Developmental Cell
Short Article

Independent Control by Each Female Gamete Prevents the Attraction of Multiple Pollen Tubes

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http://dx.doi.org/10.1016/j.devcel.2013.03.013

SUMMARY

In flowering plants, double fertilization is normally accomplished by the first pollen tube, with the fertilized ovule subsequently inhibiting the attraction of a second pollen tube. However, the mechanism of second-pollen-tube avoidance remains unknown. We discovered that failure to fertilize either the egg cell or the central cell compromised second-pollen-tube avoidance in *Arabidopsis thaliana*. A similar disturbance was caused by disrupting the fertilization-independent seed (FIS) class *polycomb*-repressive complex 2 (FIS-PRC2), a central cell- and endosperm-specific chromatin-modifying complex for gene silencing. Therefore, the two female gametes have evolved their own signaling pathways. Intriguingly, second-pollen-tube attraction induced by half-successful fertilization allowed the ovules to complete double fertilization, producing a genetically distinct embryo and endosperm. We thus propose that each female gamete independently determines second-pollen-tube avoidance to maximize reproductive fitness in flowering plants.

INTRODUCTION

Double fertilization depends on orchestrated communication among male and female gametophytic cells. Most flowering plants, including *Arabidopsis thaliana*, produce ovules containing a seven-celled female gametophyte that consists of an egg cell, a central cell, three antipodal cells, and two synergid cells that secrete attractants for the pollen tube (male gametophyte) (Maheshwari, 1950; Yadegari and Drews, 2004; Takeuchi and Higashiyama, 2011). After pollen tube arrival, one of the synergid cells degenerates and the pollen tube discharges two sperm cells, which fertilize the egg cell and central cell to produce the embryo and endosperm, respectively. Double fertilization is normally completed by the first pollen tube, but recent studies of *A. thaliana* have demonstrated that fertilization failure by the first pollen tube is recovered by the second pollen tube (Kasahara et al., 2012; Beale et al., 2012). Multiple-pollen-tube-mediated fertilization, termed polysiphonogamy (Kasahara et al., 2012), is essential for fertilization recovery; however, multiple pollen tubes approaching (or polytubey) increases the risk of multiple fertilizations. Thus, flowering plants have a "polytubey blocking system" to avoid attracting a second pollen tube after successful fertilization.

Recently, several peptides were identified as signaling molecules secreted from the egg cell. For example, ZmEAL1 determined antipodal cell fate in maize (Krohn et al., 2012), and EC1 family peptides induced activation of two sperm cells in *A. thaliana* (Sprunck et al., 2012). In particular, EC1 family peptides are also involved in the polytubey blocking system by mediating successful double fertilization. However, the role of fertilization in the polytubey blocking system in the egg cell and the central cell, which occur almost simultaneously, is poorly understood.

More than 80 years ago, a peculiar fertilization sequence that produced a genetically distinct embryo and endosperm was reported in maize (Sprague, 1929). It was termed heterofertilization. It was predicted that heterofertilization was caused by independent fertilization by two genetically distinct pollen tubes (Sprague, 1932; Kato, 2001). This prediction was supported by genetic data and observations of ovules with multiple pollen tubes (Rhoades, 1934); nevertheless, direct evidence for this association is lacking. Moreover, these reports did not show the regulatory mechanisms underlying the attraction of the second pollen tube.

In this study, we used *cdka;1* mutant pollen, which randomly induces single fertilization in either the egg cell or central cell (Aw et al., 2010), and we found that egg and central cell fertilization act independently and synergistically to induce a strong polytubey block. Genetic analysis revealed a role for FIS *polycomb*-repressive complex 2 (FIS-PRC2) in the central cell pathway of the polytubey block. We also identified examples of heterofertilization in *Arabidopsis*, including the mechanism, which involves a higher percentage of the second-pollen-tube attraction due to a weak polytubey block by the single fertilization events.

RESULTS AND DISCUSSION

Arabidopsis Has a Dual-Control Polytubey Blocking System

The egg cell and central cell are fertilized by two sperm cells carried by a single pollen tube during double fertilization

