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A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis

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Double fertilization of the egg cell and the central cell by one sperm cell each produces the diploid embryo and the typically triploid endosperm and is one of the defining characteristics of flowering plants (angiosperms). Endosperm and embryo develop in parallel to form the mature seed, but little is known about the coordination between these two organisms. We characterized a mutation of the *Arabidopsis thaliana* Cdc2 homolog *CDC2A* (also called *CDKA;1*), which has a paternal effect. In *cdc2a* mutant pollen, only one sperm cell, instead of two, is produced. Mutant pollen is viable but can fertilize only one cell in the embryo sac, allowing for a genetic dissection of the double fertilization process. We observed exclusive fertilization of the egg cell by *cdc2a* sperm cells. Moreover, we found that unfertilized endosperm developed, suggesting that a previously unrecognized positive signal from the fertilization of the egg cell initiates proliferation of the central cell.

Plants have a complex life cycle involving the alternation of a diploid sporophytic and a haploid gametophytic phase. In flowering plants, the female spore (megaspore), generated from the sporophyte, typically develops through a series of syncytial nuclear divisions followed by 'cellularization' into a seven-celled female gametophyte (embryo sac) comprising three antipodals, two synergids, a binucleated central cell and the egg cell[1]. The male spore (microspore) undergoes a defined cell division program resulting in a male gametophyte (pollen grain) comprising a vegetative cell that encloses two sperm cells[2].

Cell divisions in plants, as in other higher eukaryotes, are controlled by a class of serine-threonine kinases called cyclin-dependent kinases (CDKs). Some of the central CDKs in plants are A-type CDK proteins. *A. thaliana* contains only one A-type CDK, called CDC2A (or CDKA;1), which can rescue the fission yeast *cdc2* mutant[3–5]. Previous studies with dominant negative variants showed that CDC2A activity is required for entering both mitosis and the DNA synthesis-phase in *A. thaliana*, maize and tobacco[6–8].

RESULTS

Characterization of a *cdc2a* mutant

To analyze CDC2A function, we isolated two T-DNA insertion mutants of *A. thaliana* (*cdc2a-1* and *cdc2a-2*; **Fig. 1a**). For each allele, we observed only heterozygous mutant plants. We analyzed these plants by 3′ RACE RT-PCR and identified a class of truncated mRNAs, which were not translated into proteins (**Supplementary Fig. 1** online). Therefore, the two isolated *cdc2a* mutants represent null alleles. A rescue construct introduced into heterozygous *cdc2a* mutants was able to revert the mutant phenotypes (**Tables 1** and **2**).

A phenotypic analysis of the vegetative parts of heterozygous *cdc2a* mutant plants showed no obvious differences compared with wild-type plants. Siliques of heterozygous mutant plants, however, contained ~47% aborted seeds (**Fig. 1b** and **Table 1**). Reciprocal crosses with wild-type plants showed that pollen from heterozygous *cdc2a* mutant plants (*cdc2a* pollen) caused seed abortion (**Table 1**). Consistent with this finding, the transmission rate of the mutated allele through the male side was severely reduced, whereas transmission through the female side was not affected (**Supplementary Table 1** online).

On the basis of these findings, we investigated pollen development more closely. In the wild type, the haploid microspores derived from a microspore mother cell undergo two rounds of mitotic divisions (**Fig. 2a–e**). After the first division, the developing gametophyte contains two cells, a vegetative cell with a larger nucleus enclosing a generative cell with a smaller nucleus (**Fig. 2d**). Next, the generative cell divides again so that the mature pollen contains one large vegetative and two small sperm cells (**Fig. 2e**). In *A. thaliana*, the two sperm cell nuclei in the mature pollen enter a long S phase, which continues during pollen germination and subsequent pollen tube growth and is completed immediately before fertilization[2,9].

