold for maturation.

While (Nodine and Bartel, 2010) demonstrated, as we did, that DCL1 regulates the onset of maturation and identified some of the miRNA targets involved in the process, they did not explore their connection with the components of the maturation pathway. We, on the other hand, placed several known and novel regulators of maturation downstream of unidentified miRNA targets. If their inferences about the SPL genes are correct, it is possible that these proteins positively regulate maturation by repressing ASIL1 and other negative regulators, since SPL10 and 11 (and nine other miRNA targets) are predicted to contain transcriptional repression domains (Mitsuda and Ohme-Takagi, 2009). Further studies are now required to integrate both sets of observations, and to fully uncover the pathway by which miRNAs control the seed maturation program during embryogenesis.

Materials and methods

Plant material

Our genetic screen was performed in a mixed Ws/Ler background. Heterozygous mutant plants were backcrossed four times to Ler before further analysis. Most mutants and reporter lines have been described before: pAT2S3:GFP, pLEC2:GUS and pFUS3:GUS (Kroj et al., 2003), pLEC1:GUS (Siefers et al., 2008), dcl1-3, dcl1-5 and dcl1-9 (Schauer et al., 2002), pnh-2 and ago1-8 (Lynn et al., 1999), se-3 (Grigg et al., 2005), henl-1 (Chen et al., 2002), hyll-2 (Vazquez et al., 2004), rdr6-11 (Peragine et al., 2004), fus3-3 and lec2-1 (Meinke et al., 1994), agl23-2 (Colombo et al., 2008), asil1-1 (Gao et al., 2009) and sil1-1 (Furner et al., 1998). asil2-1 is line SAIL_258_F06 (ABRC stock CS812086). Seeds were obtained from the ABRC (Columbus, OH) or other
researchers. Plants were grown in a greenhouse or growth chamber in MetroMix 360 (Sun Gro Horticulture, Bellevue, WA) at 20-24°C, with 16 hours of light at 150 µE s⁻¹ m⁻². For transgenic selection, seeds were surface sterilized and grown on sterile media containing 4.4 g/l MS salts, 1x Gamborg’s vitamins, 0.5 g/l MES, 10 g/l sucrose and 7.5 g/l TC agar (Carolina Biological, Burlington, NC), pH 5.7, plus the appropriate selective agent. To generate fus3-3/fus3-3 and lec2-1/lec2-1 plants, homozygous bent cotyledon-stage embryos were germinated on MS plates and then transplanted to soil. All reagents were from Sigma-Aldrich (St Louis, MO) unless indicated. Primers used for PCR-genotyping of mutants are detailed in Table S1.

**Optical microscopy and histochemistry**

For morphological characterization, whole developing seeds were cleared in Hoyer’s solution (70% chloral hydrate, 4% glycerol, 5% gum arabic) and examined with DIC optics. To prevent extraction of chlorophyll, cleared embryos were analyzed within a few hours. To stain whole seeds for GUS, the developing seeds were incubated in 90% acetone for 15 minutes at room temperature, washed with water, and then transferred to GUS-staining solution (100 mM phosphate buffer pH 7, 1 mM EDTA, 1% Tween-20, 2.5 mM potassium ferro/ferricyanide and 1 mg/ml X-glucuronic acid (Sigma-Aldrich, St Louis, MO). After 1-2 hours at 37°C, the seeds were washed with water and cleared in Hoyer’s. For GUS staining or epifluorescence of isolated embryos, they were squeezed out of the seed in either GUS-staining solution and incubated at 37°C, or in 10% glycerol and observed immediately. All optical microscopy was done on a Leica DMRB microscope (Leica Microsystems, Bannockburn, IL). For observation of GFP and chlorophyll fluorescence, the excitation/emission wavelengths were 480/535 nm and 560/645 nm, respectively. Images were acquired with either a ProgRes MFcool or a ProgRes C5 camera (Jenoptik, Jena, Germany). Images taken on different channels were merged with the ProgRes software.

Total chlorophyll content in mature embryos was measured as described by (Albrecht et al., 2008).

