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A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in *Arabidopsis thaliana* double fertilization and early seed development

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SUMMARY

In plants, double fertilization requires successful sperm cell delivery into the female gametophyte followed by migration, recognition and fusion of the two sperm cells with two female gametes. We isolated a null allele (*lre-5*) of *LORELEI*, which encodes a putative glycosylphosphatidylinositol (GPI)-anchored protein implicated in reception of the pollen tube by the female gametophyte. Although most *lre-5* female gametophytes do not allow pollen tube reception, in those that do, early seed development is delayed. A fraction of *lre-5*/*lre-5* seeds underwent abortion due to defect(s) in the female gametophyte. The aborted seeds contained endosperm but no zygote/embryo, reminiscent of autonomous endosperm development in the pollen tube reception mutants *scylla* and *sirene*. However, unpollinated *lre-5*/*lre-5* ovules did not initiate autonomous endosperm development and endosperm development in aborted seeds began after central cell fertilization. Thus, the egg cell probably remained unfertilized in aborted *lre-5*/*lre-5* seeds. The *lre-5*/*lre-5* ovules that remain undeveloped due to defective pollen tube reception did not induce synergid degeneration and repulsion of supernumerary pollen tubes. In ovules, *LORELEI* is expressed during pollen tube reception, double fertilization and early seed development. Null mutants of *LORELEI-like-GPI-anchored protein 1* (*LLG1*), the closest relative of *LORELEI* among three Arabidopsis *LLG* genes, are fully fertile and did not enhance reproductive defects in *lre-5*/*lre-5* pistils, suggesting that LLG1 function is not redundant with that of LORELEI in the female gametophyte. Our results show that, besides pollen tube reception, LORELEI also functions during double fertilization and early seed development.

Keywords: *LORELEI*, glycosylphosphatidylinositol (GPI)-anchored protein, pollen tube reception, pollen tube repulsion, double fertilization, early seed development.

INTRODUCTION

During plant reproduction, a series of interactions between male and female gametophytes ensures successful fusion of male and female gametes. In flowering plants, anthers give rise to the pollen grain (male gametophyte) containing the sperm cells. The female gametophyte develops inside an ovule and in many species, including Arabidopsis, is a seven-celled structure that includes three antipodal cells, two synergid cells, an egg and a central cell. During sexual reproduction, the two gametophytes interact and facilitate fusion between the gametes (sperm and egg cell) to form a zygote and establish the sporophytic phase of the plant life cycle (Yadegari and Drews, 2004).

After pollination, the pollen tube transports the two sperm cells to its growing tip and extends through the sporophytic pistil tissues (stigma, style and transmitting tract) to reach the ovules in the ovary. The female gametophyte attracts the pollen tube by releasing guidance cues (Chen *et al.*, 2007; Higashiyama and Hamamura, 2008), and typically only one pollen tube enters the micropylar opening of the ovule and reaches the female gametophyte (Geitmann and Palanivelu, 2007). In Arabidopsis, interactions between a pollen tube and a female gametophyte occur as follows: (i) pollen tube navigation to the female gametophyte, (ii) pollen tube growth around the synergid cell, (iii) pollen tube reception involving synergid-induced pollen tube growth arrest, (iv) receptive synergid degeneration, and (v) pollen tube discharge of the two sperm cells (Huck *et al.*, 2003; Rotman *et al.*, 2003; Sandaklie-Nikolova *et al.*, 2007). An actin

cytoskeleton-based corona in the female gametophyte facilitates migration of sperm cells to the egg and central cell and completion of double fertilization (Lord and Russell, 2002; Ingouff *et al.*, 2007). After fertilization, the female gametophyte controls seed development by: (i) repressing premature central cell or egg cell proliferation until double fertilization is completed (Yadegari and Drews, 2004; Berger *et al.*, 2006; Pien and Grossniklaus, 2007), (ii) supplying factors that mediate the early stages of embryo and endosperm development (Grini *et al.*, 2002; Yadegari and Drews, 2004; Pagnussat *et al.*, 2005), and (iii) regulating imprinting of genes required for seed development (Yadegari and Drews, 2004; Baroux *et al.*, 2007).

Several Arabidopsis genes involved in pollen tube reception have been identified. FERONIA/SIRENE (FER/SRN), a receptor-like serine/threonine kinase, is essential for pollen tube reception in synergids as the pollen tube fails to arrest its growth, grows excessively in the synergids and fails to discharge the sperm cells after arriving in a *fer*/*srn* female gametophyte (Rotman *et al.*, 2003; Escobar-Restrepo *et al.*, 2007). FERONIA localizes to the synergid cell membrane and either perceives a ligand released by the pollen tube (Escobar-Restrepo *et al.*, 2007) or responds to a maturation signal from other female gametophyte cells and renders the synergid competent for mediating pollen tube reception (Capron *et al.*, 2008; Rotman *et al.*, 2008). Other mutants that disrupt female gametophyte-specific functions in pollen tube reception include *scylla* (*syl*) and *lorelei* (*lre*) (Capron *et al.*, 2008; Rotman *et al.*, 2008). While the molecular identity of *SCYLLA* (*SYL*) is not known (Rotman *et al.*, 2008), *LORELEI* (*LRE*) encodes a putative glycosylphosphatidylinositol (GPI) anchor-containing membrane protein (Capron *et al.*, 2008; also see below). Pollen tube reception also requires functions of the pollen tube; for example, in the self-sterile *abstinence by mutual consent* (*amc*) mutants, pollen tube reception fails only when an *amc* female gametophyte interacts with an *amc* pollen tube. *AMC* encodes a peroxin that is critical for importing proteins into peroxisomes (Boisson-Dernier *et al.*, 2008).

In Arabidopsis, the receptive synergid cell degenerates after mediating pollen tube reception but prior to pollen tube discharge (Sandaklie-Nikolova *et al.*, 2007). Consistent with this model, synergids in pollinated *srn* (Rotman *et al.*, 2003) and *amc* (Boisson-Dernier *et al.*, 2008) ovules fail to degenerate. By contrast, in pollinated *fer* ovules, synergid degeneration is normal (Huck *et al.*, 2003). Pollen tube-expressed proteins, including ACA9, a pollen tube plasma membrane localized calcium pump, and ANX1/ANX2, two receptor-like serine/threonine kinases closely related to FER, regulate rupture and discharge of the pollen tube (Schiott *et al.*, 2004; Miyazaki *et al.*, 2009; Boisson-Dernier *et al.*, 2009). Sperm-expressed *HAP2*/*GCS1* is critical for completion of double fertilization (Mori *et al.*, 2006; von Besser *et al.*, 2006); however, female gametophyte-expressed genes involved in sperm cell migration, recognition and fusion with the two female gametes during double fertilization have not been described (Berger *et al.*, 2008). Genes and mechanisms by which the female gametophyte represses premature central cell or egg cell proliferation until double fertilization is completed and regulates imprinting of the genes required for seed development have been described (Yadegari and Drews, 2004; Baroux *et al.*, 2007); however, only a handful of maternal-effect genes that affect early embryo (Springer *et al.*, 2000; Evans and Kermicle, 2001) and endosperm (Andreuzza *et al.*, 2010) development after fertilization have been characterized. This is particularly intriguing because nearly half of the 130 female gametophytic mutants analyzed in a study were defective in post-fertilization processes, indicating that a large number of female gametophyte-expressed genes probably have a role in early seed development (Pagnussat *et al.*, 2005).

We identified a null allele (*lre-5*) of *LRE* in Arabidopsis (Capron *et al.*, 2008). Most *lre-5* female gametophytes do not allow pollen tube reception and fail to induce synergid degeneration and repulsion of supernumerary pollen tubes. Using a zygote- and endosperm-expressed marker, we show that early seed development is delayed in *lre-5*/*lre-5* ovules that complete pollen tube reception. Additionally, we found that a small fraction of fertilized *lre-5*/*lre-5* seeds do not contain either an embryo or a growth-arrested zygote/embryo but initiate endosperm development after central cell fertilization; these seeds consequently undergo abortion. Our observations show that, besides pollen tube reception, LRE also has a role in double fertilization and early seed development.

RESULTS

A new allele of *LRE* shows reduced fertility

To identify genes important for reproduction, a mutant screen for plants with reduced fertility was performed (Figure S1 in Supporting Information). One such mutant contained a large number of undeveloped ovules and very few normal seeds (Figure 1a, Table 1). Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) revealed that this mutant is a new allele of *LRE* (*At4g26466*; Capron *et al.*, 2008) (Figure 1b; see also Data S1). Four *lre* alleles have been reported (Capron *et al.*, 2008), so this mutant was designated *lre-5*. In addition to *lre-5*, three additional *lre* alleles (*lre-4* and two new alleles, *lre-6* and *lre-7*; Figure 1b, Table S1, and Data S1) were included in this study. In all four *lre* mutants examined, self-pollinated pistils exhibited reduced fertility (Tables 1 and 2) and reciprocal crosses between *lre*/*lre* and wild-type plants showed that reduced fertility was due to defects in female reproductive tissues (Tables 1 and 2). The *LRE* transcript was not detected in ovules of any of the examined *lre* mutants, indicating that *lre-4*, *lre-5*, *lre-6* and *lre-7* are null alleles (Figure S2a).

