Sperm entry is sufficient to trigger division of the central cell but the paternal genome is required for endosperm development in Arabidopsis

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SUMMARY
Fertilization in flowering plants involves two sperm cells and two female gametes, the egg cell and the central cell, progenitors of the embryo and the endosperm, respectively. The mechanisms triggering zygotic development are unknown and whether both parental genomes are required for zygotic development is unclear. In Arabidopsis, previous studies reported that loss-of-function mutations in CYCLIN DEPENDENT KINASE A1 (CDKA;1) impedes cell cycle progression in the pollen leading to the production of a single sperm cell. Here, we report that a significant proportion of single cdka;1 pollen delivers two sperm cells, leading to a new assessment of the cdka;1 phenotype. We performed fertilization of wild-type ovules with cdka;1 mutant sperm cells and monitored in vivo the fusion of the male and female nuclei using fluorescent markers. When a single cdka;1 sperm was delivered, either female gamete could be fertilized leading to similar proportions of seeds containing either a single endosperm or a single embryo. When two cdka;1 sperm cells were released, they fused to each female gamete. Embryogenesis was initiated but the fusion between the nuclei of the sperm cell and the central cell failed. The failure of karyogamy in the central cell prevented incorporation of the paternal genome, impaired endosperm development and caused seed abortion. Our results thus support that the paternal genome plays an essential role during early seed development. However, sperm entry was sufficient to trigger central cell mitotic division, suggesting the existence of signaling events associated with sperm cell fusion with female gametes.

KEY WORDS: Arabidopsis, Fertilization, Gene expression

INTRODUCTION
Fusion of the male and female gametes leading to zygotic activation is one of the most decisive events in the life cycle of sexually reproducing organisms (Stitzel and Seydoux, 2007). Gametic fusion comprises three steps, plasma membrane fusion, mixing of gamete cytoplasms and fusion of gamete nuclei (karyogamy). In animals, plasma membrane and cytoplasmatic fusions initiate electric signals that trigger a calcium-mediated signaling cascade leading to the completion of meiosis (Santella et al., 2004). Karyogamy requires remodeling of the male chromatin (Orsi et al., 2009). In plants, calcium signaling (Antoine et al., 2000; Roberts et al., 1994) and sperm cell nucleus chromatin remodeling (Ingouff et al., 2007) also take place after fertilization. However mechanisms causing zygotic activation and controlling karyogamy remain unknown and might differ between fertilization of the egg cell and fertilization of the central cell. With a few exceptions (Bayer et al., 2009; Leroy et al., 2007; Springer et al., 2000), the importance of parentaly inherited transcripts has not been established and when the onset of transcription and translation takes place is unclear (Meyer and Scholten, 2007; Vielle-Calzada et al., 2000; Weijers et al., 2001). A global delay of paternal genome expression is expected to make early seed development independent from the presence of the paternal genome.

Impairment of the function of CYCLIN DEPENDENT KINASE A1 (CDKA;1; also known as CDC2) was initially reported to cause the production of a single sperm cell able to perform fertilization (Iwakawa et al., 2006; Nowack et al., 2006; Ungru et al., 2008). Crosses involving pollen from cdka;1 plants produce a predominant category of small abortive seeds containing a single embryo surrounded by an endosperm-like tissue. The endosperm-like tissue does not express paternally provided reporters, which led to the proposal that the single cdka;1 sperm cell causes preferential fertilization of the egg cell, producing an embryo that triggers autonomous division in the central cell (Nowack et al., 2006). However, single sperm cells produced in pollen from other genetic backgrounds can fuse with either female gamete resulting in seeds that contain either a single embryo or a single endosperm (Chen et al., 2008; Frank and Johnson, 2009). We thus hypothesized that the phenotype produced by pollination with the cdka;1 pollen was more complex than initially reported. Understanding of fertilization mechanisms has recently progressed with new imaging protocols (Berger et al., 2008), and we used in vivo imaging of fertilization with markers allowing the observation of karyogamy and the monitoring of paternal expression. We observed that a large fraction of cdka;1 pollen released two sperm cells and produced several distinct seed phenotypes. The predominant phenotype was caused by a failure of karyogamy in the central cell and non-incorporation of the paternal genome after fertilization. The cdka;1 mutation thus allowed us to dissect further the double fertilization process in flowering plants.
MATERIALS AND METHODS