**Transmission electron microscopy**
Developing seeds were fixed overnight at 4°C in 10 mM sodium phosphate pH 6.8, 100 mM sodium chloride, 4% paraformaldehyde, 2% glutaraldehyde and 0.1% Triton X-100. After washing with 10 mM sodium phosphate and 100 mM sodium chloride, the seeds were post-fixed with 1% osmium tetroxide for one hour, washed with water, and stained with 1% aqueous uranyl acetate. The stained seeds were gradually dehydrated with a dilution series of ethanol and acetone, and embedded in Spurr’s resin according to the manufacturer’s instructions. After thin-sectioning (80 nm), the sections were stained with uranyl acetate in 50% ethanol, washed with water, and treated with Reynold’s lead citrate. Thin sections were visualized on a JEOL JEM-100SX electron microscope (JEOL USA, Peabody, MA). Photographs were taken at 10,000X or 50,000X on Kodak 4489 film. All the reagents for TEM were from Electron Microscopy Sciences (Hatfield, PA).

Microarray experiments

Sample collection, RNA isolation, and labeled target preparation. Three hundred torpedo stage mutant embryos from siliques of DCL1/dcl1-15 plants and 300 wild-type torpedo stage embryos from DCL1/DCL1 siblings were collected and ground in liquid nitrogen. RNA was extracted from the tissue using the RNeasy Micro kit (Qiagen, Valencia, CA) according to the directions for extracting RNA from tissues, with modifications, as follows. The samples were ground in 350 μl RLT alone and then with 25 μl of Plant RNA Isolation Aid (Ambion, Austin, TX) added. The samples were homogenized by passing the material over a Qiashredder column (Qiagen). The rest of the RNeasy Micro protocol was performed according to the instructions, including the on-column DNA digestion. The eluted RNA was precipitated overnight at -20°C in 0.1 μg/μl glycogen, 0.6 M ammonium acetate, and 70% EtOH, spun at 4°C for 50 minutes at 18,000 x G, and eluted in 10 μl water. The RNA concentration and purity were verified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA).

Biotin-labeled cDNA targets for hybridization to Affymetrix Arabidopsis ATH1 microarrays were made from 50 ng starting RNA that was reverse-transcribed and amplified using the Ovation™ RNA Amplification System V2, and fragmented and labeled using the FL-Ovation™ cDNA Biotin Module V2 (NuGEN Technologies, San
Carlos, CA) per the manufacturer’s instructions. Three biological replicates each of wild-type and dcl1-15 embryos were performed. The University of Pennsylvania Microarray Core Facility hybridized the arrays. Raw data have been deposited at Gene Expression Omnibus under accession number GSE24887.

Normalization and identification of differentially expressed genes. The microarrays were gcRMA-normalized in R and filtered using MAS 5.0 presence/absence calls to remove any probesets not expressed in at least one sample. The remaining 17,159 probesets were tested for differential expression in R using Limma with a Benjamini-Hochberg multiple test correction (MTC) ≤ 0.05. Quality control verification was done using the simpleaffy package in R (http://www.bioconductor.org/). Principal components analysis (PCA) performed in Partek Genomics Suite 6.5 showed that the replicates clustered together well. GO term and SP-PIR keyword enrichment was performed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp; (Dennis et al., 2003; Huang et al., 2009).

