

# DNA LIGASE I exerts a maternal effect on seed development in *Arabidopsis thaliana*

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## SUMMARY

Maternal effects are defined by mutations that affect the next generation when they are maternally inherited. To date, most in-depth studies of maternal effects in plants have attributed their origin to genomic imprinting that restricts expression to the maternal allele. The DNA glycosylase *DEMETER* (*DME*) removes methylated cytosine residues, causing transcriptional activation of the maternal allele of imprinted genes. In this study, we show that loss-of-function of the major DNA LIGASE I (*AtLIG1*) in *Arabidopsis thaliana* causes maternal effects in the endosperm, which is the seed tissue that nurtures embryo development. *AtLIG1* expression is not imprinted and has a limited impact on imprinted gene expression. Genetic interaction analyses further indicate that *AtLIG1* acts downstream of *DME*. The removal of methylated cytosine residues by *DME* involves the creation of DNA single-strand breaks and our results suggest that *AtLIG1* repairs these breaks.

**KEY WORDS:** Sexual reproduction, *Arabidopsis*, Seed, Maternal effect, Endosperm

## INTRODUCTION

Maternal effects have been genetically defined by mutations that affect the next generation when inherited from the mother. Maternal effect genes play an essential role in early development in many animal species (Riechmann and Ephrussi, 2001; Sardet et al., 2004). In plants, the maternal contribution to early developmental phases is still unclear. The flowering plant life cycle alternates between the vegetative phase of the diploid sporophyte and the reproductive phase of the haploid gametophyte. The sporophyte is represented by the vegetative phase of plant life and produces flowers in which specialised cells undergo meiosis. Meiosis in turn initiates the gametophytic phase. The haploid products of meiosis develop as gametophytes, which produce the gametes. The female gametophyte (embryo sac) produces the two female gametes: the egg cell and the central cell (Drews and Yadegari, 2002). The male gametophyte (pollen) produces two sperms cells (Borg et al., 2009). One sperm cell fuses with the egg cell to produce the embryo, whereas the other sperm cell activates the central cell to develop as the endosperm, which nurtures the developing embryo (Berger et al., 2008). The developing embryo and the endosperm inherit maternal cytoplasm and are supplied with nutrients from the mother plant. Hence, in flowering plants, several distinct maternal contributions can arise from the sporophytic diploid tissues as well as from the haploid female gametophyte (Chaudhury and Berger, 2001).

The maternal sporophyte affects the provision of nutrients to the endosperm and controls seed size via the integuments (Garcia et al., 2005; Ingouff et al., 2006; Schruff et al., 2006). Deregulation of such transfer activities causes maternal sporophytic effects on seed development (Cheng, 1996; Gutierrez-Marcos et al., 2004; Hueros et al., 1999; Miller and Chourey, 1992).

Maternal gametophytic controls originate from the activity of genes expressed in the gametophyte that are required for the development of the embryo or the endosperm. A limited number of mutations in genes associated with a gametophytic maternal effect have been studied in plants (Chaudhury et al., 1997; Evans, 2007; Grini et al., 2002; Grossniklaus et al., 1998; Guitton et al., 2004; Ngo et al., 2007; Pagnussat et al., 2005; Springer et al., 2000; Springer et al., 1995). In *Arabidopsis*, a group of six gametophytic maternal effect mutations comprise the *FERTILIZATION INDEPENDENT SEED* (*FIS*) class. Maternally inherited loss-of-function in *FIS* genes causes a complex pleiotropic phenotype in the endosperm (Guitton et al., 2004; Ingouff et al., 2005; Kiyosue et al., 1999). Four *FIS* genes encode Polycomb group (PcG) proteins (Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999; Guitton et al., 2004; Kohler et al., 2003). The maternal effects associated with *FIS* genes are caused by parental genomic imprinting. *MEDEA* (*MEA*) and *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*) expression in the endosperm is contributed by their maternal allele (Jullien et al., 2006; Kinoshita et al., 1999). As a consequence, a paternal wild-type *MEA* or *FIS2* allele is unable to rescue the maternal inheritance of a null mutant allele that causes a maternal effect on endosperm development. Activation of the maternal alleles of *FIS2* and *MEA* depends on the function of another *FIS* gene, *DEMETER* (*DME*) (Choi et al., 2002; Gehring et al., 2006; Jullien et al., 2006). The expression of two other *FIS* genes, *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) and *MULTICOPY SUPPRESSOR OF IRA 1* (*MSI1*), in the central cell and during the initiation of endosperm development is sufficient to rescue the maternal effect associated with loss-of-function *fie* (Kinoshita et al., 2001) and *msi1* (Leroy et al., 2007) alleles. It is thus possible that the maternal effects associated with

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*fie* and *msi1* might not originate from imprinting. However, in transient periods only the paternal alleles of *MSI1* (Leroy et al., 2007) and *FIE* (Yadegari et al., 2000) are expressed and the maize ortholog *FIE1* is imprinted (Gutierrez-Marcos et al., 2006; Hermon et al., 2007). The origins of the maternal effects associated with other mutations remain unclear (Chaudhury et al., 1997; Grini et al., 2002; Ngo et al., 2007; Pagnussat et al., 2005; Springer et al., 2000; Springer et al., 1995).

We have isolated four mutant lines that display a maternal effect on seed development with a phenotype distinct from that of *fis*. The mutant lines were shown to be allelic, and positional cloning identified mutations affecting the *Arabidopsis* DNA LIGASE I (*AtLIG1*) gene, which is essential for DNA replication and repair (Taylor et al., 1998). Detailed expression analyses show that *AtLIG1* is not imprinted, as it is expressed from both parental alleles in the embryo and endosperm. Additional experiments suggest that loss-of-function of *AtLIG1* in the central cell acts in the DME pathway and directly impacts on endosperm development.

## MATERIALS AND METHODS

### Plant material and growth conditions

Seeds of all wild-type ecotypes and from the Salk line SALK\_013442 and the Sail line SAIL\_0716\_G06 were obtained from the *Arabidopsis* Biological Resource Center ([www.arabidopsis.org](http://www.arabidopsis.org)). The marker lines carrying *MEA-GUS* (Luo et al., 2000), the *FWA-GFP* reporter line (Kinoshita et al., 2004) and the tetraploid line in C24 ecotype (Scott et al., 1998) were kind gifts from Abed Chaudhury, Tetsu Kinoshita and Rod Scott, respectively. After 3 days at 4°C in the dark, seeds were germinated and grown on soil. Plants were cultured in a growth chamber under short days (8 hours of light at 20°C/16 hours of dark at 16°C; 60–70% hygrometry) until rosettes were formed. Plants were transferred to long days at 20°C (14 hours of light/10 hours of dark) to induce flowering.

### Microscopy and image processing

Developing seeds were isolated from individual siliques at different stages of development. Seeds were mounted in Hoyer's medium and observed microscopically using differential interference contrast (DIC) optics (Boisnard-Lorig et al., 2001). Images were acquired with a DXM1200F digital camera (Nikon, Tokyo, Japan) and processed using MetaMorph (version 6.2; Universal Imaging, Irvine, CA, USA).

Patterns of expression of *pAtLIG1-AtLIG1::GFP* in gametes and seeds were analysed with a LSM 510 confocal microscope (Zeiss, Jena, Germany) with objectives Plan Neofluar ×20 0.8 numerical aperture (n.a.) and Plan Neofluar ×40 1.3 n.a.

### Genetic mapping

The lines JF1781 and JF1630 (C24 accession background) were obtained from the previously described genetic screen of γ-ray mutagenised *Arabidopsis* seeds (Guitton et al., 2004) and were crossed with wild-type Columbia (Col) to generate F1 plants heterozygous for polymorphic markers between Col and C24. The F1 mutant plants were self-pollinated to generate F2 mapping populations. A small mapping population of 300 F2 plants from the JF1630 line was analysed. A total of 2285 F2 plants from the F2 population derived from the JF1781 line were analysed by PCR. Markers for mapping were designed using polymorphisms described by the Cereon online database ([www.arabidopsis.org](http://www.arabidopsis.org)) or derived from our own sequence data (see Table S1 in the supplementary material). The mapping interval was reduced to an interval spanning ten open reading frames between the markers T6D22\_12/13 and T6D22\_24/25. SALK\_013442 and SAIL\_0716\_G06 T-DNA insertion lines in the gene coding for *Arabidopsis* DNA LIGASE I, Atlg08130 ([signal.salk.edu/cgi-bin/tdnaexpress](http://signal.salk.edu/cgi-bin/tdnaexpress)), were analysed and found to display a phenotype similar to JF1781 and JF1630. Crosses between these lines produced brown aborted seeds, indicating allelism. Genomic DNA extracted from JF1781 and JF1630 heterozygous plants was used to sequence Atlg08130. Since no mutation could be identified from the sequences obtained, we inferred that a deletion or

genomic rearrangement encompassing Atlg08130 had occurred in JF1781 and JF1630, and the region was further analysed by Southern blot (see Fig. S1 in the supplementary material).