During the first stages of pollen development, we observed no differences between *cdc2a* pollen and wild-type pollen (**Fig. 2a–d**). In ~42% of *cdc2a* pollen, however, the generative nucleus failed to progress through the second mitosis, resulting in pollen with one vegetative and only one other cell (**Fig. 2f** and **Table 2**). Yet this defect influenced neither pollen viability nor ability to germinate in *in vitro* assays (**Supplementary Fig. 2** online).

In the wild type, sperm cell development is marked by nuclear DNA condensation (**Fig. 2d,e**). The single mutant cell of *cdc2a* pollen

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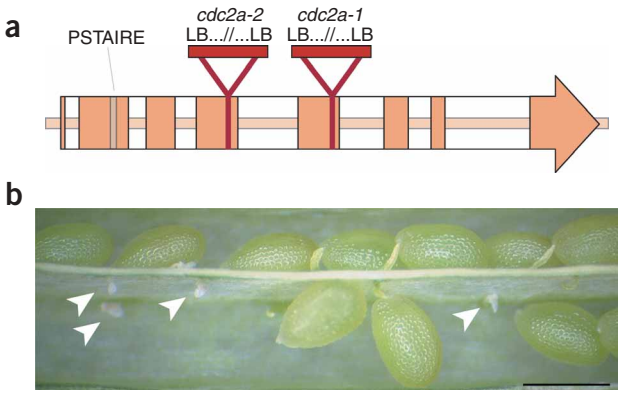

Figure 1 T-DNA insertion mutations of the *A. thaliana* gene *CDC2A*. (**a**) In *cdc2a-1* and *cdc2a-2* alleles, the T-DNA disrupts the coding frame downstream of the highly conserved and necessary for CDK function PSTAIRE domain in the fifth and fourth exons, respectively. (**b**) Siliques of heterozygous *cdc2a*$^{+/-}$ mutant plants with early aborted seeds (arrowheads). Scale bar, 500 μm. LB, left T-DNA border sequence.

appeared slightly larger and less condensed than sperm cell nuclei of wild-type pollen in DAPI staining (**Fig. 2e**,**f**). To investigate further the fate of this single cell, we analyzed the ultrastructure of *cdc2a* pollen (**Fig. 2g–l**). We compared mature *cdc2a* pollen with two-celled and mature three-celled wild-type pollen and found that the single mutant cell in *cdc2a* pollen was distinct from a sperm cell with a less compact nucleus. Overall, the ultrastructure of the mutant cell was similar to that of a generative cell in two-celled wild-type pollen (**Fig. 2h**,**j**,**l**). Conversely, the vegetative cell of *cdc2a* pollen resembled a vegetative cell in mature three-celled wild-type pollen with many electron light vacuoles (**Fig. 2g**,**i**,**k**). Taken together, these data suggest that the development of the generative cell is arrested or retarded in the mutant, whereas the vegetative cell continues to differentiate as in the wild type.

To determine at what stage the mutant cell is arrested, we analyzed the DNA content of *cdc2a* pollen at anther dehiscence. The single generative-like nuclei of mature mutant pollen had a slightly but significantly higher DNA content than sperm cells in wild-type pollen, which have progressed halfway through the final S phase at this stage and have a DNA content of ~1.5C (**Fig. 2m**)[9]. Conversely, we could easily separate the DNA content of the single generative-like cell in *cdc2a* pollen from that of pollen from tetraploid plants with sperm nuclei with a presumed average DNA content of 3C (**Fig. 2m**). Therefore, our data suggest that *cdc2a* pollen progresses through the second DNA-synthesis phase during gametophytic development but fails to undergo the second mitosis and remains at the level of 2C.