Meta-analysis. We compared our dataset to a set of 10 ATH1 microarrays of laser capture microdissection samples of preglobular, globular, heart, linear cotyledon (torpedo), and mature green cotyledon stage Arabidopsis embryos (two replicates of each) from the labs of Bob Goldberg (UCLA) and John Harada (UC Davis) (http://www.seedgenenetwork.net/arabidopsis). To identify temporally expressed genes in embryos—genes whose expression varies with embryonic stage—the 10 arrays were normalized, as described above, but all probesets not expressed based on MAS 5.0 P/A calls in at least two arrays were filtered out. Differential expression analysis was done using Limma on the remaining 15,010 probesets, as above, with all pairwise contrasts between different stages. The union of the significantly different genes in these different contrasts (Benjamini-Hochberg MTC ≤ 0.01) was used as a unified list of temporally expressed genes in embryos. These genes were then clustered in Partek into nine partitions using Pearson’s dissimilarity, four temporally increasing (2654 total genes increasing in subsequent developmental stages), three temporally decreasing (2825), one peaking in heart stage embryos (335), and one peaking in torpedo stage embryos (325).
Overlaps between differentially expressed genes in *dcl1-15* embryos and these datasets were tested for statistical significance by a Fisher’s Exact test using only the sets of genes expressed in both experiments by MAS 5.0 P/A calls. For the purposes of hierarchical clustering (in Partek) with our own arrays, these were renormalized with our six arrays with all probesets not expressed based on MAS 5.0 P/A calls in at least two arrays filtered out. To verify that we identified the most appropriate hierarchical clustering pattern, trees were drawn using Euclidean or Pearson’s dissimilarity measures of distance with single, complete, or average linkage measures. All six trees were evaluated in R by calculating the average silhouette widths at each node, which is a classic method for evaluating dendrograms (Rousseeuw, 1987). The tree with the highest average silhouette widths was that produced using Pearson’s dissimilarity with average linkage (Figure S3). Silhouette widths range from -1 (definitely wrong cluster assignment) to 1 (absolutely correct cluster assignment) with 0 being neutral. Values above 0 suggest proper assignment.

Acknowledgements

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Literature cited


is critical for dsRNA cleavage', Proceedings of the National Academy of Sciences 105: 2391-2396.


**Figure legends**

**Figure 1. Phenotypes of miRNA pathway mutants.** (A-C) Full size embryos, showing the late morphological phenotypes of (A) WT, (B) dcl1-15 and (C) dcl1-5. (D) Chlorophyll autofluorescence in a mid-globular stage dcl1-15 embryo. (E-H) DIC optics and chlorophyll autofluorescence images of heart stage embryos. WT embryos are white (E), and chlorophyll is starting to accumulate in the protodermis (arrow) (F). dcl1-15 embryos at this stage are green (G) and show very strong fluorescence throughout (H). (I-L) Expression of pAt2S3:GFP (green, magenta is chlorophyll autofluorescence). (I) WT heart stage embryos do not express the reporter. (J) dcl1-15 early heart stage embryos show strong expression. (K) In WT embryos expression is first observed around the early torpedo stage. (L) In dcl1-15 early torpedo stage embryos expression is much higher. (M) Chlorophyll autofluorescence in a dcl1-5 late globular embryo. (N) pnh-2 ago1-8 embryos show chlorophyll autofluorescence at the late globular stage. Scale bar: 40 μm (A-H, M-N), 20 μm (I-L).

**Figure 2. TEM analysis of maturation phenotypes.** (A, B) Chloroplasts in WT heart stage embryos (A) are less developed than those of dcl1-15 embryos at the same stage (B). Arrows point to a thylakoid stack. (C, D) Chloroplasts in WT (C) and dcl1-15 (B) bent-cotyledon stage embryos look very similar. (E, F) Early accumulation of lipid bodies (arrowheads) is higher in dcl1-15 heart stage embryos (E) compared to WT embryos (F). Scale bar: 1 μm (A-D), 5 μm (E-F).

**Figure 3. Microarray analysis of torpedo-stage dcl1-15 embryos reveals heterochronic changes in gene expression patterns.** (A) Hierarchical clustering of WT and dcl1-15 microarrays with arrays of preglobular, globular, heart, torpedo, and green cotyledon stage embryos from the Goldberg and Harada laboratories using the genes differentially expressed between WT and dcl1-15 embryos. WT samples cluster with same stage embryos (torpedo), but dcl1-15 samples cluster with developmentally more mature embryos (green cotyledon). (B) The 7146 temporally expressed genes (genes whose expression varies with embryonic stage) identified from the Goldberg-Harada
dataset fall into 9 clusters of unique expression patterns based on Pearson’s dissimilarity. Each line shows the average expression pattern for all genes in that cluster. Values from replicate arrays were averaged. For all clusters with significant differences between torpedo and green cotyledon samples, the \textit{dcl1-15} samples looked more like the green cotyledon samples when all temporally expressed genes were considered. (C) There is a significant positive enrichment for temporally increasing genes in \textit{dcl1-15} upregulated genes (significant Fisher’s exact right-tail p-value) and a significant absence of temporally decreasing genes (significant Fisher’s exact left-tail p-value). Only genes expressed in both datasets were considered in C and D. (D) There is a significant positive enrichment for temporally decreasing genes in \textit{dcl1-15} downregulated genes and a significant absence of temporally increasing genes. Temporally increasing genes increase in expression with stage from preglobular through green cotyledon, while temporally decreasing genes decrease in expression with stage from preglobular through green cotyledon.