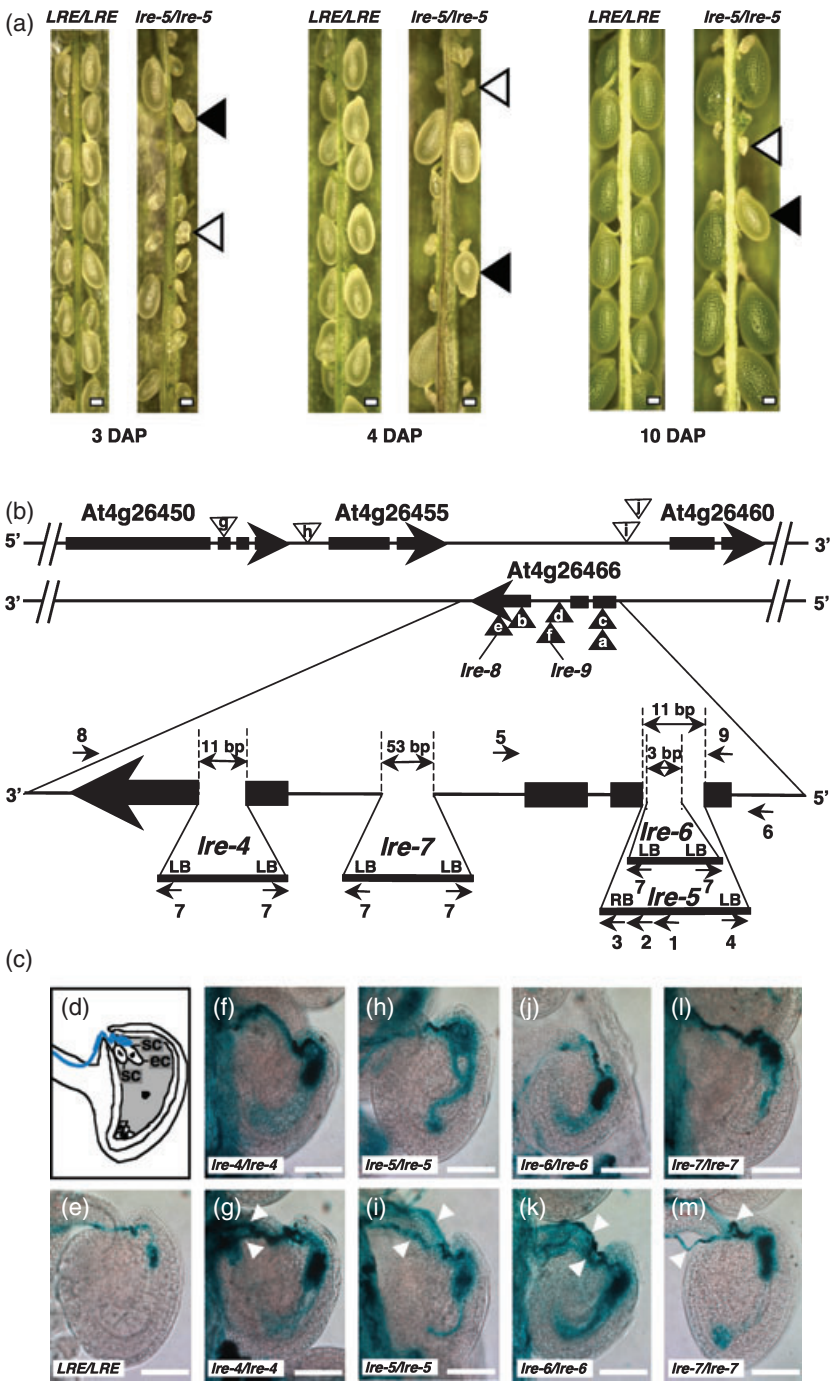

Figure 1. Characterization of *lre-5*, a new allele of *LORELEI* (*LRE*).
(a) Light micrographs of wild-type and *lre-5*/*lre-5* siliques, opened at indicated days after pollination (DAP). In *lre-5*/*lre-5* siliques, undeveloped ovules (open triangles) and aborted seeds (solid triangles) are marked. Scale bars, 100 μm.
(b) T-DNA insertions in and around *LRE*; only insertions in *LRE* (shaded triangles), but not in flanking regions (white triangles), segregated reduced seed-setting plants. Genomic DNA/T-DNA junctions in *lre* alleles and primers (Table S7) used in characterizing these insertions and *LRE* expression are shown.
(c) Pollen tube reception and repulsion are defective in *lre-5*/*lre-5* ovules:
(d) diagram depicting pollen tube reception by one of the two synergid cells (sc) flanking an egg cell (ec) in a female gametophyte;
(e–m) micrographs of *LAT52*:GUS pollen tube growth in ovules. (e) *LAT52*:GUS pollen tube undergoing reception in a wild-type ovule. (f, h, j, l) *LAT52*:GUS pollen tube exhibiting a reception defect in the indicated *lre*/*lre* ovule. (g, i, k, m) Multiple pollen tubes (white arrowheads) entering the indicated *lre*/*lre* ovule. Scale bars, 50 μm.

lre-5 disrupts function of the female gametophyte in pollen tube reception

The Arabidopsis *LRE* gene encodes a putative GPI anchor containing membrane protein (Capron *et al.*, 2008). It contains an N-terminal signal sequence for secretion and a highly hydrophobic C-terminus that is removed and replaced with a GPI anchor (Udenfriend and Kodukula, 1995; Figure S3a). The GPI anchors serve as alternatives to transmembrane domains for anchoring proteins in cell membranes and GPI-anchored proteins have many functions including cell–cell signaling (Schultz *et al.*, 1998).

Two lines of evidence demonstrate that *lre-5* specifically disrupts female gametophyte function, as reported for other *lre* alleles (Capron *et al.*, 2008). First, in reciprocal crosses between *LRE*/*lre-5* and wild-type plants, reduced fertility was observed only when the *LRE*/*lre-5* plant was used as the female parent (Table 1). Second, reduced

Table 1 Reduced and aborted seed-set phenotypes in *lre-5* are specific to the female gametophyte

Female parent	Male parent	Pollination	Undeveloped ovules (%)	Aborted seeds (%)	Normal seeds (%)
LRE/*LRE*	*LRE*/*LRE*	Self	9 (1.2)	0 (0.0)	721 (98.8)
LRE/*LRE*	*LRE*/*LRE*	Manual self	31 (4.3)	2 (0.3)	683 (95.4)
lre-5/*lre-5*	*lre-5*/*lre-5*	Self	683 (70.6)	27 (2.8)	258 (26.6)
lre-5/*lre-5*	*lre-5*/*lre-5*	Manual self	769 (78.2)	31 (3.2)	183 (18.6)
lre-5/*lre-5*	*LRE*/*LRE*	Manual	551 (80.4)	12 (1.8)	122 (17.8)
LRE/*LRE*	*lre-5*/*lre-5*	Manual	25 (2.6)	2 (0.2)	952 (97.2)
LRE/*lre-5*	*LRE*/*lre-5*	Self	634 (34.7)	27 (1.5)	1165 (63.8)
LRE/*lre-5*	*LRE*/*LRE*	Manual	587 (44.1)	12 (0.9)	731 (55.0)
LRE/*LRE*	*lre-5*/*LRE*	Manual	16 (1.9)	1 (0.1)	823 (98.0)

Parent genotypes were confirmed by PCR and progeny segregation.

Table 2 Reduced and aborted seed-set phenotypes in *lre-4*, *lre-6* and *lre-7* are specific to female reproductive tissue

Female parent	Male parent	Pollination	Undeveloped ovules (%)	Aborted seeds (%)	Normal seeds (%)
LRE/*LRE*	*LRE*/*LRE*	Self	9 (1.2)	0 (0.0)	721 (98.8)
LRE/*LRE*	*LRE*/*LRE*	Manual self	31 (4.3)	2 (0.3)	683 (95.4)
lre-4/*lre-4*	*lre-4*/*lre-4*	Self	950 (74.4)	43 (3.4)	284 (22.2)
lre-6/*lre-6*	*lre-6*/*lre-6*	Self	834 (69.5)	26 (2.2)	339 (28.3)
lre-7/*lre-7*	*lre-7*/*lre-7*	Self	847 (71.0)	27 (2.3)	319 (26.7)
LRE/*lre-4*	*LRE*/*lre-4*	Self	420 (39.8)	11 (1.0)	625 (59.2)
LRE/*lre-6*	*LRE*/*lre-6*	Self	374 (36.4)	5 (0.5)	649 (63.1)
LRE/*lre-7*	*LRE*/*lre-7*	Self	409 (39.3)	11 (1.0)	621 (59.7)
lre-4/*lre-4*	*LRE*/*LRE*	Manual	644 (78.3)	25 (3.1)	153 (18.6)
lre-6/*lre-6*	*LRE*/*LRE*	Manual	583 (81.5)	13 (1.8)	119 (16.7)
lre-7/*lre-7*	*LRE*/*LRE*	Manual	483 (78.3)	16 (2.6)	118 (19.1)
LRE/*LRE*	*lre-4*/*lre-4*	Manual	15 (1.6)	1 (0.1)	921 (98.3)
LRE/*LRE*	*lre-6*/*lre-6*	Manual	38 (4.0)	2 (0.2)	908 (95.8)
LRE/*LRE*	*lre-7*/*lre-7*	Manual	22 (2.2)	0 (0.0)	960 (97.8)

Parent genotypes were confirmed by PCR and progeny segregation.

numbers of heterozygous plants in the F_1 progeny of these crosses demonstrated that transmission of the *lre-5* allele through the female gametophyte, but not the male gametophyte, was specifically affected (Tables S2 and S3). The structure of the female gametophyte (Figures 2a,c and 3a,c) and expression analysis of cell-specific markers (Figure S4, Table S4, Data S1) of *lre-5* ovules revealed that cell specification and differentiation in *lre-5* female gametophytes were comparable to that of the wild type. Thus, similar to other *lre* alleles (Capron *et al.*, 2008), the *lre-5* mutation also appears to disrupt late stages of female gametophyte function, including pollen tube reception.

To investigate pollen tube reception in *lre* ovules, we pollinated pistils with wild-type pollen expressing GUS (GUS^+) from the pollen-specific *LAT52* promoter (Twell *et al.*, 1989; Johnson *et al.*, 2004). *LAT52*:GUS pollen tubes reached the female gametophyte at a similar frequency and rate in wild-type and *lre-5*/*lre-5* pistils (Figure S5a,b). However, in the wild type, the GUS^+ pollen tubes entered each ovule singly, stopped growth and burst in a synergid cell (Figure 1d,e, Table 3). By contrast, a majority of GUS^+ pollen tubes failed to cease growth, coiled excessively after entering *lre*/*lre* ovules and sometimes reached the location of the central cell (Figure 1f,h,j,l, Table 3). These results demonstrate that pollen tube reception is defective in *lre-4*, *lre-5*, *lre-6* and *lre-7* ovules.

Pollen tube reception defects in *lre-5* female gametophytes can be recapitulated *in vitro*

To observe pollen tube reception in *lre-5* gametophytes in real time, we employed an Arabidopsis *in vitro* pollen tube guidance assay that recapitulates much of the *in vivo* pollen tube behavior in ovules (Palanivelu and Preuss, 2006). For this assay, pollen tubes were marked with DsRed driven by the *LAT52* promoter (Twell *et al.*, 1989; Francis *et al.*, 2007) and synergids were marked with GFP expressed from the synergid-specific *DD3* promoter (Steffen *et al.*, 2007). Similar to their behavior in the wild type (Movie S1; $n = 89$), in *lre-5*/*lre-5* ovules *LAT52*:DsRed pollen tubes always

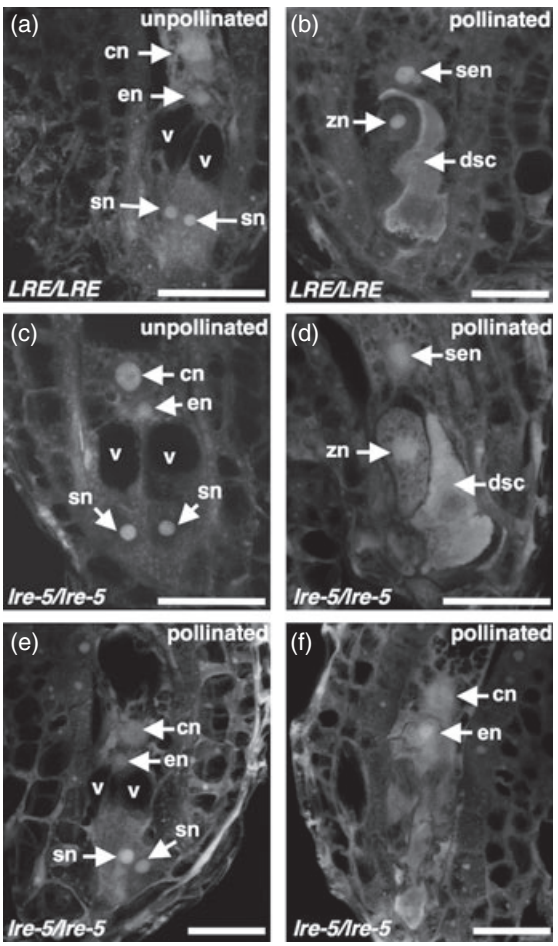

Figure 2. Confocal laser scanning microscopy (CLSM) analysis of female gametophyte development and synergid cell death.
(a, c) The CLSM images of unfertilized stage 7 female gametophytes.
(b, d–f) The CLSM images of embryo sacs in pollinated ovules.
(a–f) Only the micropylar end of an ovule is shown and each image is a projection of several 1μm optical sections: (a, c) confocal images of wild-type (a) and *lre-5* (c) female gametophytes containing synergid, egg and central cells. The gametophytic cell nuclei appear as bright fluorescent spots (arrows). (b, d) Pollinated wild-type (b) and *lre-5* (d) embryo sacs containing a highly autofluorescent, degenerating synergid cell (dsc). (e, f) Two pollinated *lre-5* ovules that do not contain a highly autofluorescent, degenerated synergid cell but contain egg and central cell nuclei.
(g) Table showing unpollinated and pollinated ovules analyzed by CLSM. Letters within parentheses refer to images (a–f).
Abbreviations: cn, central cell nucleus; en, egg cell nucleus; sen, secondary endosperm nucleus; sn, synergid cell nucleus; v, vacuole; zn, zygote nucleus. Scale bars, 10 μm.