Table 1 Percentage of aborted seeds in *cdc2a* mutant lines

Parental genotype (female × male)	Normal (%)	Aborted (%)	Undeveloped (%)	*n*	Expected aborted (within 95% confidence limits)
Col-0 × Col-0	95.8	2.3	1.9	406	NA
cdc2a-1$^{+/-}$ selfed	51.3	46.7	1	452	43.6 < 47.5%[a] < 52.4
cdc2a-1$^{+/-}$ × Col-0	96.9	0.4	2.6	602	4.2 < 6.0%[a] < 8.5
Col-0 × *cdc2a-1*$^{+/-}$	57.8	42.2	0.5	472	34.8 < 39.0%[a] < 43.4
cdc2a-2$^{+/-}$ selfed	51.6	47	1.4	230	41.0 < 47.5%[b] < 55.0
Rescue hetero selfed	54.2	44.9	0.9	128	38.1 < 47.5%[b] < 58.0
Rescue homo selfed	98.5	0	1.5	134	0.0 < 0.0%[c] < 3.8

[a]Expected abortion as determined by transmission rate. [b]Expected abortion as in *cdc2a-1*$^{+/-}$. [c]Expected abortion in the wild type. *n*, number of seeds scored. NA, not applicable. Rescue hetero, homozygous *cdc2a-1*$^{-/-}$ mutant plants, complemented with a *proCDC2A:CDC2A* construct in heterozygous condition; Rescue homo, homozygous *cdc2a-1*$^{-/-}$ mutant plants, complemented with a *proCDC2A:CDC2A* construct in homozygous condition.

Egg cell fertilization promotes endosperm proliferation

Pollen with one vegetative and only a single generative-like cell is also formed in *duo* mutant plants[10]. The single cell, however, reaches a DNA content of >2.5C at anthesis, and even though the *duo* pollen can arrive at the embryo sac, it is unable to fertilize the egg cell. Because the single generative-like nucleus in *cdc2a* pollen had a DNA content of ~2C, mirroring the DNA content of wild-type sperm cells at the time of fertilization, we considered whether *cdc2a* pollen could accomplish fertilization.

In the wild type, one of the two sperm cells arriving at the embryo sac fertilizes the egg cell, generating the embryo, and the other sperm cell fertilizes the central cell, giving rise to the endosperm. Analysis of wild-type plants pollinated with *cdc2a* pollen (in which ~40% have only a single generative-like cell) showed that in all embryo sacs embryos were formed and developed normally until, in ~38% of cases, they were arrested at the globular stage (**Fig. 3a–i**,**p**). Notably, we found also developing endosperm (more than two nuclei) in 92% of all seeds ($n = 374$) of wild-type plants 36 h after pollination with *cdc2a* pollen. To test whether the central cell or the egg cell was fertilized by the single mutant cell of *cdc2a* pollen, we introduced a fertilization-marker construct comprising the *CDC2A* promoter fused to the gene β-GLUCURONIDASE (*GUS*) into wild-type and heterozygous *cdc2a* mutant plants. Pollen from these plants was used to fertilize wild-type flowers. In control plants fertilized with pollen from wild-type plants carrying the *CDC2A* promoter–*GUS* construct, GUS activity was found in both the endosperm and the embryo in 98% of cases (**Fig. 3j**). In contrast, in plants fertilized with pollen from heterozygous *cdc2a* mutant plants containing the *CDC2A* promoter–*GUS* construct, GUS activity could be detected exclusively in embryos in ~34% of cases (**Fig. 3k**,**l**). Therefore, the single male gamete of *cdc2a* pollen fertilized the egg cell exclusively, giving rise to a zygote and a developing embryo.

We confirmed that, although the central cell was not fertilized, endosperm started to develop by analyzing wild-type plants pollinated by *cdc2a* mutant plants containing the *CDC2A* promoter–*GUS* construct. In at least 50% of embryo sacs, which contained an embryo expressing the GUS reporter, proliferating and unstained endosperm could be recognized (**Fig. 3k** and **Supplementary Table 2** online). Central cell proliferation without fertilization has also been found in the fertilization independent seed (*fis*) and in the retinoblastoma protein related (*rbr*) mutants[11–17]. But the FIS group and RBR repress the proliferation of the central cell nucleus in the absence of fertilization, whereas the *cdc2a* mutant suggests that an unexpected positive signal from the fertilization of an egg cell initiates endosperm proliferation.