Plant material

The wild-type ecotype Columbia (Col-0) was provided by the Arabidopsis Stock Centre (http://www.arabidopsis.org). We used the allele cdka;1-1. The same Salk allele that was analyzed in detail in previous publications (Nowack et al., 2006; Nowack et al., 2007). The seeds were provided by A.S. The marker lines used originate from our previous studies (Ingouff et al., 2007; Ingouff et al., 2009). After 3 days at 4°C in the dark, seeds were germinated and grown on soil or plates. Plants were grown at 18°C in a growth room with an 8-hour day/16-hour night cycle until they formed rosettes. Flowering was then induced at 22°C in Conviron growth chambers with a 16-hour day/8-hour night cycle.

Imaging of fixed materials

For confocal microscopy, seeds were stained with Feulgen as described previously (Garcia et al., 2003) and examined with a Zeiss (Jena, Germany) LSM 510 microscope using the 488-nm excitation line of an argon laser and an emission filter LP 510 nm, with a ×63 Plan-Apochromat oil immersion objective (NA 1.4). Serial optical sections were recorded with a 0.4-0.6 μm depth and projections were realized using the Zeiss LSM 510 software.

Imaging of fresh material

Emasculated pistils expressing either promFWA::FWA-GFP (Kinoshita et al., 2004) or promEC1::HISTONE2B-mRFP1 (Ingouff and Berger, 2009) were crossed with pollen from the HTR10::HTR10-RFP line or the HTR12::HTR12-GFP line (Fang and Spector, 2005). Female markers allowed the nuclei of the female gametes to be visualized.

Fluorescence was sequentially acquired using laser scanning confocal microscopy, and live imaging of the fertilization process of wild type and cdka;1 mutants was performed as previously described (Ingouff et al., 2007). It is important to note that the percentages in Fig. 2 were established based on the predominant population of ovules, which gave signals that could be analyzed without doubt. Still, we failed to record a signal in about 15-20% ovules. Because the HTR10-RFP signal from cdka;1 sperm cell is lower than that from wild type, it is likely that we were unable to observe the signal from ovules fertilized by cdka;1 sperm cells, thus causing an underestimation of the proportion of fertilization by cdka;1 sperm cells. It is also important to note that fertilization events in the central cell are more difficult to score than those in the egg cell because the HTR10-RFP signal becomes comparatively more dilute. These technical problems can cause an under-evaluation of the percentage of fertilization by cdka;1 single sperm cells with the central cell.

Pollen from cdka;1-1; HTR10-RFP/HTR10-RFP plants were germinated in vitro as described previously (Boavida and McCormick, 2007).

RESULTS

Impairment of cell cycle progression affects chromatin composition in sperm cells

Early pollen development is initiated by an unequal mitotic division, producing a vegetative cell and a generative cell (McCormick, 2004). In Arabidopsis and maize, the generative cell divides into two sperm cells before pollen release at anthesis. In some other species, the generative division takes place in the pollen tube after germination. The loss of CYCLIN DEPENDENT KINASE A1 (CDKA;1) activity was reported to produce single sperm cells, but it was unclear whether this resulted from an arrest or a delay of generative cell division (Gusti et al., 2009; Iwakawa et al., 2006; Kim et al., 2008; Nowack et al., 2006). We germinated in vitro pollen from cdka;1-1/+ plants that expressed the sperm-specific marker HTR10-RFP (Ingouff et al., 2007). We could directly monitor the number of sperm cells per pollen tube and measured the proportion of pollen tubes containing two sperm cells. At anthesis, 6% of the cdka;1 pollen contained two gametes (3% of the total pollen population; Fig. 1A). The proportion of cdka;1 pollen tubes containing a single sperm cell continuously decreased during germination assays, suggesting that the cdka;1 generative cell underwent mitosis during the journey from the pollen tube to the female gametes (Fig. 1A). After 20 hours, 66% of pollen tubes contained two sperm cells. As cdka;1 does not cause pollen lethality before maturity and does not impair pollen germination (Iwakawa et al., 2006; Nowack et al., 2006), half of the pollen tubes produced by cdka;1-1/+ plants carry sperm with the cdka;1 mutation. We estimated that 16% of the total pollen tube population originated from cdka;1 pollen and carried two sperm cells. Hence, 32% of the cdka;1 pollen tubes contained two sperm cells. We thus concluded that cdka;1 delayed but did not prevent division of the generative cell.
We tested the effect of *cdka;1* mutation on the expression of genes associated with sperm cell differentiation, *DUO1* (Rotman et al., 2005), *GEX1* (Engel et al., 2005) and *HTR10* (Ingouff et al., 2007; Okada et al., 2005). The *cdka;1* mutation did not alter the expression of the sperm cell markers *GEX1* (Table 1; Fig. 1B,C) and *DUO1* (Table 1; Fig. 1D,E). This was consistent with previous data reporting that *cdka;1* pollen express *HTR10* (Brownfield et al., 2009). Although our cell fate study was limited by the number of sperm cell fate markers currently available, it suggested that *cdka;1* did not affect sperm cell fate.

