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# Arabidopsis *LORELEI*, a Maternally Expressed Imprinted Gene, Promotes Early Seed Development[1][OPEN]

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In flowering plants, the female gametophyte controls pollen tube reception immediately before fertilization and regulates seed development immediately after fertilization, although the controlling mechanisms remain poorly understood. Previously, we showed that *LORELEI* (*LRE*), which encodes a putative glycosylphosphatidylinositol-anchored membrane protein, is critical for pollen tube reception by the female gametophyte before fertilization and the initiation of seed development after fertilization. Here, we show that *LRE* is expressed in the synergid, egg, and central cells of the female gametophyte and in the zygote and proliferating endosperm of the Arabidopsis (*Arabidopsis thaliana*) seed. Interestingly, *LRE* expression in the developing seeds was primarily from the matrigenic *LRE* allele, indicating that *LRE* expression is imprinted. However, *LRE* was biallelically expressed in 8-d-old seedlings, indicating that the patrigenic allele does not remain silenced throughout the sporophytic generation. Regulation of imprinted *LRE* expression is likely novel, as *LRE* was not expressed in pollen or pollen tubes of mutants defective for MET1, DDM1, RNA-dependent DNA methylation, or MSI-dependent histone methylation. Additionally, the patrigenic *LRE* allele inherited from these mutants was not expressed in seeds. Surprisingly, and contrary to the predictions of the parental conflict hypothesis, LRE promotes growth in seeds, as loss of the matrigenic but not the patrigenic *LRE* allele caused delayed initiation of seed development. Our results showed that *LRE* is a rare imprinted gene that functions immediately after double fertilization and supported the model that a passage through the female gametophyte establishes monoalleleic expression of *LRE* in seeds and controls early seed development.

The female gametophyte in flowering plants controls the transition from the gametophyte to the sporophyte by multiple mechanisms. Before fertilization, gene expression in the female gametophyte (hereafter called maternal expression) controls pollen tube reception and sperm release. After double fertilization, maternally derived components maintain housekeeping functions, while matrigenic expression (arising from the maternally transmitted allele in the seed) plays a major role in embryo and endosperm development (Chaudhury et al., 2001). Expression of a gene primarily or exclusively from either the matrigenic or the patrigenic allele is called genomic imprinting (Gehring, 2013). Imprinted genes that control developmental processes through both maternal and matrigenic expression remain poorly characterized; identifying these genes and their roles before and after fertilization will help us understand how the female gametophyte controls early seed development.

Mutant analysis led to the identification of an initial set of maternally expressed genes (MEGs) and paternally expressed genes (PEGs), and the advent of transcriptomic analysis has revealed numerous MEGs and PEGs in Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), and maize (*Zea mays*; Gehring et al., 2011; Hsieh et al., 2011; Luo et al., 2011; Nodine and Bartel, 2012; Raissig et al., 2013; Xin et al., 2013). Imprinting appears more common in endosperm but also occurs in embryos (Jahnke and Scholten, 2009; Luo et al., 2011; Ngo et al., 2012; Nodine and Bartel, 2012; Raissig et al., 2013; Pignatta et al., 2014). However, the interpretation of transcriptomic studies can be confounded by contamination from the maternal seed coat (Schon and Nodine, 2017). A few of the MEGs or PEGs that function in seeds also are expressed in the mature female or male gametophyte, respectively, where they might mediate double fertilization.

Genomic imprinting is controlled by differential epigenetic modification of the matrigenic and patrigenic alleles (Gehring, 2013; Kawashima and Berger, 2014). In the case of MEGs, inhibitory epigenetic modifications

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are maintained on the paternal allele in the male gametophyte and selectively removed from the maternal allele in the female gametophyte. Consequently, in the seed, only the matrigenic allele is expressed and the patrigenic allele remains silenced. Epigenetic modifications that underlie imprinting are typically associated with DNA or histone methylation (Gehring, 2013).

The evolutionary and functional significance of imprinting remains unclear. As per the parental conflict hypothesis, parent-of-origin effects are the outcome of conflict between matrigenic and patrigenic alleles in resource allocation: matrigenic alleles favor limited but equitable growth among sibling seeds that have the same mother, while patrigenic alleles enhance growth at the expense of siblings with different fathers (Haig, 2013). The loss of matrigenic expression of some MEGs promotes seed growth, providing support for this hypothesis; however, not all MEGs follow this pattern (Bai and Settles, 2015). Identifying all the imprinted genes is critical to test the parental conflict hypothesis and explore alternative theories of imprinting (Spencer and Clark, 2014).

*LORELEI* (*LRE*), which encodes a putative glycosylphosphatidylinositol-anchored membrane protein, is critical for pollen tube reception by the female gametophyte before fertilization (Capron et al., 2008; Tsukamoto et al., 2010; Liu et al., 2016). Early seed development is delayed in *lre-5* ovules that successfully induce pollen tube reception, indicating that *LRE* also plays a role in the timely initiation of seed development after fertilization (Tsukamoto et al., 2010). Consistent with these LRE functions, using RT-PCR experiments, we previously showed that *LRE* expression is temporally and spatially regulated during reproduction (Tsukamoto et al., 2010). Before fertilization, *LRE* is expressed in mature unfertilized ovules but not in pollen or pollen tubes. After fertilization, *LRE* is expressed in ovules up to 24 h after pollination (HAP) and is not detectable in ovules at 36 and 48 HAP. Yet, important questions remain to be answered. Like some female gametophyte-expressed genes that play a role in seed development after fertilization (Evans and Kermicle, 2001; Yadegari and Drews, 2004; Gehring, 2013; Bai and Settles, 2015; Chettoor et al., 2016), it is not known if only the matrigenic allele of *LRE* is expressed in the seeds. Additionally, it is not clear if the loss of *LRE* expression in the maternal sporophyte or female gametophyte or seed leads to the delayed seed development.

Here, we showed that *LRE* is expressed in the zygote and in the proliferating endosperm at least up to 24 HAP. We also showed that *LRE* expression is imprinted in both zygote and endosperm soon after double fertilization. A novel mechanism might control the imprinting of *LRE* expression, as the patrigenic allele of *LRE* remained silent when inherited from mutants defective in DNA or histone methylation. Loss of matrigenic *LRE*, but not the patrigenic *LRE*, caused delays in the initiation of embryo and endosperm development, indicating that *LRE* is a rare imprinted gene that functions immediately after double fertilization. Our study showed that *LRE* mediates the maternal control of two critical events during the transition from gametophyte to sporophyte generation: pollen tube reception and seed development.

## RESULTS

### *LRE* Expression in Seeds Is Primarily from the Matrigenic Allele

Some female gametophyte-expressed genes that play a role in seed development after fertilization are imprinted, resulting in the synthesis of transcripts primarily from the matrigenic allele in the seed (Evans and Kermicle, 2001; Yadegari and Drews, 2004; Gehring, 2013; Bai and Settles, 2015; Chettoor et al., 2016). *LRE* functions in both the female gametophyte and the seed (Tsukamoto et al., 2010). Therefore, we tested if *LRE* expression is monoallelic in seeds by reciprocally crossing *lre-5* with the wild type. We chose the *lre-5* mutant for this experiment, as *lre-5* is a null allele of *LRE* and *LRE* transcripts are not produced (Tsukamoto et al., 2010). RT-PCR analysis of ovules isolated 24 HAP showed that *ACTIN2* transcripts were expressed whether *lre-5* or the wild type was used as the female parent. However, *LRE* transcripts were detected only when the female parent was the wild type (Fig. 1A), suggesting that primarily the matrigenic *LRE* allele, but not the patrigenic *LRE* allele, is expressed after fertilization.

To obtain additional evidence in support of this finding, we performed reciprocal crosses between two Arabidopsis accessions (Columbia and C24) with a single-nucleotide polymorphism (SNP) in *LRE*. At 24 HAP, we isolated ovules and used allele-specific (AS)-PCR involving locked nucleic acid (LNA; Latorra et al., 2003) primers to SNP genotype and distinguish whether endogenous *LRE* transcripts originated from the Columbia or the C24 allele. Control PCRs using Columbia and C24 genomic DNA identified the annealing temperature at which LNA primers can be reliably used to perform SNP genotyping by AS-PCR (Fig. 1B, left).

We used *GRP23* expression as a biallelic expression control in SNP genotyping by AS-PCR (Fig. 1B) because *GRP23* is expressed during early seed development, starting from the zygote and endosperm nuclear proliferation stages (Ding et al., 2006; Tsukamoto et al., 2010). We chose to SNP genotype ovules at 24 HAP, as *GRP23* is expressed in seeds at least from 16 HAP, even when transmitted through pollen (Tsukamoto et al., 2010). As expected, in seeds, *GRP23* was biallelically expressed (Fig. 1B, top two right gels). Importantly, the identification of accession-specific expression of *GRP23* from the patrigenic allele in seeds confirmed the sensitivity of this assay in detecting contributions from the patrigenic allele in the embryo sac despite the presence of a large amount of maternal sporophytic tissues in ovules. In this assay, Columbia-specific LNA *LRE* primers amplified an *LRE* PCR product only when Columbia was the female parent and not when it was used as the male parent (Fig. 1B, bottom two right gels). Conversely, C24-specific LNA primers amplified an *LRE* PCR product only when C24 was the female parent and not when it was used as the



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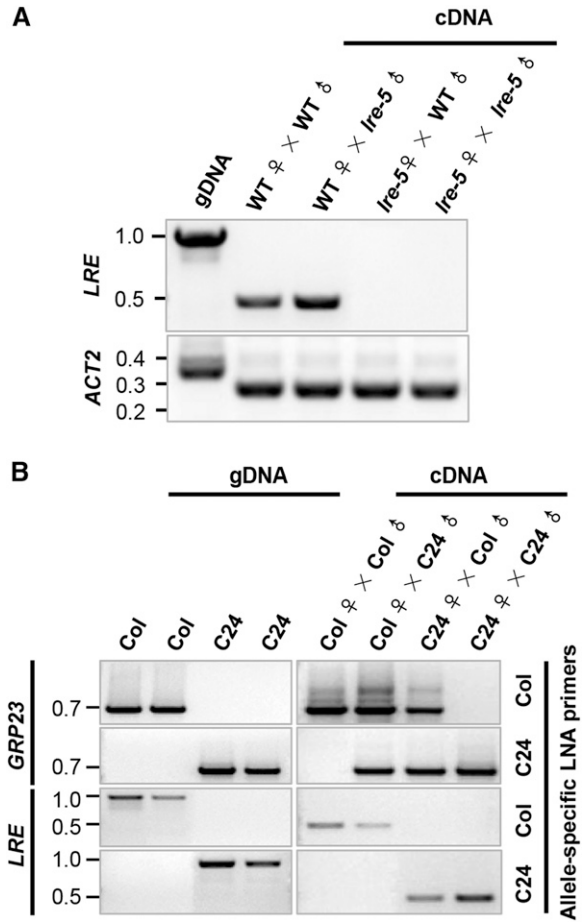


**Figure 1.** *LRE* is a maternally expressed imprinted gene. A, RT-PCR analysis of *LRE* expression in 24-HAP ovules from the indicated crosses between the wild type (WT) and *lre-5*. *ACTIN2* (*ACT2*) expression was used as a positive control (An et al., 1996). B, LNA primer-based AS-PCR analysis of *LRE* expression in 24-HAP ovules from the indicated crosses involving two accessions of Arabidopsis, Columbia (Col) and C24. Amplification of target genes from Col and C24 genomic DNA (gDNA) in an accession-specific manner (Latorra et al., 2003) confirmed the allele specificity of the AS-PCR assay. *GRP23* in seeds was used as a control gene that is expressed from both matrigenic and patrigenic alleles. Marker sizes (in kb) are shown on the left. ♀, Female parent; ♂, male parent.

male parent (Fig. 1B, bottom two right gels). Based on these results, we concluded that endogenous *LRE* transcripts in 24-HAP ovules primarily originated from the matrigenic *LRE* allele.