\textbf{Figure 4. Expression of master regulators of maturation in \textit{dcl1-15}.} (A, B) \textit{pFUS3:GUS} at the mid-globular stage is expressed only in the suspensor in WT embryos (A), but throughout the embryo proper in \textit{dcl1-15} (B). (C, D) \textit{pLEC2:GUS} shows similar expression to \textit{pFUS3:GUS} at the mid-globular stage in WT (C) and \textit{dcl1-15} (D) embryos. (E, F) At the mid-torpedo stage \textit{pLEC1:GUS} is expressed in WT (E) but not in \textit{dcl1-15} (F) embryos. Scale bar: 20 µm (A-D), 40 µm (E-F). Arrowheads point to the embryo proper.

\textbf{Figure 5. Phenotypes of mutants that regulate maturation downstream of \textit{dcl1-15}.} (A-C) \textit{lec2-1 dcl1-15} double mutant embryos. No chlorophyll autofluorescence is observed at the globular stage (arrowhead points at the embryo proper) (A), but is intense at the late heart stage (B). \textit{pAt2S3:GFP} expression is seen at the late heart stage (C). (D-F) \textit{fus3-3 dcl1-15} double mutant embryos. DIC optics (D) and chlorophyll autofluorescence (E) images of a heart stage embryo showing a patch without chlorophyll (arrows). \textit{pAt2S3:GFP} expression is seen at the late heart stage (F). (G) A \textit{agl23-2 dcl1-15} heart stage embryo, showing a \textit{dcl1-15}-like morphology but no chlorophyll. (H) Early
chlorophyll autofluorescence throughout an early heart stage asil1-1 embryo. (I) Early pAt2S3:GFP expression in a heart stage asil1-1 asil2-1 embryo. Scale bar: 40 µm (A-H), 20 µm (I).

**Supplemental Figures and Tables**

**Figure S1.** Mutations in DCL1.

**Figure S2.** Accumulation of seed storage proteins in WT and agl23-2 mature seeds.

**Figure S3.** Hierarchical clustering validation of the cladogram in Figure 3 using average silhouette widths. A. The tree shown was clustered using Pearson’s dissimilarity with average linkage, and had the highest average silhouette widths among the six trees tested (Pearson’s dissimilarity or Euclidean distance measures with single, complete, or average linkage). Each cluster is numbered at the branch point. B. Average silhouette widths for each cluster (using the numbers in A). All average silhouette widths are well above 0, validating the tree.

**Table S1.** PCR primers used for genotyping.

**Table S2.** Genes upregulated in dcl1-15 torpedo-staged embryos, based on Limma with a Benjamini-Hochberg adjusted p-value < 0.05.

**Table S3.** Genes downregulated in dcl1-15 torpedo-staged embryos, based on Limma with a Benjamini-Hochberg adjusted p-value < 0.05.

**Table S4.** GO term and SP-PIR keyword enrichment of genes upregulated and downregulated in dcl1-15 torpedo-staged embryos.

**Table S5.** Genes temporally expressed in embryos based on the Goldberg-Harada dataset.
Table S6. Genes upregulated in *dcl1-15* torpedo-staged embryos and temporally increasing in embryos.

Table S7. Genes downregulated in *dcl1-15* torpedo-staged embryos and temporally decreasing in embryos.

Table S8. Probesets on the ATH1 array recognizing miRNA targets.

Table S9. Temporally expressed miRNA targets with probesets on the ATH1 array.

Table S10. miRNA targets upregulated in *dcl1-15* torpedo stage embryos with temporal genes designated.