However, we observed that the expression level of *HTR10-RFP* was variable in pollen produced by *cdka;1/+* plants (Fig. 1F) and estimated that 70% of sperm cells of *cdka;1* pollen express *HTR10* at levels lower than those of wild type (Table 1; Fig. 1F). We thus investigated whether the level of expression of another component of sperm cell chromatin was affected by *cdka;1*. We studied the expression of CENTROMERIC HISTONE 3 (CENH3) (Talbert et al., 2002) in *cdka;1* pollen. We also observed a reduction of CENH3 loading in *cdka;1* pollen carrying one or two sperm cells (Table 1; Fig. 1G). Because wild-type sperm cells initiate a new S phase before anthesis (Durbarry et al., 2005), it is likely that when the *cdka;1* generative cell divides it does not enter S phase, resulting in low levels of chromatin components, including HTR10 and CENH3.

In order to establish the proportion of *cdka;1* pollen tubes delivering two sperm cells, we monitored in vivo fertilization between wild-type ovules and *cdka;1/+* plants (*HTR10-RFP/HTR10-RFP* plants). We observed that 26.1% (*n=211, s.d.=8.3*) fertilization events by pollen from *cdka;1/+* plants correspond to delivery of single sperm cells (Fig. 2; see also Movies 1 and 2 in the supplementary material). We thus deduced that approximately 48% of *cdka;1* pollen delivered two sperm cells.

In conclusion, we propose that *cdka;1* does not affect sperm cell fate but delays the pace of the cell cycle, leading to the production of one or two sperm cells with altered chromatin composition.

**Determination of the timing of transcriptional onset after fertilization**

*HTR10* is a very divergent H3 variant (Okada et al., 2005) and its predominant incorporation in sperm cell chromatin indicates that it could be involved in large-scale expression regulation or chromatin compaction (Ingouff and Berger, 2010). We thus investigated in detail fertilization and early seed development following pollination from *cdka;1/+* plants.

In order to evaluate the success of fertilization, we required a marker reporting paternal expression as early as possible after karyogamy. We selected CENH3-GFP because it produces a distinct fluorescence from the expression of HTR10-RFP from the sperm cell (arrow) fertilizes the egg cell (e; the egg cell nucleus is marked by red fluorescence in the euchromatin). Additional strong green fluorescence from the synergids is autofluorescence (s). Arrowheads indicate a sperm nucleus delivering two sperm cells. Scale bar: 15 μm.

**Table 1. Effect of *cdka;1* on pollen development**

<table>
<thead>
<tr>
<th>Phenotype categories (see Fig. 2F,G)</th>
<th>+ (two sperm cells with wild-type expression level)</th>
<th>tw (two sperm cells with low expression level)</th>
<th>s (one sperm cell with wild-type expression level)</th>
<th>sw (one sperm cell with low expression level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR10</td>
<td>0.8</td>
<td>19.8</td>
<td>34.2</td>
<td>45.2</td>
</tr>
<tr>
<td>s.d. (<em>n=1500</em>)</td>
<td>0.2</td>
<td>6.6</td>
<td>7.4</td>
<td>18.2</td>
</tr>
<tr>
<td>CENH3</td>
<td>14.3</td>
<td>8.1</td>
<td>7.8</td>
<td>69.8</td>
</tr>
<tr>
<td>s.d. (<em>n=500</em>)</td>
<td>6.2</td>
<td>5.2</td>
<td>4.6</td>
<td>17.8</td>
</tr>
<tr>
<td>DUO1</td>
<td>98%, <em>n=150</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GEX1</td>
<td>95%, <em>n=75</em></td>
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*The measurements were made after orthogonal projections of confocal sections, such as those shown in Fig. 1 (the percentage was normalized to the *cdka;1* pollen population; s.d., standard deviation).*