### The Matrigenic *LRE* Allele Is Expressed in Proliferating Endosperm and Zygote-Like Cell

Ovules from pollinated pistils used in RT-PCR assays in Figure 1 comprise both maternal sporophytic tissues and embryo sacs. *LRE* expression after fertilization can arise from expression in either one or both tissues. Additionally, parent-of-origin-dependent gene expression has been reported in both endosperm (Gehring, 2013; Bai and Settles, 2015) and embryo (Jahnke and Scholten, 2009; Nodine and Bartel, 2012; Raissig et al., 2013; Del Toro-De León et al., 2014), two distinct cell types within an embryo sac. The RT-PCR and AS-PCR assays in Figure 1 could not distinguish *LRE* expression in maternal sporophytic tissues of ovules from expression in the developing embryo sac, nor can it rule out contamination of seeds with RNA from maternal tissues, as was reported for endosperm and early embryo transcriptomes in Arabidopsis (Schon and Nodine, 2017). We overcame this shortcoming by performing cell-specific expression analysis using a promoter:reporter fusion. To examine the spatial and temporal expression of *LRE* expression in seeds, we generated plants carrying a *pLRE*::*GFP* reporter transgene (Fig. 2A). Prior to performing expression analysis in seeds, we checked for GFP signal in synergid cells of unfertilized ovules, where LRE functions in pollen tube reception (Capron et al., 2008; Tsukamoto et al., 2010; Liu et al., 2016). The GFP expression was strong in synergid cells of unfertilized ovules (Fig. 2, B–D; Supplemental Table S1; Supplemental Movies S1 and S2), consistent with LRE function in pollen tube reception in the synergid cell. No GFP expression was detected in pollen or pollen tubes carrying the *pLRE*::*GFP* transgene ($n >$ 1,000), consistent with RT-PCR analysis and phenotypic analysis that showed no function for LRE in pollen tubes (Tsukamoto et al., 2010).

When the *pLRE*::*GFP* transgene was maternally transmitted in a cross with wild-type pollen, GFP expression was detected in the proliferating endosperm (Fig. 2, E–I and O; Supplemental Table S1; Supplemental Movie S3). Additionally, when the *pLRE*::*GFP* transgene was maternally transmitted, GFP expression was detected in the zygote-like cell in the micropylar end of seeds (Supplemental Movie S3; also see below). *LRE* promoter activity is dynamic in the seed, as GFP expression level increased (Fig. 2, E–G; Supplemental Table S1) and then decreased (Fig. 2, G–I; Supplemental Table S1) over time after pollination. GFP expression in the endosperm and the zygote-like cell was not detected when the *pLRE*::*GFP* transgene was paternally contributed (Fig. 2, J–O), confirming that *LRE* expression in the seeds is primarily from the matrigenic allele. These results also show that the *LRE* promoter used in the *pLRE*::*GFP* transgene is sufficient to recapitulate the matrigenic allele-specific expression of *LRE* in seeds and likely contains all of the cis-elements required for the monoallelic expression of *LRE* in seeds. Additionally, there was no GFP expression in the maternal sporophytic tissues of ovules/seeds such as integuments/seed coat and funiculus either before or after fertilization (Fig. 2, E–I). Based on these results, we infer that the *LRE* transcripts detected in RT-PCR experiments using seeds (Fig. 1) must have been primarily from the embryo sac rather than the maternal sporophytic tissues. Taken together, our results indicate that *LRE* expression lasts only for a short duration after double fertilization and that *LRE* is a MEG in both fertilization products.



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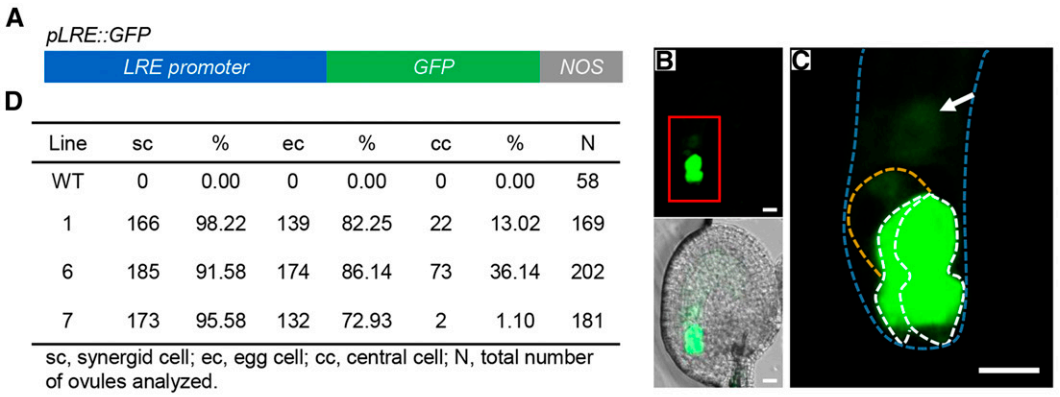
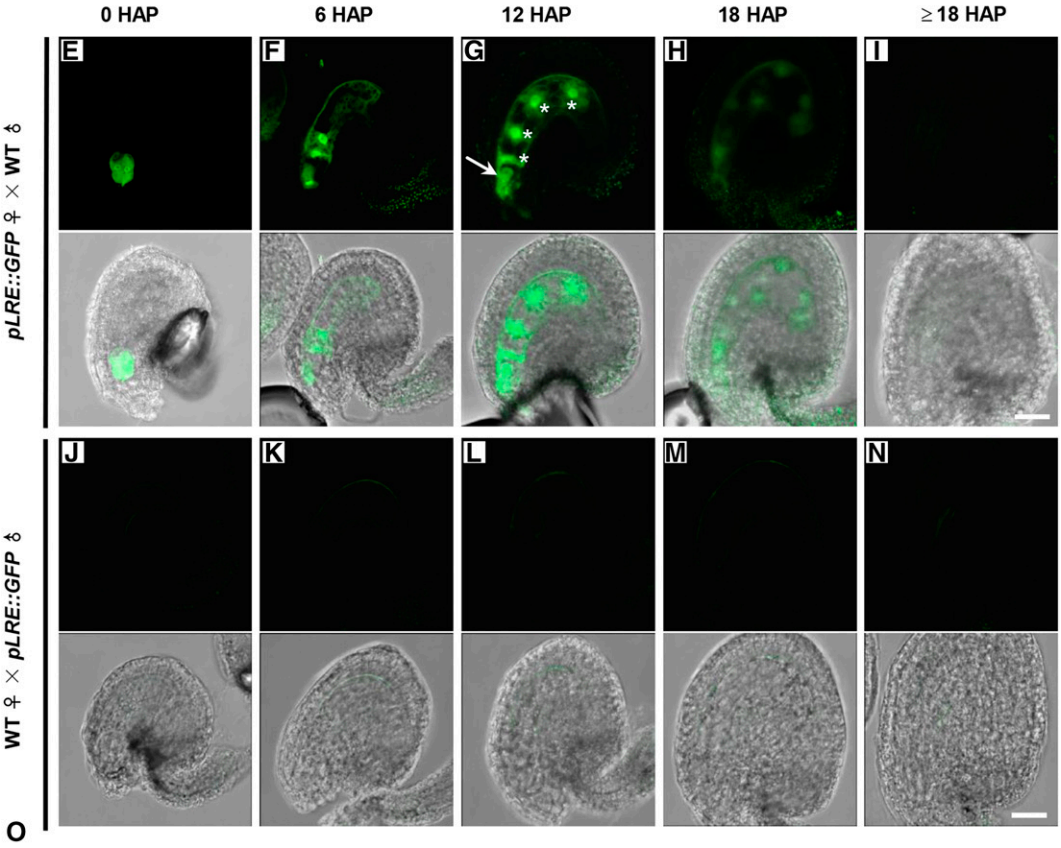



D

| Line | sc | % | ec | % | cc | % | N |
|---|---|---|---|---|---|---|---|
| WT | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 58 |
| 1 | 166 | 98.22 | 139 | 82.25 | 22 | 13.02 | 169 |
| 6 | 185 | 91.58 | 174 | 86.14 | 73 | 36.14 | 202 |
| 7 | 173 | 95.58 | 132 | 72.93 | 2 | 1.10 | 181 |

sc, synergid cell; ec, egg cell; cc, central cell; N, total number of ovules analyzed.




| Crosses (♀ × ♂) | Endosperm expression (n) * | % | N ^ |
|---|---|---|---|
| WT × WT | 0 | 0.00 | 300 |
| Line 1 × WT | 161 | 70.61 | 228 |
| Line 6 × WT | 189 | 72.14 | 262 |
| Line 7 × WT | 192 | 62.14 | 309 |
| WT × Line 1 | 0 | 0.00 | 236 |
| WT × Line 6 | 0 | 0.00 | 221 |
| WT × Line 7 | 0 | 0.00 | 224 |

* Number of seeds with GFP expression in the endosperm containing ≥2 endosperm nuclei.
^ Total number of ovules/seeds observed.