### Cloning and complementation

For the complementation construct, the entire Atlg08130 locus and its promoter was amplified by KOD Plus Taq polymerase (Toyobo, Nagoya, Japan) as specified by manufacturer, with primers Lig1-attB1, 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGATTAGTCTGGAGG-TCTTGTGCTC-3' and Lig1-attB2, 5'-GGGGACCACTTTGTACAA-GAAAGCTGGGTAATCATCGTCACCTTTGACTTCATTAC-3'. In the latter primer the stop codon TGA was removed. BAC T6D22 containing Atlg08130 was used as the DNA template. The 7 kb PCR product was cloned into pDONR/Zeo vector (Invitrogen, Madison, WI, USA), the entry clone vector of the GATEWAY system, by BP reaction. The fragment was then inserted into the destination GFP fusion vector pMDC107 (Curtis and Grossniklaus, 2003) by LR reaction. The construct *pAtLIG1-AtLIG1::GFP* was introduced into *Agrobacterium* clones and *AtLIG1/atlig1-4* plants were transformed by the floral dip method. For Southern blot analysis of the *AtLIG1/atlig1-4* plants carrying the construct *pAtLIG1-AtLIG1::GFP*, genomic DNA was isolated from leaves. The DNA was digested with restriction enzymes, size fractionated through a 0.7% agarose gel and then blotted onto Hybond-N<sup>+</sup> nylon membrane (Amersham, UK). A 699 bp DNA probe was amplified from the BAC T6D22 using primers 5'-CACTTCGGCCCAAATCCAGT-3' and 5'-GAAGCCCCGAGGAAG-CAACATCAT-3' and labelled using the AlkPhos Direct System (Amersham). DNA gel blot hybridisation was performed at 56°C overnight and the probe detected by CDP-Star Reagent (Amersham). The membranes were exposed to Kodak BioMax Light Film overnight at room temperature.

### RT-PCR analyses

Reverse transcription from *Arabidopsis* RNA was performed as described (Jullien et al., 2006). Primers used to amplify *AtLIG1* mRNA were AtLIG1\_3'UTR-For, 5'-GATGTCAATCAGGGCAGTGT-3' and AtLIG1\_3'UTR-Rev, 5'-CTGGCCTCAGAGCACATTTCT-3'. Primers used to amplify the control *GAPDH* mRNA were GAPDH3', 5'-GTAGCCCCACTCGTTGTCGTA-3' and GAPDH5', 5'-AGGGTGGT-GCCAAGAAGGTTG-3'. *GAPDH* primers were designed to span introns, such that genome-specific and cDNA-specific PCR products could easily be distinguished by size, which was not possible for *AtLIG1* because it differs from its close homologue *AtLIG1a* only in its 3' UTR. A negative control without reverse transcriptase was used for each RT-PCR experiment.

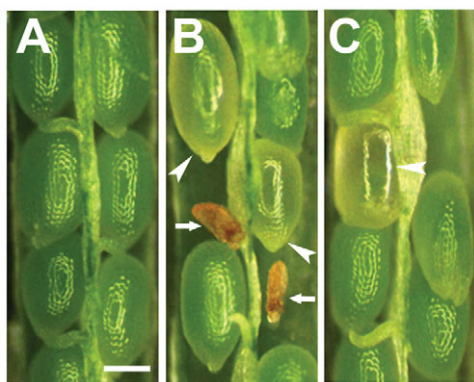
### Polymorphism analysis

Total RNA was extracted from seeds at 25 and 34 hours after pollination (HAP) using the RNeasy Mini Kit (Qiagen, Cambridge, UK) and 1 µg treated with DNaseI; the resulting DNA-free RNA was reverse transcribed using oligo(dT) primers and AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene, La Jolla, CA, USA). The cDNA was used as template for PCR with primer P-Lig+, 5'-CGGCCACTTTCATTGAGAT-3' and the Col polymorphism-specific primer P-LigG, 5'-ATACA-TCGTCAATTGCGGAG-3'. The last base should be A in the Cvi sequence. To ensure high-fidelity results, Hotstar Taq DNA polymerase (Qiagen, Germantown, MD, USA) was used.

## RESULTS

### Isolation of two mutant lines carrying two genetically linked but distinct seed phenotypes

We produced M1 plants from γ-ray-irradiated wild-type seeds and isolated heterozygous lines carrying maternal effect mutations that caused the production of more than 25% abnormal seeds after self-pollination. The two mutant lines, JF1781 and JF1630, produced two types of abnormal seeds: (1) white, translucent, late-aborting seeds; and (2) shrivelled, brown, early-aborting seeds (Fig. 1A,B). For each line, the two phenotypes remained linked after five consecutive backcrosses. Reciprocal crosses between the two lines showed the persistence of both phenotypes, potentially indicating



**Fig. 1. Altered seed set in JF1781 plants.** (A) Wild-type *Arabidopsis* silique 10 days after pollination. All the seeds are mature with large green embryos that occupy most of the seed. (B) A silique resulting from JF1781 self-pollination. Two types of mutant phenotypes are observed: white to pale green translucent seeds (arrowheads) and shrivelled brown seeds (arrows). (C) A silique resulting from the pollination of ovule from a plant heterozygous for JF1781 by wild-type pollen. Mutant seeds are translucent. In some cases the green embryo can be distinguished (arrowhead). Scale bar: 200 µm.

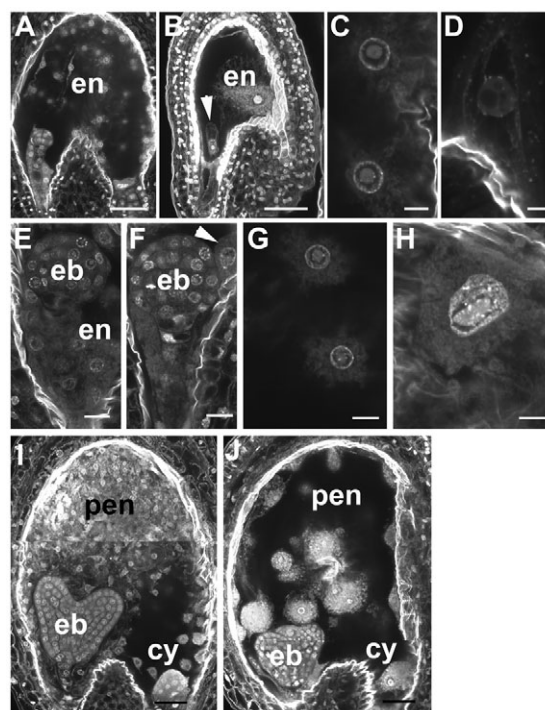
that the two lines were allelic ( $n=679$ ). Crosses between wild-type ovules and pollen from the heterozygous mutant lines produced only wild-type seeds (Table 1). Surprisingly, crosses between ovules from heterozygous mutants with wild-type pollen still produced white translucent seeds (Fig. 1C; Table 1). The dependence of the late-aborting phenotype on the maternal origin of the mutation suggested that we had isolated maternal effect mutations.

Microscopic examination at the wild-type dermatogen stage showed that the mutant early-aborting seeds hardly increased in size after fertilisation and usually contained an arrested zygote and an endosperm containing a single enlarged nucleus (Fig. 2A,B). In less than 25% of cases the embryo arrested after the first division and the endosperm contained two or four enlarged nuclei ( $n=56$  out of 231 arrested seeds) (Fig. 2C,D and see Fig. S2A,B in the supplementary material). After the heart stage, the mutant small seeds had collapsed and it was no longer possible to perform phenotypic analysis (Fig. 1B). During the globular stage, an

**Table 1. Analysis of seed development in reciprocal crosses between wild type (WT) and JF1781 (*atlig1-3*) and JF1630 (*atlig1-4*)**

Female × male	Seed phenotype (%)			n
	Wild-type	Late aborting	Early aborting	
<i>atlig1-3/+</i> × <i>atlig1-3/+</i>	53.7	22.8	23.5	785
<i>atlig1-3/+</i> × WT	64.2	35.8	0	946
WT × <i>atlig1-3/+</i>	98.6	0	1.4	420
<i>atlig1-4/+</i> × <i>atlig1-4/+</i>	67.6	14.9	17.5	416
<i>atlig1-4/+</i> × WT	84.3	14.2	1.5	330
WT × <i>atlig1-4/+</i>	97.8	0.5	1.7	400
WT × WT	98.9	0.4	0.7	560
WT 2n × WT 4n	97.6	2.4	0	123
<i>atlig1-3/+</i> × WT 4n*	61.5	38.5	0	161

The siliques resulting from the indicated crosses were opened 10 days after pollination and the phenotype of the seeds assessed. \*When *atlig1-3/+* plants were crossed using pollen taken from a tetraploid (4n) wild-type plant, the ratio of late-aborting seeds obtained did not significantly differ from that obtained when *atlig1-3/+* plants were crossed using pollen taken from a diploid (2n) wild-type plant ( $\chi^2=0.68$ ,  $P>0.25$ ).



**Fig. 2. Microscopic analysis of seeds produced from heterozygous JF1781 plants.** (A) Wild-type *Arabidopsis* seed at the dermatogen stage of embryo development showing syncytial endosperm. (B) Early-abortion phenotype segregating in siliques from self-pollinated JF1781/+ plants. The zygote (arrowhead) is arrested and the endosperm contains one large nucleus. (C) Two wild-type endosperm nuclei at the dermatogen stage. (D) Single large endosperm nucleus in aborted in homozygous mutant seed at the wild-type dermatogen stage. (E) Wild-type late globular stage embryo surrounded by endosperm nuclei. (F) At the equivalent late globular stage, a seed with an enlarged endosperm nucleus (arrowhead) that segregated with seeds shown in E from a cross between wild-type pollen and ovules from JF1781/+ plants. (G) Detail of peripheral endosperm nuclei from the seed shown in E. (H) Detail of peripheral endosperm nuclei from the seed shown in F. The nucleolus is larger and irregular in shape in comparison to the wild type. (I) Wild-type seed at the mid-heart stage of embryo development. Cellularisation has occurred in the peripheral endosperm, while the cyst remains syncytial. (J) At the equivalent of mid-heart stage, a seed with enlarged endosperm nuclei that segregated with seeds shown in I from a cross between wild-type pollen and ovules from JF1781/+ plants. eb, embryo; en, endosperm; pen, peripheral endosperm; cy, cyst. Scale bars: 50 µm in A,B,I,J; 5 µm in C,D,G,H; 10 µm in E,F.

additional class of phenotype was characterised by endosperm nuclei that were larger than those of the wild type and appeared to result from the fusion of nuclei (Fig. 2G,H). The embryo, however, was not affected (Fig. 2E,F). At the heart stage, the mutant endosperm did not undergo cellularisation (Fig. 2I,J and see Fig. S2E,F in the supplementary material), the process that partitions the syncytial wild-type endosperm into individual cells ( $n>250$ ). The endosperm contained a reduced number of nuclei, ranging from 28 to 38 ( $n=30$ ), by comparison with wild-type seeds that typically contained 200 nuclei at this stage ( $n=30$ ) (Fig. 2I,J and see Fig. S2C,D in the supplementary material).