**Fig. 2. Sperm entry in wild-type ovules fertilized by *cdka;1* pollen.** Wild-type ovules expressing the central cell (c) chromatin marker Histone2B-GFP (green) receive sperm cells delivered by pollen from *cdka;1* plants expressing *HTR10-RFP* in sperm cells (red). (A) Two sperm cells fuse with the egg cell (e; the nucleus is marked by a dotted circle) and the central cell (75.3%). (B) A single *cdka;1* sperm cell (arrow) fertilizes the egg cell (e; the egg cell nucleus is marked by red fluorescence from the expression of *HTR10-RFP* from the sperm chromatin; 18.9%). (C) A single *cdka;1* sperm cell (arrowhead) fertilizes the central cell (c; 5.8%). Arrows in A–C indicate a sperm nucleus fertilizing the egg cell. Additional strong green fluorescence from the synergids is autofluorescence (s). Arrowheads indicate a sperm nucleus delivering two sperm cells.
Single sperm cells from cdka;1 pollen fertilize either female gamete

We examined the phenotype of every seed produced by crosses between wild-type ovules and pollen from cdka;1/+ plants (Fig. 4). We also crossed ovules of a wild-type transgenic line expressing the HISTONE 2B-RFP in the egg cell to pollen from cdka;1/+ transgenic plants expressing the CENH3-GFP fusion protein (Fig. 5). Half of the seeds from crosses between wild-type ovules and pollen from cdka;1/+ plants differed markedly from the controls (n=736, 48% wild-type phenotype, s.d.=4.5). We documented the phenotypes of each class of abnormal seed development contrasting with the wild-type phenotype (Fig. 4A, Fig. 5A). Approximately 10-25% of the abnormal seeds contained either only fertilized endosperm with an unfertilized egg cell (Fig. 4B) or an elongated zygote with an unfertilized central cell (Fig. 4C). These seeds are likely to have originated from fertilization by cdka;1 pollen delivering a single sperm cell to the central cell or to the egg cell, respectively (Fig. 2). The fertilization markers showed a similar proportion of single fertilization of the egg cell (Fig. 5D) and the central cell (Fig. 5B), indicating that single sperm cells from cdka;1 pollen had an equal chance of fertilizing either female gamete. These minor classes of seeds had not been recognized in the previous report (Nowack et al., 2006), and their conclusion of preferential fertilization of the egg cell is not supported by our analysis. Earlier studies of other mutants had also concluded that both female gametes could be fertilized by single sperm cells (Chen et al., 2008; Frank and Johnson, 2009), or that both wild-type sperm cells were able to fertilize multiple egg cells from mutant ovules (Ingouff et al., 2010; Pagnussat et al., 2007). Our results thus further support that, in Arabidopsis, both sperm cells are able to fertilize either the egg cell or the central cell. However, because mutants were used as either the female or the male in all studies performed to date, it is still not possible to completely exclude some degree of specialization of wild-type Arabidopsis sperm cells regarding their ability to fuse with either female gamete, similar to that reported for other species (Lord and Russell, 2002; Russell, 1992).

cdka;1 prevents fusion between sperm and central cell nuclei leading to seed abortion