**Figure 2.** *LRE* is expressed in the female gametophyte and seeds. A to D, *LRE* is expressed in the mature female gametophyte. A, Diagram of the *pLRE*::*GFP* construct. B to D, *LRE* expression in unfertilized ovules. B, The top image is a fluorescent image, and the bottom image is a merged image of a bright-field image (not shown) and the fluorescent image. An enlarged version of the portion within the red rectangle in B is shown in C. B and C, *LRE* is expressed in the synergid cell (sc; white dashed line). To a lower



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### *LRE* Is Expressed in the Zygote

The GFP expression in the zygote-like cell in the micropylar end of the seed could be either from the zygote or continued expression in the persistent synergid cell that briefly lingers after double fertilization (Völz et al., 2013; Maruyama et al., 2015). To distinguish between these two possibilities, we performed colocalization of *LRE* expression from *pLRE*::*DsRed* with a zygote marker (*pWOX8*::*gWOX8-YFP*; Ueda et al., 2011). The colocalization analysis was done at a developmental stage (seeds with four or more endosperm nuclei) when the persistent synergid in most of the seeds has already degenerated (Völz et al., 2013) and any residual *pLRE*:*DsRed* expression in a single persistent synergid cell will not confound our analysis of *pLRE*:*DsRed* expression in the zygote. We reasoned that, in ovules with four or more endosperm nuclei, any *DsRed* expression in the micropylar end must be from a cell in the embryo sac of a seed (i.e. zygote).

We established a *pLRE*::*DsRed* transgene (Fig. 3A), in which *DsRed* reporter was expressed from the same *LRE* promoter used in *pLRE*::*GFP*, and transformed it into plants carrying the *pWOX8*::*gWOX8-YFP* transgene. In the transgenic plants carrying both markers, we chose three lines for colocalization analysis. Unlike in *pLRE*:*GFP* transgenic lines, in *pLRE*::*DsRed* lines, the *DsRed* levels were barely above the background autofluorescence in the endosperm nuclei of the seed. However, in the zygote-like cell in the micropylar end of the seed, the *DsRed* signal was clearly above the background autofluorescence (Fig. 3C; Supplemental Table S2), which is sufficient to complete the colocalization experiments with WOX8-YFP and determine if *LRE* is expressed in the zygote. Lack of clear *pLRE*::*DsRed* transgene expression in the proliferating endosperm did not prevent us from staging the seed development, as we were able to overcome this shortcoming by scoring WOX8-YFP expression, which also is expressed in proliferating endosperm nuclei (our analysis of the *pWOX8*::*gWOX8-YFP* lines in this study) in addition to the zygote (Ueda et al., 2011).

Pistils carrying both reporter genes were pollinated with wild-type pollen, and ovules were scored for colocalization of *DsRed* and YFP expression at 13.5 HAP, a time point at which ~70% of the ovules (314 of 478) are fertilized, as indicated by developing endosperm in them. Of these 314 seeds, 210 contained four or more endosperm nuclei. Our analysis showed that 89% of these ovules with four or more endosperm nuclei (187 of 210) expressed both *DsRed* and YFP in a single cell in the micropylar end of the ovule (Fig. 3, C and D), indicating that *LRE* is expressed in the zygote. Similar to the *pLRE*::*GFP* construct, *pLRE*::*DsRed* expression also was dynamic in the seed, as *DsRed* expression level in the zygote increased and then decreased over time after pollination (Supplemental Table S2). *DsRed* expression in the zygote was not detected when the *pLRE*::*DsRed* transgene was paternally contributed (Supplemental Table S3), confirming that *LRE* expression in the zygote is primarily from the matrigenic allele. Based on these results, in conjunction with the matrigenic allele-specific expression of *LRE* (Figs. 1 and 2; Supplemental Table S3), we concluded that *LRE* expression is paternally imprinted in both the zygote and the proliferating endosperm of seeds.

### De Novo Transcription after Fertilization Likely Results in Increased Expression of *LRE* in the Zygote and Proliferating Endosperm

*LRE* expression in proliferating endosperm and zygote (Figs. 2 and 3) could be due to transcripts that were transcribed in the two female gametes and inherited into the corresponding fertilization products and/or to de novo transcription of *LRE* after fertilization in the zygote and proliferating endosperm. To distinguish between these possibilities, we examined *pLRE*::*GFP* expression in the two female gametes before fertilization. We found that the *LRE* promoter is active in the egg (Fig. 2, B and C; Supplemental Movies S1 and S2) and central cells (Fig. 2, B and C), although the level of GFP expression in these cells was noticeably lower than that in the synergid cells (Fig. 2, B and C; Supplemental Table S1; Supplemental Movies S1 and S2) but clearly above the background fluorescence in nontransgenic Columbia ovules (Supplemental Fig. S1). GFP expression in the central cells was observed in noticeably fewer ovules compared with those that expressed GFP in the synergid and egg cells (Fig. 2D; Supplemental Table S1). Importantly, GFP expression was higher in the proliferating

**Figure 2.** (*Continued.*)
extent, *LRE* is expressed in the egg cell (ec; yellow dashed line). In some ovules, *LRE* also is expressed in the central cell (cc; arrow). The embryo sac is outlined with a blue dashed line. D, Quantification of microscopic observations of *LRE* expression, as shown in B and C. WT, Wild type. Bars = 15 $\mu$m. E to N, *pLRE*::*GFP* expression up to 24 HAP in the unfertilized mature ovule (E) and in developing seeds (F–I) carrying only maternally transmitted *pLRE*::*GFP*. Double fertilization is asynchronous in Arabidopsis; hence, a crossed pistil contains seeds at various stages of development. Only representative images of seeds that have developmentally progressed the furthest in a crossed pistil at the indicated HAP are shown. A complete distribution of seeds at different stages of development in these crosses is provided in Supplemental Table S1. Asterisks in G point to developing endosperm in the embryo sac. The arrow in G refers to a zygote-like cell in the embryo sac. J to N, *pLRE*::*GFP* signal was not detected either in the unfertilized mature ovule (J) or in seeds carrying only paternally transmitted *pLRE*::*GFP* (K–N). In E to N, the top images are fluorescent images while the bottom images are merged images of bright-field images (not shown) and the fluorescent images. Images in E to N were captured using identical camera settings. Bars = 30 $\mu$m. O, Quantification of microscopic observations of *LRE* expression in 13.5-HAP ovules from crosses involving three independent *pLRE*::*GFP* transgenic lines. Only the developing seeds with GFP expression are reported here; unfertilized or just fertilized ovules in the crossed pistil are not shown.



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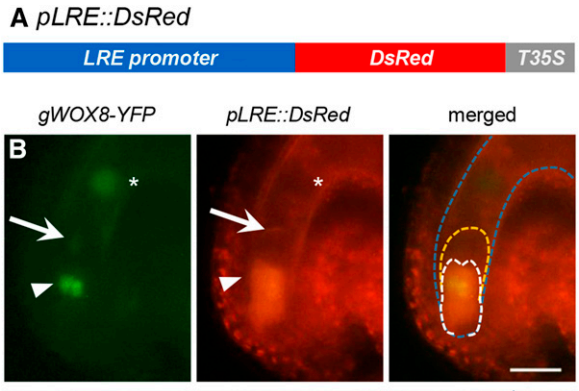


D

| Line | Zygote only (n)[a] | % | Zygote + psc (n)[b] | % | Not clear (n)[c] | % | N |
|---|---|---|---|---|---|---|---|
| 22 | 63 | 90.00 | 1 | 1.43 | 6 | 8.57 | 70 |
| 14 | 35 | 77.78 | 0 | 0.00 | 10 | 22.22 | 45 |
| 26 | 89 | 93.68 | 2 | 2.11 | 4 | 4.21 | 95 |
| Total | 187 | 89.05 | 3 | 1.43 | 20 | 9.52 | 210 |

[a] Number of seeds with both *gWOX8-YFP* expression (C, left panel) and *pLRE::DsRed* expression (C, middle panel) in zygote only.

[b] Number of seeds with both *gWOX8-YFP* expression and *pLRE::DsRed* expression in both zygote and persistent synergid cell (psc). A representative image of this type is not shown.

[c] Number of seeds with unclear *pLRE::DsRed* expression in the zygote. A representative image of this type is not shown.

**Figure 3.** *LRE* is expressed in the zygote. A, Diagram of the *pLRE*::*DsRed* construct. B, Unfertilized ovules with noticeable *LRE* expression in the synergid cells. The left image is a YFP channel fluorescent image of the micropylar end of the unfertilized ovule showing *gWOX8-YFP* expression in the two synergid cell nuclei (white arrowhead), the egg cell nucleus (white arrow), and the central cell nucleus (white asterisk). The middle image is a red channel fluorescent image of the micropylar end of the unfertilized ovule showing *pLRE*::*DsRed* expression in the synergid cells (white arrowhead). The location of the egg cell (white arrow) also is shown. Due to autofluorescence, *LRE* expression in the central cell (asterisk) is not visible. The right image is the merged image of the two images on the left. C, Seed with *LRE* expression in the zygote (at the elongating stage). The left image is a YFP channel fluorescent image of the micropylar end of the seed showing *gWOX8-YFP* expression in the zygote nucleus (white arrowhead). The middle image is a red channel fluorescent image of the micropylar end of the seed showing *pLRE*::*DsRed* expression in the zygote cell (white arrowhead). The right image is the merged image of the two images on the left. D, Quantification of the colocalization of *gWOX8-YFP* and *pLRE*::*DsRed* expression in 13.5-HAP ovules from crosses involving three independent *pLRE*::*DsRed* lines carrying the *pWOX*::*gWOX8-YFP* transgene. Only those ovules with *gWOX8-YFP* expression in four or more endosperm nuclei were included in the colocalization analysis and reported in this table. Bars = 20 $\mu$m.

endosperm than that in the central cell, the cell from which endosperm is derived after fertilization (compare Fig. 2G with Fig. 2E, or compare Supplemental Movies S1 and S2 with Supplemental Movie S3). Similarly, GFP expression was higher in the zygote than that in the egg cell, the cell from which the zygote is derived after fertilization (compare Fig. 2G with Fig. 2E, or compare Supplemental Movies S1 and S2 with Supplemental Movie S3). These results suggest that there is de novo expression of the matrigenic *pLRE*::*GFP* allele in the proliferating endosperm and zygote after fertilization.