(g)

	Unpollinated female gametophytes				Manually self-pollinated embryo sacs					
							No zygote, no dsc (but contain cn and en)			
Plant genotype	Normal (a, c)	(%)	Unclear	(%)	Zygote + dsc (b, d)	(%)	Both sn detected (e)	(%)	Both sn not detected (f)	(%)
LRE/LRE	106	(97.2)	3	(2.8)	151	(94.4)	9	(5.6)	0	(0.0)
lre-5/lre-5	127	(96.9)	4	(3.1)	43	(26.4)	35	(21.5)	85	(52.1)

navigated to the synergids (Movies S2–S4). After reaching the synergids in *lre-5*/*lre-5* ovules, some *LAT52:*DsRed pollen tubes underwent pollen tube reception (Movie S2; $n = 67$) while others did not arrest growth in a synergid and occasionally (in 10/108 ovules) elongated further into the female gametophyte (Movie S3; $n = 108$). Thus, pollen tube reception defects in *lre-5* female gametophytes can be recapitulated *in vitro* and events prior to reception – pollen tube entry into the ovule and navigation to the synergid cells – are normal in *lre-5* female gametophytes.

Failure to repel supernumerary pollen tubes is linked with defective pollen tube reception

Besides the pollen tube reception defect, multiple *LAT52:* GUS-tagged pollen tubes entered ovules in *lre*/*lre* or *LRE*/*lre* pistils (Figure 1g,i,k,m, Table 3). This phenotype in *lre-5*/*lre-5* ovules was also recapitulated *in vitro* (Movie S4; in 38/108 ovules). Pollen tube repulsion defects have been reported in *fer*/*srn* and *amc*, in which the pollen tube reception phenotype is fully penetrant (Huck *et al.*, 2003; Rotman *et al.*, 2003; Escobar-Restrepo *et al.*, 2007; Boisson-Dernier *et al.*, 2008; Capron *et al.*, 2008). We thus exploited the partial penetrance of pollen tube reception defects in *lre* female gametophytes to examine whether pollen tube repulsion is linked to pollen tube reception. In all four *lre* alleles, every instance of multiple pollen tube entry was observed in an ovule that was also defective in pollen tube reception (Table 3). Thus, in *lre* female gametophytes, failure to repel supernumerary pollen tubes is linked with defective pollen tube reception.

Table 3 Multiple pollen tubes enter *lre* ovules

	Targeted ovules						
	Normal pollen tube reception[a]			Defective pollen tube reception[b]			
Female parent	More than one pollen tube	One pollen tube (%)	NC (%)	More than one pollen tube (%)	One pollen tube (%)	NC (%)	Untargeted ovules[c] (%)
LRE/*LRE*	0	85 (95.5)	3 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1)
lre-4/*lre-4*	0	38 (21.2)	0 (0.0)	40 (22.3)	95 (53.1)	5 (2.8)	1 (0.6)
lre-5/*lre-5*	0	57 (26.1)	1 (0.5)	39 (17.9)	98 (45.0)	18 (8.3)	5 (2.3)
lre-6/*lre-6*	0	57 (26.3)	1 (0.5)	32 (14.7)	110 (50.7)	16 (7.4)	1 (0.5)
lre-7/*lre-7*	0	40 (21.5)	2 (1.1)	40 (21.5)	92 (49.5)	10 (5.4)	2 (1.1)
LRE/*lre-4*	0	167 (59.6)	8 (2.9)	21 (7.5)	70 (25.0)	7 (2.5)	7 (2.5)
LRE/*lre-5*	0	165 (57.3)	8 (2.8)	41 (14.2)	57 (19.8)	14 (4.9)	3 (1.0)
LRE/*lre-6*	0	110 (57.6)	5 (2.6)	22 (11.5)	44 (23.0)	8 (4.2)	2 (1.0)
LRE/*lre-7*	0	116 (60.1)	1 (0.5)	31 (16.1)	41 (21.1)	0 (0.0)	4 (2.1)

[a]GUS staining as in Figure 1e.
[b]Abnormal GUS staining as in Figure 1f–m.
[c]Not approached by a pollen tube.
NC, not clear if more than one pollen tube entered an ovule.
Male parent in all crosses: *LRE*/*LRE, LAT52*:GUS/*LAT52*:GUS.
Parent genotypes were confirmed by PCR and progeny segregation.

Pollen tube arrival does not lead to initiation of synergid degeneration in most *lre-5* female gametophytes

In flowering plants, one of the two synergids degenerates prior to fertilization (Faure *et al.*, 2002; Lord and Russell, 2002; Weterings and Russell, 2004). In *Torenia* and Arabidopsis, synergid cell death is initiated following direct interaction with the pollen tube (Higashiyama, 2002; Sandaklie-Nikolova *et al.*, 2007). Given that pollen tube arrival, but not pollen tube reception, in the synergid cell of *lre-5* female gametophytes is comparable to that of the wild type (Movie S3, Figure 1c), we examined synergid degeneration in *lre-5* mutants. We scored synergid cell death in self-pollinated wild-type and *lre-5*/*lre-5* pistils using confocal laser scanning microscopy (CLSM). In wild-type pistils, 94.4% of female gametophytes had a zygote and a single degenerated synergid cell with a distinct autofluorescent region between the micropyle and the central cell (Figure 2b,g). In contrast, in *lre-5*/*lre-5* pistils, two types of female gametophytes were observed. Some had a highly autofluorescent, degenerated synergid cell and a zygote (Figure 2d,g), but a majority of the female gametophytes did not contain a degenerated synergid cell (compare Figure 2d with Figure 2e,f); instead, they either contained four nuclei at the micropylar end – one egg nucleus, two synergid nuclei and one central cell nucleus (Figure 2e; about five times more than in wild-type pistils, Figure 2g) or only the central and egg cell nuclei (Figure 2f). The latter category of female gametophytes (Figure 2f) lacked both synergid nuclei and had irregular synergid cells, perhaps obscured and affected, respectively, by excessive pollen tube growth. Nevertheless, the higher percentage of *lre-5*/*lre-5* ovules without a highly autofluorescent degenerated synergid cell suggested that synergid cell death, as observed in the wild type, did not occur in a majority of pollinated *lre-5* female gametophytes.

To directly score pollen tube arrival and synergid cell death within the same ovule, thick plastic sections of self-pollinated wild-type and *lre-5*/*lre-5* ovules were examined. All wild-type ovules contained a degenerated synergid cell and a zygote (Figure 3b; $n = 79$). In contrast, in *lre-5*/*lre-5* pistils, two types of ovules were observed. Some had a degenerated synergid cell and a zygote (Figure 3d; $n = 114$). The remaining ovules lacked synergid degeneration (note the absence of dark-stained regions in Figure 3e,f); yet, in every instance, at least one pollen tube was seen in a synergid cell (Figure 3e,f; $n = 40$). This category of female gametophytes (Figure 3e,f) was not observed in the wild type, demonstrating that synergids do not degenerate in most *lre-5* female gametophytes.

Aborted seeds in lre pistils lack an embryo but contain proliferating endosperm nuclei

Nearly 10% of seeds are aborted in mature self-fertilized *lre-5*/*lre-5* siliques (for example, 27/285 = 9.5%; third row, Table 1). Reciprocal crosses between *LRE*/*lre-5* and wild-type plants showed that aborted seeds resulted from defects in the female gametophyte (Table 1). Aborted seeds are due to abnormal embryo and/or endosperm development (Yadegari and Drews, 2004; Berger *et al.*, 2006). Therefore, we examined seed development in manually self-pollinated *lre*/*lre* pistils 3 days after pollination (DAP), the earliest point in development when aborted seeds are distinguishable from normal seeds (Figure 1a). In wild-type pistils, nearly every developing seed had an embryo and endosperm

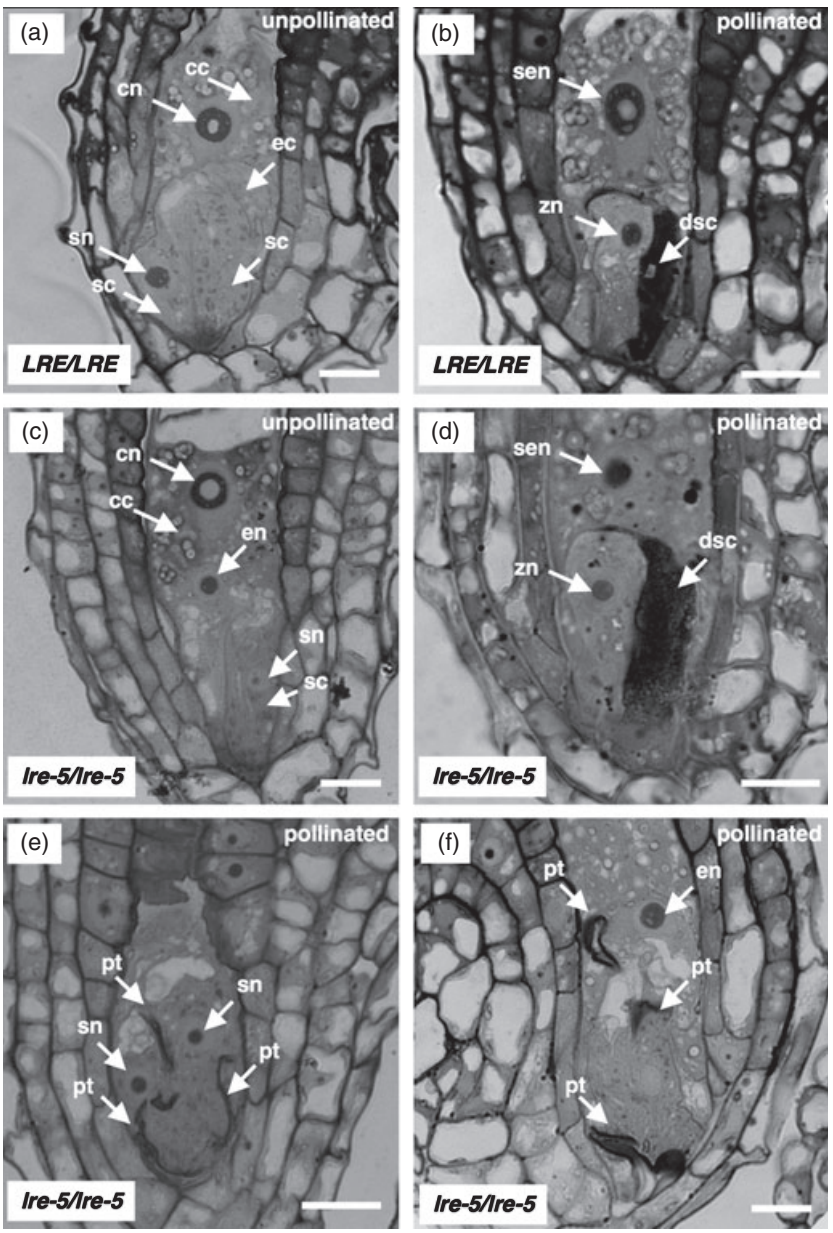

Figure 3. Analysis of female gametophyte development and synergid cell death using light microscopy of thick plastic sections.
(a, c) Sections of stage female gametophyte 7 in unfertilized ovules.
(b, d–f) Sections of ovules 16 h after pollination (HAP).
(a, c) Wild-type (a) and *lre-5* (c) female gametophytes containing intact synergid (sc), egg (ec) and central (cc) cells.
(b, d) Wild-type (b) and *lre-5* (d) female gametophytes with a degenerating synergid cell (dsc).
(e, f) Two *lre-5* ovules showing abnormal interaction between pollen tube (pt) and intact synergid cells (sc).
In (a–f) Only the micropylar end of an ovule is shown. Abbreviations used are the same as in Figure 2. Scale bars, 10 μm.