In addition to seeds produced by fertilization with single sperm cells, crosses between wild-type ovules and cdka;1 pollen also produced two other types of aborted seeds. The most abundant class of abnormal seeds contained a fertilized developing embryo surrounded by an enlarged central cell, which did not divide further than three or four divisions (Fig. 4D, Fig. 5C), similar to that reported in previous studies (Iwakawa et al., 2006; Nowack et al., 2006; Unguru et al., 2008). CENH3-GFP was not expressed in the endosperm nuclei (Fig. 5C), indicating that some step of fertilization of the central cell failed, although autonomous development, including nuclear division and cell growth, was initiated. In addition, we observed a class of seeds that showed no evidence of double-fertilization, as CENH3-GFP was expressed...
neither in the egg cell nor in the central cell, but the central cell still contained several nuclei (Fig. 5E). We could thus conclude that the autonomous onset of central cell division in absence of fertilization does not depend on the fertilization of the egg cell. Conversely, the absence of autonomous division of the central cell in seeds containing a fertilized zygote (Fig. 5D) indicated that the developing zygote does not trigger central cell division in the absence of fertilization. Previous studies had also reported that the development of a single embryo produced by pollen carrying a single sperm is not correlated with central cell division (Frank and Johnson, 2009; Chen et al., 2008; Pagnussat et al., 2007). In conclusion, the current data do not support that a signal from the fertilized zygote triggers central cell development, as had been proposed previously (Nowack et al., 2006).

What caused autonomous central cell division in the predominant class of seeds produced by the fertilization of wild-type ovules with cdka;1 sperm cells thus remained to be explained. In wild-type fertilization observed in crosses between wild-type ovules expressing the central cell marker pFWA-FWA-GFP and pollen from HTR10-RFP/HTR10-RFP plants, karyogamy takes place simultaneously in both fertilization products (Fig. 6A), as
reported earlier (Ingouff et al., 2007). By contrast, we observed single karyogamy events following fertilization in the seed population produced by crosses with pollen from cdka;1/+; HTR10-RFP/HTR10-RFP plants (31%, n=126). In these cases, the sperm nucleus in the central cell did not decondense and did not fuse with the central cell nucleus (Fig. 6B). In spite of failure of karyogamy in the central cell, mitosis was triggered (Fig. 6C,D). The proportion of failed karyogamy (62% of cdka;1 pollen) was comparable to the proportion of seeds with a fertilized embryo and a proliferative abortive endosperm amongst abnormal seeds produced by cdka;1 pollination (60 to 70%) (Fig. 4D, Fig. 5C). This proportion was higher than the proportion of fertilization events involving two cdka;1 sperm cells estimated from the direct observation of HTR10-RFP after sperm release (48%, Fig. 2). This discrepancy could be explained by the fact that cdka;1 sperm chromatin was loaded with low amounts of HTR10-RFP (Fig. 1), which hinders imaging fertilization, thus causing an underestimation of the percentage of two sperm cells released by cdka;1 pollen tubes. This hypothesis was also supported by the proportion of seeds produced by single cdka;1 sperm cells (26%-36%; Fig. 4B,C; Fig. 5B,D,E), which was lower than was expected from the proportions established by the direct inspection of HTR10-RFP at fertilization (52%, Fig. 2). We thus estimated that 60 to 70% of cdka;1 pollen delivers two sperm cells, which both fuse with each female gamete, but karyogamy usually fails in the central cell, preventing incorporation of the paternal genome and causing seed abortion.

**DISCUSSION**

**cdka;1 sperm allows insight into the early events of double-fertilization**

Our results indicate that the major seed phenotype caused by pollination with cdka;1/+ plants does not result from the delivery of a single mutant sperm cell to the egg cell followed by the induction of autonomous division of the central cell as reported previously (Nowack et al., 2006). In a predominant proportion, cdka;1 pollen produces two sperm cells, one of which successfully fertilizes the egg cell while the other activates central cell division, but karyogamy fails and endosperm development is subsequently aborted.

The mutant cdka;1 sperm cell is able to activate the onset of the cell cycle in the central cell. It is still not clear whether sperm entry also triggers transcriptional onset in the central cell. Our results thus suggest that mitotic activation in the central cell is not dependent on a successful karyogamy, or on expression of the paternal genome, or on embryo development. This conclusion is also supported by the central cell activation in the absence of fertilization observed in mutants deficient for the Polycomb group pathway (Chaudhury et al., 1997; Guitton et al., 2004). The signal causing activation of the central cell by cdka;1 sperm cells could originate from sperm recognition through the GCS1 pathway (Mori et al., 2006; von Besser et al., 2006), from delivery of a cytoplasmic activation signal (Bayer et al., 2009), membrane fusion, or from calcium signaling, as shown in the brown alga *Fucus* and in maize (Antoine et al., 2000; Roberts et al., 1994).

