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 cytoplasmic 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 parentally 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.

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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/+*; *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/+* 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
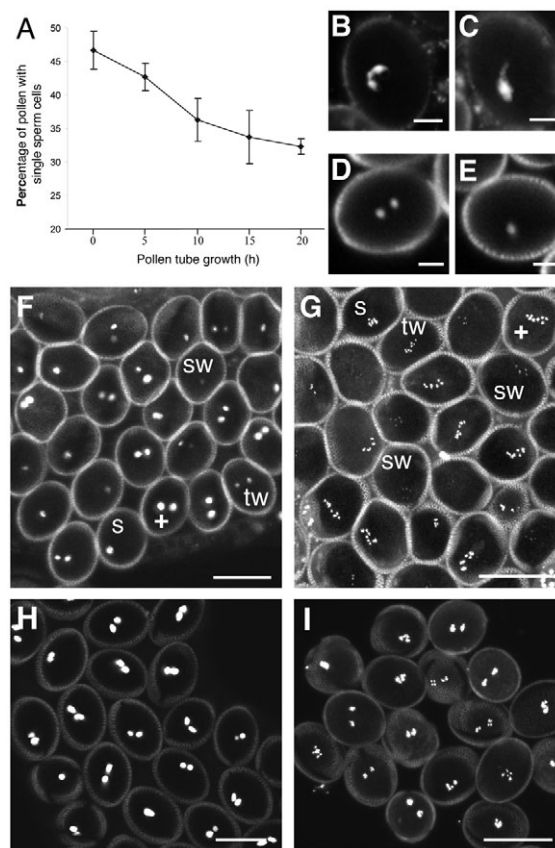

Fig. 1. Impact of the mutation *cdka;1* on sperm cells. (**A**) Percentage of growing pollen tubes with single sperm cells. Pollen from *cdka;1/+* plants were germinated in vitro and the number of sperm cells marked by HTR10-RFP was counted in each pollen tube (n=300 for each data point, error bars indicate s.d.). (**B**) Expression of *PromGEX1-GFP* in wild-type pollen marks cytoplasm of the two sperm cells. (**C**) Expression of *PromGEX1-GFP* in *cdka;1* pollen marks the single sperm cell. (**D**) Expression of *PromDUO1-DUO1-RFP* in wild-type pollen marks the nucleus of the two sperm cells. (**E**) Expression of *PromDUO1-DUO1-RFP* in *cdka;1* pollen marks the nucleus of the single sperm cell. (**F**) Pollen samples from *cdka;1/+*; *HTR10-RFP/HTR10-RFP* plants contain pollen with wild-type high signal in two sperm cells (+), but also weaker expression in two sperm cells (tw), and high (s) and weak (sw) expression in single sperm cells from *cdka;1* pollen. (**G**) Pollen samples from *cdka;1/+*; *CENH3-GFP/CENH3-GFP* plants show pollen with wild-type high signal in two sperm cells (+), but also weaker expression in two sperm cells (tw), and high (s) and weak (sw) expression in single sperm cells from *cdka;1* pollen. (**H**) Pollen from *+/+*; *HTR10-RFP/HTR10-RFP* plants. (**I**) Pollen from *+/+*; *CENH3-GFP/CENH3-GFP* plants. B-I are confocal sections; F-I are projections of 10-14 confocal sections. Scale bars: 8 µm.

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/+* 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/+*; 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 discrete signal at each centromere and could allow a distinction
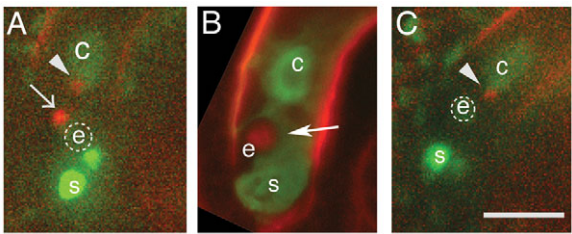

Fig. 2. Sperm entry in wild-type ovules fertilized by cdka;1 pollen. Wild-type ovules expressing the central cell (c) chromatin marker Histone2B-GFP (green) receive sperm cells delivered by pollen from cdka;1 plants expressing HTR10-RFP in sperm cells (red). (**A**) Two sperm cells fuse with the egg 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 fertilizing the central cell. Scale bar: 15 µm.