(Figure 4a,b and Table 4). However, self-pollinated *lre*/*lre* pistils contained two types of developing seeds: about 90% contained an embryo and endosperm (Figure 4c,d, Table 4), and the remainder (about 10%) contained proliferating endosperm nuclei but no embryo (Figure 4e–h, Table 4). Even upon examining a Z-stack of 3-μm thick optical sections of seeds in whole-mount preparations, neither a growth-arrested zygote nor an embryo was found in aborted seeds (data not shown).

To check for embryos in aborted seeds, we used the embryo-expressed reporter gene *DD45:GFP* (Steffen *et al.*, 2007). We pollinated wild-type pollen onto pistils carrying *DD45:GFP* (Figure S4) and examined reporter expression 3 DAP. In nearly every wild-type seed, GFP expression was specifically observed in developing embryos (Figure 4i, $n = 110$). Among the fertilized seeds in *lre-5*/*lre-5* pistils, normal-looking seeds showed GFP expression specifically in embryos (Figure 4j, $n = 50$). In contrast, among the aborted seeds no GFP expression was detected in the micropylar end of the ovule, where an embryo is expected (Figure 4k, $n = 8$), demonstrating that no developing or growth-arrested embryo is present in aborted seeds.

The *DD45:GFP* marker is expressed in the egg cell in addition to the embryo (Figure S4a,b and Steffen *et al.*, 2007). Because undeveloped ovules within *lre-5*/*lre-5*,

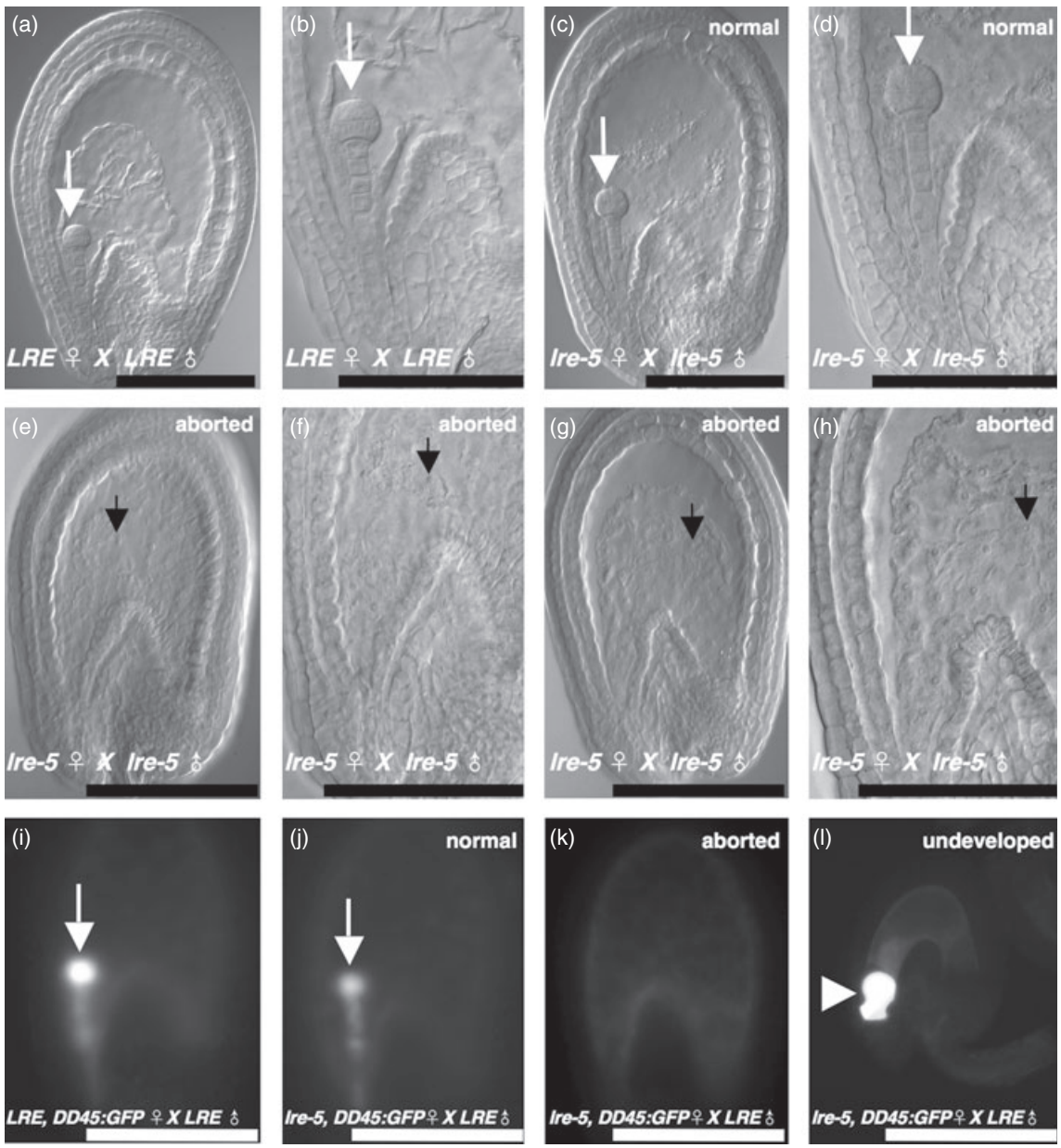

Figure 4. Analysis of aborted seeds in *lre-5/lre-5* pistils. (a–d) Normal embryo (white arrows) and endosperm development in wild-type (a, b) and a majority of *lre-5/lre-5* seeds (c, d).
(e–h) An embryo is absent in aborted *lre-5/lre-5* seeds but the seeds contain proliferating endosperm nuclei (black arrows). Two aborted seeds (e and g) are shown.
(a–h) Magnified view of the micropylar pole of seeds in (a, c, e and g) are shown in (b, d, f and h), respectively.
(j–m) Fluorescent images of *DD45:GFP* expression in normal and aborted seeds in wild-type (j) or *lre-5/lre-5* pistils (k–m) crossed to *DD45:GFP/DD45:GFP* pollen and observed 3 days after pollination (DAP). White arrows, embryos (j, k) and white arrowhead, egg cell (m).
Scale bars: (a, c, e, g, i–m), 100 μm; (b, d, f, h), 50 μm.

DD45:GFP/DD45:GFP pistils pollinated with wild-type pollen showed *DD45:GFP* expression in unfertilized egg cells (Figure 4l, $n = 150$), the absence of *DD45:GFP* expression in aborted seeds indicated that these seeds do not contain an unfertilized egg cell. Lack of *DD45:GFP* expression in the aborted seed is not due to defective egg cell expression in *lre-5* female gametophytes; there was near identical *DD45:GFP* expression in egg cells in both wild-type (96% of all ovules in stage 14 emasculated *LRE/LRE*, *DD45:GFP/DD45:GFP* pistils, $n = 278$) and *lre-5* female gametophytes (95.5% of all ovules in stage 14 emasculated *lre-5/lre-5*, *DD45:GFP/DD45:GFP* pistils, $n = 280$). Consistent with the absence of egg cell marker expression in aborted seeds, no unfertilized egg cells were observed in a Z-stack of 3-μm thick optical sections of aborted seeds in whole-mount preparations (data not shown).

Table 4 Aborted ovules in pollinated *lre* pistils contain proliferating endosperm nuclei but not an embryo

Genotype	Number of enlarged ovules with an embryo and endosperm nuclei proliferation[a] (%)	Number of enlarged ovules without an embryo but with endosperm nuclei proliferation[b] (%)	Number of pistils scored[c]
Self-pollinated pistils			
LRE/*LRE*	252 (100.0)	0 (0.0)	6
lre-4/*lre-4*	61 (88.4)	8 (11.6)	5
lre-5/*lre-5*	128 (91.8)	13 (9.2)	13
lre-6/*lre-6*	130 (89.7)	15 (10.3)	13
lre-7/*lre-7*	54 (90.0)	6 (10.0)	5
Manually self-pollinated pistils			
LRE/*LRE*	316 (100.0)	0 (0.0)	6
lre-5/*lre-5*	121 (91.3)	13 (9.7)	8
Emasculated pistils			
LRE/*LRE*	0 (0.0)	2 (0.6)[d]	6
lre-5/*lre-5*	0 (0.0)	3 (0.8)[d]	7

[a]Enlarged ovules as shown in Figure 4a–d.
[b]Enlarged ovules as shown in Figure 4e–h.
[c]Pistils were scored 3 days after pollination or 4 days after emasculation.
[d]Remaining ovules in emasculated *LRE*/*LRE* (314, 99.4%) and *lre-5*/*lre-5* (364, 99.2%) pistils contained neither an embryo nor proliferating endosperm nuclei.
Parent genotypes were confirmed by PCR and progeny segregation.

Unfertilized *lre-5* ovules do not initiate autonomous endosperm development

Proliferation of endosperm nuclei in the absence of an embryo in aborted seeds in *lre*/*lre* pistils is reminiscent of autonomous endosperm development (Ohad *et al.*, 1999; Berger *et al.*, 2006) in the pollen tube reception mutants, *syl* and *srn*/*fer* (Rotman *et al.*, 2008). To test if *lre* ovules can initiate endosperm development without fertilization, we emasculated *lre-5*/*lre-5* pistils and visually examined ovules several days after emasculation (DAE). Ovules that undergo autonomous endosperm development are distinctly larger than those that do not (Rotman *et al.*, 2008). In *lre-5*/*lre-5* pistils, similar to wild-type pistils, none of the ovules enlarged either 4 or 10 DAE (n = 10 pistils, data not shown). Similarly, in whole-mount preparations of chloral hydrate-cleared ovules from 4 DAE *lre-5*/*lre-5* pistils, proliferation of endosperm nuclei was not observed (Table 4). These results suggest that endosperm development observed in aborted seeds in *lre-5*/*lre-5* pistils is pollination-dependent.