Since the two seed phenotypes were very distinct from each other and appeared to be under different types of genetic control we concluded that either the lines JF1630 and JF1781 carried a single mutation in a single gene that causes both early-aborting and late-



aborting seed phenotypes, or that each line carried a deletion encompassing at least two genes, each linked to one of the two phenotypes.

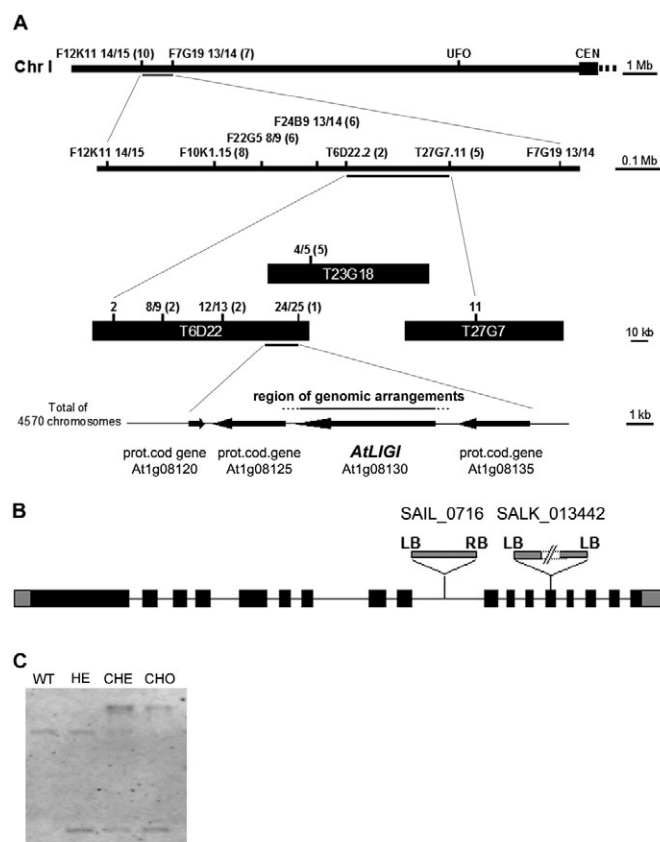
### Identification of *AtLIG1* as the single gene responsible for the phenotypes in JF1781 and JF1630

Preliminary genetic mapping of the mutation in each line identified the same genetic interval, which further supported the proposal that the two mutant lines were allelic. Using the JF1781 mapping population, the mutation was mapped to an interval comprising ten open reading frames on chromosome 1 (Fig. 3A and see Materials and methods). We analysed the phenotype of T-DNA insertion lines in genes likely to be essential for viability and located within the mapping interval. In lines SALK\_013442 and SAIL\_0716\_G06, a T-DNA insertion in the gene encoding the *Arabidopsis* DNA LIGASE I (*AtLIG1*) between exons 9 and 10, and in exon 13, respectively, may result in a partial loss-of-function of *AtLIG1* (Fig. 3B). Seeds produced by each line after self-pollination showed the two phenotypes leading to seed arrest, similar to JF1781 and JF1630 ( $n=256$  for SALK\_013442 and  $n=278$  for SAIL\_0716\_G06). Crosses between the T-DNA insertion lines and JF1781 produced small, brown, aborted seeds ( $n=345$  seeds for SALK\_013442 and  $n=245$  seeds for SAIL\_0716\_G06). The absence of complementation indicated that JF1781 and the T-DNA insertion lines were alleles for the mutation causing the early seed abortion.

The *AtLIG1* gene was cloned and sequenced in lines JF1781 and JF1630, but no mutation was identified. We hypothesised that a deletion encompassing the *AtLIG1* locus was caused by the  $\gamma$ -ray mutagenesis in the lines JF1781 and JF1630, leaving only wild-type DNA to be sequenced from each heterozygous mutant line. A Southern blot analysis using the entire *AtLIG1* genomic locus as a probe identified in both lines a possible deletion accompanied by a genomic rearrangement (see Fig. S1 in the supplementary material). To confirm that mutations in *AtLIG1* were responsible for the two seed phenotypes observed in the JF1630 line, JF1630 heterozygous plants were transformed with a construct comprising the *AtLIG1* genomic locus fused to the sequence encoding green fluorescent protein (GFP) and placed under the endogenous 5' region that controls *AtLIG1* expression (*pAtLIG1-AtLIG1::GFP*). We produced three transgenic lines and obtained plants homozygous for both the *pAtLIG1-AtLIG1::GFP* construct and the JF1630 mutation (Fig. 3C), indicating complementation. Plants homozygous for JF1630 and the *pAtLIG1-AtLIG1::GFP* construct produced only seeds with a wild-type phenotype ( $n=695$ ), in contrast to the 30% of seeds bearing mutant phenotypes that were produced by heterozygous JF1630 plants ( $n=232$ ). The construct encoding the protein fusion *AtLIG1::GFP* was thus able to complement both the early- and late-aborting seed phenotypes observed in the JF1630 line. We concluded that the loss-of-function of *AtLIG1* was responsible for the two seed phenotypes observed in the JF1630 mutant line. Since allelism was established between the four mutant lines studied, they were renamed *atlig1-1* for Salk line SALK\_013442, *atlig1-2* for Salk line SAIL\_0716\_G06, *atlig1-3* for JF1781 and *atlig1-4* for JF1630.

### Expression pattern of *AtLIG1*

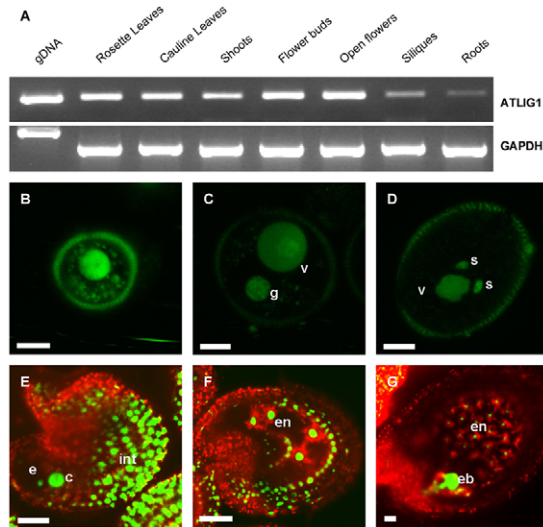
RT-PCR analysis showed that *AtLIG1* was expressed in all vegetative and reproductive tissues (Fig. 4A), confirming previous observations (Taylor et al., 1998). We used transgenic lines carrying the complementing construct *pAtLIG1-AtLIG1::GFP* to analyse the dynamics of the expression of *AtLIG1* in reproductive tissues. The



**Fig. 3. Identification of *AtLIG1* as responsible for the phenotype of JF1630 and JF1781 plants.** (A) The genetic interval, molecular markers, BAC clones and the region of genomic rearrangements where the JF1781 mutation was mapped in the *Arabidopsis* genome. The numbers in parentheses indicate the number of recombinants obtained at each molecular marker, for a total of 4570 chromosomes. (B) Schematic representation of *AtLIG1*, indicating T-DNA insertion sites. Black boxes represent exons. (C) Complementation of *atlig1-4* by *pAtLIG1-AtLIG1::GFP*. A Southern blot distinguishes between wild-type plants (WT, one band of medium size), *atlig1-4/+* plants (HE, two small bands), and plants carrying the complementing construct (CHE, upper band). The gel shows that homozygous *atlig1-4* plants (CHO, upper and lower bands) are obtained from a population of *atlig1-4/+*; *pAtLIG1-AtLIG1::GFP/pAtLIG1-AtLIG1::GFP* plants (CHE, three bands).

fusion protein mainly localised to the nucleus (Fig. 4). *AtLIG1::GFP* expression was observed throughout male gametogenesis (Fig. 4B–D). In the mature male gametophyte, the reporter gene was expressed in the vegetative cell as well as in the two sperm cells (Fig. 4D;  $n=87$ ). Similarly, *AtLIG1::GFP* was expressed in the mature female gametes in the embryo sac (Fig. 4E;  $n=58$ ). The reporter gene showed strong expression in the central cell nucleus and weaker expression in the egg cell and synergids (Fig. 4E). After fertilisation, *AtLIG1::GFP* was expressed in the syncytial endosperm (Fig. 4F,G;  $n=34$ ) and in the embryo (Fig. 4G;  $n=46$ ).