Coordination of early seed development

Because the development of the unfertilized endosperm in plants pollinated with *cdc2a* pollen was retarded and eventually ceased (**Fig. 3f–i**,**l**), we considered whether the arrest of embryo development was caused by an

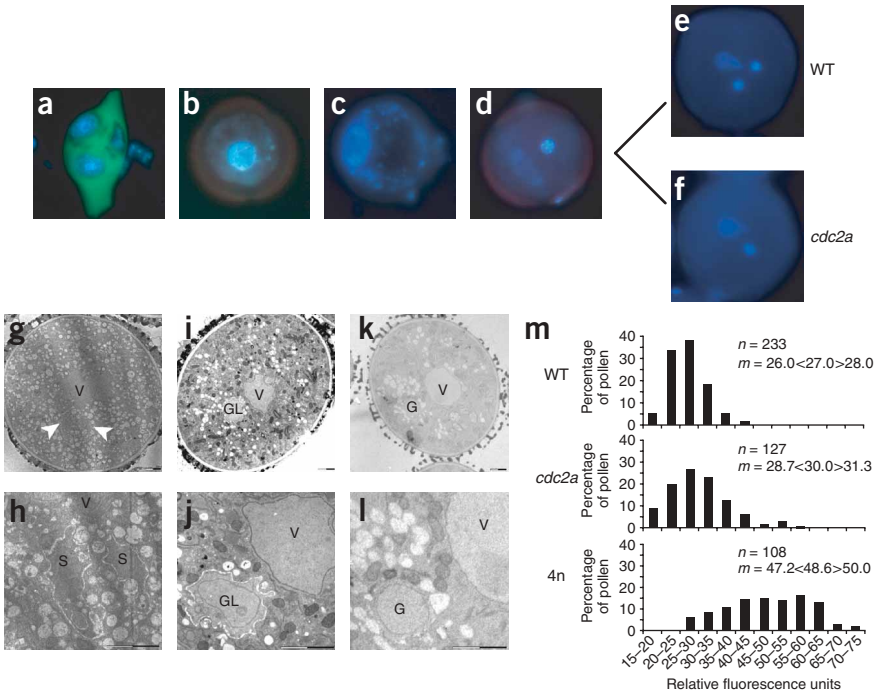

Figure 2 Phenotype of *cdc2a* pollen. (**a**–**f**) DAPI staining of wild-type and *cdc2a* pollen during gametophyte development. (**a**) Tetrad comprising four haploid microspores (counterstained with aniline blue). (**b**) Microspore. (**c**) Vacuolized microspore. (**d**) Two-celled pollen grain. (**e**) Mature wild-type pollen comprising one large vegetative and two small sperm cells. (**f**) Mature *cdc2a* pollen with a vegetative and only one other cell. (**g**–**l**) Transmission electron micrographs of wild-type and *cdc2a* pollen. (**g**) Mature three-celled wild-type pollen. Arrowheads indicate the two sperm cells. (**h**) Close-up view of **g**, showing the two characteristic sperm cells. (**i**) Mature *cdc2a* pollen with one vegetative and only one generative-like cell. (**j**) Close-up view of **i**, showing the vegetative cell nucleus and the single generative-like mutant cell. (**k**) Two-celled wild-type pollen. (**l**) Close-up view of **k**, showing the generative cell. (**m**) DNA measurements of sperm nuclei DNA content of wild-type and tetraploid plants (4n) in comparison with *cdc2a* mutant plants with only one generative-like cell at anther dehiscence. Scale bar, 2 μm. G, generative cell nucleus; GL, generative-like mutant nucleus; S, sperm cell nucleus; V, vegetative cell nucleus. *m*, mean value of relative fluorescence units in the 95% confidence interval determined by ANOVA; *n*, number of sperm nuclei measured. WT, wild-type.