Proliferation of endosperm nuclei in aborted seeds in *lre-5*/*lre-5* pistils initiates after central cell fertilization

To determine if proliferation of endosperm nuclei in aborted seeds in *lre-5*/*lre-5* pistils occurs after fertilization of the central cell, we examined an endosperm-specific marker (*KS117:GFP*; Sorensen *et al.*, 2001) which was transmitted through the pollen. The observed *KS117:GFP* expression pattern in endosperm during wild-type seed development (Figure 5e–h) was as reported in Sorensen *et al.* (2001). In *lre-5*/*lre-5* pistils pollinated with *KS117:GFP* pollen (observed 3 and 4 DAP), both aborted and normal seeds showed *KS117:GFP* expression (Figure 5i–l), indicating that endosperm development in aborted seeds initiated after central cell fertilization.

To directly determine the absence of embryos and the expression of endosperm markers in the same aborted seed (i.e. score all three phenotypes in the same seed), we first visually identified normal and aborted seeds (Figure 1a) in pistils pollinated with *KS117:GFP* pollen 3 DAP and then, in each seed, we scored endosperm-specific expression of the *KS117:GFP* marker followed by embryo development in whole-mount preparations. Normal seeds in wild-type (Figure S6a–c, n = 127) and *lre-5*/*lre-5* pistils (Figure S6d–f, n = 115) that showed wild-type *KS117:GFP* expression in the posterior chalazal endosperm also contained a globular stage embryo. In contrast, every aborted seed in *lre-5*/*lre-5* pistils pollinated with *KS117:GFP* pollen that showed *GFP* expression in proliferating endosperm nuclei lacked an embryo (Figure S6g–i, n = 22). In a second approach, we visually separated normal and aborted seeds 3 and 4 DAP, and monitored expression of a paternally transmitted embryo and endosperm marker (*GRP23:*GUS; Ding *et al.*, 2006). In normal-looking seeds in wild-type [Figure 5m (n = 56) and 5n (n = 34)] and *lre-5*/*lre-5* [Figure 5o (n = 27) and 5p (n = 24)] pistils crossed with *GRP23:*GUS pollen, both the embryo and the posterior chalazal endosperm showed GUS expression. However, in aborted seeds, GUS staining was observed only in the endosperm and embryos were not detected [Figure 5q,r (n = 4) and 5s,t (n = 6)]. Based on expression of paternally transmitted markers in the endosperm (Figures 5 and S6), we concluded that endosperm development in embryo-less, aborted seeds in *lre-5*/*lre-5* pistils initiated after central cell fertilization.

Early seed development is delayed in *lre-5*/*lre-5* ovules that induce pollen tube reception

In *lre-5*/*lre-5* pistils crossed with pollen containing reporter genes, embryo development in normal seeds was delayed (compare embryo sizes in Figures 4i,j, 5m–p). Additionally, in aborted seeds within *lre-5*/*lre-5* pistils crossed with pollen containing reporter genes, delay in endosperm development was observed (compare *KS117:GFP* expression in endosperm in Figures 5e,f,i,j and *GRP23:*GUS expression in endosperm in Figure 5m,n,q,r). This lag could originate from a delay in: (i) arrival of the pollen tube in the female gametophyte, (ii) completion of pollen tube reception, (iii) double fertilization, or (iv) initiation of zygote and endosperm development. *In vivo*, *LAT52:*GUS and *GRP23:*GUS pollen tubes arrived at the female gametophyte at

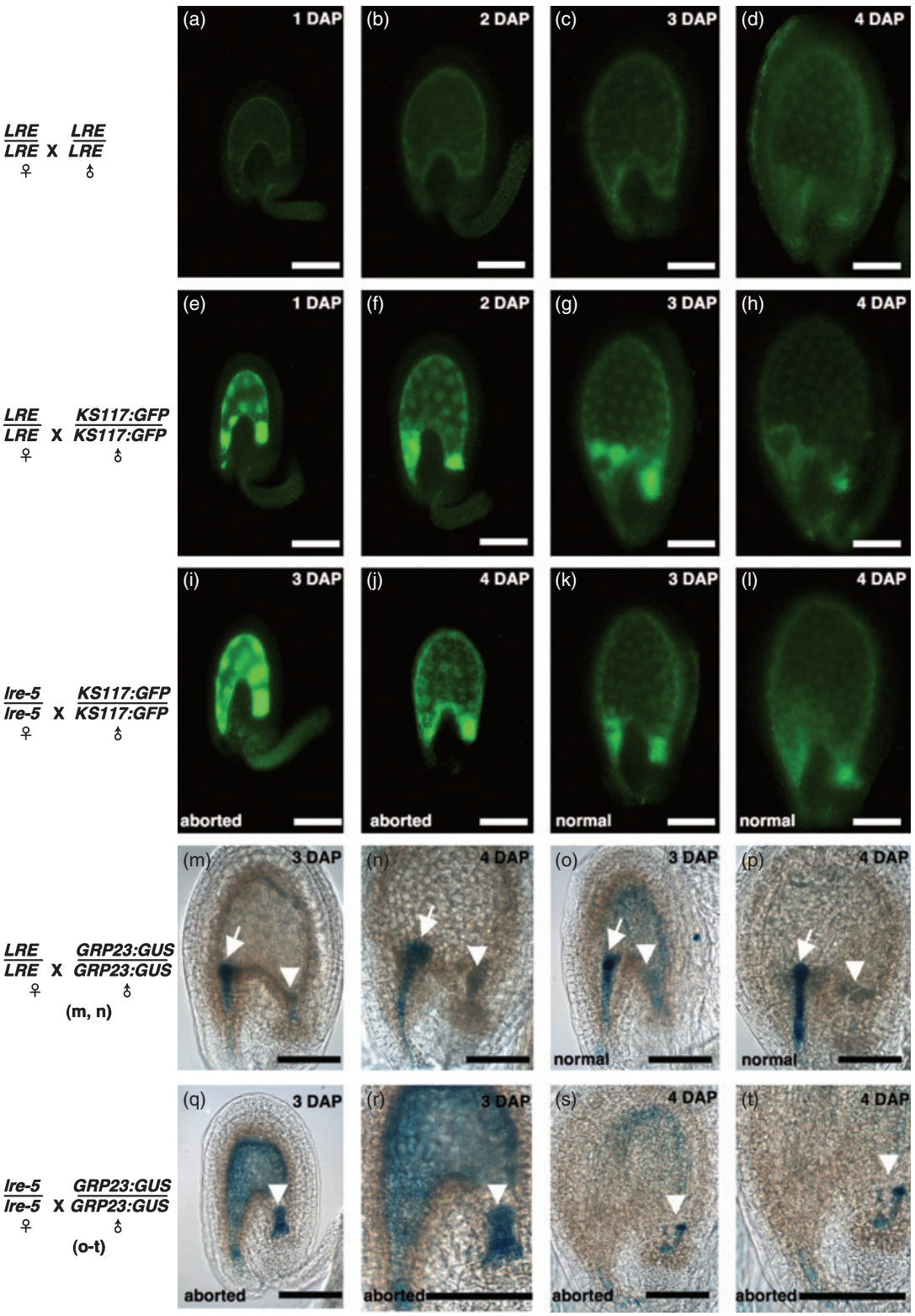

Figure 5. Analysis of central cell fertilization in aborted seeds in *lre-5*/*lre-5* pistils. (a–d) At indicated times after crossing, basal autofluorescence in wild-type seeds was monitored.
(e–h) Fluorescent images of *KS117:GFP* expression in endosperm after crossing *KS117:GFP*/*KS117:GFP* pollen onto wild-type (e–h) or *lre-5*/*lre-5* (i–l) pistils.
(m–t) Bright field images of *GRP23:*GUS expression during seed development in wild-type (m, n) or *lre-5*/*lre-5* (o–t) pistils. Arrows (m–p), GUS staining in embryos; arrowheads (m–t), GUS staining in the chalazal endosperm. Magnified views of the micropylar pole of aborted seeds in (q and s) are shown in (r and t), respectively. Scale bars: (a–l, m–q, s), 100 μm; (r, t), 50 μm.

comparable rates in wild-type and *lre-5*/*lre-5* pistils (Figure S5a–c). Additionally, *in vitro*, functional *lre-5* female gametophytes completed pollen tube reception in the same amount of time (146 ± 30.62 min, n = 10; Movie S2) as the wild type (142 ± 25.73 min, n = 10; Movie S1). Finally, by 16 hours after pollination (HAP), based on zygote and endosperm formation (Faure *et al.*, 2002), there are no differences in the completion of double fertilization between wild-type and *lre-5*/*lre-5* ovules that induce pollen tube reception (Figure 2b,d). Thus, the lag in embryo and endosperm development in *lre-5*/*lre-5* pistils was not likely to be caused by delays in events leading to double fertilization.

To investigate if early seed development is delayed in fertilized *lre-5*/*lre-5* ovules, we monitored *GRP23:*GUS expression in *lre-5*/*lre-5* ovules after fertilization. The *GRP23:*GUS marker is expressed during early seed development, starting from the zygote and endosperm nuclear proliferation stages (Ding *et al.*, 2006). We transmitted the *GRP23:*GUS marker only through pollen to avoid expression in unfertilized ovules (Ding *et al.*, 2006). In wild-type seeds, *GRP23:*GUS expression was detected in proliferating endosperm nuclei in a majority of the ovules 16 HAP (Figure 6a). Subsequently, *GRP23:*GUS expression was observed in the zygote and the chalazal endosperm (24 HAP, Figure 6c) and in the proembryo and the suspensor (30 HAP, Figure 6e). Ultimately, by 36 and 48 HAP, all seeds showed *GRP23:*GUS expression in the embryo proper, the suspensor and the chalazal endosperm (Figure 6g,h).

We reasoned that in *lre-5*/*lre-5* pistils the ~27% of ovules that induce pollen tube reception (Table 3) would be useful in monitoring post-fertilization *GRP23:*GUS expression. By 16 HAP, 27.1% of all *lre-5*/*lre-5* ovules induced pollen tube reception (Figure 6i), while the remainder (72.9%) showed evidence of abnormal pollen tube reception (Figure 6j). However, none of the *lre-5*/*lre-5* ovules showed *GRP23:*GUS expression in proliferating endosperm nuclei like that in the wild type (Figure 6a). Even by 24 HAP, 25.4% of all ovules continued to show GUS staining that is only indicative of completion of pollen tube reception and lacked expression elsewhere in the embryo sac (Figure 6k), indicating a delay in the initiation of early seed development.