**Karyogamy is placed under distinct genetic controls in the central cell and in the egg cell**

In plants, there is limited knowledge of mechanisms controlling karyogamy. Failure of polar nuclei fusion during central cell differentiation can be associated with karyogamy failure but the mechanisms involved remain unclear (Maruyama et al., 2010; Portereiko et al., 2006). The fact that karyogamy with cdka;1 sperm fails preferentially in the central cell is intriguing. This specific defect might originate from the fact that the fertilized central cell enters mitosis immediately after fertilization, contrary to the zygote (Faure et al., 2002). The cell cycle delay caused by cdka;1 might prevent synchronization of the sperm nucleus with the central cell nucleus leading to karyogamy failure. An alternative hypothesis is based on the fact that following karyogamy, the sperm cell-specific histone3 HTR10 is actively removed in the fertilized egg cell but not in the fertilized central cell (Ingouff et al., 2007). It is possible that improper loading of HTR10 in the chromatin of cdka;1 sperm cells prevents male chromatin decondensation in the central cell, leading to karyogamy failure. By contrast, the active chromatin remodeling machinery in the zygote might be able to overcome the chromatin composition defect in cdka;1 sperm cells. Thus, we conclude that distinct mechanisms control karyogamy in the two events of double-fertilization.

**Determination of the timing of transcriptional activation**

The timing of expression of the paternal genome during seed development has been debated (Berger et al., 2008). It is difficult to assess precisely when the paternal allele of a gene is expressed because this depends on parental mRNAs and protein inheritance, and on the timing of translational and transcriptional activation. Several studies have argued for early transcriptional onset after fertilization (Andreuzza et al., 2010; Pillot et al., 2010; Ronceret et al., 2008), but the precise timing remained unclear. Here, we use CENH3-GFP to monitor the expression from the paternally provided copy and observe that CENH3-GFP proteins are produced in the endosperm and in the zygote within a few hours of fertilization. If CENH3-GFP proteins were inherited from the sperm cell proteins or from translated sperm cell mRNAs, we should detect CENH3-GFP in the centromeres of nuclei in proliferating central cells in seeds in which sperm cell cytoplasm was inherited and karyogamy failed. As this is not the case, we conclude that we observed de novo transcription from the paternal copy of the CENH3-GFP transgene. We thus propose that transcriptional and translational activation takes place in the endosperm and the zygote by about one hour and eight hours after fertilization, respectively.

**Importance of the paternal genome during early seed development**

The cdka;1 mutation that prevents incorporation of the paternal genome after fertilization of the central cell allowed us to assess the requirement of the paternal genome contribution to the early development of the endosperm. Prevention of incorporation of one parental genome had been used in mice (McGrath and Solter, 1984; Surani et al., 1984), leading to the demonstration of parental genomic imprinting. Our data suggest that endosperm development requires the contribution of the paternal genome to complete its development beyond the initial rounds of mitotic divisions. Our results thus support that essential loci are expressed in the endosperm from the paternal genome immediately after fertilization and do not support an extensive silencing of the paternal genome as has been proposed previously (Vielle-Calzada et al., 2000). This dependence on paternal genome expression might be specific to the endosperm, as elimination of the paternal genome from the embryo does not appear to affect seed development (Ravi and Chan, 2010), although in this study the timing and mechanisms of paternal genome elimination are not known.
In the autonomously proliferating central cell activated by cdk2;1 sperm entry, some of the paternally expressed loci are probably not expressed, or are not expressed at sufficient levels from the maternal genome, causing endosperm development failure and seed abortion. Only a couple of paternally expressed imprinted genes have been identified in Arabidopsis (Gehring et al., 2009; Makarevich et al., 2008) but the essential aspect of their function remains to be established. In Arabidopsis, depletion of Polycolyb group activity releases silencing of the paternally expressed gene PHERES1 (Makarevich et al., 2006). A central cell deficient for Polycolyb group activity is able to bypass the absence of paternal expression when activated by cdk2;1 sperm cell entry, leading to the production of viable seeds (Nowack et al., 2007). This might indicate that several paternally expressed imprint genes controlled by Polycolyb group activity are essential for endosperm development. We thus propose that, in the wild type, paternal genome activation after fertilization is essential for endosperm and seed development.

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