We further investigated the parental origin of *AtLIG1* expression in crosses between wild-type ovules and pollen from the *AtLIG1::GFP*-expressing line (Fig. 5A–C). The paternal allele of *AtLIG1::GFP* was expressed in the zygote and in the endosperm 8–10 hours after pollination (HAP) (Fig. 5A;  $n=37$ ) and subsequently remained expressed in the syncytial endosperm and embryo (Fig. 5B,C;  $n=36$ ). We performed symmetrical crosses between ovules

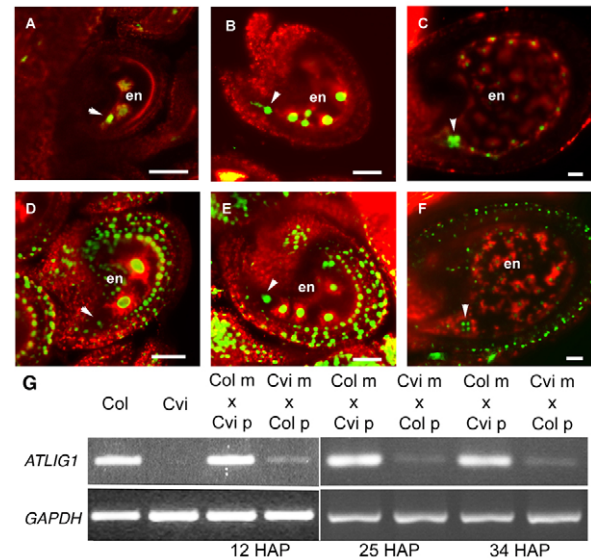


**Fig. 4. Expression of *AtLIG1*.** (A) Expression of *AtLIG1* in a range of tissues as assessed by RT-PCR. *GAPDH* provides a loading control. (B-D) Expression of *pAtLIG1-AtLIG1::GFP* during pollen development. *AtLIG1::GFP* marks the nucleus of the microspore (B) of both the vegetative (v) and generative (g) cells in the bicellular pollen (C) and of both the vegetative and sperm (s) cells in the mature tricellular pollen (D). (E) Expression of *pAtLIG1-AtLIG1::GFP* in the ovule marks nuclei in the maternal integuments (int) and in both female gametes, the central cell (c) and the egg cell (e). Strong expression is also observed in the maternal integuments surrounding the two female gametes. (F,G) Expression of *pAtLIG1-AtLIG1::GFP* after fertilisation in the endosperm at the four-nuclei stage (F) and in the embryo (eb) and the endosperm (en) at the embryo globular stage (G). Scale bars: 10  $\mu$ m.

expressing *AtLIG1::GFP* and wild-type pollen (Fig. 5D-F). The maternal allele of *AtLIG1::GFP* was also expressed at 8-10 HAP in both zygotic products (Fig. 5D;  $n=49$ ) and remained expressed throughout seed development (Fig. 5E,F;  $n=48$ ).

In order to confirm the early expression of the paternal allele of *AtLIG1*, we identified a polymorphism between two wild-type accessions such that we could distinguish the transcripts from each parental allele. We observed that *AtLIG1* was expressed from the paternal allele as early as 12 HAP (the zygotic stage corresponding to the two- to four-nuclei endosperm) and at 25 and 34 HAP (Fig. 5G). The intensity of the signal originating from the paternally derived transcripts was significantly reduced compared with the maternal signal, most likely because most of the maternal transcripts are contributed by the maternal integuments and not by the fertilisation products. Thus, the difference in intensity does not reflect a difference in the levels of expression of the parental copies. In support of this interpretation, we did not detect any significant differences in the levels of GFP fluorescence contributed maternally or paternally by the reporter *pAtLIG1-AtLIG1::GFP*.

In conclusion, both the *AtLIG1* locus and the *pAtLIG1-AtLIG1::GFP* reporter show biparental expression before the first zygotic division and as early as the two-nuclei stage in endosperm. Since *AtLIG1* is also expressed in both male and female gametes, it is not possible to deduce whether the mRNA detected in the early zygotic products originates from the gametes or from de novo transcription in the zygotic products after double fertilisation. However, the fact that seeds deprived of both parental copies of *AtLIG1* (*atlig1/atlig1*) abort at the zygotic stage with one to two



**Fig. 5. Parental expression of *pAtLIG1-AtLIG1::GFP* after fertilisation in the endosperm and embryo.** (A-C) Paternal expression of *pAtLIG1-AtLIG1::GFP* in seeds produced by crosses between wild-type ovules and pollen from plants carrying *pAtLIG1-AtLIG1::GFP*. The red autofluorescence surrounds endosperm nuclei and is not present in the embryo. *pAtLIG1-AtLIG1::GFP* is paternally expressed in the zygote and two-nuclei endosperm (A), the elongated zygote (arrowhead) and the endosperm (en) (B) and in the octant stage embryo (arrowhead) and the syncytial endosperm (en) at stage VIII (C). (D-F) Maternal expression of *pAtLIG1-AtLIG1::GFP* in seeds produced by crosses between wild-type pollen and ovules from plants carrying *pAtLIG1-AtLIG1::GFP*. *AtLIG1::GFP* is expressed in the ovule integuments. The red autofluorescence outlines the endosperm (en) and is not present in the embryo (arrowhead). *pAtLIG1-AtLIG1::GFP* is maternally expressed in the zygote and in the two-nuclei endosperm (D), the elongated zygote and the four-nuclei endosperm (E) and in the octant stage embryo and the syncytial endosperm at stage VIII (F). Scale bars: 25  $\mu$ m. (G) Paternal expression of *AtLIG1* at 12, 25 and 34 hours after pollination (HAP). A polymorphism enables detection of *AtLIG1* transcripts in plants from Col, but not Cvi, accession. When reciprocal crosses are performed between Col and Cvi accessions as mothers (m) and fathers (p), the paternal transcripts of *AtLIG1* are detected. *GAPDH* was used as a loading control.

nuclei in the endosperm leads us to conclude that transcripts of *AtLIG1* from both parents are required immediately after fertilisation and that the *atlig1* loss-of-function mutations have a sporophytic recessive effect on embryo development.

### Origin of the of the maternal effect of *AtLIG1*

In addition to early-aborting seeds, *atlig1* mutant alleles produced an additional class of late-aborting seeds that presented major defects in the endosperm. When reciprocal crosses were performed between wild-type and each *atlig1* allele, the white seed phenotype was observed only when the mutation was maternally inherited (Fig. 1C, Table 1). The genetic transmission of *atlig1-3*, which produced the highest proportion of abnormal seeds (Table 1), was not affected when pollen from *atlig1-3/+* plants was crossed to wild-type ovules [ $49.8 \pm 1.9\%$  (s.d.);  $n=253$ ], but was significantly reduced when ovules from *atlig1-3/+* plants were crossed to wild-type pollen ( $37.5 \pm 5.8\%$ ;  $n=261$ ). If *atlig1-3* had a completely penetrant gametophytic effect, 50% of seeds should display the phenotype in

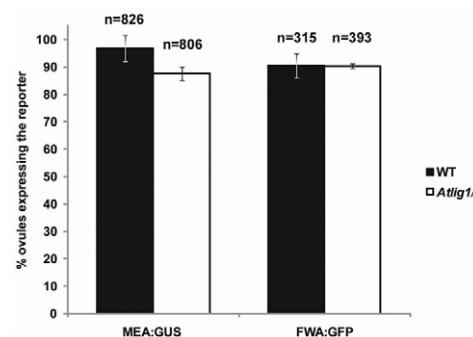
crosses involving *atlig1* as female. By contrast, a dominant fully penetrant maternal sporophytic effect would cause abnormal development in 100% seeds. Together, the proportion of seeds that displayed a mutant phenotype and the genetic transmission data indicated that the maternal effect linked to *atlig1* was incompletely penetrant and most likely had a gametophytic origin.

In the seeds affected by the maternal loss of *AtLIG1*, endosperm development was affected by reduced nuclear division as early as the embryo globular stage, although the embryo did not show any phenotype (Fig. 2E,F). From the late heart stage onwards, embryo growth was retarded but did not show patterning or major morphological defects (Fig. 2I,J). These defects resulted in shrivelled seeds, some of which carried viable embryos, allowing a limited maternal transmission of *atlig1*. Since embryo growth is affected by defects in endosperm development (Garcia et al., 2005; Ingouff et al., 2005; Liu and Meinke, 1998; Sorensen et al., 2002), we hypothesised that *atlig1* exerts a direct maternal effect on endosperm development and indirectly delays embryo development.

Abnormal development of the integuments can affect endosperm development (Colombo et al., 1997) and it is possible that a reduced dosage of *atlig1* activity in the *atlig1/+* integuments could indirectly affect endosperm development. However, we could not detect any phenotypic alteration in the *atlig1/+* integuments (see Fig. S3 in the supplementary material), arguing against a maternal effect derived from surrounding maternal vegetative tissues. The maternal loss of *AtLIG1* is therefore likely to primarily affect endosperm development.

The endosperm is a triploid tissue, as it inherits two doses of the maternal genome (2m) and one copy of the paternal genome (1p). Hence, the maternal effect caused by the maternal inheritance of *atlig1* in endosperm could result from a low dosage of active *AtLIG1* protein in the endosperm, which inherits two maternal mutant *atlig1* alleles with a single wild-type paternal *AtLIG1* allele. We tested this hypothesis by introducing an additional wild-type paternal allele of *AtLIG1* in crosses between mutant *atlig1* ovules and pollen from tetraploid plants to produce seeds with a 2m:2p parental dosage in endosperm. If the maternal effect of *atlig1* was the result of a dosage imbalance in the endosperm, the restoration of a 1:1 balanced dosage between mutant and wild-type copies of *AtLIG1* should rescue the maternal effect of *atlig1*. Seeds resulting from the cross *AtLIG1/atlig1* 2n<sub>mat</sub> × *AtLIG1/AtLIG1* 4n<sub>pat</sub> (dosage *atlig1* versus *AtLIG1*=1:1) showed a proportion of white seeds that was comparable to that among seeds resulting from the cross *AtLIG1/atlig1* 2n<sub>mat</sub> × *AtLIG1/AtLIG1* 2n<sub>pat</sub> (dosage *atlig1* versus *AtLIG1*=2:1) (Table 1). Thus, the addition of a supplementary wild-type copy of *AtLIG1* in the endosperm did not rescue the maternal effect phenotype. We concluded that the maternal effect of *atlig1* does not result from a dosage effect.