between de novo transcription and protein inheritance. This marker thus could provide a means to evaluate the timing of transcriptional activation after fertilization. We crossed ovules of a wild-type transgenic line expressing the HISTONE2B-RFP in the egg cell to pollen from wild-type or *cdka;1/+* transgenic plants expressing the CENH3-GFP fusion protein (Fig. 3). The control cross with wild-type pollen carrying the *CENH3-GFP* reporter produced seeds with the zygote nucleus marked by red fluorescence in the euchromatin and by green fluorescence in centromeres, resulting from paternal expression of CENH3-GFP (Fig. 3; *n*=72). In the endosperm, we observed that paternal CENH3-GFP expression marked centromeres as early as the first mitotic division (Fig. 3A). We observed this expression during subsequent nuclear divisions in the syncytial endosperm (Fig. 3B,C) (Ingouff et al., 2007). In the zygote, we detected paternal CENH3-GFP expression when the endosperm contained eight nuclei (Fig. 3C), corresponding to 8 hours after fertilization (Boisnard-Lorig et al., 2001; Faure et al., 2002). Given the limited amount of cytoplasm and proteins inherited from sperm cells, it is most likely that the high level of *CENH3-GFP* expression originates from de novo transcription from the paternal allele, rather than from paternally inherited mRNAs. We concluded that transcriptional onset takes place as early as the first division in the endosperm and from 8 hours after fertilization in the zygote.

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

Phenotype categories (see Fig. 2F,G)	+ (two sperm cells with wild-type expression level)	tw (two sperm cells with low expression level)	s (one sperm cell with wild-type expression level)	sw (one sperm cell with low expression level)
HTR10	0.8	19.8	34.2	45.2
s.d. (*n*=1500)	0.2	6.6	7.4	18.2
CENH3	14.3	8.1	7.8	69.8
s.d. (*n*=500)	6.2	5.2	4.6	17.8
DUO1	98%, *n*=150		96%, *n*=146	
GEX1	95%, *n*=75		93%, *n*=68	

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).
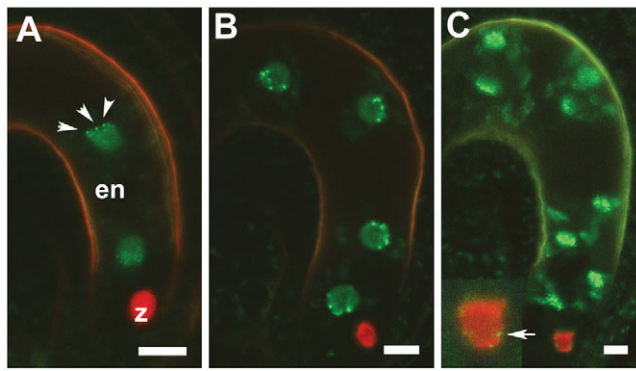
Fig. 3. Paternal expression of CENH3-GFP at the initiation of seed development. Crosses were made between wild-type ovules expressing the egg cell nucleus marker H2B-RFP (red) and pollen from wild-type plants carrying the *CENH3-GFP* transgene. (**A**) Immediately after fertilization the first endosperm (en) division is marked by the expression of CENH3-GFP localized to the centromeres (some are marked by arrowheads). No expression is observed in the zygote nucleus (red; z). (**B**) At 4 hours after fertilization, the endosperm contains four nuclei marked with paternal *CENH3-GFP* expression. No expression is detectable in the zygote. (**C**) At 8 hours after fertilization, the endosperm contains eight nuclei (here at telophase) and CENH3-GFP marks centromeres in the endosperm and in the zygote nucleus (inset, one arrow points to one of the centromeres). Only a fraction of centromeres is marked in each confocal section, hence not all ten centromeres are shown. Scale bars: 10 μm.