We also examined *pLRE*::*DsRed* expression in the female gametophyte and found that *DsRed* levels were barely above the background autofluorescence in the central cell of the female gametophyte (Fig. 3B; Supplemental Table S2). Since *DsRed* expression also was barely detectable in the proliferating endosperm, *DsRed* expression could not be used to examine the de novo transcription in the proliferating endosperm. However, *DsRed* expression was higher in the zygote than in the egg cell, the cell from which the zygote is derived after fertilization (compare Fig. 3, C and B). Increased *pLRE*::*GFP* expression in the zygote and proliferating endosperm and the increased *pLRE*::*DsRed* expression in the zygote indicate that de novo transcription after fertilization is likely a major contributing factor toward the increased *LRE* expression in the seed.

### *LRE* Is Biallelically Expressed in 8-d-Old Seedlings

Our results showed that the paternal and patrigenic allele is silenced in the male gametophyte and during early seed development, respectively, raising the possibility that the patrigenic allele remains silent throughout the sporophytic generation. Examining *LRE* expression in vegetative tissues is one way to test this possibility. Previous RT-PCR experiments showed that *LRE* is expressed in 8-d-old seedlings (Tsukamoto et al., 2010). Therefore, we examined if the patrigenic *LRE* allele is expressed in 8-d-old seedlings. We reciprocally crossed the wild type and *lre* mutants and did RT-PCR experiments using 8-d-old seedlings of F1 progeny from these crosses. As in Figure 1A, for this experiment, we also chose a null allele of *LRE* (*lre-5*) to reliably identify the source of detected *LRE* transcripts. RT-PCR experiments showed that *LRE* is expressed in 8-d-old seedlings of the F1 progeny regardless of whether the wild type is used as a male or female parent (Fig. 4), indicating that *LRE* is biallelically expressed in 8-d-old seedlings. Similar results were obtained when a second null allele (*lre-7*) was used in this experiment (Fig. 4). This finding is in marked contrast to the monoallelic expression of *LRE* in seeds (Fig. 1A). These results, combined with our observation that *LRE* is expressed in the female gametophyte but not the male gametophyte, indicate that *LRE* expression is imprinted during gametophytic generation (at some point during male gametogenesis) and that the restoration of biallelic



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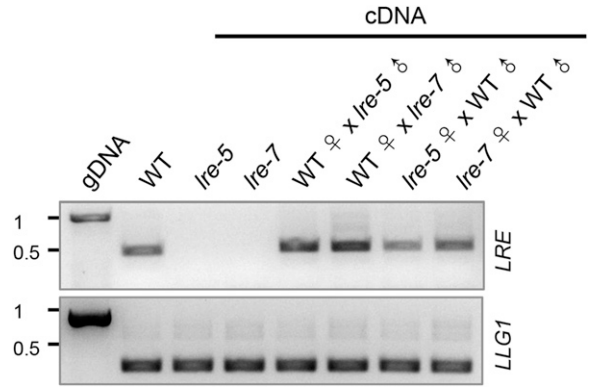


**Figure 4.** Both matrigenic and patrigenic *LRE* are expressed in 8-d-old seedlings. WT (wild type), *lre-5*, and *lre-7* indicate 8-d-old seedlings from selfed seeds of the indicated genotypes. WT ♀ × *lre-5* ♀ or WT ♀ × *lre-7* ♂ indicates 8-d-old F1 seedlings raised from a cross in which the wild type was the female parent and *lre-5* (or *lre-7*) was the male parent. Marker sizes are shown in kb. gDNA, Genomic DNA.

*LRE* expression occurs during sporophytic generation (at some point during embryogenesis or after seed development or germination).

### DNA Methylation Pathways That Regulate MEGs Do Not Control the Imprinted Expression of *LRE*

During development, DNA methylation of both maternal and paternal alleles of many genes is primarily maintained by *METHYLTRANSFERASE1* (*MET1*; CG sites; Kishimoto et al., 2001) and *CHROMOMETHYLASE3* (*CMT3*; CHG sites; Lindroth et al., 2001). The chromatin remodeler *DECREASE IN DNA METHYLATION1* (*DDM1*) also is required for the maintenance of CG and non-CG (CHG and CHH) methylation (Johnson et al., 2002; Stroud et al., 2013). At many MEG loci, selective removal of DNA methylation at the maternal allele is achieved by the activity of the DNA glycosylase DEMETER (DME) in the female gametophyte, resulting in differential methylation of maternal and paternal alleles (Choi et al., 2002; Gehring et al., 2006). At some MEG loci, RNA-directed DNA methylation silences the paternal allele and, thereby, sets up the MEG expression pattern (Vu et al., 2013). Such methylation differences in the two alleles are the basis of differences in transcription, as the hypermethylated patrigenic allele and hypomethylated matrigenic allele are usually associated with inactive and active transcriptional states, respectively.

To test if differential DNA methylation establishes the imprinting of *LRE*, we examined whether the patrigenic allele of *LRE* is expressed in seeds when inherited from pollen of three DNA hypomethylation mutants: *met1-1* (defective in CG methylation), *ddm1-2* (defective in CG and non-CG methylation), and *drm1-2 drm2-2 cmt3-11* (*ddc*; defective in CHG and CHH methylation; Kakutani et al., 1996; Yadegari et al., 2000; Kankel et al., 2003; Chan et al., 2006). We pollinated *lre-5* pistils with pollen from hypomethylated mutants and examined endogenous *LRE* expression in the ovules from pollinated pistils in an RT-PCR assay. We examined the expression of *PHERES1*, a gene that is primarily expressed from the patrigenic allele after fertilization (Köhler et al., 2005; Makarevich et al., 2008), as a control for the sensitivity of this RT-PCR assay to detect expression from a patrigenic allele in seeds. *ACTIN11* expression was examined as a control for a gene that is biallelically expressed in seeds (Huang et al., 1997). Our results showed that hypomethylation is not sufficient to induce expression from the patrigenic allele of *LRE* (Fig. 5A; Supplemental Fig. S2A), even though we detected *PHERES1* (Fig. 5A) or *ACTIN11* (Supplemental Fig. S2A) expression in these crosses.

When each of the three hypomethylated mutant pollen carrying *pLRE*::*GFP* was crossed onto wild-type pistils, we did not detect *pLRE*::*GFP* expression in the seeds, indicating that the patrigenic *pLRE*::*GFP* allele remained silent following demethylation (Supplemental Table S4). GFP expression from *pLRE*::*GFP* was detected in seeds from corresponding reciprocal crosses in which the hypomethylated pollen carrying *pLRE*::*GFP* was used as a female parent (Supplemental Table S4). We performed these crosses with pollen from a heterozygous hypomethylated mutant carrying *pLRE*::*GFP*, as (1) it will help examine potential roles of the male gametophyte in the silencing of the patrigenic allele and (2) sibling wild-type pollen can serve as an internal control for GFP expression in seeds in each cross. Furthermore, there was no ectopic expression of the *pLRE*::*GFP* transgene in mature pollen ($n > 1{,}000$) or pollen tubes ($n > 200$) grown through a cut pistil (Palanivelu and Preuss, 2006) in any of the three hypomethylated mutants, indicating that demethylation is not sufficient to express paternal

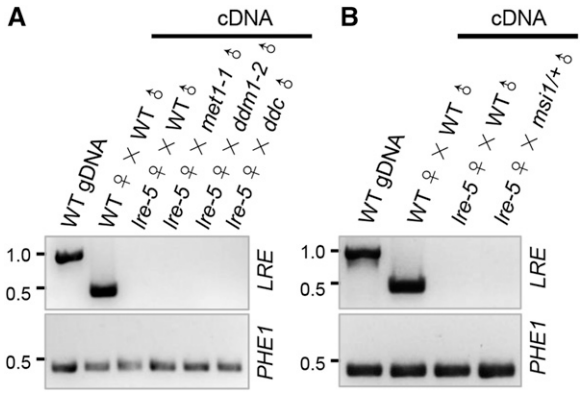


**Figure 5.** Defects in DNA and histone methylation pathway genes do not lead to *LRE* expression from the patrigenic allele in seeds. A, RT-PCR analysis of *LRE* expression in 12-HAP seeds from crosses between the wild type (WT) and *lre-5*. B, RT-PCR analysis of *LRE* expression in 13.5-HAP seeds from the indicated crosses. *PHERES1* (*PHE1*), whose patrigenic allele is preferentially expressed in seeds, was used as a positive control. ♀ and ♂ represent the female and male parent, respectively. A 0.83% RT reaction was used as a template in each PCR. gDNA, Genomic DNA. Marker sizes (in kb) are shown on the left. The genotype of *ddc* used in this experiment was *drm1-2/+ drm2-2/drm2-2 cmt3-11/cmt3-11*.



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*pLRE*::*GFP*. Additionally, we also found that demethylation mediated by DME in the female gametophyte is not required to activate *LRE* expression from the matrigenic allele in the seed (Supplemental Table S5).

Female gametophyte-derived factors also are known to regulate the expression of patrigenic alleles in seeds. For example, GLAUCE function in the female gametophyte is required for the expression of the patrigenic allele of RPS5a and FAC1 in developing embryos (Ngo et al., 2007). Recently, it was shown that the maternal RdDM pathway mediates the silencing of the patrigenic allele at MEG loci; loss of the maternal DCL3 resulted in the activation of the patrigenic *AtBMI1C* allele in the endosperm, but not in the embryo, of hybrid seeds from a cross between *dcl3-1* and the wild type (Bratzel et al., 2012). To investigate if the function of maternal RdDM pathways as well as other DNA methylation pathways is required to repress expression from the patrigenic *LRE* allele, we crossed wild-type pollen carrying *pLRE*::*GFP* onto the DNA hypomethylation mutant pistils. To increase the chance of identifying a seed with *pLRE*::*GFP* expression from the patrigenic allele, we performed these crosses between homozygous hypomethylation mutant pistils and pollen from plants that are homozygous for the *pLRE*::*GFP* transgene. At 13.5 HAP, we scored GFP expression in the seeds of these crosses. None of the hypomethylation mutants when used as a female parent induced the expression of *pLRE*::*GFP* in seeds (Table I). These results demonstrate that DNA methylation by pathways known for some MEGs does not control the imprinting of *LRE*.