underdeveloped endosperm. To examine this possibility, we used the *fis1* mutant, in which endosperm develops autonomously[18]. fis1 mutant plants were pollinated with *cdc2a* pollen and, as a control, with pollen of Columbia wild-type plants (**Fig. 3m**). As a further control, wild-type L*er* plants were pollinated with *cdc2a* mutant pollen (**Fig. 3n**). Only in the cross of homozygous fis1 mutant plants with *cdc2a* pollen was the *cdc2a* mutant phenotype partially rescued, reducing the fraction of embryos that aborted at globular stage by approximately half (43% versus 22%; **Fig. 3o** and **Table 3**).

Notably, the onset of autonomous endosperm development in unpollinated *fis1* mutants is delayed with respect to flower development and is also not found in all embryo sacs; under our growth conditions, endosperm started to develop autonomously in only 11% of all ovules when inspected 5 d after flowers had reached maturity (**Table 4**). In contrast, *fis1* mutants pollinated with *cdc2a* pollen, in which ~40% of the pollen grains contain only a single generative-like cell, endosperm developed in 92% of all seeds 5 d after pollination of mature flowers (**Table 4**). This result indicates that the presumed signal generated by the fertilization of the egg cell promotes immediate endosperm development in a *fis1* mutant background. Furthermore, this finding suggests that in *fis1* mutant plants, endosperm development is retarded owing to lack of an instructive signal (**Fig. 3q**).

Table 2 Phenotype of *cdc2a* pollen at anther dehiscence

	Normal (two sperm cells; %)	Aberrant (one generative-like cell; %)	*n*	Expected aberrant (within 95% confidence limits)
Col-0	98.5	1.5	1,380	NA
cdc2a-1$^{+/-}$	58.1	41.9	2,195	36.0% < 39%[a] < 42.1%
cdc2a-2$^{+/-}$	59.9	40.1	282	34.8% < 39%[b] < 43.4%
Rescue hetero	61.5	38.5	200	34.8% < 39%[b] < 43.4%
Rescue homo	98.5	1.5	200	0.7% < 1.5%[c] < 5.1%

[a]Expected aberrant as determined by transmission rate. [b]Expected aberrant as in *cdc2a-1*$^{+/-}$. [c]Expected aberrant as in the wild type. *n*, number of pollen grains scored. NA, not applicable. Rescue hetero and Rescue homo are defined in **Table 1**.

DISCUSSION

Here we presented the analysis of a mutant in the *A. thaliana* Cdc2 homolog *CDC2A*. We could not recover homozygous mutant plants; sequence homology and the study of dominant negative versions suggest that CDC2A is one of the main kinases controlling cell cycle progression in *A. thaliana*[3–8]. The primary *cdc2a* mutant phenotype is a failure to progress through the second mitotic division during male gametophyte development. *cdc2a* pollen undergoes the first mitosis without apparent disturbances. Development of the female gametophyte, which includes three rounds of nuclear divisions, was not affected in the mutant. Furthermore, in a fraction of the progeny of self-pollinated plants, probably representing the homozygous mutant offspring, embryo development arrested after only a few cell divisions. Other kinases may compensate for the loss of CDC2A during these developmental stages. But CDC2A is the only A-type CDK present in the *A. thaliana* genome and the only kinase found to rescue a yeast cdc2 mutant[3–5]. Taking into account the fact that CDKs are regulated at the post-translational level by phosphorylation and cofactor binding rather than by protein degradation[19], we favor the hypothesis that surplus CDK protein or stored mRNA from premeiotic stages supplies enough activity for a few cell cycle rounds that would allow completing the developmental program of the female gametophyte and even a few cell divisions during embryo growth.