*GRP23:*GUS staining was not observed in the endosperm of fertilized *lre-5*/*lre-5* ovules until 30 HAP (Figure 6n). Additionally, only a minority of fertilized *lre-5*/*lre-5* ovules showed *GRP23:*GUS expression in the zygote by 30 HAP (Figure 6m), which is strikingly different from the zygotic *GRP23:*GUS expression in a majority of wild-type embryo sacs observed by 24 HAP (Figure 6c). These results point to a 6–14 h delay in *GRP23:*GUS expression in the zygote and endosperm of fertilized *lre-5*/*lre-5* ovules compared with the wild type. The *GRP23:*GUS staining pattern in fertilized *lre-5*/*lre-5* ovules at 36 HAP (Figure 6o) and 48 HAP (Figure 6q) was similar to that observed in the wild type at 30 HAP (Figure 6e) and 36 HAP (Figure 6g), respectively. In addition, a smaller proportion of fertilized *lre-5*/*lre-5* ovules showed *GRP23:*GUS expression in the entirety of the endosperm without any discernible staining in the zygote even at 36 and 48 HAP (Figure 6p,r). The delay in *GRP23:*GUS expression in zygote and endosperm of *lre-5*/*lre-5* ovules that induce pollen tube reception indicates that LRE plays a role in the initiation of early seed development.

In flowers, *LRE* is specifically expressed during female gametophyte development

We performed RT-PCR analysis to examine *LRE* expression during reproduction. *LRE* is not expressed in pollen, pollen tubes or in the stigma and style portions of pistils (Figure 7a), consistent with its female gametophyte-specific function (Table 1). Therefore, we next monitored *LRE* expression during previously described stages of ovule development (Smyth *et al.*, 1990). *LRE* transcription was not detected in ovaries collected from stage 11 or 12a floral buds (Figure 7a and Experimental Procedures). In ovaries from stage 12b buds (stage 5 female gametophyte; Yadegari and Drews, 2004) *LRE* is expressed at low levels (only after 50 cycles, data not shown), indicating that *LRE* expression initiates before the female gametophyte reaches stage 7. In ovaries from stage 12c and mature female gametophyte-containing stage 14 flowers, *LRE* expression was readily detectable (Figure 7a). These results coupled with *LRE* expression in unfertilized, mature ovules (Figures 7c and S2) indicate that *LRE* expression is independent of pollination.

To correlate *LRE* expression with the development of synergid cells, which regulate pollen tube reception, we examined its relationship to *MYB98*, a synergid-specific transcription factor (Kasahara *et al.*, 2005). As reported (Kasahara *et al.*, 2005), *MYB98* expression was detected in floral development stages 12b, 12c and 14, but not in 11 or 12a (Figure 7a), similar to *LRE*. Because many synergid-expressed genes are regulated by MYB98 (Punwani *et al.*, 2007), we used quantitative RT-PCR (qRT-PCR) to determine if expression of *LRE* is affected by loss of MYB98 (Figure S2a,b). *LRE* and *AMC* are expressed at normal levels in *myb98*/*myb98* ovules (Figure S2a,b). However, *FER* expression is half its wild-type level in *myb98*/*myb98* ovules (Figure S2b), suggesting that *FER* may be partially regulated by MYB98.

LRE expression rapidly decreases following pollen tube reception

Loss of LRE function results in a small percentage of embryo-less, aborted seeds. Although seed abortion occurs after fertilization, it results from loss of LRE function in the female gametophyte (Table 1). Additionally, early seed development is delayed in *lre-5*/*lre-5* seeds (Figure 6). We therefore monitored the expression of *LRE* in ovules before, during and after fertilization. In wild-type pistils, *LAT52:*GUS-tagged pollen tubes reached the transmitting tract 3

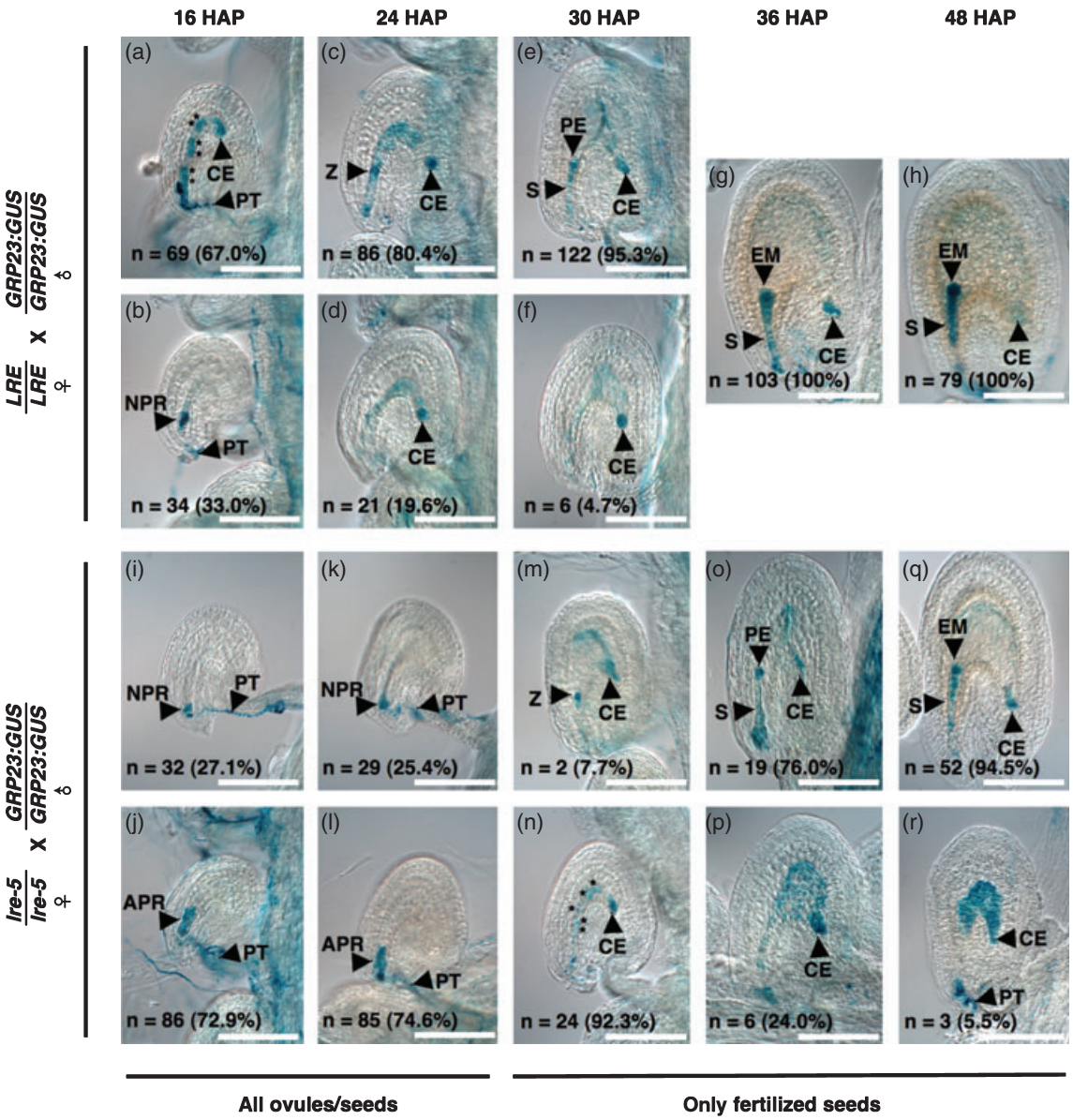

Figure 6. Analysis of early seed development in *lre-5*/*lre-5* ovules that induce pollen tube reception.
(a–r) Bright field images of *GRP23*:GUS expression in ovules in wild-type (a–h) or *lre-5*/*lre-5* (i–r) pistils crossed with *GRP23*:GUS pollen. Only 15 rows of ovules, from the stigma end, were analyzed.
(a–d, i–l) Number (*n*) and %, the number and percentage of seeds, respectively showing GUS staining in all wild-type seeds (a–d) or *lre-5*/*lre-5* (i–l) ovules.
(e–h, m–r) Number (*n*) and %, the number and proportion, respectively of GUS staining only in fertilized wild-type (a–h) or *lre-5*/*lre-5* (m–r) ovules.
(a, n) Black asterisks, proliferating endosperm nuclei.
Abbreviations: APR, abnormal pollen tube reception; CE, chalazal endosperm; EM, embryo proper; NPR, normal pollen tube reception; PE, proembryo; PT, pollen tube; S, suspensor; Z, zygote. Scale bars: (a–r), 100 μm.

HAP (Figure 7b) and pollen tube reception was completed in nearly every ovule in a pistil by 16 HAP (Figures 7b and S5). Zygote formation was complete in a majority of wild-type embryo sacs by 24 HAP (Figures 2g and 6c).

Consistent with its function during early seed development, *LRE* is expressed in pollinated ovules (16 and 24 HAP; Figure 7c). However, *LRE* expression in ovules 16 and 24 HAP was lower compared with ovules in unfertilized or pistils 3 HAP (Figure 7c). Consistent with these results, qRT-PCR experiments showed a ~22-fold decrease in *LRE* expression in ovules 16 HAP compared with unfertilized ovules (Figure 7d). The dramatic decrease in *LRE* expression in pollinated ovules does not appear to be part of a global down-regulation of pollen tube reception genes as expression of *FER* and *AMC* did not decrease to the same extent as that of *LRE* (Figure 7c,d). *LRE* transcripts could not be