**Table I.** *The paternal silencing of LRE is not affected by maternally inherited mutants in the DNA methylation pathway and in PRC2 function*

| Crosses (♀ × ♂)[a] | Endosperm Expression[b] | Percentage | Total[c] |
|---|---|---|---|
| WT × WT | 0 | 0.00 | 156 |
| *pLRE*::*GFP* × WT | 240 | 76.92 | 312 |
| WT × *pLRE*::*GFP* | 0 | 0.00 | 228 |
| *met1-1* × *pLRE*::*GFP* | 0 | 0.00 | 412 |
| WT × WT | 0 | 0.00 | 125 |
| *pLRE*::*GFP* × WT | 181 | 69.88 | 259 |
| WT × *pLRE*::*GFP* | 0 | 0.00 | 350 |
| *ddm1-2* × *pLRE*::*GFP* | 0 | 0.00 | 546 |
| WT × WT | 0 | 0.00 | 170 |
| *pLRE*::*GFP* × WT | 245 | 69.41 | 353 |
| WT × *pLRE*::*GFP* | 0 | 0.00 | 45 |
| *ddc*[d] × *pLRE*::*GFP* | 0 | 0.00 | 193 |
| *ddc*[e] × *pLRE*::*GFP* | 0 | 0.00 | 194 |
| WT × WT | 0 | 0.00 | 143 |
| *pLRE*::*GFP* × WT | 262 | 72.18 | 363 |
| WT × *pLRE*::*GFP* | 0 | 0.00 | 266 |
| *msi1*/+ × *pLRE*::*GFP* | 0 | 0.00 | 493 |

[a]Line 6 of *pLRE*::*GFP* (Fig. 2O; homozygous) was used in the crosses reported in this table. WT, Wild type. [b]Number of seeds with GFP expression in endosperm (≥2n) 13.5 HAP. [c]Number of total ovules analyzed. [d]The genotype of the *ddc* triple mutant was *drm1-2 drm2-2 cmt3-11*. [e]The genotype of the *ddc* triple mutant was *drm1-2/+ drm2-2 cmt3-11*.

### Histone Methylation Pathways That Regulate MEGs Do Not Control the Imprinted Expression of *LRE*

Gene imprinting also can be mediated by differential histone modification. In Arabidopsis endosperm and embryo, differential methylation of Lys-27 on histone H3 (H3K27me3) establishes the monoallelic expression of some MEGs (Jullien et al., 2006; Raissig et al., 2013). At the paternal alleles of these loci, H3K27me3 is selectively maintained by Polycomb Repressive Complex2 (PRC2) to repress patrigenic expression after fertilization (Jullien et al., 2006; Raissig et al., 2013). To determine if H3K27me3 silences the paternal or patrigenic allele of *LRE*, we used the *msi1* mutant, which is defective in one of the four core subunits of the PRC2 complex (Köhler et al., 2003; Guitton et al., 2004). RT-PCR experiments, in which *lre-5* pistils were pollinated with pollen from the *msi1* mutant, revealed that the patrigenic *LRE* allele is not expressed even when inherited from *msi1* mutant pollen (Fig. 5B; Supplemental Fig. S2B). Our results also showed that *msi1* does not cause ectopic expression from the paternal *pLRE*::*GFP* transgene in mature pollen ($n > 1{,}000$) or pollen tubes grown through a cut pistil ($n > 200$) or from the patrigenic *pLRE*::*GFP* allele in seeds (Supplemental Table S6).

Loss of SUVH4 histone methyltransferase KRYPTONITE (KYP) function in the female gametophyte led to increased and earlier expression of the patrigenic alleles of RPS5a, AGP18, PROLIFERA, and GRP23 in the seeds of crosses between kyp mutant pistils and wild-type pollen (Autran et al., 2011). In other instances, maternal histone methylation activity is required for continued repression of the silent patrigenic allele of two MEGs after fertilization (Raissig et al., 2013). To investigate if maternal PRC2 activity is required to repress expression from the patrigenic *LRE* allele, we crossed wild-type pollen carrying *pLRE*::*GFP* onto *msi1* mutant pistils. At 13.5 HAP, we scored seeds in these crosses for GFP expression. Even when the *msi1* mutant was used as a female parent, there was no induction of expression of *pLRE*::*GFP* in the seeds (Table I). Based on these results, we concluded that *LRE* is not imprinted through any histone modification pathways that are known to regulate some MEGs in Arabidopsis.

### Loss of Expression from the Matrigenic *LRE* Allele Results in Delayed Early Seed Development

Previously, using embryo and/or endosperm markers, we showed that the initiation of embryo and endosperm development is delayed in homozygous *lre* mutant seeds (Tsukamoto and Palanivelu, 2010; Tsukamoto et al., 2010). Importantly, we showed that this late start was not caused by a delay in (1) pollen tube arrival at the female gametophyte, (2) completion of pollen tube reception, or (3) double fertilization; instead, the delay was in the initiation of early seed development after double fertilization (Tsukamoto et al., 2010). RT-PCR and genetic assays also established that *LRE* is not expressed and does not function, respectively, in the



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male gametophyte (Tsukamoto et al., 2010). Above, we demonstrated that the patrigenic allele of *LRE* is silenced in seeds (Figs. 1 and 2), indicating that loss of the matrigenic *LRE* allele, rather than the patrigenic *LRE* allele, leads to the delayed initiation of seed development. Still, this possibility remains to be tested. Additionally, complementation experiments to conclusively demonstrate that *LRE* is required for the timely initiation of seed development have not been performed.

To address these questions, we examined endosperm and embryo development in seeds using a well-established chloral hydrate-based clearing assay (Yadegari et al., 1994), as it offered two advantages over marker-based analysis of seed development. First, by facilitating the counting of cells/nuclei in developing embryo and endosperm, this clearing assay allowed accurate determination of the embryo and endosperm development stage (Supplemental Fig. S3, A and B). Second, this assay also allowed the scoring pollen tube reception (manifested as coiled tubes) in every ovule, even if it is fertilized. By scoring both pollen tube reception and seed development simultaneously in an ovule, we identified seeds that initiated embryo development after undergoing normal pollen tube reception (type 1) and distinguished them from two other types of ovules: unfertilized *lre* mutant ovules that do not undergo pollen tube reception (type 2; Supplemental Fig. S3C) and those in which embryo development has initiated but had a coiled tube in it (type 3; Supplemental Fig. S3D). In this study, to examine embryo and endosperm development without any confounding effects from pollen tube reception defects, we excluded type 2 and 3 ovules from our analysis and used only type 1 ovules (reported in Figs. 6 and 7; Supplemental Figs. S4 and S5).

Using a chloral hydrate-based clearing assay, we first confirmed the delayed initiation of seed development in *lre* seeds (reported previously using *GRP23*:*GUS* reporter-based experiments; Tsukamoto et al., 2010) by crossing *lre-7* pistil with wild-type pollen and monitoring the development of endosperm and embryo in seeds at 48 HAP. Loss of *LRE* delayed endosperm nuclear proliferation in 48-HAP ovules, as is evident from the significantly higher number of *lre-7* seeds with five or fewer nuclear divisions compared with wild-type ovules (Supplemental Fig. S4). Embryo development also was delayed in 48-HAP ovules; significantly more *lre-7* seeds contained an embryo with less than two cell divisions (zygote or proembryos with one or two cells of embryo proper [EP]; see "Materials and Methods" for details) compared with wild-type ovules (Fig. 6A). Next, we used this assay to perform in-depth analysis of seed development by focusing only on embryo development. The delay in embryo development was not observed if the *lre-7* mutation was paternally contributed to the seeds; instead, the delay was observed in 48-HAP ovules only if they carried the matrigenic *lre-7* allele (Fig. 6, A and C). A similar delay in embryo development also was observed when we used *lre-5*, another loss-of-function *LRE* allele (Tsukamoto and Palanivelu, 2010; Tsukamoto et al., 2010); like our observations using *lre-7*, a delay in embryo development was seen only with the loss of the matrigenic *lre-5* allele but not the patrigenic *lre-5* allele (Supplemental Fig. S5).

To complement the delay in the initiation of the embryo development defect in *lre-7* seeds, we generated the *pLRE*::*LRE-HA* transgene, transformed it into *lre-7* plants, identified single insertion lines, and demonstrated, first, that the transgene is functional; the presence of the *pLRE*::*LRE-HA* transgene restored seed set in *lre-7* plants to wild-type levels, presumably by rescuing the pollen tube reception defect in these plants (Supplemental Tables S7–S9). We then reciprocally crossed the transgenic line carrying *pLRE*::*LRE-HA* with wild-type pollen and found that the delayed embryo development defect can be complemented by supplying the matrigenic, but not the patrigenic, *pLRE*::*LRE-HA* transgene (Fig. 6, A and C), indicating that loss of expression from the matrigenic *LRE* allele results in delayed early seed development.

### Delay in Seed Development Is Not Caused by the Loss of *LRE* Expression in the Maternal Sporophyte

An alternative explanation for the observations in Figure 6 is that the loss of *LRE* expression in the maternal sporophyte resulted in delayed seed development, as homozygous *lre* ovules were used in these experiments and they contain mutant sporophytic tissues enclosing mutant gametophytes. However, this possibility is unlikely, because *LRE* is not expressed in the maternal sporophyte (Fig. 2). To confirm that the effect on embryo development is due to maternal or matrigenic *LRE* expression and not to *LRE* expression in the female sporophyte, we crossed *lre* heterozygous pistils with wild-type pollen and scored embryo development in cleared ovules. In heterozygous pistils, there is a functional *LRE* allele in only 50% of female gametophytes, even though the diploid female sporophyte surrounding every ovule has one functional *LRE* allele. To survey the effects of the loss of *LRE* in the female sporophyte on seed development, we first crossed pollen carrying the *pGRP23*::*GUS* transgene (Ding et al., 2006) with wild-type and *lre-5* heterozygous pistils. In wild-type pistils crossed with *pGRP23*::*GUS* pollen, 98.4% of seeds showed normally developed embryos (Fig. 7A). However, in *lre-5* heterozygous pistils (Fig. 7B), among all the seeds, 18.8% (70 of 373) of seeds were delayed in embryo development (Fig. 7D), while the remainder had normal embryo development (Fig. 7C). The detection of seeds with delayed embryo development in *lre-5* heterozygous pistils indicates that the loss of *LRE* in the maternal sporophyte is not related to delayed embryo development.