The missing mitosis on the male side creates a special situation that results in pollen with one vegetative cell and only one instead of two gametes. The single gamete had features of a generative cell, yet its DNA content of 2C mirrored that of a mature sperm cell. Mutant

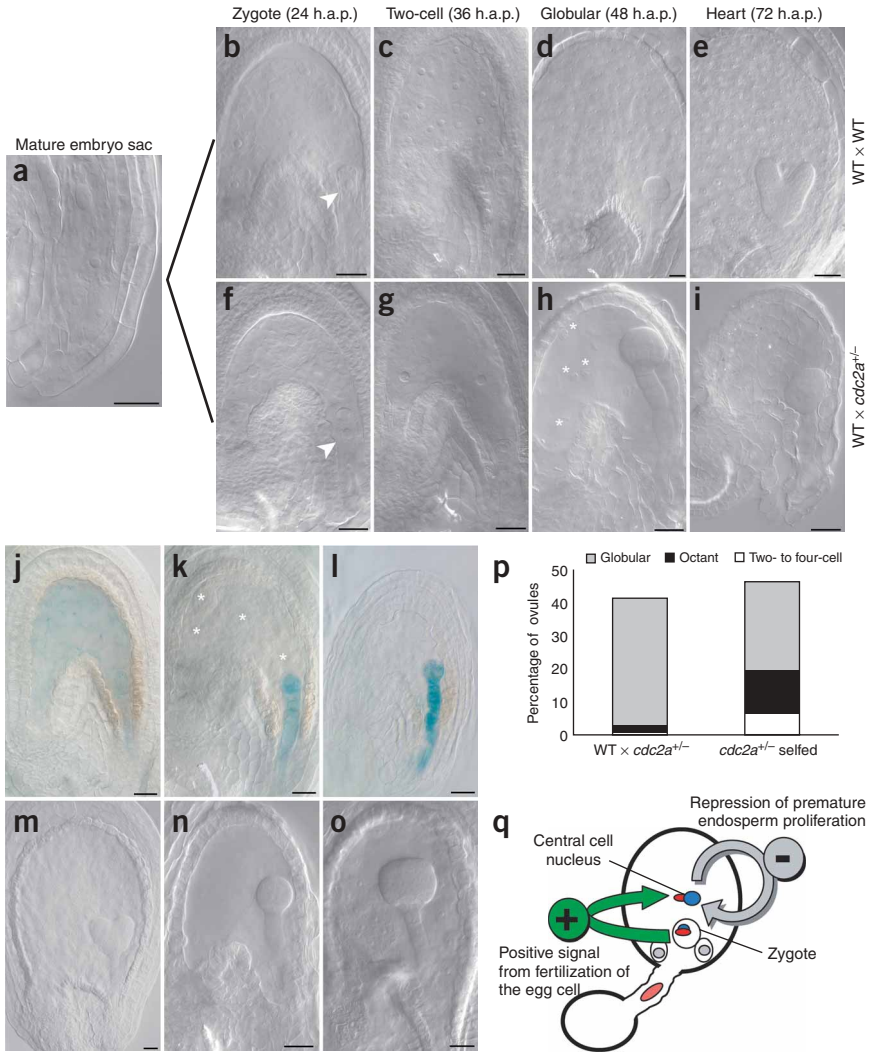

Figure 3 Embryo sac development in plants fertilized with *cdc2a* pollen. (**a**) Wild-type mature embryo sac immediately before fertilization. (**b**–**e**) Wild-type embryo development during the first 72 h after pollination (h.a.p.). (**b**) 24 h.a.p., zygote (arrowhead) with endosperm that has undergone three to four rounds of nuclear divisions. (**c**) 36 h.a.p., two-celled embryo; at this stage, 96% of the seeds contained endosperm with 32 or more nuclei ($n = 230$). (**d**) 48 h.a.p., globular stage embryo with syncytial endosperm nuclei evenly distributed over the central cell. (**e**) 72 h.a.p., heart-stage embryo; the endosperm started to 'cellularize'. (**f**–**i**) Seed development in wild-type plants pollinated with *cdc2a* pollen. No evidence for aneuploidy or aborted mitoses (*i.e.*, enlarged cells with multiple nuclei or irregular mitotic figures) was found. (**f**) 24 h.a.p., zygote (arrowhead) and central cell with four large endosperm nuclei. (**g**) 36 h.a.p., two-celled embryo surrounded by retarded endosperm with only 4–16 nuclei (24% of all seeds, $n = 374$). (**h**) Seed 48 h.a.p., globular stage embryo and remnants of endosperm nuclei and cytoplasm in the central cell (asterisks). (**i**) 72 h.a.p.; the embryo and the surrounding sporophytic tissue started to decay. (**j**) Wild-type seed 36 h.a.p. with pollen expressing a *proCDC2A:GUS* fusion construct, with blue staining in both the embryo and the endosperm. (**k**) Wild-type seed 36 h.a.p. with *cdc2a* pollen expressing a *proCDC2A:GUS* fusion construct, with blue staining exclusively in the developing embryo, as found in 34% of seeds with GUS staining ($n = 59$). Asterisks mark endosperm nuclei. (**l**) Wild-type seed 72 h.a.p. with *cdc2a* pollen expressing a *proCDC2A:GUS* fusion construct. Aborting seed with blue staining in the embryo. (**m**) Seed of a homozygous *fis1* mutant after pollination with Col-0 wild-type pollen. Seed with a heart-staged embryo and slightly overproliferated chalazal endosperm. (**n**) Seed of a L*er* wild-type plant after pollination with *cdc2a* pollen showed the same abortion phenotype as Col-0 wild type after pollination with *cdc2a* pollen. (**o**) Seed of a homozygous *fis1* mutant after pollination with *cdc2a* pollen. Note the proliferating endosperm in comparison with seeds