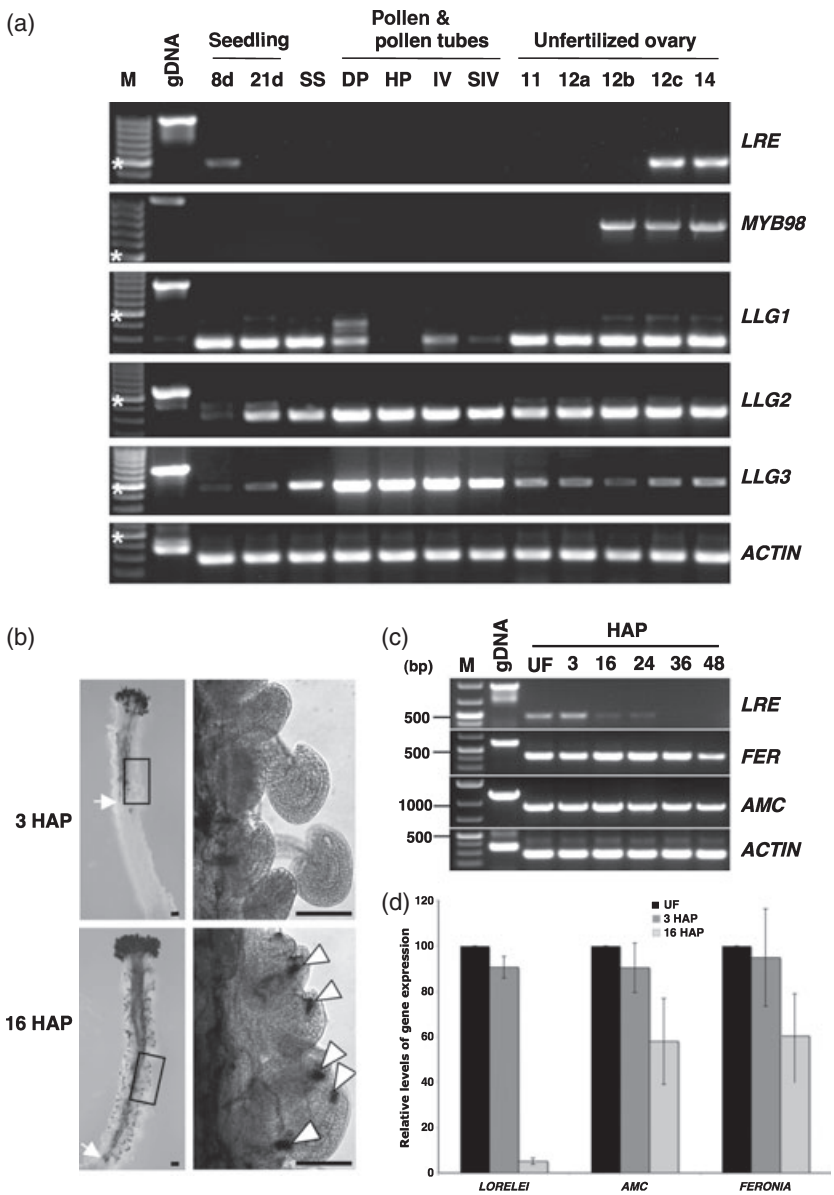

Figure 7. *LORELEI* expression during plant development.
(a) The RT-PCR analysis of gene expression in indicated tissues. Eight-day-old (8 day) and 21-day-old (21 day) seedlings, stigma and style portion of pistils (SS), dry pollen (DP), hydrated pollen (HP), *in vitro* (IV)- and semi-*in vitro* (SIV)-grown pollen tubes, and developmental stages of unfertilized ovaries. M, DNA size standard (white asterisk = 500 bp).
(b) Bright field images of *LAT52:*GUS pollen tube growth in wild-type pistils. Magnified portions within black rectangles in the left panels are shown in the right panels. White arrows, pollen tube front in the transmitting tract; white arrowheads, pollen tube reception within ovules. Scale bars, 100 μm.
(c) The RT-PCR analysis of the expression of indicated genes in wild-type unfertilized (UF) or pollinated ovules harvested at the indicated hours after pollination (HAP).
(d) Quantitative RT-PCR analysis of the expression of the indicated genes in pollinated ovules harvested at the indicated HAP. For each gene, expression in different samples was calculated relative to a normalized expression value of 100 in wild-type UF ovules.

detected by 36 and 48 HAP (Figure 7c), indicating that *LRE* is probably not expressed during seed development starting from the embryo stage (Figure 6g).

Loss of LLG1 function does not enhance *lre* female gametophyte defects

Three genes in the Arabidopsis genome are predicted to encode proteins that are very similar to LRE (Figure S3a; Capron *et al.*, 2008); we refer to these as LORELEI-like-GPI-anchored proteins (LLG1-At5g56170; LLG2-At2g20700 and LLG3-At4g28280). While LLG1 is most closely related to LRE, LLG2 and LLG3 are more closely related to each other than either to LRE or LLG1 (Figure S3b).

Unlike *LRE*, the *LLG1–LLG3* genes are expressed in pollen, pollen tubes, sporophytic pistil tissues and in the early stages of female gametophyte development (stages 11 and 12a; Figure 7a). However, during ovule development, expression of *LLG1*, *LLG2* and *LLG3* genes overlaps with *LRE* (stages 12b, 12c and 14; Figure 7a). The overlap between expression of *LLG* and *LRE* in ovules and the incomplete penetrance of *lre* mutant phenotypes prompted us to investigate whether LLGs are redundant to the function of LRE in the female gametophyte.

First, no compensatory increase in expression was observed in any of the three *LLG* genes in any *lre* allele (Figure S2). Next, we isolated null mutant alleles in *LLG1* (*llg1-1* and *llg1-2*; Figure 8a,b) but did not observe reduced seed set or aborted seed phenotypes in *llg1-1* and *llg1-2* single mutants (Figure 8c) indicating that loss of *LLG1* is tolerated during reproduction. Moreover, no increase in the

(a)

(b)

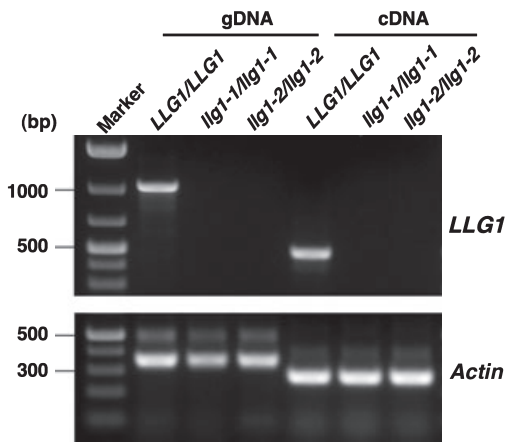

Figure 8. Functional redundancy between LLG1 and LRE during reproduction.
(a) The T-DNA insertions in *LLG1*. Genomic DNA/T-DNA junctions in *llg1-1* and *llg1-2* and primers (Table S7) used for characterizing these insertions and *LLG1* expression are shown.
(b) The RT-PCR analysis (50 cycles) of *LLG1* expression in 21-day-old wild-type, *llg1-1/llg1-1* and *llg1-2/llg1-2* seedlings.
(c) Seed-set in single and double mutants involving *llg1-1*, *llg1-2*, *lre-5* and *lre-7* mutations.

(c)

Plant genotype	Undeveloped ovules	(%)	Aborted seeds	(%)	Normal seeds	(%)
LRE/LRE, LLG1/LLG1	5	(0.8)	0	(0.0)	623	(99.2)
LRE/LRE, LLG1/llg1-1	58	(2.0)	0	(0.0)	2839	(98.0)
LRE/LRE, llg1-1/llg1-1	30	(2.6)	2	(0.2)	1097	(97.2)
LRE/LRE, LLG1/llg1-2	4	(0.4)	0	(0.0)	988	(99.6)
LRE/LRE, llg1-2/llg1-2	5	(0.9)	0	(0.0)	567	(99.1)
LRE/lre-5, LLG1/LLG1[a]	634	(34.7)	27	(1.5)	1165	(63.8)
LRE/lre-5, LLG1/llg1-1	369	(38.0)	19	(1.9)	584	(60.1)
LRE/lre-7, LLG1/LLG1[b]	409	(39.3)	11	(1.0)	621	(59.7)
LRE/lre-7, LLG1/llg1-2	317	(34.9)	7	(0.8)	584	(64.3)
lre-5/lre-5, LLG1/LLG1[a]	683	(70.6)	27	(2.8)	258	(26.6)
lre-5/lre-5, llg1-1/llg1-1	426	(73.6)	20	(3.4)	133	(23.0)

[a]Data from Table 1; [b]Data from Table 2.

frequency of undeveloped and aborted ovules was observed in the siliques of double mutants of *lre* and *llg1* compared with single mutants (Figure 8c), indicating that *LLG1* function is not redundant with the functions of *LRE* in the female gametophyte.

DISCUSSION

Pollen tube repulsion and pollen tube reception

Pollen tube repulsion in wild-type ovules (Movie S1) could be due to cessation of ovule attraction after pollen tube reception (passive model) or initiation of repulsion (active model). Our observations with wild-type ovules in our *in vitro* assay support the active model. First, repulsion of the late-arriving tube initiated rapidly (within 10 min of the first tube entering the ovule; Movie S1), a time-frame too short for cessation of attraction signal(s) to have a repulsive effect. Second, the unsuccessful tube actively approached and then abruptly altered its path near the micropyle of the ovule that had been penetrated by another pollen tube (compare time points 80 and 100 min in Movie S1).

This short-range repulsion is defective in *lre-5*/*lre-5* ovules (Figure 1, Movie S4). Linkage between pollen tube reception and repulsion in *lre*/*lre* ovules demonstrated that pollen tube reception is pivotal in preventing supernumerary pollen tubes from entering an ovule. Characterization of pollen tube repulsion in mutants defective in synergid degeneration (*gfa2*; Christensen *et al.*, 2002) or pollen tube discharge (*aca9*; Schiott *et al.*, 2004) will show whether repulsion is initiated following pollen tube reception or after completion of downstream events such as synergid degeneration or pollen tube discharge.

Synergid cell death and pollen tube reception

Using two different approaches, we found that synergids did not undergo degeneration in most of the *lre-5* female gametophytes. Notably, the proportion of *lre-5* female gametophytes containing degenerated and non-degenerated synergid cells (26.4% and 73.6%, respectively; Figure 2g) is similar to: (i) the ratio of normal and defective pollen tube reception in self-pollinated *lre-5*/*lre-5* pistils (26.6 and 71.2% respectively; Table 3), and (ii) the ratio of

normal seeds and undeveloped ovules in self-pollinated *lre-5*/*lre-5* pistils (26.6% and 70.6%, respectively; Table 1) suggesting that synergid cell degeneration does not occur in *lre-5* female gametophytes that remain undeveloped due to pollen tube reception defects. Synergid degeneration was also not observed in pollen tube reception mutants *amc* (Boisson-Dernier *et al.*, 2008) and *srn* (Rotman *et al.*, 2003). These observations favor a model where pollen tube reception occurs prior to synergid degeneration in Arabidopsis.

Synergid degeneration after pollen tube reception could be triggered by several mechanisms. First, synergid degeneration could be initiated mechanically by pollen tube penetration and growth arrest in the synergid cell (Sandaklie-Nikolova *et al.*, 2007). However, this is unlikely because in *lre-5* (Figure 3e,f), *amc* (Boisson-Dernier *et al.*, 2008) and *srn* (Rotman *et al.*, 2003) mutants synergids did not degenerate despite interacting extensively with pollen tubes. Alternative mechanisms to initiate synergid degeneration include a contact-mediated signal between the growth-arrested pollen tube and a synergid cell or events downstream of pollen tube reception, for example pollen tube discharge (Higashiyama, 2002).

Down-regulation of *LRE* expression after pollen tube reception

Levels of *FER* and *AMC* mRNA in ovules decrease about two fold after pollen tube reception (Figure 7d). As reported for *MYB98* (Kasahara *et al.*, 2005), the remaining *FER* and *AMC* mRNA in ovules that have interacted with pollen tubes is probably from expression in the persistent synergid, which does not undergo degeneration. By contrast, *LRE* expression decreases about 22-fold after pollen tube reception (Figure 7d). The *LRE* expression in an ovule is primarily due to its expression in the two synergid cells (Capron *et al.*, 2008). Therefore, the rapid decline in expression we observed can be accounted for only if loss of *LRE* expression occurs in both synergids. These observations raise the possibility that gene expression in the persistent synergid might be actively regulated following pollen tube reception.

The functional significance of a rapid decrease in *LRE* expression in both synergids after pollen tube reception could be that loss of *LRE* expression might serve as a signal to rapidly terminate certain synergid functions following pollen tube reception. Disabling synergids from releasing chemoattractants (Okuda *et al.*, 2009) so that pollinated ovules do not continue to attract pollen tubes into ovules (Higashiyama *et al.*, 1998; Palanivelu and Preuss, 2006) could be one such function. Both synergids are equally capable of attracting a pollen tube into an ovule (Higashiyama *et al.*, 2001) and consequently, after pollen tube reception, cessation of pollen tube attraction by both synergids may be essential.