Because *AtLIG1* is expressed from both parental alleles in the endosperm as early as the one- to two-nuclei stage (Fig. 5), parental genomic imprinting of *AtLIG1* is unlikely to account for the maternal effect of *atlig1* on endosperm. Still, we envisaged that the expression of imprinted genes could be perturbed by *atlig1* loss-of-function in the central cell. In the *atlig1-3/+* background, we observed normal expression of the imprinted gene *FLOWERING WAGENINGEN* (*FWA*) and a limited, but significantly altered, expression of *MEA* (Fig. 6). Given the mild effect of *atlig1* on the expression of *MEA*, it is possible that we failed to detect a decrease in the expression of *FWA*. The expression of *MEA* and *FWA* depends on the DNA glycosylase DME, which is expressed specifically in the central cell and removes methylated cytosine residues, leaving behind a single-strand break that must be repaired by a DNA ligase



**Fig. 6. Expression of *MEA* and *FWA* in *atlig1*.** The percentage of central cells expressing *MEA-GUS* in self-pollinated *atlig1-3/+*; *MEA-GUS/MEA-GUS* plants differs significantly from the expression of *MEA-GUS* in a wild-type background (*t*-test, *t*=3.77, *P*<0.001). The percentage of central cells expressing *FWA-GFP* in self-pollinated *atlig1-3/+*; *FWA-GFP/+* plants does not significantly differ from the expression of *FWA-GFP* in a wild-type background (*t*=0.045, *P*>0.1). The total number of mature ovules observed (*n*) is indicated above each bar. Error bars indicate s.e.m.

(Choi et al., 2002; Gehring et al., 2006). The absence of DME in the central cell causes a maternal effect on endosperm development with a *fis* mutant phenotype that is distinct from the maternal effect caused by *atlig1* (Choi et al., 2002) (see Fig. S4 in the supplementary material).

We further investigated the possibility of a genetic interaction between *atlig1-3* and *dme* and analysed the phenotypes of the seeds produced by crossing the double mutant *dme/+*; *atlig1-3/+* with wild-type pollen (Table 2). The association of *dme* and *atlig1* did not cause phenotypes other than those observed in *dme* and *atlig1* single mutants (see Fig. S4 in the supplementary material) and did not cause ovule or early seed abortion (see Table S2 in the supplementary material). We calculated the proportion of seeds displaying each phenotype in the population of seeds derived from the female gametes carrying both mutations. This calculation was based on the fact that *dme/+*; *atlig1/+* plants were predicted to produce the following proportions of genetic classes: 25% wild-type, 25% *dme*, 25% *atlig1* and 25% *dme*; *atlig1* ovules. The penetrance calculated from a dataset obtained under the same growth conditions was 89% for *dme* and 70% for *atlig1* (Table 2). From the total population of 523 seeds produced by ovules from *dme/+*; *atlig1/+* plants fertilised with wild-type pollen, 131 (25%) *dme* ovules were therefore expected to produce 117 seeds with the *dme* mutant phenotype and 131 *atlig1* ovules were expected to produce 92 seeds with the *atlig1* mutant phenotype. This population

**Table 2. Phenotypic analysis of *dme*; *atlig1* double mutant**

Female × male	Phenotypic classes (% ± s.d.)			n
	<i>dme</i>	<i>atlig1</i>	<i>dme</i> ; <i>atlig1</i>	
<i>dme/+</i> × WT	44.5±10.8	0	0	506
<i>atlig1-3/+</i> × WT	0	35.1±8.3	0	486
<i>atlig1-3/+</i> ; <i>dme/+</i> × WT	36.9±11.5	21.5±10.1	0	523

Seeds resulting from the indicated crosses were cleared and the phenotype analysed 6 days after pollination (see Fig. S4 in the supplementary material). Statistical analyses indicate that the loss-of-function of *dme* is epistatic over the maternal effect caused by *atlig1* ( $\chi^2=0.12$ , *P*>0.9; see Table S2 in the supplementary material), indicating that *AtLIG1* acts downstream of *DME*.

*n*, number of seeds analysed.

WT, wild type.



of seeds should also contain  $[131 + (131 - 92) + (131 - 117)] = 184$  seeds with a wild-type phenotype. Hence, from the number of seeds obtained for each phenotypic class (see Table S3 in the supplementary material), we could estimate that the 131 ovules carrying both the *dme* and *atlig1* mutations produced 34 (218 – 184) seeds with a wild-type phenotype (26% of this population), 75 (192 – 117) seeds with a *dme* phenotype (57% of this population) and 21 (113 – 92) seeds with a *atlig1* phenotype (16% of this population). Synthetic rescue between the two mutations could not be accounted for by the low percentage of double-mutant seeds with a wild-type phenotype (see Table S3 in the supplementary material;  $\chi^2 = 242.4$ ,  $P < 0.001$ ). However, the low percentage of double-mutant seeds of wild-type phenotype could be explained by the reduced penetrance associated with both the *atlig1* and *dme* mutations. We thus observed that *dme; atlig1* ovules produce a large majority of seeds with a *dme* phenotype, indicating that *DME* acts upstream of *AtLIG1*. This conclusion was supported by statistical tests for various genetic interactions (see Table S3 in the supplementary material;  $\chi^2 = 1.1$ ,  $P > 0.9$ ). The persistence of the *atlig1* phenotype in a minor proportion of seeds derived from *dme; atlig1* ovules could be explained by the redundant activity of *ROS1* (also known as *DML1*), *DME-LIKE2* (*DML2*) and *DME-LIKE3* (*DML3*), which encode DME-like proteins (Ortega-Galisteo et al., 2008; Penterman et al., 2007). However, changes in *DME* expression did not detectably affect the expression of *ROS1*, *DML2* or *DML3* in ovules (see Fig. S5 in the supplementary material). It is still possible that residual DME-like activity is present in *dme* ovules, as a consequence of which a small proportion of *dme; atlig1* ovules exhibit a phenotype similar to that of *atlig1* ovules. Alternatively, the persistence of the *atlig1* phenotype could be explained by the incomplete penetrance of the *dme* mutation. In conclusion, our genetic data suggest that *AtLIG1* repairs the DNA single-strand breaks introduced by *DME* in the central cell.

## DISCUSSION

### DNA LIGASE I is encoded by a unique essential gene in *Arabidopsis*

In mammals and yeast, four different types of ATP-dependent DNA ligases (I to IV) have been identified, each with a specialised function (Martin and MacNeill, 2002; Timson et al., 2000). DNA ligase I is required for Okazaki fragment joining during DNA replication and for nucleotide-excision repair and base-excision repair (BER) pathways. DNA ligase II has no known function and might result from alternative splicing of DNA ligase III transcripts. DNA ligase III has several isoforms that are involved in repair and recombination, and DNA ligase IV is necessary for non-homologous end-joining and for V(D)J recombination in mammals.

The genome of *Arabidopsis* does not appear to contain any DNA ligase II or III homologues and only four genes with any similarity to eukaryotic ATP-dependent DNA ligases (Sunderland et al., 2006) (see Fig. S6 in the supplementary material). At5g57160 encodes the *Arabidopsis* homologue of yeast and mammalian DNA ligase IV, and is required for DNA repair following exposure to damaging agents [methyl methanesulphonate (MMS) and  $\gamma$ -ray], but is not required for T-DNA integration (van Attikum et al., 2009). At1g08130 (*AtLIG1*) encodes the *Arabidopsis* homologue of yeast and mammalian DNA ligase I (Taylor et al., 1998). The *Arabidopsis* genome contains two other genes encoding DNA ligases, At1g49250 and At1g66730, which share 71% and 38% homology with *AtLIG1*, respectively (see Fig. S4 in the supplementary material). These two genes have been named *AtLIG1a* and *AtLIG6*, respectively (Sunderland et al., 2006), and their functions remain

unknown. Transcriptome analyses ([http://csbdb.mpimp-golm.mpg.de/csbdb/dbxp/ath/ath\\_xpmgq.html](http://csbdb.mpimp-golm.mpg.de/csbdb/dbxp/ath/ath_xpmgq.html)) reveal that *AtLIG1a* (At1g49250) is probably not expressed, indicating that *AtLIG1* is likely to be the sole source of DNA ligase I activity in *Arabidopsis*. The essential role played by *AtLIG1* is likely to account for the absence of division of the embryo and endosperm after fertilisation in the loss-of-function mutant *atlig1*. In addition, we observed that *AtLIG1* is expressed from either parental allele within hours after fertilisation in the endosperm and zygote. We are unable to entirely discount the possibility that *AtLIG1* mRNA is inherited from the gametes, although the levels of *AtLIG1* expression from the paternal allele are unlikely to be accounted for by mRNA inherited from the limited amount of sperm cytoplasm. Similar conclusions can be reached from an analysis of *MSI1* expression dynamics (Leroy et al., 2007). Paternal expression of genes within a few hours or days after fertilisation has been reported for other essential genes in *Arabidopsis* and maize (Ingouff et al., 2007; Meyer and Scholten, 2007; Weijers et al., 2001) and we thus consider that the paternal transcriptional delay reported previously (Vielle-Calzada et al., 2000) is restricted to certain loci and might reflect a delay in zygotic activation of the gene from both parental alleles, which is masked by the presence of mRNAs inherited from the female gametes.

### *AtLIG1* exerts a direct maternal effect on seed development

Maternal inheritance of the *atlig1* mutation specifically affects endosperm development. Two days after pollination, when the endosperm has undergone five to six syncytial nuclear divisions, the *atlig1<sub>mat</sub>/+* endosperm nuclei tend to enlarge and stop dividing. The *atlig1<sub>mat</sub>/+* endosperm never cellularises and does not properly support embryo growth, leading to seed abortion. We show that the maternal effect associated with *atlig1* results from neither a dominant effect of *atlig1*, a genomic dosage imbalance, nor imprinted expression of *AtLIG1*. We conclude that *atlig1* mutations cause a maternal gametophytic effect on endosperm development that is linked to a defect during female gametogenesis in the central cell lineage. The *atlig1* central cell is able to undergo fertilisation and expresses *FWA*, which marks the differentiation of the central cell in the wild type. Hence, the defects caused by *atlig1* do not affect the fate of the central cell.