To confirm these results, we performed this experiment using the chloral hydrate assay and *lre-7* heterozygous pistils. The number of seeds with delayed embryo development in crosses with *lre-7* heterozygous pistils is significantly higher than in crosses with wild-type pistils (Fig. 7E), indicating that delayed early embryo development is not caused by the female sporophyte. Still, these



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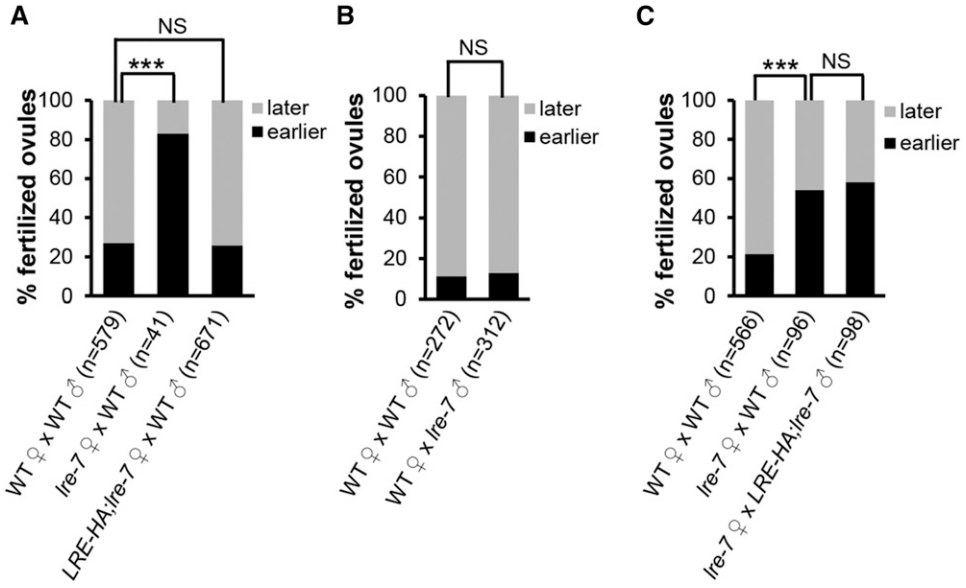


**Figure 6.** Loss of matrigenic *LRE* causes delayed embryo development in 48-HAP seeds. A, Graph showing that embryo development was delayed in 48-HAP seeds from a cross in which *lre-7* was the female (♀) parent. This delay in embryo development was rescued in crosses with female *lre-7* mutant plants carrying the *pLRE*::*LRE-HA* transgene (progeny of lines 12-7 and 12-17; Supplemental Table S5). B, Graph showing that embryo development was not delayed in 48-HAP seeds from a cross in which the wild type (WT) was the female parent. C, Graph showing that the delayed embryo development in 48-HAP seeds using *lre-7* mutant as the female parent was not rescued if pollinated with pollen from *lre-7* mutant plants carrying the *pLRE*::*LRE-HA* transgene. In these graphs, black columns show the first category of embryo development stages (earlier), which includes zygote and proembryos with one- or two-cell EP, while gray columns represent the second category (later), which includes proembryos with 4-, 8-, or 16-cell EP. The wild type and *lre-7* single mutant were included in every experiment to account for the variation in the penetrance of the delayed embryo development phenotype and allow direct comparisons between wild-type and mutant or wild-type and complemented lines or mutant and complemented lines. Fisher's exact test *P* values in A (*** and NS [not significant]) are 1.022e-12 and 0.6998, respectively. Fisher's exact test *P* value in B (NS) is 0.6143. Fisher's exact test *P* values in C (*** and NS) are 3.835e-10 and 0.6644, respectively.

experiments cannot distinguish between loss of maternal *LRE* expression in the female gametophyte and loss of matrigenic *LRE* expression in the developing seed. However, our observations of increased *LRE* expression in the zygote and endosperm after fertilization compared with *LRE* expression in the egg and central cell before fertilization (Fig. 2) suggest that the loss of expression from the matrigenic *LRE* allele in seeds causes delayed early seed development.

## DISCUSSION

### *LRE* Expression Is Imprinted in the Zygote and Endosperm Immediately after Double Fertilization

Soon after fertilization, in the zygote and proliferating endosperm, there is preferential expression from the matrigenic allele of *LRE*. This expression is likely from de novo *LRE* transcripts generated after fertilization, as the GFP expression level in the zygote and endosperm is higher than that in the egg cell and the central cell, respectively (Fig. 2). *LRE* expression in the seeds is not only primarily from the matrigenic allele but also is detectable only for a short duration. *LRE* transcripts were detected at 24 HAP but not at 36 or 48 HAP in seeds of manually selfed crosses (Tsukamoto et al., 2010) or up to 24 HAP in reciprocal crosses between *pLRE*::*GFP* and the wild type (Fig. 2), suggesting that sometime between 24 and 36 HAP, *LRE* expression ceases in seeds. Additionally, based on publicly available RNA-seq or microarray data, *LRE* is not expressed in embryos at approximately 40, 64, and 78 HAP (one-/two-cell, eight-cell, and 32-cell stages; Nodine and Bartel, 2012) and in the endosperm of seeds 6 to 8 d after pollination (Gehring et al., 2011; Hsieh et al., 2011). Subsequently in development, *LRE* is expressed in 8-d-old seedlings, at which point it is bialleically expressed. Still, without determining the expression of *LRE* in seeds from 9 d after pollination to the mature seed, and seedlings before 8 d old, the precise stage of plant development when biallelic expression of *LRE* is restored cannot be established.

Imprinted expression of *LRE* shares similarities and differences with other MEGs in Arabidopsis. Monoallelic expression during reproduction (Fig. 2) and biallelic expression of *LRE* in 8-d-old seedlings (Fig. 4) indicate that, like other MEGs, epigenetic reprogramming for imprinted expression and subsequent restoration of biallelic *LRE* expression must occur during gametogenesis and vegetative growth, respectively (Jahnke and Scholten, 2009; Kawashima and Berger, 2014; Boavida et al., 2015). Additionally, like other MEGs, *LRE* is not expressed in pollen (Tsukamoto et al.,



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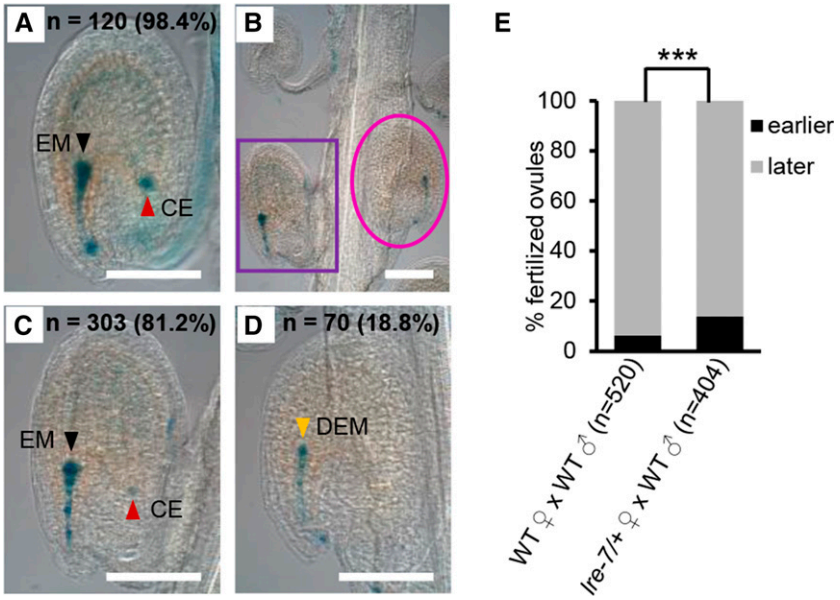


**Figure 7.** Loss of *LRE* in the female sporophyte does not result in delayed embryo development in 48-HAP seeds. A, A seed with normal embryo development from crosses using the wild type as the female parent (♀) and *pGRP23*::*GUS* as the male parent (♂). B to D, Seeds from crosses using *lre-5/+* as the female parent and *pGRP23*::*GUS* as the male parent. B, A portion of the heterozygous silique containing a seed undergoing normal (purple rectangle) and delayed (pink oval) embryo development. C, An enlarged view of the seed in the purple rectangle in B. D, An enlarged view of the seed in the pink oval in B. CE, Chalazal endosperm (red arrowheads); DEM, delayed embryo (orange arrowhead); EM, embryo (black arrowheads). Bars = 100 $\mu$m. E, Graph showing that embryo development is delayed in 48-HAP seeds from a cross in which *lre-7/+* was the female parent and the wild type (WT) was the male parent. In this graph, black columns show the first category of embryo development stages (earlier), which includes zygote and proembryos with one or two cells, while gray columns represent the second category (later), which includes proembryos with four, eight, or 16 cells. Fisher's exact test *P* value (***) in the graph is 0.0001038.

2010; Loraine et al., 2013; this study) or pollen tubes (Tsukamoto et al., 2010; this study). *LRE* also is distinct from other MEGs in other respects, as its expression is imprinted in both zygote and proliferating endosperm. Additionally, monoallelic expression of *LRE* expression after fertilization in the developing seed is earlier than reported for other MEGs (Jahnke and Scholten, 2009; Ngo et al., 2012; Nodine and Bartel, 2012; Raissig et al., 2013). Because the patrigenic allele of *LRE* remains silent in various stages of seed development after 24 HAP (Tsukamoto et al., 2010; Gehring et al., 2011; Hsieh et al., 2011; Nodine and Bartel, 2012; Raissig et al., 2013), our observations also cannot be explained by delayed paternal genome activation (Autran et al., 2011; Del Toro-De León et al., 2014; García-Aguilar and Gillmor, 2015). Based on these observations, we conclude that *LRE* expression is imprinted in both zygote and endosperm.

### Silencing of the Paternal Allele of *LRE* May Be Controlled by a Novel Pathway

Release of silencing of *LRE* likely occurs at some point during female gametogenesis, which suggests that passing through the female gametophyte could result in differential modification of the maternal *LRE* allele compared with the paternal *LRE* allele. For example, the paternal allele of *LRE* could be preferentially hypermethylated, either in the untranslated region or the gene body region, as is the case in many MEGs (Gehring, 2013; Bai and Settles, 2015). However, our data showed that this is not the case for *LRE*, as paternal imprinting of *LRE* is not affected in DNA methylation pathway mutants, including *met1-1*, *ddm1-2*, and *ddc*. Conversely, maternal imprinting of *LRE* also is not affected in the *dme* mutant (Supplemental Table S2), which disrupts the DNA demethylation pathway in the female gametophyte. Known pathways that differentially modify histone methylation also are likely not involved in imprinting *LRE* expression, as silencing of the paternal allele of *LRE* is not reversed in the *msi1* mutant, which disrupts PRC2-dependent histone modification (H3K27me3). These results indicate that imprinting of *LRE* expression is controlled by a yet to be characterized novel pathway. For example, it could be due to an imprinting-like phenomenon proposed for certain transcripts in early embryos of Arabidopsis (Nodine and Bartel, 2012), as imprinted *LRE* expression in seeds is short-lived. Nevertheless, our results point to the fact that the maternal *LRE* allele is relieved of silencing at some point during female gametogenesis, which then sets up monoallelic expression of the matrigenic allele in the seeds.