shown in **h** and **l** with a globular stage embryo. (**p**) Diagram comparing the stages of embryo arrest in wild-type plants pollinated with *cdc2a* pollen to selfed *cdc2a*$^{+/-}$ plants. In selfed *cdc2a*$^{+/-}$ plants, in which one-quarter homozygous *cdc2a*$^{-/-}$ offspring is expected, a higher proportion of earlier embryo arrest (two- to four-celled and octant embryo) was found, consistent with a requirement of CDC2A for cell cycle progression. (**q**) Model for crosstalk between embryo and endosperm during wild-type seed development. Endosperm proliferation is blocked before fertilization by the FIS group and RBR. Fertilization of the egg cell promotes non-cell-autonomous endoperm proliferation. Only after fusion of the second sperm cell with the central cell nucleus is the locally acting FIS and RBR block removed and does full endosperm development commence. Scale bars, 20 μm. WT, wild-type.

pollen was viable and could reach and fertilize a female gamete. This fertilization, however, led to a paternal effect and caused seed abortion.

By analyzing a fertilization marker, we observed that the single gamete of *cdc2a* pollen fertilized only the egg cell and not the central cell. Three scenarios could explain this preferential fertilization. First, physical constraints in the embryo sac may be responsible, such as the relative position or accessibility of the egg cell nucleus versus the central cell nucleus for the arriving sperm cell. Second, active signaling could be involved, and the egg cell might attract the first sperm nucleus. Finally, sperm cells could be predetermined for fertilization with the egg and the central cell, respectively. To our knowledge, no reports pointing toward a sperm cell dimorphism in *A. thaliana* have been published thus far. To identify the molecular nature of the disparity of fertilization processes that we detected, the *cdc2a* mutant might be of use, because it allows a genetic dissection of the double fertilization process.

We found that upon exclusive fertilization of the egg cell, the endosperm also started to develop, suggesting that a positive signal is the 'starting gun' for proliferation of the central cell. Endosperm proliferation is also regulated by the action of the *FIS* gene complex[11–17]. Our data suggest that early seed development is

Table 3 Seed development in *fis* × *cdc2a-1*$^{+/-}$ plants

Parental genotype (female × male)	Normal (%)*	Undeveloped (%)	Autonomous endosperm (%)	Aborted (%)	*n*
L*er* × *cdc2a-1*$^{+/-}$	49	7	0	43	203
fis1$^{-/-}$ × *cdc2a-1*$^{+/-}$	68	5	4	22	222
fis1$^{-/-}$ × Col-0	89	5	1	5	336

*Embryos at globular stage.