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
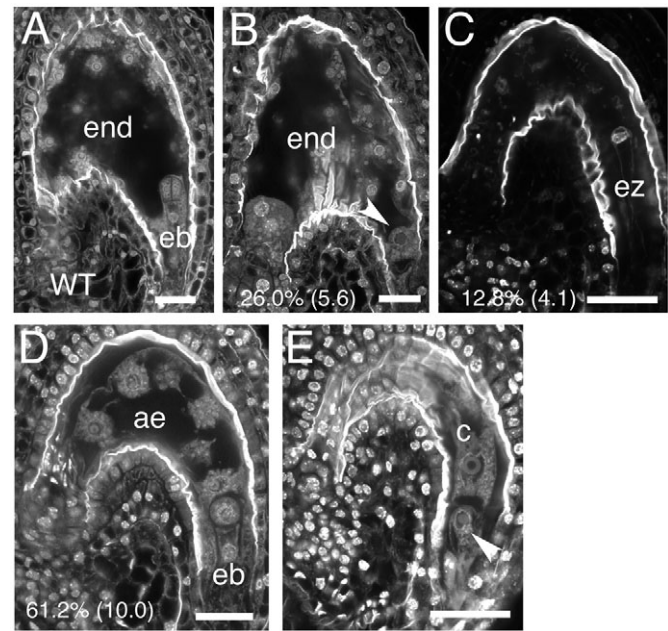

Fig. 4. Impact of pollination with *cdka;1* pollen on seed development analyzed from fixed samples. Crosses were made between wild-type ovules and pollen from *cdka;1/+*; plants. Seeds were fixed at 2 days after pollination. (**A**) Wild-type seeds contain an embryo with two apical cells (eb) surrounded by nuclei of the syncytial endosperm (end), showing successful double-fertilization. (**B**) A category of seeds fertilized by *cdka;1* pollen shows only endosperm development, similar to that of fertilized endosperm. The absence of egg cell fertilization is marked by the round shape of the nucleus, which can be compared to the egg cell nucleus from unfertilized ovules (**E**) (arrowheads in B and E). (**C**) A category of seeds fertilized by *cdka;1* pollen shows an elongated zygote (ez) but no proliferation from the central cell. (**D**) A category of seeds fertilized by *cdka;1* pollen shows an embryo with a single apical cell (eb) and proliferation of the central cell as autonomous endosperm (ae), limited in comparison with fertilized endosperm. The percentage of each class of seed from pollination by *cdka;1* pollen is shown (s.d. is shown in brackets, *n*=510). Confocal sections. Scale bars: 20 μm.

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; Ungru 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
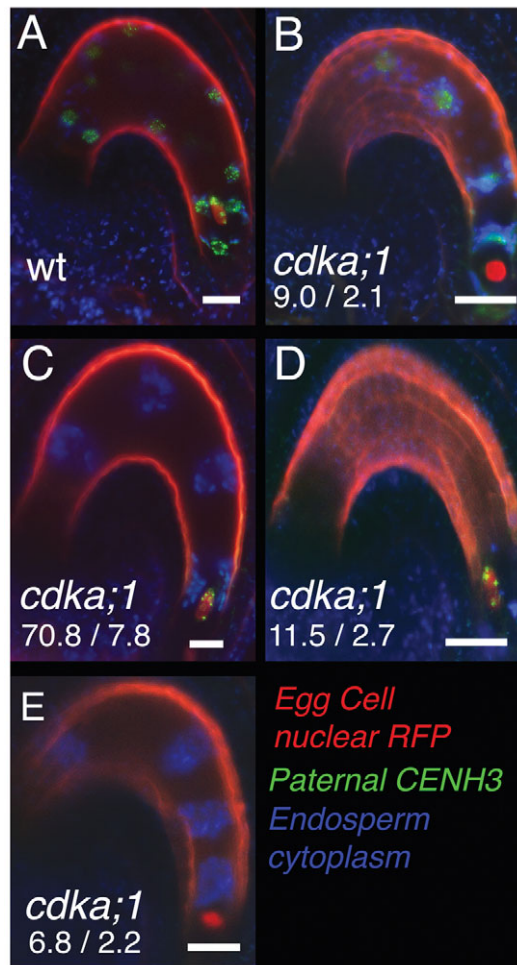
Fig. 5. Impact of pollination with *cdka;1* pollen on seed development analyzed in vivo. Crosses were made between wild-type ovules expressing the egg cell nucleus marker H2B-RFP (red) and pollen from *cdka;1/+*; *CENH3-GFP/CENH3-GFP* plants. Fertilization is marked by the expression of CENH3-GFP localized to the centromeres (green dots). The cytoplasm around endosperm nuclei is marked by autofluorescence (blue). Seeds were observed at 1 day after pollination. (**A**) Wild-type seeds show the nucleus of the zygote elongating (red with green dots) surrounded by nuclei of the syncytial endosperm (green dots surrounded by blue), showing successful double-fertilization. (**B**) A category of seeds fertilized by *cdka;1* pollen shows only fertilized endosperm development. The absence of egg cell fertilization is marked by the round shape of the nucleus and the absence of CENH3-GFP expression. (**C**) The predominant category of seeds fertilized by *cdka;1* pollen shows an elongated zygote surrounded by proliferating nuclei from the central cell, although no CENH3-GFP is expressed indicating that fertilization did not take place in the central cell. (**D**) A category of seeds fertilized by *cdka;1* pollen shows a fertilized zygote but no proliferation from the central cell. (**E**) A category of seeds fertilized by *cdka;1* pollen shows only proliferation of the central cell, although they show no sign of fertilization. The percentage of each class of seed from pollination by *cdka;1* pollen is shown (1.9% unfertilized ovules are not shown; s.d. is shown in parentheses brackets, *n*=226). Confocal sections. Scale bars: 20 μm.