The restriction of the maternal effect of *atlig1* to the endosperm is striking, as *AtLIG1* is expressed in both the egg cell and central cell and later from both parental alleles in the embryo and the endosperm. The maternal effect of *atlig1* on endosperm development is therefore likely to depend on the activity of *DME* expressed specifically in the central cell. This interaction probably explains the slightly reduced *MEA* expression observed in *atlig1* central cells. *DME* creates DNA single-strand breaks as it removes methylated cytosine residues, and a repair mechanism is therefore required to seal these nicks (Gehring et al., 2006). In mammals, nicks introduced by DNA glycosylases are repaired by a short-patch or a long-patch BER pathway (Sancar et al., 2004). Both BER pathways involve neo-synthesis of DNA at the site of base excision by DNA polymerases and ligation of the newly synthesized DNA fragment by DNA ligases, but differ in the length of the neo-synthesized DNA fragment. In the short-patch BER pathway, a single nucleotide is synthesized by DNA polymerase  $\beta$ , and DNA ligase III seals the nick. In the long-patch BER pathway, two to ten nucleotides are synthesized by DNA polymerases  $\delta$  and  $\epsilon$ , and DNA ligase I seals the nick. In contrast to mammals, the *Arabidopsis* genome contains neither DNA polymerase  $\beta$  nor DNA ligase III homologues (Sunderland et al., 2006). Therefore, it is possible that

only the long-patch BER pathway is conserved in *Arabidopsis*. Our genetic data support the contention that AtLIG1 participates in the repair machinery acting downstream of DME in the central cell, such that the absence of maternal AtLIG1 would lead to an absence of nick repair. The resulting genome damage would be gradually amplified with the rapid rounds of syncytial division in the endosperm, resulting in defective nuclear architecture.

In conclusion, we propose that the maternal effect associated with the loss-of-function of AtLIG1 in the central cell reflects its function in association with DME. In animals, genes homologous to DME have not been identified. However, a recent report in the zebrafish embryo has shown that DNA demethylation involves the association of a deaminase and a glycosylase, which might also necessitate a functional DNA ligase activity (Rai et al., 2008). Confirmation of similarities between DNA demethylation mechanisms in vertebrates and plants awaits further investigation and would represent a further example of the convergence of imprinting mechanisms between the two major eukaryotic phylogenetic branches.

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#### Supplementary material

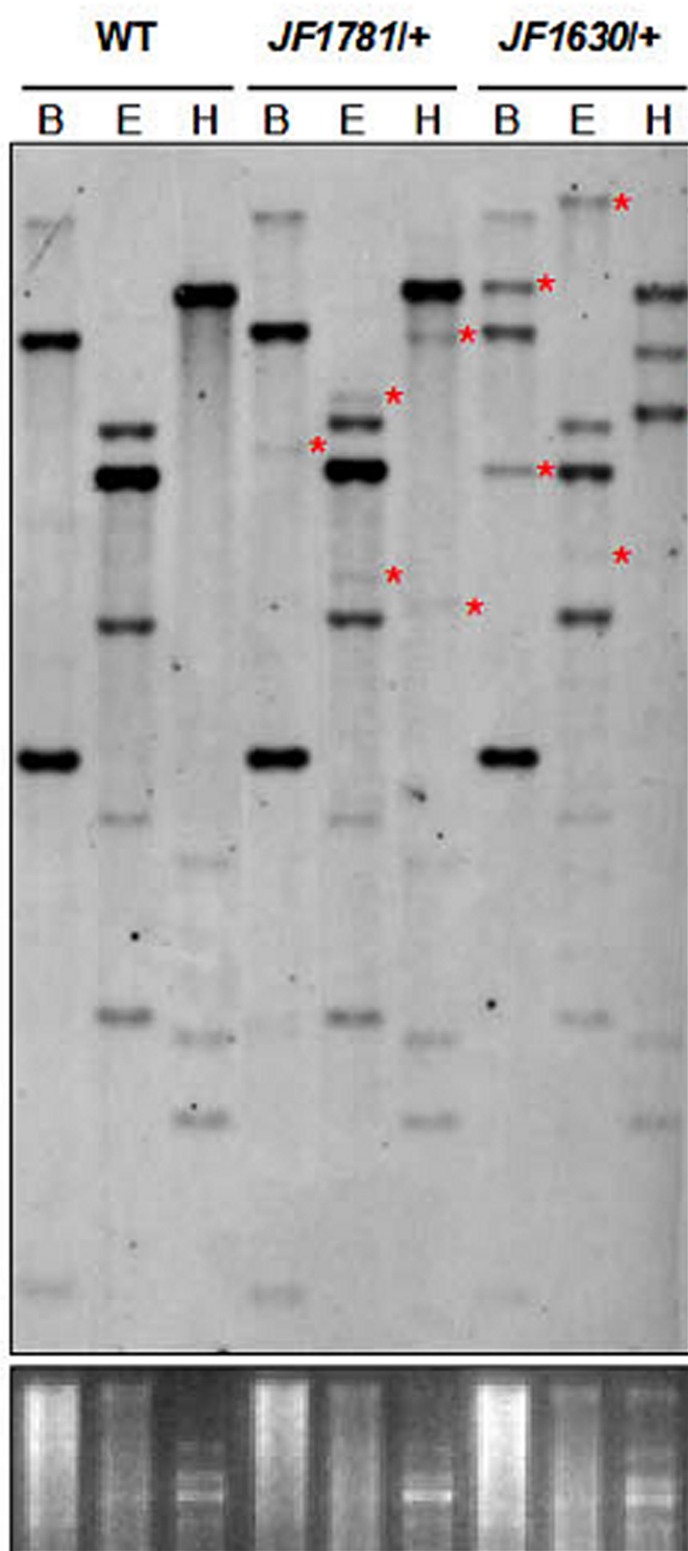
Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.041020/-DC1>