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### Loss of Expression from the Matrigenic *LRE* Allele Causes Delayed Seed Development

Imprinting is well established in several genes; however, its evolutionary and functional significance remain unclear. The parental conflict/kinship conflict theory provides a plausible reason for the prevalence of imprinting (Haig and Westoby, 1989, 1991; Haig, 2013). As per this theory, parent-of-origin effects are the outcome of the conflict between patrigenic and matrigenic alleles in influencing resource allocation: matrigenic alleles favor equitable resource allocation among all sibling seeds and thus tend to promote smaller seeds, while the patrigenic alleles favor larger seeds and are less constrained by costs to sibling seeds (Haig and Westoby, 1989, 1991; Haig, 2013). Endosperm proliferation and increase in seed growth when mutations in maternally expressed imprinted genes, including *FIS2*, *FIE*, and *MEA*, are maternally inherited (Grossniklaus et al., 1998; Köhler and Makarevich, 2006) provide support for this hypothesis.

Contrary to the expectations of this theory, loss-of-function mutations in *lre* resulted in delayed seed development, revealing a positive role for maternal expression of *LRE* during seed development. *LRE* is expressed in the zygote and the proliferating endosperm; therefore, we hypothesize that loss of *LRE* in the zygote and the proliferating endosperm caused the delay in zygote and endosperm development. Alternatively, the delay of early embryogenesis could be due to an indirect effect of loss of *LRE* in the endosperm. Nevertheless, our study adds *LRE* to a growing list of MEGs whose loss negatively impacts seed development, including *ZIX*, *FH5*, and *NUWA* in Arabidopsis (Ingouff et al., 2005; Fitz Gerald et al., 2009; Ngo et al., 2012; He et al., 2017) and *MEG1* in maize (Gutiérrez-Marcos et al., 2004; Costa et al., 2012). Additionally, mutations in MEGs like *FWA* and *AGL36* do not result in endosperm phenotypes (Kinoshita et al., 2004; Shirzadi et al., 2011). These MEGs highlight the need to explore alternative theories for imprinting. For example, the maternal-offspring coadaptation theory suggests that imprinting evolved to increase the adaptive integration of offspring and maternal genomes, leading to higher offspring fitness (Wolf and Hager, 2006).

## CONCLUSION

Double fertilization in flowering plants occurs in the female gametophyte, which is located within an ovule. During this critical step in flowering plant reproduction, the two female gametes (the egg cell and the central cell) in the female gametophyte fuse with the two male gametes (two sperm cells) delivered by the male gametophyte. The fusion of egg with a sperm cell results in the embryo, and the fusion of the central cell with the second sperm cell gives rise to the endosperm. The initiation of embryo and endosperm development occurs in the seed after double fertilization. Since double fertilization and seed development occur in the female gametophyte of an ovule, the female gametophyte controls events immediately before and soon after fertilization.

The experiments reported in this study lead to three major conclusions. First, we show that *LRE* expression is imprinted, as the matrigenic *LRE* allele contributes nearly all the *LRE* expression after fertilization. Second, it is likely that the imprinting of *LRE* is mediated by a novel pathway, as histone and DNA methylation pathways known to regulate MEGs do not control the imprinted expression of LRE. Finally, we show that the loss of the matrigenic but not the patrigenic *LRE* allele caused delayed embryo and endosperm development and revealed a growth-promoting role for LRE in seeds. Our study shows that *LRE* is a rare imprinted gene that functions immediately after double fertilization. Coupled with our prior study of the role of LRE in pollen tube reception (Liu et al., 2016), this study demonstrates that maternal and matrigenic expression of *LRE* in the female gametophyte and seeds, respectively, allows the female gametophyte to exert control over pollen tube reception before fertilization and seed development after fertilization.

Many interesting questions remain to be addressed. It needs to be confirmed if the loss of matrigenic or maternal *LRE* expression in the seed results in the delayed initiation of seed development. The pathway that controls imprinting in the gametophyte generation also needs to be deciphered. The molecular mechanism by which LRE controls early seed development is another important area of future research. LRE might be part of a signaling complex in embryo and endosperm analogous to signaling that induces pollen tube reception in the synergid (Li et al., 2015; Liu et al., 2016). Expression of the matrigenic *LRE* allele in both fertilized products perhaps allows the female gametophyte to extend the control of seed development beyond fertilization and points to *LRE*'s utility as a marker to characterize the maternal control of molecular events taking place during this critical yet poorly characterized developmental phase in sexual plant reproduction, the transition from gametophytic to sporophytic generation.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) seeds were plated on Murashige and Skoog (MS) medium (Carolina Biological Supply Company; 195703) and incubated in the growth chamber at 20°C and 24 h of illumination. For segregation analysis, seedlings were grown for 2 weeks in MS medium supplemented with hygromycin B (20 $\mu$g mL$^{-1}$; PhytoTechnology Laboratories; H397) and Basta (10 $\mu$g mL$^{-1}$; Fisher Scientific; 50-240-693). For other experiments, 7- to 10-d-old seedlings were transplanted from plates to soil and grown in chambers at 20°C and 24 h of illumination. Unless indicated, the wild-type and mutant accession used in this study is Columbia. In AS-PCR, wild-type Columbia and C24 accessions were used. The *dme-1* mutant is in the Landsberg *erecta* background. All mutant plants were confirmed by genotyping and/or phenotyping: *lre-5* and *lre-7* (Tsukamoto et al., 2010), *dme-1* (Choi et al., 2002), *met1-1* (Kankel et al., 2003), *ddm1-2* (Kakutani et al., 1996; Yadegari et al., 2000), *drm1-2 drm2-2 cmt3-11* (Chan et al., 2006), and *msi1* (SAIL_429_B08; Köhler et al., 2003).



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## RT-PCR

To analyze *LRE* gene expression (Fig. 1), RT-PCR was performed as described (Tsukamoto et al., 2010). RT-PCR involving hypomethylation mutants was performed as follows. The indicated mutant pollen was crossed onto *lre-5* pistils. In the case of *met1-1* and *ddm1-2*, only first-generation homozygous plants from segregating progeny of a heterozygote were used. The genotype of *ddc* triple mutant plants used in this experiment was *drm1-2/+ drm2-2/drm2-2 cmt3-11/cmt3-11* unless indicated. For *msi1*, pollen from only heterozygous plants was used in the crosses, as homozygous mutants cannot be established (Köhler et al., 2003). In each case, 25 to 30 pollinated pistils were excised from the plant 12 to 14 HAP, and ovary walls were removed before freezing. The RNeasy Plant Mini Kit (Qiagen; catalog no. 74904), DNase I (RNase free; Life Technologies; catalog no. AM2222), and the RNeasy MinElute Cleanup Kit (Qiagen; catalog no. 74204) were used for RNA isolation, digestion of contaminating genomic DNA, and RNA purification, respectively. The Thermoscript RT-PCR System (Life Technologies; catalog no. 11146-024) was used for RT. In all crosses, 4 $\mu$g of total RNA was used as a template for each RT reaction. TaKaRa Ex Taq DNA Polymerase (Fisher Scientific; catalog no. TAK_RR01BM) was used to carry out RT-PCR as follows: 94°C (denaturation) for 15 s, 55°C (annealing) for 15 s, and 72°C (extension) for 1 min for 41 cycles. RNA isolation and RT-PCR for *LRE* expression in 8-d-old seedlings, including the roots, were performed similarly (Fig. 4).

## Allele-Specific RT-PCR

Seeds at 24 HAP were collected from reciprocal crosses between Columbia and C24 ecotypes. RNA isolation, purification, and cDNA synthesis were performed as above, and AS-PCR was performed as follows: 3 min at 94°C; 50 cycles of 30 s at 94°C, 30 s at the chosen annealing temperature (below), and 1 min at 72°C; followed by 10 min at 72°C. LNA primers (Latorra et al., 2003) used to amplify Columbia or C24 alleles of *LRE* and *GRP23* are listed (Supplemental Table S10). The optimal annealing temperature, at which the LNA primer pair can amplify a PCR product from only one allele but not the other, was determined using genomic DNA as a template in a 50-cycle PCR and conditions as described above: 67°C and 66°C for Columbia and C24 alleles of *LRE*, respectively, and 68°C for both Columbia and C24 alleles of *GRP23*.

## *pLRE*::*GFP* and *pLRE*::*DsRed* Expression Analysis

The *pLRE*::*GFP* construct was generated by amplifying the *LRE* promoter, 959 bp upstream of the *LRE* start codon, which was sufficient to complement reproductive defects in *lre-7* (Liu et al., 2016; this study using *pLRE*::*LRE-HA* [see below]), and cloning it into the *pCAMBIA1300-GFP* vector between *Bam*HI and *Sal*I sites. The sequence-verified *pLRE*::*GFP* construct was introduced into wild-type plants (Clough and Bent, 1998). Transformants were selected on MS plates with hygromycin. GFP fluorescence in mature ovules (24–48 HAE) and developing seeds (0–48 HAP) was observed and imaged in a confocal laser scanning microscope (Leica TCL SP5) using 488-nm excitation and 510-nm emission. Plants homozygous for the transgene from three independent transformants, with at least two plants per line and two pistils per plant, were scored for GFP expression in an epifluorescence microscope (Zeiss Axiophot) using a GFP filter with excitation HQ 470/40 and emission HQ 525/50. The sequence-verified *pLRE*::*DsRed* construct was introduced into plants carrying *pWOX8*::*gWOX8-YFP* (Ueda et al., 2011). The *pLRE*::*DsRed* construct was generated by cloning a 959-bp fragment upstream of the *LRE* start codon and PCR-amplified *DsRed* sequence (678 bp from a *pLAT52*::*DsRed* construct) into the *pH7WG* vector cut with *Spe*I (New England Biolabs; catalog no. R0133S) and *Asc*I (New England Biolabs; catalog no. R5558S) using the In-Fusion HD Cloning Plus System (Clontech; catalog no. 638909). Transformants were selected on MS plates with hygromycin. Expression of both *gWOX8-YFP* and *pLRE*::*DsRed* was first confirmed in unfertilized ovules 24 to 48 h after emasculation in an epifluorescence microscope (Zeiss Axiophot) using a GFP filter (as above) and a rhodamine filter with excitation BP 546/12 and emission LP 590. Wild-type pollen was crossed onto pistils expressing both markers, and seeds were collected at 13.5 HAP, mounted on slides using 5% glycerol, and visualized with the epifluorescence microscope. Three independent transformants, with at least two plants per line and two pistils per plant, were scored for *DsRed* expression.