Table S11. miRNA targets downregulated in *dcl1-15* torpedo stage embryos with temporal genes designated.
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<td>(Reyes and Chua, 2007)</td>
</tr>
<tr>
<td><strong>AGAMOUS-LIKE18 (AGL18)</strong></td>
<td>At3g57390</td>
<td>3.42</td>
<td>0.0042</td>
<td>(Zheng et al., 2009)</td>
</tr>
<tr>
<td><strong>MYB115</strong></td>
<td>At5g40360</td>
<td>2.98</td>
<td>0.0111</td>
<td>(Wang et al., 2009)</td>
</tr>
<tr>
<td><strong>MYB101</strong></td>
<td>At2g32460</td>
<td>2.67</td>
<td>0.0014</td>
<td>(Reyes and Chua, 2007)</td>
</tr>
<tr>
<td><strong>FUSCA3 (FUS3)</strong></td>
<td>At3g26790</td>
<td>2.61</td>
<td>0.0224</td>
<td>(Meinke et al., 1994)</td>
</tr>
<tr>
<td><strong>MYB44</strong></td>
<td>At5g67300</td>
<td>2.18</td>
<td>0.0035</td>
<td>(Kirik et al., 1998)</td>
</tr>
<tr>
<td><strong>AtbZIP12/ENHANCED EM LEVEL (EEL)</strong></td>
<td>At2g41070</td>
<td>1.84</td>
<td>0.0048</td>
<td>(Bensmihen et al., 2002)</td>
</tr>
<tr>
<td><strong>LEAFY-COTYLEDON1-LIKE (L1L)</strong></td>
<td>At5g47670</td>
<td>1.84</td>
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<td>(Yamamoto et al., 2009)</td>
</tr>
<tr>
<td><strong>AtbZIP10</strong></td>
<td>At4g02640</td>
<td>1.75</td>
<td>0.0492</td>
<td>(Alonso et al., 2009)</td>
</tr>
<tr>
<td><strong>LEAFY-COTYLEDON2 (LEC2)</strong></td>
<td>At1g28300</td>
<td>1.71</td>
<td>0.0798</td>
<td>(Meinke et al., 1994)</td>
</tr>
<tr>
<td><strong>NF-YC6</strong></td>
<td>At5g50480</td>
<td>1.67</td>
<td>0.0127</td>
<td>(Yamamoto et al., 2009)</td>
</tr>
<tr>
<td><strong>WRINKLED1 (WRI1)</strong></td>
<td>At3g54320</td>
<td>1.57</td>
<td>0.0223</td>
<td>(Cernac and Benning, 2004)</td>
</tr>
<tr>
<td><strong>NF-YC2</strong></td>
<td>At1g56170</td>
<td>1.42</td>
<td>0.0453</td>
<td>(Yamamoto et al., 2009)</td>
</tr>
<tr>
<td><strong>LEAFY-COTYLEDON1 (LEC1)</strong></td>
<td>At1g21970</td>
<td>-2.02</td>
<td>0.0059</td>
<td>(Meinke et al., 1994)</td>
</tr>
<tr>
<td><strong>GOLDEN-LIKE2 (GLK2)</strong></td>
<td>At5g44190</td>
<td>-2.82</td>
<td>0.0044</td>
<td>(Waters et al., 2008)</td>
</tr>
<tr>
<td><strong>ABA INSENSITIVE4 (ABI4)</strong></td>
<td>At2g40220</td>
<td>-3.15</td>
<td>0.0016</td>
<td>(Brocard-Gifford et al., 2003)</td>
</tr>
</tbody>
</table>