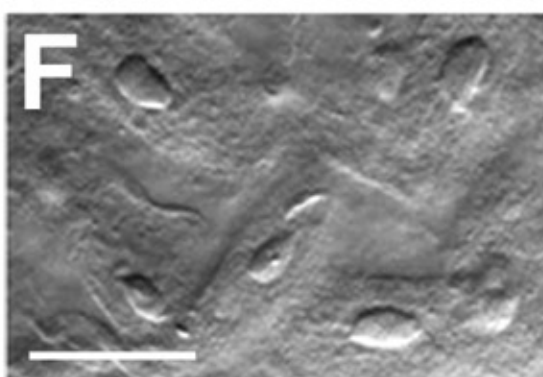
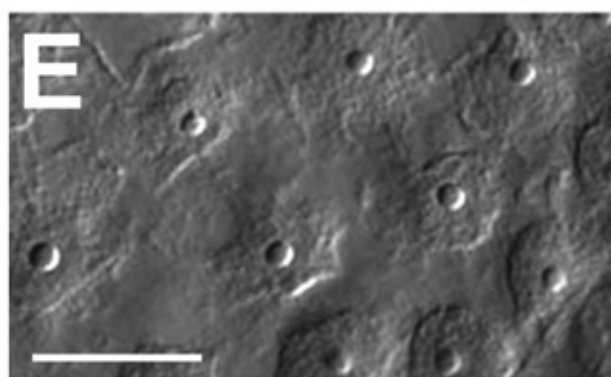
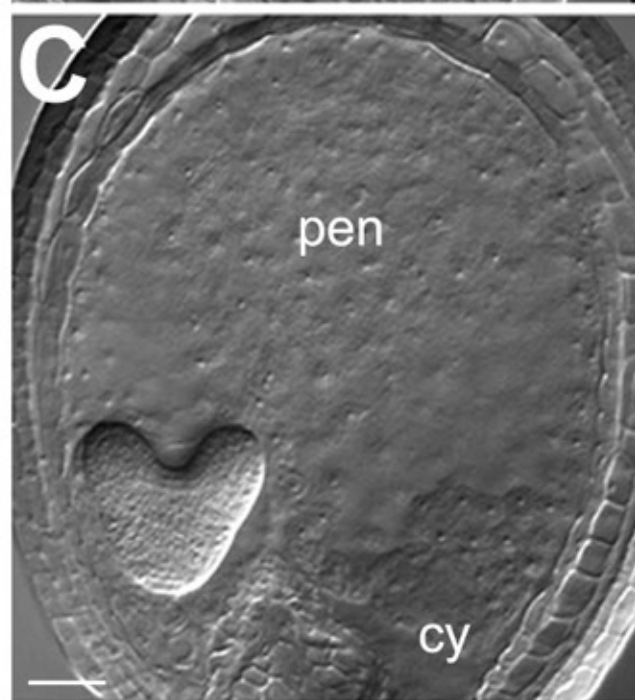
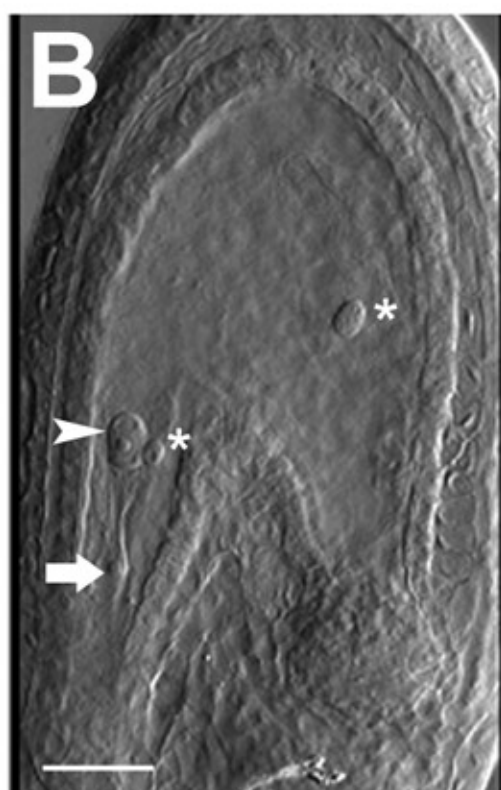
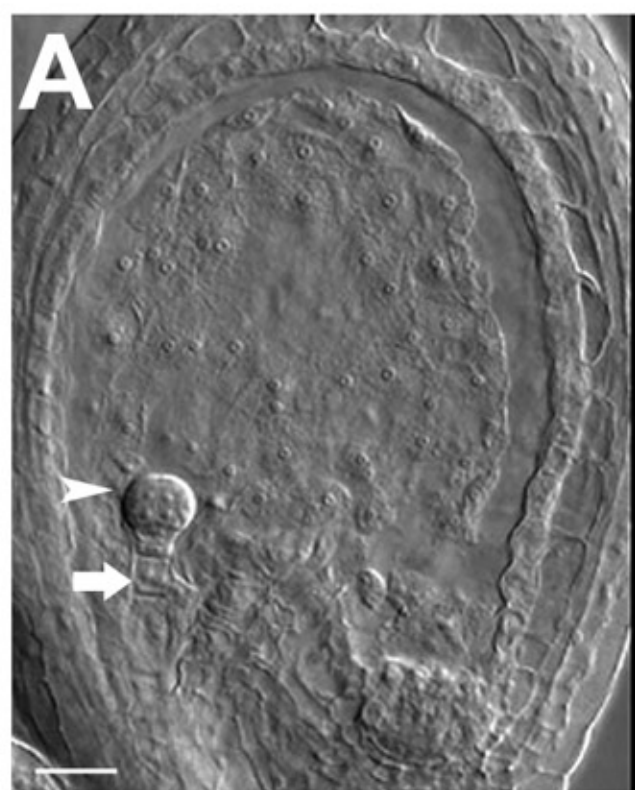
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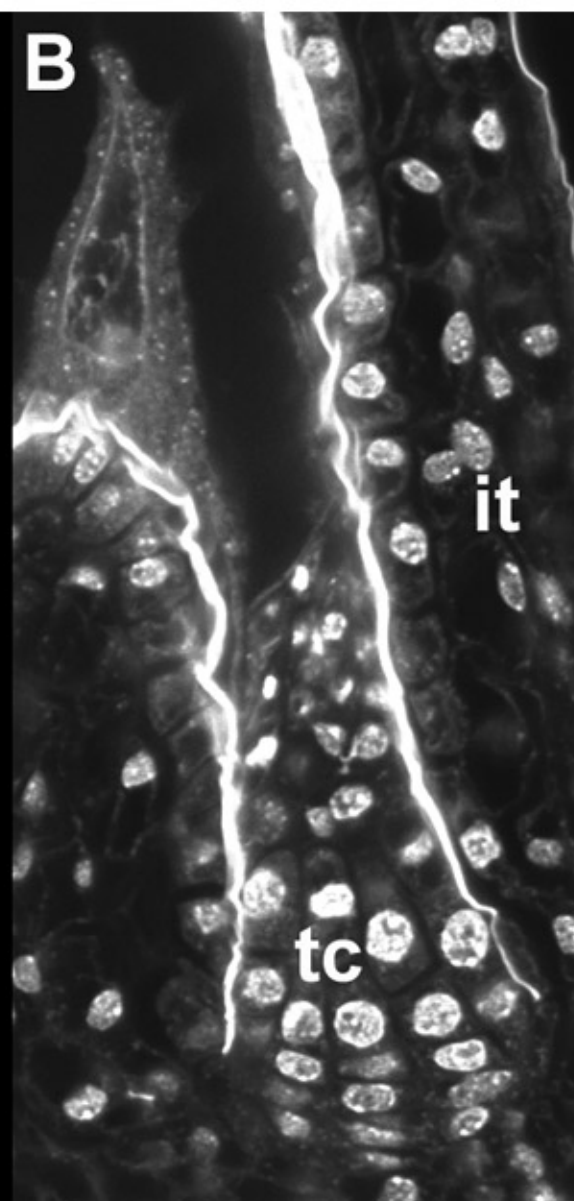


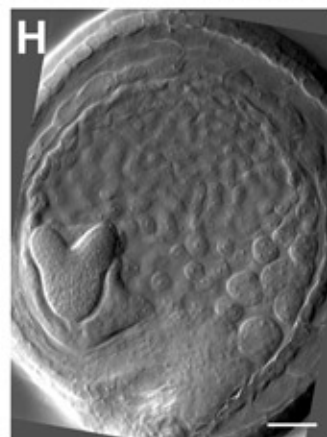
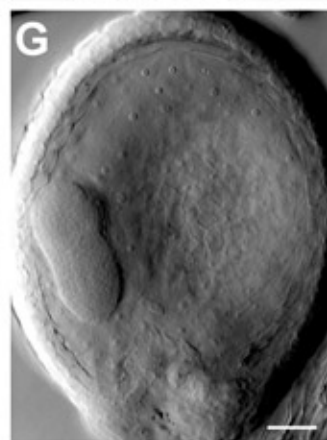
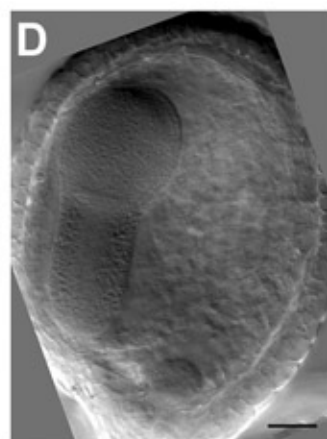
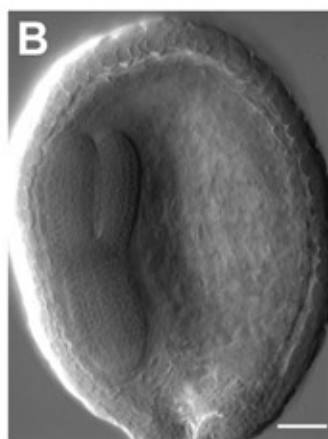
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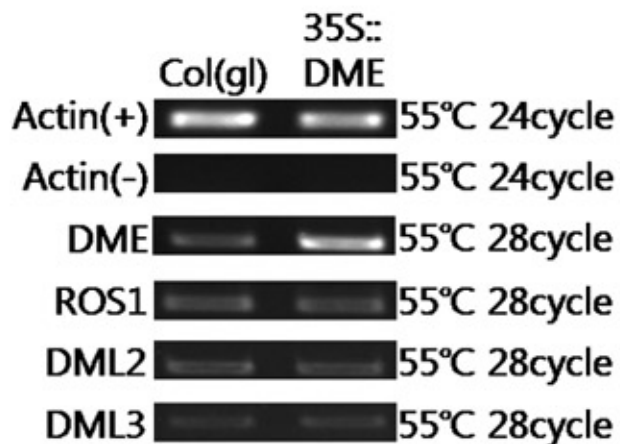
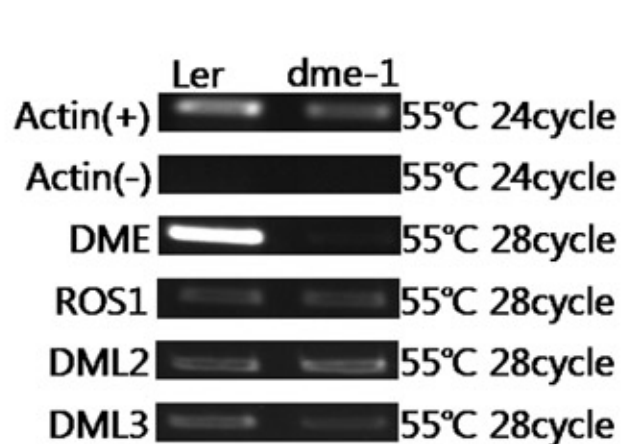