## Complementation of the Delayed Seed Development Phenotype Using *pLRE*::*LRE-HA*

The *pLRE*::*LRE-HA* construct was generated as follows. The 959-bp sequence upstream of the *LRE* start codon, *LRE* coding sequence (888 bp), and 3′ untranslated region sequence (131 bp) were PCR amplified (primers are listed in Supplemental Table S7). Primers used to amplify the *LRE* coding sequence contained the HA sequence (27 bp). The fragments were fused by overlapping PCR and cloned into *pENTR/D-TOPO* (Invitrogen; catalog no. K2400-20) and then swapped into Gateway vector *pH7WG* using Clonase II enzyme mix (Life Technologies; catalog no. 11791020). The sequence-verified *pLRE*::*LRE-HA* construct was transformed into *lre-7* plants. Transformants were selected on MS plates containing both hygromycin (*pLRE*::*LRE-HA* transgene) and Basta (*lre-7* mutation).

Candidate single-insertion lines were selected from among the T1 transformants as follows: if the T1 transformant generated in the *lre-7* background had a single insertion of the transgene (*pLRE*::*LRE-HA/+*, *lre-7/lre-7*) and if it complemented the partially penetrant female gametophytic defects in *lre*, then seed set frequency (~65%) in the selfed pistils will resemble that of *lre-7/+* pistils (Supplemental Table S7). Seed set was scored in five to eight siliques per plant in 10 plants for every line. Based on this criterion, we selected four T1 lines (12, 13, 15, and 20) as putative single-insertion lines (Supplemental Table S7). To further test the presence of single insertions in these lines, we plated T2 seeds from these lines on hygromycin. If the transgenic plant is expected to contain a single transgene and is heterozygous in that locus, the hygromycin resistance-to-susceptibility ratio of the T2 selfed seeds from that plant is expected to be ~8:1 to 13:1, provided that the transgene complements partially penetrant *lre* female gametophytic defects in inducing pollen tube reception (Liu et al., 2016). If there is no complementation, the single-insertion line will be expected to produce T2 seeds that have a segregation ratio of 3:1. Based on this test, line 12 (335 resistant:32 sensitive; 10.5:1) and line 13 (484 resistant:37 sensitive; 13.1:1) were identified as single-insertion lines.

T2 plants from line 12 were raised and scored for seed set. Those plants that showed complete seed set were considered to be homozygous for the *pLRE*::*LRE-HA* transgene and that the seed set defect in these plants has been completely rescued (Supplemental Table S8). T3 seeds from two plants (12-7 and 12-17) were tested further if they were homozygous for the *pLRE*::*LRE-HA* transgene; indeed, T3 seeds from both of these plants were all resistant to hygromycin (Supplemental Table S9). Progeny of 12-7 and 12-17 were then used in parent-of-origin complementation experiments (Fig. 6).

## Analysis of Embryo and Endosperm Development

GUS assay and microscopic analysis using *pGRP23*::*GUS* in 48-HAP seeds were performed as described (Tsukamoto et al., 2010). For the seed-clearing assay, stage 12c flowers (Smyth et al., 1990) were emasculated and pollinated 24 h later. Crossed pistils were excised from the plant 48 HAP; silique walls were removed and fixed in ethanol:acetic acid (9:1) solution overnight, followed by successive incubations in 90% ethanol and 70% ethanol each for 30 min. Clearing of seeds was performed as described (Yadegari et al., 1994) after minor modifications: siliques were cleared overnight in a clearing solution (chloral hydrate:glycerol:water, 4:1:2, m/v/v [chloral hydrate; Sigma-Aldrich; catalog no. C8383]), mounted using the same clearing solution, and scored with a differential interference contrast microscope (Zeiss Axiophot) within 1 week after mounting the slides.

Embryo development in Arabidopsis is asynchronous, and the different stages of embryo development were scored at 48 HAP as zygote, two-cell proembryo, proembryo with two-, four-, or eight-cell EP, and embryo with 16-cell EP as described (Goldberg et al., 1994) based on the number of cells/nuclei in the developing embryo.

The wild-type and *lre-7* single mutant were included in every experiment to account for variation in the penetrance of the delayed embryo development phenotype, which is caused by the variation in the seed set (14.29%–42.44%; $n$ = 3,561 from 10 plants; Supplemental Table S7). Accounting for this variability, in every experiment we performed, we included as controls a fresh set of wild-type × wild-type and *lre* × wild-type crosses using wild-type and *lre* mutant plants grown simultaneously under the same conditions. As a result, in each experiment, we only compared results from the experimental crosses with those in concurrently performed control crosses, and experimental crosses in one experiment were never compared with the control crosses performed as part of another experiment. This strategy allowed direct comparisons between wild-type and mutant lines or wild-type and complemented lines or mutant and complemented lines.



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## Statistical Analysis

In wild-type and *lre* crosses, the majority of embryos were at proembryo with four-cell EP and proembryo with two-cell EP, respectively. Therefore, we grouped embryo developmental stages into two categories. Embryos in the earlier developmental stages, including zygote, two-cell proembryo, and proembryo with two-cell EP (after one cell division in EP) are part of the first category (black columns in graphs of Figs. 6 and 7 and Supplemental Fig. S5). Embryos in the later developmental stages, including proembryos with four- and eight-cell EP and embryos with 16-cell EP (i.e. two or more cell divisions in EP), were grouped in the second category (gray columns in graphs of Figs. 6 and 7 and Supplemental Fig. S5).

Endosperm development stage was scored at 48 HAP only based on the number of endosperm nuclei, as cellularization in endosperm does not initiate until the embryo reaches the heart stage (Berger, 2003). In wild-type and *lre* crosses, the majority of endosperm were at 33 to 128 endosperm nuclei (six to seven nuclear divisions of the primary endosperm nucleus) and at 32 or fewer endosperm nuclei (five or fewer nuclear divisions of the primary endosperm nucleus), respectively. Therefore, endosperm developmental stages were grouped into two categories. Endosperm with 32 or fewer endosperm nuclei was included in the first category (earlier; black columns in graphs of Supplemental Fig. S4). Later developmental stages of endosperm, including those with 33 to 128 endosperm nuclei, were grouped in the second category (later; gray columns in graphs in Supplemental Fig. S4).

Fisher's exact test for count data was performed for the earlier and later category data in a $2 \times 2$ contingency table using the R package (version 3.2.3) with fisher.test() function (R Core Team, 2015). We hypothesized that the true odds ratio is equal to 1. If $P < 0.05$, the hypothesis was rejected. In graphs (Figs. 6 and 7; Supplemental Figs. S4 and S5), three asterisks are used to represent statistically significant differences in Fisher's exact test ($P < 0.001$) and NS indicates when there was no statistical difference in Fisher's exact test ($P > 0.05$).

The number of samples in each experiment was determined to be large enough for a statistical analysis using Power and Sample Size. The seed number analyzed in each category of every cross reported in this study is provided in Supplemental Table S11.

## Image Processing

Photoshop CS4 (Adobe) and ImageJ were used to assemble image panels and prepare figures.

## Accession Numbers

Accession numbers of the genes studied in this work are as follows: *LRE* (*At4g26466*), *GRP23* (*At1g10270*), *ACTIN2* (*At3g18780*), *ACTIN11* (*At3g12110*), *LLG1* (*At5g56170*), *PHERES1* (*At1g65330*), *MSI1* (*At5g58230*), *DME1* (*At5g04560*), *CMT3* (*At1g69770*), *DRM2* (*At5g14620*), *DRM1* (*At5g15380*), and *MET1* (*At5g49160*).

## Supplemental Data

The following supplemental materials are available

**Supplemental Figure S1.** *LRE* is expressed in the female gametophyte.

**Supplemental Figure S2.** Defects in DNA and histone methylation pathway genes did not lead to *LRE* expression from the patrigenic allele in seeds.

**Supplemental Figure S3.** A clearing procedure to monitor embryo and endosperm development in seeds.

**Supplemental Figure S4.** Loss of matrigenic *LRE* allele leads to delayed endosperm development.

**Supplemental Figure S5.** Loss of matrigenic *LRE*, but not patrigenic *LRE*, causes delayed embryo development in 48-HAP seeds.

**Supplemental Table S1.** GFP expression in the seeds from crosses of *pLRE*::*GFP* ♀ × Columbia ♂.

**Supplemental Table S2.** *DsRed* expression in the seeds from crosses of *pLRE*::*DsRed*, *pWOX8*::*WOX8-YFP* ♀ × Columbia ♂.

**Supplemental Table S3.** *DsRed* expression in the seeds from crosses of *pWOX8*::*WOX8-YFP* ♀ × *pLRE*::*DsRed*, *pWOX8*::*WOX8-YFP* ♂.

**Supplemental Table S4.** Defects in DNA methylation pathway genes do not lead to expression from the patrigenic *pLRE*::*GFP* allele in fertilized ovules.

**Supplemental Table S5.** The *dem* mutation does not result in a decrease in the expression of the matrigenic *pLRE*::*GFP* allele in fertilized ovules.

**Supplemental Table S6.** Defects in a histone methylation pathway gene (*MSI1*) do not lead to expression from the patrigenic *pLRE*::*GFP* allele in fertilized ovules.

**Supplemental Table S7.** The seed set defect in *lre-7* plants is complemented if they carry the *pLRE*::*LRE-HA* transgene, establishing that *pLRE*::*LRE-HA* is functional.

**Supplemental Table S8.** The reduced seed set defect seen in *lre-7* plants is rescued in T2 segregants, establishing that *pLRE*::*LRE-HA* is functional.

**Supplemental Table S9.** T3 segregation on plates containing both hygromycin and Basta confirm that tested lines are homozygous for the *pLRE*::*LRE-HA* transgene.

**Supplemental Table S10.** List of primers used in this study.

**Supplemental Table S11.** Seed number analyzed in each category of every cross reported in this study.

**Supplemental Movie S1.** *LRE* is expressed in the synergid and egg cells of the female gametophyte.

**Supplemental Movie S2.** *LRE* is expressed in the synergid, egg, and central cells of the female gametophyte.

**Supplemental Movie S3.** *LRE* is expressed in the zygote-like cell and the proliferating endosperm of a developing seed.

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