**Negative regulators of maturation (known or proposed)**

<table>
<thead>
<tr>
<th></th>
<th>Accession</th>
<th>Log2 Fold Change</th>
<th>p-value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ENHANCER(ZESTE)A1/SWINGER (EZA1/SWN)</strong></td>
<td>At4g02020</td>
<td>2.32</td>
<td>0.0197</td>
<td>(Makarevich et al., 2006)</td>
</tr>
<tr>
<td><strong>HISTONE DEACETYLASE-TUIN1 (HDT1)</strong></td>
<td>At3g44750</td>
<td>-1.38</td>
<td>0.0430</td>
<td>(Zhou et al., 2004)</td>
</tr>
<tr>
<td><strong>HISTONE DEACETYLASE10 (HDA10)</strong></td>
<td>At3g44660</td>
<td>-1.53</td>
<td>0.0312</td>
<td>(Hollender and Liu, 2008)</td>
</tr>
<tr>
<td><strong>CURLY LEAF (CLF)</strong></td>
<td>At2g23380</td>
<td>-1.69</td>
<td>0.0357</td>
<td>(Makarevich et al., 2006)</td>
</tr>
<tr>
<td><strong>HISTONE DEACETYLASE6/ (HDA6/SIL1)</strong></td>
<td>At5g63110</td>
<td>-1.83</td>
<td>0.0042</td>
<td>(Tanaka et al., 2007)</td>
</tr>
<tr>
<td><strong>HISTONE DEACETYLASE9 (HDA9)</strong></td>
<td>At3g44680</td>
<td>-1.94</td>
<td>0.0299</td>
<td>(Hollender and Liu, 2008)</td>
</tr>
</tbody>
</table>
Table I. Transcripts significantly misregulated in dcl1-15 torpedo-stage embryos. Within each category genes are sorted by fold change (dcl1-15 vs. WT).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession</th>
<th>Fold Change</th>
<th>P-value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARABIDOPSIS 6B-INTERACTING PROTEIN 1-LIKE2 (ASIL2)</td>
<td>At3g14180</td>
<td>-1.97</td>
<td>0.0096</td>
<td>(Gao et al., 2009)</td>
</tr>
<tr>
<td>ARABIDOPSIS 6B-INTERACTING PROTEIN 1-LIKE1 (ASIL1)</td>
<td>At1g54060</td>
<td>-2.30</td>
<td>0.0123</td>
<td>(Gao et al., 2009)</td>
</tr>
<tr>
<td>HISTONE DEACETYLASIS5 (HDA5)</td>
<td>At5g61060</td>
<td>-2.73</td>
<td>0.0005</td>
<td>(Hollender and Liu, 2008)</td>
</tr>
<tr>
<td>APETALA2 (AP2)</td>
<td>At4g36920</td>
<td>-6.28</td>
<td>0.0001</td>
<td>(Ohto et al., 2005)</td>
</tr>
<tr>
<td>Genotype of selfed parental plant</td>
<td>Fraction of fluorescent embryos: globular stages</td>
<td>Fraction of fluorescent embryos: early heart/heart stage</td>
<td>Expected fraction of double mutant embryos</td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------------------------------------</td>
<td>--------------------------------------------------------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>0% (n &gt; 100)</td>
<td>0% (n &gt; 100)</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>sil1-1/sil1-1</td>
<td>0% (n &gt; 100)</td>
<td>0% (n &gt; 100)</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td>asil1-1/asil1-1</td>
<td>0% (n &gt; 100)</td>
<td>43.5% (n = 230)</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td>asil2-1/asil2-1</td>
<td>9.2% (n = 261)</td>
<td>27.6% (n = 87)</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td>ASIL1/asil1-1</td>
<td>27% (n = 209)</td>
<td>38% (n = 279)</td>
<td>6.25%</td>
<td></td>
</tr>
<tr>
<td>ASIL1/sil1-1</td>
<td>41.7% (n = 48)</td>
<td>70% (n = 80)</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>ASIL2/asil2-1</td>
<td>5.2% (n = 328)</td>
<td>27% (n = 271)</td>
<td>6.25%</td>
<td></td>
</tr>
<tr>
<td>ASIL1/asil1-1/ASIL2/asil2-1</td>
<td>25% (n = 354)</td>
<td>60% (n = 319)</td>
<td>6.25%</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Fractions of embryos showing early chlorophyll fluorescence in single and double mutants of putative repressors of the maturation program. n.a.: not applicable.
Figure 1. Phenotypes of miRNA pathway mutants. (A-C) Full size embryos, showing the late morphological phenotypes of (A) WT, (B) dcl1-15 and (C) dcl1-5. (D) Chlorophyll autofluorescence in a mid-globular stage dcl1-15 embryo. (E-H) DIC optics and chlorophyll autofluorescence images of heart stage embryos. WT embryos are white (E), and chlorophyll is starting to accumulate in the protodermis (arrow) (F). dcl1-15 embryos at this stage are green (G) and show very strong fluorescence throughout (H). (I-L) Expression of pAt2S3:GFP (green, magenta is chlorophyll autofluorescence). (I)