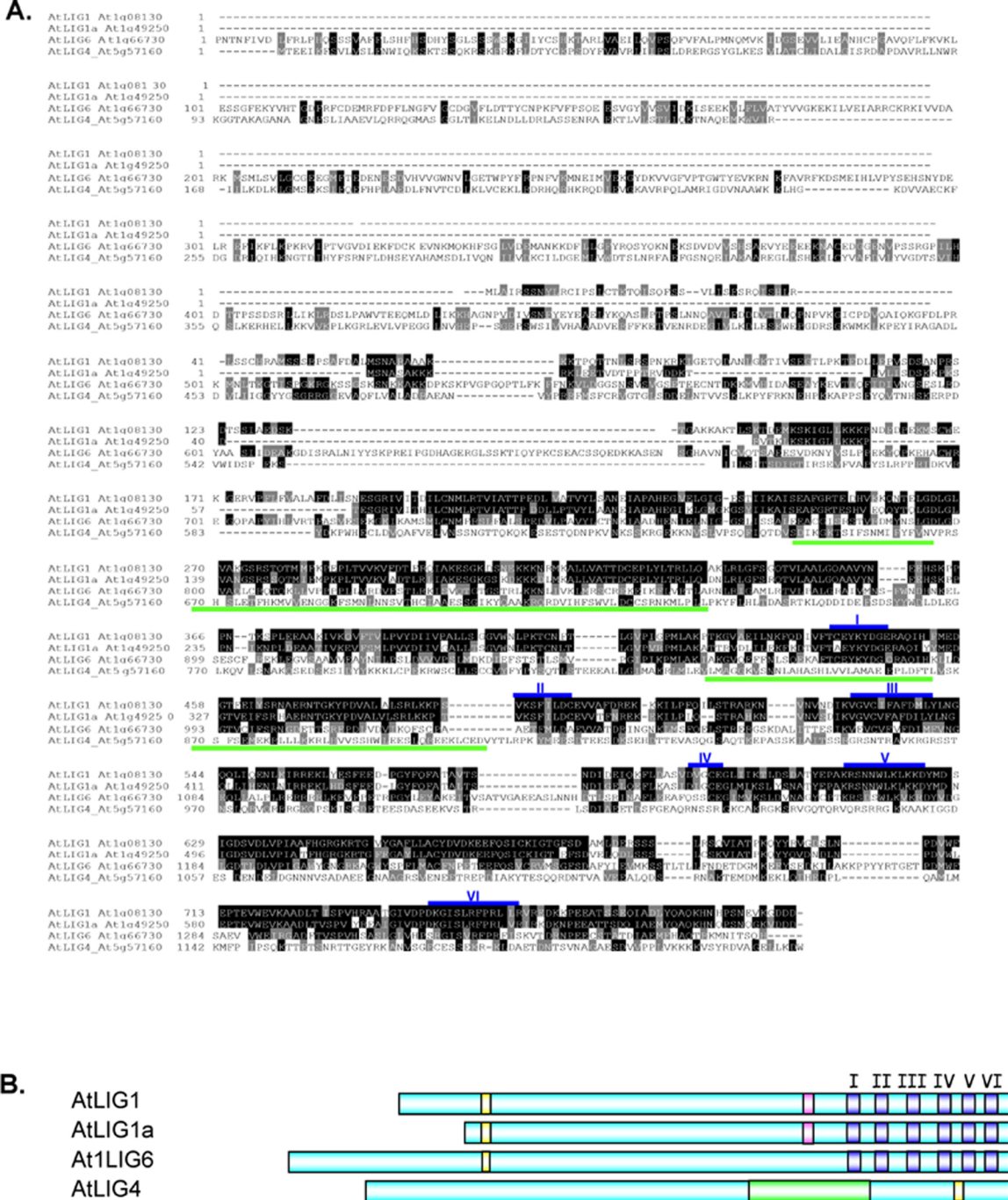












**Table S1. Markers and primers generated for mapping the mutation in the line JF1781**

Marker	Primer sequence (5' to 3')	Polymorphism	Enzyme	Amplicon (bp)	
				Col	C24
F12K11 14/15	AAGAAGAGCCTGTCACCAACTAC TTGACTCTAACCTAATCGCAGAT	In/del	–	300	250
F7G19 13/14	TTCATCGGTCGGCACAAAAGTGT CAAACCTCGCTTATTCAACAAGAGAC	In/del	–	350	300
F10K1.15	TGCTGATGGTGAAGTCAAGG CAGTCGACCATGTTGGCATA	In/del	–	350	400
F22G5 8/9	TCTTAGTCGGGTCAACGAAAA TTTTTGTGTGGTGAGACCAATC	In/del	–	325	275
T23G18 4/5	AAGAGGAAAATGAAGGCAACAG TCCCTAACTACTGGTTTTCCACC	In/del	–	275	300
T27G7.11	TCAGGCCCATATTCGTTTGG AGGCTTTCAGTTTGACCCCA	In/del	–	250	275
F24B9 13/14	GCAGTATAGGCTGATGTGTGTGT CCAAACCGAATCCGACTCTA	In/del	–	300	320
T6D22 24/25	GCAGAAATATGTTCCCCTGCT GTCGATGAACATGTTTGGAAGA	CAPS	<i>Bst</i> NI	–	+
T6D22 8/9	TCAAGAAGTTGGAGTGTTGGAA AAGGTAAAGCAGGGGAGGAA	CAPS	<i>Nsi</i> I	–	+
T6D22.2	ATCGTTTGCGTGAGGTTAGC ATGCTGCTCCAGAATTACCC	CAPS	<i>Mwo</i> I	–	+
T6D22 12/13	GGTGTTTTTAGTTAGGCTGAATTTGGA- TCTTATTGTATAATCTTATT <u>C</u> AGCT GGTCTGTGATTGCTGGTT	CAPS	<i>Pvu</i> II	+	–

The markers have been named according to the BAC and the ORF in which they were identified. In/del indicates an insertion/deletion has taken place, and the size of PCR fragments obtained for each ecotype is specified. CAPS, cleavage amplified polymorphic sequence. Plus and minus signs indicate from which ecotype the corresponding PCR fragment will be cut or not, respectively. Underlined bases in the T6D22 12/13 forward primer were introduced to create a CAPS between the Col and the C24 sequence.

**Table S2. Ovule and early seed abortion in *atlig1*<sub>mat</sub>/+, *dme*<sub>mat</sub>/+ and the double mutant**

Female × male	Aborted (% ± s.d.)	<i>n</i>
WT × WT	9±5.7	404
<i>dme-4</i> /+ × WT	13±8.3	390
<i>atlig1-3</i> /+ × WT	10.5±13.6	264
<i>atlig1-3</i> /+; <i>dme-4</i> /+ × WT	11.8±4.4	416

Siliques were dissected 6 days after pollination and the proportion of aborted seeds was measured. No significant increase in the rate of seed abortion was observed in the *atlig1*; *dme* double mutant indicating that the two mutations are not synthetic lethal.

*n*, number of seeds analysed.



**Table S3.  $\chi^2$  calculations for double-mutant seeds**

Hypotheses		Phenotypic classes				$\chi^2$	Degree of freedom	<i>P</i>
		[WT]	[ <i>dme</i> ]	[ <i>atlig1</i> ]	[ <i>atlig1/dme</i> ]			
<i>AtLIG1</i> and <i>DME</i> are in independent pathways and <i>atlig1</i> ; <i>dme</i> seeds show both phenotypes	Obs	34	75	21	0	242.3888889	4–1=3	<i>P</i> <0.001
	Exp (%)	26	13.7	13.7	46.6			
	Exp ( <i>n</i> =131)	34	18	18	61			
	(Obs-Exp) <sup>2</sup> /Exp	0	180.5	0.888888889	61			
The <i>atlig1</i> and <i>dme</i> mutations are synthetic lethal and <i>atlig1</i> ; <i>dme</i> seeds abort	Obs	34	75	21	0	242.3888889	4–1=3	<i>P</i> <0.001
	Exp (%)	26						
	Exp ( <i>n</i> =131)	34	18	18	61			
	(Obs-Exp) <sup>2</sup> /Exp	0	180.5	0.888888889	61			
<i>atlig1</i> is epistatic over <i>dme</i> and <i>atlig1</i> ; <i>dme</i> seeds have an <i>atlig1</i> phenotype	Obs	34	75	21	N.E.	221.6265823	3–1=2	<i>P</i> <0.001
	Exp (%)	26	60.3	13.7	–			
	Exp ( <i>n</i> =131)	34	18	79	–			
	(Obs-Exp) <sup>2</sup> /Exp	0	180.5	41.12658228	–			
<i>dme</i> is epistatic over <i>atlig1</i> and <i>atlig1</i> ; <i>dme</i> seeds have a <i>dme</i> phenotype	Obs	34	75	21	N.E.	1.091420534	3–1=2	<i>P</i> >0.5
	Exp (%)	26	13.7	60.3	–			
	Exp ( <i>n</i> =131)	34	79	18	–			
	(Obs-Exp) <sup>2</sup> /Exp	0	0.202531646	0.888888889	–			
Both mutations complement each other and <i>atlig1</i> ; <i>dme</i> seeds have a WT phenotype	Obs	34	75	21	N.E.	220.5573099	3–1=2	<i>P</i> <0.001
	Exp (%)	72.6	13.7	13.7	–			
	Exp ( <i>n</i> =131)	95	18	18	–			
	(Obs-Exp) <sup>2</sup> /Exp	39.16842105	180.5	0.888888889	–			

(WT) male will produce 25% WT seeds, 25% *dme* seeds and 25% *atlig1*; *dme* seeds. Owing to the incomplete penetrance of each mutation observed in the single-mutant control crosses (see Table 2), the proportion of *atlig1* and *dme* seeds showing a phenotype will be 22.25% ( $0.25 \times 0.89$ ) and 17.5% ( $0.25 \times 0.7$ ), respectively. Thus, the proportion of expected WT seeds is 35.25% [ $0.25 + (0.25 - 0.2225) + (0.25 - 0.175)$ ]. However, 35.25% of 523 is 184 seeds, and we obtained 218 WT seeds. The 34 extra seeds (218 – 184) are most likely to be double-mutant *atlig1*; *dme* seeds that show a WT phenotype due to the incomplete penetrance of each mutation. However, it is not possible to predict the degree of penetrance for each mutation in this double-mutant background as the penetrance is expected to vary depending on the genetic background. Thus, we calculated that out of 131 genetically *atlig1*; *dme* seeds (25% of 523), 34 are phenotypically WT, which enables us to calculate that the penetrance of both mutations amounts to 74% [ $(131 - 34)/131$ ]. Based on this result, we estimated that the penetrance of each mutation in the double-mutant background was 86% (sqrt of 0.74). Therefore, out of 131 genetically double-mutant seeds, we expect that 34 will have a WT phenotype, 18 will have a *dme* phenotype, 18 will have an *atlig1* phenotype, and 61 will show a phenotype resulting from the interaction of both mutations. The obtained data only fit a model in which *dme* is epistatic over *atlig1* ( $\chi^2=1.09$ , *P*>0.5), which indicates that *AtLIG1* acts downstream of *DME*, most likely by repairing the DNA breaks created by *DME* in the central cell.

Obs, observed.  
Exp, expected.  
N.E., none expected.

*A* female *atlig1-3/+*; *dme-4/+* crossed with a wild-type