Table 4 Endosperm development in *fis1* × *cdc2a-1*$^{+/-}$ plants

Parental genotype (female × male)	Developed endosperm (%)	Undeveloped endosperm (%)	Not analyzable (%)	*n*
fis1$^{-/-}$ unfertilized	11	74	15	153
fis1$^{-/-}$ × *cdc2a-1*$^{+/-}$	92	7	1	60

administrated by two signaling pathways: the release of the FIS-dependent proliferation block of the central cell and the positive signal from the fertilization of the egg cell that we identified. These results introduce a new concept in angiosperm seed development.

METHODS

Plant material and growth conditions. *A. thaliana* plants used in this study were derived from the Columbia-0 (Col-0) and the Landsberg *erecta* (L*er*) accessions. We obtained the *cdc2a-1* allele (SALK_106809.34.90.X) from the SALK T-DNA insertion collection and the *cdc2a-2* allele (Koncz_51209) from Koncz collection[20]. We obtained seeds homozygous with respect to the *fis1* allele from A. Chaudhury (Commonwealth Scientific and Industrial Research Organization (CSIRO) Plant Industry, Australia); these are in the L*er* accession[18]. Seeds were germinated on soil or MS-2 medium and grown in a Percival chamber with a 14 h:10 h light/dark cycle at 20 °C. We determined all genotypes by PCR. Primer sequences are given in **Supplementary Table 3** online.

DNA and RNA analysis. We carried out allele-specific PCRs to determine the T-DNA insertion sites using the primers J504 (left border T-DNA primer for *cdc2a-1*) and hook1 (left border T-DNA primer for *cdc2a-2*) and N034 and N035 (for wild-type *CDC2A*). To identify homozygous knockout plants rescued by a *proCDC2A:CDC2A* construct, we used the primers N048 and N049. For the rescue construct, we used a region of 2,000 bp 5′ upstream of the *CDC2A* start codon together with the *CDC2A* cDNA. To obtain a *CDC2A* promoter reporter construct, we fused the same 5′ region to *GUS*. Primer sequences are given in **Supplementary Table 3**.

Histology. We prepared pistils and siliques of different developmental stages as described previously[21]. We fixed dissected siliques on ice with FAA (10:7:2:1 ethanol:distilled water:acetic acid:formaldehyde (37%)) for 30 min, hydrated them in a graded ethanol series to 50 mM $NaPOH_4$ buffer (pH 7.2) and mounted them on microscope slides in a clearing solution of 8:2:1 chloral hydrate:distilled water:glycerol. We cleared the specimens for 1 h at 4 °C before inspecting them. We carried out light microscopy with a Zeiss Axiophot microscope using DIC optics.

We carried out whole-mount GUS assays as previously described[22] followed by chloral hydrate clearing as described above.

We stained mature pollen at the stage of anther dehiscence with a DAPI solution (2.5 μg ml^{-1} DAPI in 50 mM phosphate-buffered saline (pH 7.2) with 0.01% Tween20 and 5% dimethylsulfoxide) for 1 h. We quantified the DAPI fluorescence intensity and subtracted the background fluorescence using the DISKUS software package (Carl H. Hilgers–Technisches Büro, version 4.30.19). We normalized the obtained values against wild type and analyzed them statistically by Analysis of Variance Between Groups (ANOVA) using the STATISTICA software package.

URL. The SALK T-DNA insertion collection is available at http://signal.salk.edu/.

Accession codes. GenBank: T-DNA insertion sites for *cdc2a-1*, DQ156166 and DQ156167; T-DNA insertion sites for *cdc2a-2*, DQ156168 and DQ158862.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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