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
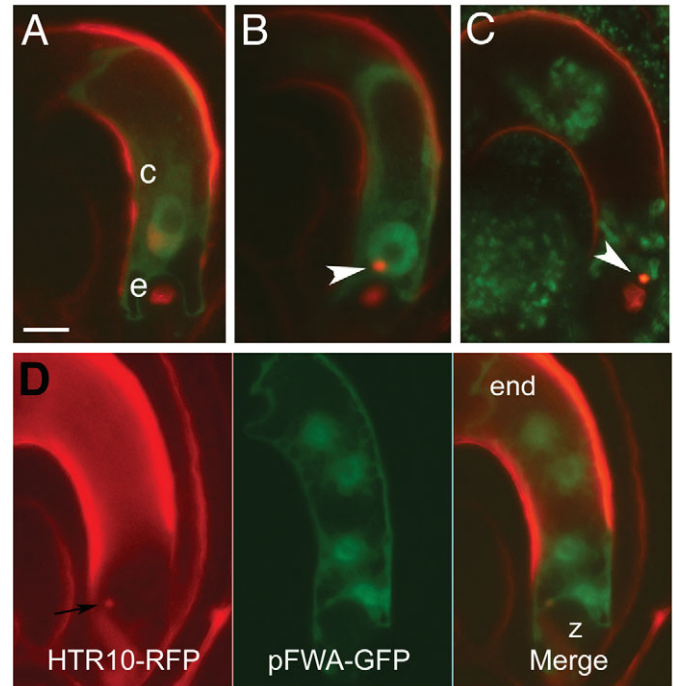

Fig. 6. Impact of fertilization with *cdka;1* sperm cells on gamete nuclei fusion. (**A**,**B**) Crosses were made between wild-type ovules expressing the central cell marker *pFWA-GFP* (green) and pollen from *cdka;1/+*; *HTR10-RFP/HTR10-RFP* (red) plants. Observations were made between 7 to 10 hours after pollination. (A) In the wild type, fertilization is marked by the decondensation of male chromatin in the egg cell (e) and in the central (c) cell nuclei after gamete fusion takes place. (B) An ovule fertilized with *cdka;1* pollen, where karyogamy does not take place in the central cell and the sperm nucleus remains condensed (arrowhead). (C) In spite of the absence of incorporation of the male chromatin, the central cell nucleus divides, leaving a residual condensed male chromatin by the side of the fertilized egg cell (arrowhead; this cross involves wild-type ovules and the green autofluorescence marks the cytoplasm surrounding endosperm nuclei). (**D**) Cross between ovules from a wild-type plant line carrying the transgene *pFWA-GFP* and pollen from *cdka;1/+*; *HTR10-RFP/HTR10-RFP* plants. The residual condensed male chromatin can be still observed (black arrow) when the endosperm contains four nuclei, marked by expression of *FWA*. At that stage, HTR10-RFP has been evicted from the zygote nucleus (z). Scale bar: 15 μm.

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

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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 *cdka;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 Polycomb group activity releases silencing of the paternally expressed gene *PHERES1* (Makarevich et al., 2006). A central cell deficient for Polycomb group activity is able to bypass the absence of paternal expression when activated by *cdka;1* sperm cell entry, leading to the production of viable seeds (Nowack et al., 2007). This might indicate that several paternally expressed imprinted genes controlled by Polycomb 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.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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