WT heart stage embryos do not express the reporter. (J) dcl1-15 early heart stage embryos show strong expression. (K) In WT embryos expression is first observed around the early torpedo stage. (L) In dcl1-15 early torpedo stage embryos expression is much higher. (M) Chlorophyll autofluorescence in a dcl1-5 late globular embryo. (N) pnh-2 ago1-8 embryos show chlorophyll autofluorescence at the late globular stage. Scale bar: 40 µm (A-H, M-N), 20 µm (I-L).
Figure 2. TEM analysis of maturation phenotypes. (A, B) Chloroplasts in WT heart stage embryos (A) are less developed than those of dcl1-15 embryos at the same stage (B). Arrows point to a thylakoid stack. (C, D) Chloroplasts in WT (C) and dcl1-15 (B) bent-cotyledon stage embryos look very similar. (E, F) Early accumulation of lipid bodies (arrowheads) is higher in dcl1-15 heart stage embryos (E) compared to WT embryos (F). Scale bar: 1 µm (A-D), 5 µm (E-F).
Figure 3. Microarray analysis of torpedo-stage dcl1-15 embryos reveals heterochronic changes in gene expression patterns. (A) Hierarchical clustering of WT and dcl1-15 microarrays with arrays of preglobular, globular, heart, torpedo, and green cotyledon stage embryos from the Goldberg and Harada laboratories using the genes differentially expressed between WT and dcl1-15 embryos. WT samples cluster with same stage embryos (torpedo), but dcl1-15 samples cluster with developmentally more mature embryos (green cotyledon). (B) The 7146 temporally expressed genes identified from the Goldberg-Harada dataset fall into 9 clusters of unique expression patterns based on Pearson’s dissimilarity. Each line shows the average expression pattern for all genes in that cluster. Values from replicate arrays were averaged. For all clusters with significant differences between torpedo and green cotyledon samples, the dcl1-15 samples looked more like the green cotyledon samples when all temporally expressed genes were considered. (C) There is a significant positive enrichment for temporally increasing genes in dcl1-15 upregulated genes (significant Fisher’s exact right-tail p-
value) and a significant absence of temporally decreasing genes (significant Fisher’s exact left-tail p-value). Only genes expressed in both datasets were considered in C and D. (D) There is a significant positive enrichment for temporally decreasing genes in dcl1-15 downregulated genes and a significant absence of temporally increasing genes.
Figure 4. Expression of master regulators of maturation in dcl1-15. (A, B) 
*pFUS3:GUS* at the mid-globular stage is expressed only in the suspensor in WT embryos (A), but throughout the embryo proper in *dcl1-15* (B). (C, D) *pLEC2:GUS* shows similar expression to *pFUS3:GUS* at the mid-globular stage in WT (C) and *dcl1-15* (D) embryos. (E, F) At the mid-torpedo stage *pLEC1:GUS* is expressed in WT (E) but not in *dcl1-15* (F) embryos. Scale bar: 20 µm (A-D), 40 µm (E-F). Arrowheads point to the embryo proper.
Figure 5. Phenotypes of mutants that regulate maturation downstream of dcl1-15.

(A-C) lec2-1 dcl1-15 double mutant embryos. No chlorophyll autofluorescence is observed at the globular stage (arrowhead points at the embryo proper) (A), but is intense at the late heart stage (B). pAt2S3:GFP expression is seen at the late heart stage (C). (D-F) fus3-3 dcl1-15 double mutant embryos. DIC optics (D) and chlorophyll autofluorescence (E) images of a heart stage embryo showing a patch without chlorophyll (arrows). pAt2S3:GFP expression is seen at the late heart stage (F). (G) A agl23-2 dcl1-15 heart stage embryo, showing a dcl1-15-like morphology but no chlorophyll. (H) Early chlorophyll autofluorescence throughout an early heart stage asil1-1 embryo. (I) Early pAt2S3:GFP expression in a heart stage asil1-1 asil2-1 embryo. Scale bar: 40 µm (A-H), 20 µm (I).