Aborted seeds in *lre* pistils

We identified four characteristics of aborted seeds in *lre* pistils: (i) aborted seeds result from loss of *LRE* function in the female gametophyte, (ii) aborted seeds lack an embryo, but have proliferating endosperm nuclei, (iii) proliferation of endosperm nuclei in aborted seeds is initiated after central cell fertilization, and (iv) aborted seeds do not harbor an unfertilized egg cell by 3 DAP. At least three scenarios could be envisioned for events in *lre* female gametophytes that result in aborted seeds with the above-described attributes. First, invasive pollen tube growth into the central cell (Figure 1h,i,k,l, Movie S3) could lead to accidental or inappropriate pollen tube reception by the *lre-5* central cell, resulting in fertilization events in the central cell only that typically result in seed abortion (Chen *et al.*, 2008; Frank and Johnson, 2009). However, in time-lapse observations and *LAT52:*GUS pollen tube–*lre-5*/*lre-5* ovule interactions, we did not observe any instance in which growth arrest and discharge of the pollen tube occurred in the central cell.

The second and third scenarios are based on the observation that *LRE* is also expressed in the egg cell (Capron *et al.*, 2008). In these scenarios, pollen tube reception occurs in the *lre-5* synergid cell and one of the two released sperm cells fuses normally with the central cell. However, the second sperm cell either: (i) fuses with the *lre-5* egg and completes fertilization but cannot develop due to lack of *LRE* or (ii) remains unfertilized as loss of LRE function in the *lre-5* egg cell has rendered it incapable of completing fertilization. In the former case, a growth-arrested zygote/proembryo would have been detected in whole-mount preparations of aborted seeds as reported for the *capulet1* mutant (Grini *et al.*, 2002). However, we did not observe any growth-arrested zygote/embryo in any of the more than 100 aborted seeds in *lre-5*/*lre-5* pistils that were analyzed (Figures 4 and 5). Based on these observations, we propose that due to loss of LRE function in some *lre* female gametophytes, only central cell fertilization is completed and the unfertilized egg cell degenerates by 3 DAP, and these seeds undergo abortion. Future experiments involving time-lapse imaging of double fertilization (Ingouff *et al.*, 2007) and confirmation of unfertilized egg cells using egg cell-specific markers in aborted seeds in *lre*/*lre* pistils within 24 HAP will be essential for testing these predictions. Prior to these experiments, however, it is essential to develop vital staining or other assays to identify aborted seeds in *lre*/*lre* pistils earlier (1 or 2 DAP) as current methods cannot distinguish aborted seeds from normal seeds in *lre*/*lre* pistils until 3 DAP.

Early seed development delay in *lre-5*/*lre-5* ovules that induce pollen tube reception

We demonstrated a significant delay in initiation of early seed development in *lre-5*/*lre-5* ovules that complete pollen

tube reception (Figure 6). Incomplete penetrance of the pollen tube reception phenotype in *lre-5*/*lre-5* ovules results in about 27% of mutant ovules inducing pollen tube reception (Figure 6i). However, lack of *GRP23:*GUS marker expression by 16 and 24 HAP in all the *lre-5*/*lre-5* ovules suggests that seed development is delayed even in those ovules that induced pollen tube reception. These results, coupled with the finding that *lre-5*/*lre-5* ovules that induce pollen tube reception completed double fertilization (Figure 2d,g), indicate that delays to seed development originate after *lre-5*/*lre-5* ovules complete double fertilization.

Some paternally transmitted genes are not expressed until several hours after fertilization, primarily due to the silencing of paternal alleles (Vielle-Calzada *et al.*, 2000). One interpretation of the results in Figure 6 is that activation of paternally transmitted *GRP23:*GUS expression alone is delayed in fertilized *lre-5*/*lre-5* ovules. However, our observations do not support this model; the smaller size of embryos (Figure 6q) and the lag in endosperm development in fertilized *lre-5*/*lre-5* ovules expressing *GRP23:*GUS (Figure 6p,r) compared with the wild type demonstrate that initiation of overall seed development is delayed in fertilized *lre-5*/*lre-5* ovules. Characterization of seed development phenotypes in reciprocal crosses between *LRE*/*lre-5* and wild-type plants and expression analysis of maternal and paternal alleles of *LRE* in early seed development are essential to evaluate if LRE exerts a gametophytic maternal effect on seed development similar to PROLIFERA (Springer *et al.*, 2000) and DNA LIGASE I (Andreuzza *et al.*, 2010).

EXPERIMENTAL PROCEDURES

Plant growth, seeds and mutants

Plant growth was as described (Qin *et al.*, 2009). For kanamycin and Basta sensitivity assays, growth media contained 50 μg ml^{-1} kanamycin sulfate or 10 μg ml^{-1} glufosinate ammonium (Basta, Crescent Chemical Company, http://www.creschem.com/), respectively. Seventy pools of 100 SALK T-DNA mutant lines (T_4 seeds, CS75001–CS75070) and individual SALK and SAIL T-DNA insertions in *LRE* and *LLG1* and *KS117:GFP* seeds (CS9341) were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/).

The T-DNA insertion site in *lre-5* was mapped (Lukowitz *et al.*, 2000) and identified (see Data S2) by modified TAIL-PCR (Liu *et al.*, 1995). The T-DNA insertion in *lre-5* has a RB and LB deletion; consequently, *lre-5* was not indexed in the SALK T-DNA database (see Data S2). Single locus T-DNA insertion SAIL, (*lre-4*, *lre-6*, *lre-7*; Table S1) and (*llg1-1*; SAIL_47_G04), were isolated according to Johnson *et al.* (2004). Briefly, a Basta-resistant heterozygous plant containing a T-DNA insertion in the target gene was identified by PCR. Next, in progeny of the selfed heterozygote, linkage among Basta resistance, pollen tetrad GUS expression and mutant phenotypes was confirmed (Table S5 and data not shown for *llg1-1*). Segregation of the *LAT52:*GUS transgene in pollen tetrads and seed-set phenotypes were then used to assign genotypes and monitor T-DNA transmission (Table S6 and data not shown for *llg1-1*). For *llg1-2* (SALK_086036), a heterozygous plant and the homozygotes in its progeny were identified by PCR using the primers listed in Table S7.

Thick plastic sections and light microscopy

Stage 12c flowers (Smyth *et al.*, 1990) were emasculated. Twenty-four hours later pistils were either fixed directly (unfertilized ovules) or at 16–24 HAP (pollinated ovules) sectioned (1 μm thickness) as described (Sandaklie-Nikolova *et al.*, 2007) using a Sorvall MT-7000 microtome, stained with 1% toluidine blue O and observed under bright-field optics with a Leica TCS SP5 confocal microscope (http://www.leica.com/).

CLSM analysis of female gametophytes and double fertilization

Stage 12c buds were emasculated. Twenty-four hours later pistils were either observed directly (unfertilized ovules) or hand-pollinated and, 16 HAP, prepared for CLSM to score unfertilized female gametophytes (Christensen *et al.*, 1997) or double fertilization (Faure *et al.*, 2002). For each genotype, ovules in six pistils were observed using a Leica TCS SP5 confocal microscope.

In vivo pollen tube guidance

*LAT52:*GUS pollen was crossed to at least three emasculated stage 14 *lre* or wild-type pistils, harvested at the indicated HAP, stained for GUS activity (Johnson *et al.*, 2004) and imaged using a Zeiss Axiovert 200 differential interference contrast (DIC) microscope (Carl Zeiss, http://www.zeiss.com/).

Microscopy of embryos and endosperm

For whole-mount preparations (Figures 4 and S6) siliques were slit open, fixed, cleared (Yadegari *et al.*, 1994) and observed using Zeiss Axiophot DIC microscope. In processed *lre-5*/*lre-5* pistils, fertilized seeds were distinguished from undeveloped ovules by size.

For *KS117:GFP* and *DD45:GFP* expression, ovules mounted in distilled water were observed using a Zeiss Axiophot epifluorescence microscope. For sequential scoring (Figure S6), after *KS117:GFP* expression scoring, embryo development was scored in a DIC microscope by replacing distilled water with clearing solution (Yadegari *et al.*, 1994) and incubating for 30 min at 23°C. For *GRP23:*GUS expression, pistils were stained for GUS activity (Johnson *et al.*, 2004) for 4 days, incubated in acetic acid:ethanol (1:1) for 16 h at 23°C and cleared for 16 h at 23°C either in chloral hydrate:glycerol:water (4:1:2) (16 and 24 HAP samples) or chloral hydrate:glycerol:water (8:1:2) (30, 36, 48, 72 and 96 HAP samples). Samples mounted in 50% glycerol were observed with a Zeiss Axiophot DIC microscope.

RT-PCR and qRT-PCR

Seedlings, stigma and style portions of pistils and pollen samples were collected for RNA isolation (Qin *et al.*, 2009). For *LRE* expression during female gametophyte development, ovaries without stigma and style tissue from staged flowers (Smyth *et al.*, 1990) were harvested for RNA isolation. For post-pollination gene expression experiments, emasculated stage 14 wild-type or *lre-5*/*lre-5* flowers were manually self-fertilized. At the indicated HAP, about 800–900 ovules were excised from 15 pistils for total RNA isolation for each replicate. Unless indicated, RT-PCR (36 cycles) was performed as described (Qin *et al.*, 2009). A gene was considered not to be expressed if there is no amplification in PCR reactions after 50 cycles.

Quantitative RT-PCR (45 cycles) was performed as described (Qin *et al.*, 2009). *ACTIN2* [threshold cycle (C_T) value of 18–19] was used

to normalize mRNA levels in each experiment. Since *LRE* is not expressed in *lre-5*/*lre-5* ovules, a C_T value of 45 was used to calculate gene expression changes. For each gene, four reactions were carried out, including two technical replicates and two biological replicates using the primers listed in Table S7.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Identification of *lre-5* in a mutant screen.

Figure S2. RT-PCR analysis of gene expression in *lre* and *myb98* ovules.

Figure S3. Phylogenetic analysis of LORELEI and related proteins.

Figure S4. Analysis of cell specification and differentiation of female gametophytic cells in the *lre-5* female gametophyte.

Figure S5. Pollen tube arrival in wild-type and *lre-5* gametophytes.

Figure S6. Sequential scoring of the expression of *KS117:GFP* endosperm marker and seed development in fertilized wild-type and *lre-5*/*lre-5* ovules.

Table S1. T-DNA alleles in the *LORELEI* gene, but not those in flanking genomic sequences, segregate reduced seed-setting plants.

Table S2. The *lre-5* mutation is transmitted at a reduced frequency through the female gametophyte.

Table S3. The *lre-5* mutation is transmitted at a reduced frequency through female gametophytes and kanamycin resistance is linked to the reduced fertility phenotype in *lre-5* mutants.

Table S4. The *lre-5* mutation does not affect cell-specification of female gametophytic cells.

Table S5. Basta resistance, pollen tetrad GUS expression and reduced seed-set phenotypes are linked in *lre-4*, *lre-6* and *lre-7* mutants.

Table S6. Distorted segregation of pollen tetrad GUS expression and reduced seed-set phenotypes in progeny of self-fertilized *lre-4*, *lre-6* and *lre-7* mutants.

Table S7. Primers used in this study.

Movie S1. Pollen tube interaction with a wild-type ovule in an *in vitro* guidance assay.

Movie S2. Pollen tube interaction with a *lre-5*/*lre-5* ovule that induces normal pollen tube reception in an *in vitro* guidance assay.

Movie S3. Pollen tube reception defect in a *lre-5*/*lre-5* ovule is recapitulated in an *in vitro* guidance assay.

Movie S4. Multiple pollen tubes enter a single *lre-5*/*lre-5* ovule in an *in vitro* guidance assay.

Data S1. Results.

Data S2. Methods.

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