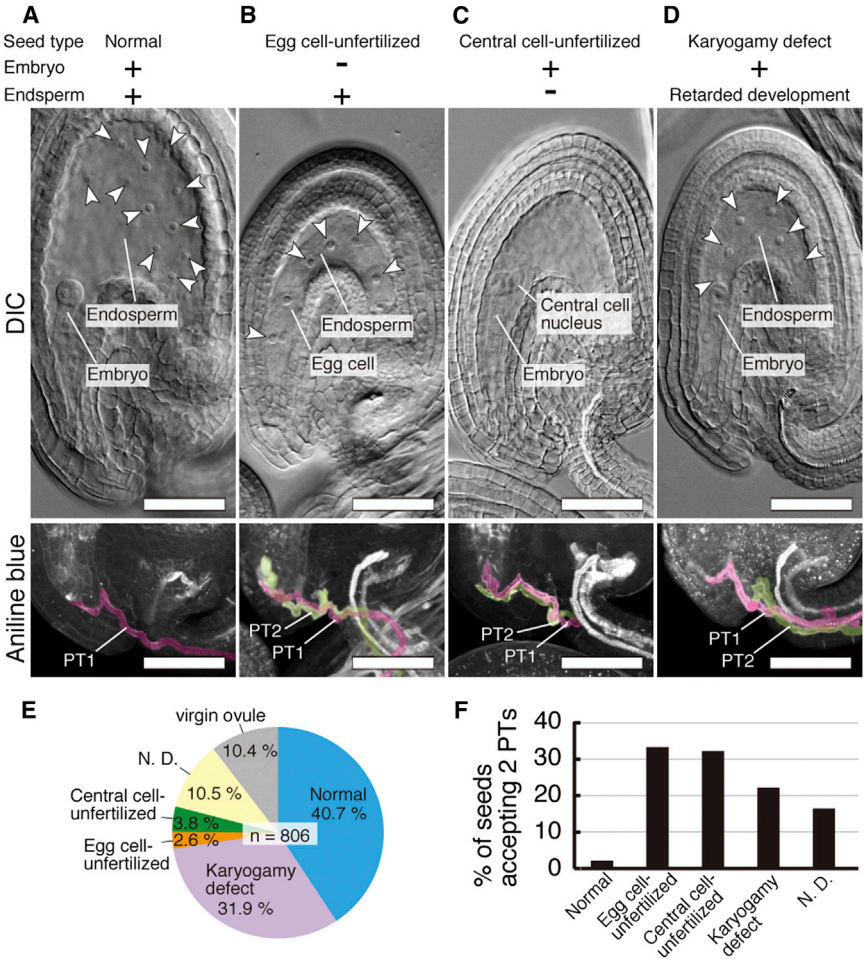

Figure 1. Second-Pollen-Tube Attraction Induced by *cdka;1*/+ Pollination

(A–D) Seed development (DIC) and pollen tube insertion (aniline blue) were analyzed in each seed obtained from the pollination between wild-type pistil and *cdka;1*/+. A single pollen tube was usually received in a normal seed (A). However, insertions of two pollen tubes were frequently observed in egg-cell-unfertilized seeds (B), central-cell-unfertilized seeds (C), and seeds with a karyogamy defect in the central cell (D). Positive (+) or negative (−) symbols indicate development of the embryo and endosperm in each seed type. Arrowheads indicate the endosperm nuclei.

(E and F) Frequency of seeds (E) and percentages of second-pollen-tube insertions (F) observed in the *cdka;1*/+ pollination. The frequencies of single fertilizations in (E) were less than those previously reported (Aw et al., 2010), which might be due to differences in growth conditions. ND, not identified (determined) due to seed abortion. PT1/PT2, pollen tube 1/pollen tube 2. Scale bars, 50 μm.

(Figure S1 available online). We examined whether the signal(s) for the initiation of the polytubey block originated from the egg cell and/or central cell after fertilization in *A. thaliana*. To dissect the two fertilization events, we used *cdka;1* mutant pollen, a subset of which contains a single sperm-like cell that randomly fertilizes either the egg cell or the central cell (Aw et al., 2010). As the pollen donor was heterozygous for *cdka;1*, half of the siblings had normal seeds with an embryo and multinucleate endosperm, indicating fertilization of the egg cell and central cell, respectively. The normal seeds displayed a strong polytubey block, because only 2.1% of the seeds accepted two pollen tubes (Figures 1A and 1F). In contrast, two pollen tubes were inserted at a higher percentage in those seeds that had an endosperm and undeveloped egg cell (33%, n = 21, Figures 1B and 1F). This suggested that egg cell fertilization plays an important role in blocking polytubey. A similarly high percentage was observed in seeds containing an embryo and undeveloped central cell nucleus (32%, n = 31, Figures 1C and 1F). These surprising data indicate the "dual control" nature of the polytubey block by the fertilized egg cell and fertilized central cell. Importantly, two pollen tubes were targeted with higher percentages when both female gametes were unfertilized (~80%, reported previously by Kasahara et al., 2012). This implies that the two signaling pathways, the egg cell pathway and central cell pathway, are independent of each other and act synergistically to induce a strong polytubey blocking response.

The involvement of central cell fertilization was supported by the other type of seed displaying retarded endosperm development and a morphologically normal embryo (n = 257, Figures 1D and 1E). This type of seed was reported to be produced by a defect in central-cell-specific nuclear fusion (or karyogamy) after successful double fertilization (plasmogamy); two sperm cells were frequently generated from a single sperm-like cell during pollen tube growth in *cdka;1* (Aw et al., 2010). This type of seed displayed a high percentage of two-pollen-tube insertions, indicating the importance of the postfertilization events, including karyogamy, for polytubey blockade activation, at least in the central cell.

The Polytubey Block Is Rapidly Initiated after Double Fertilization

Pollen tubes of *A. thaliana* are guided by diffusible signals from the ovules, including AtLURE1 attractant peptides secreted from the two synergid cells in the female gametophyte (Figure S1; Takeuchi and Higashiyama, 2012). After the pollen tube bursts and the death of a synergid cell occurs, the persistent synergid cell either ceases to attract pollen tubes or continues to attract them, depending on its fertilization state (Kasahara et al., 2012; Beale et al., 2012). To evaluate the attraction activity of ovules, we performed semi-in-vitro pollen-tube attraction assays (Palanivelu and Preuss, 2006; Hamamura et al., 2011). First, wild-type pistils were pollinated with a heterozygous mutant of *GENERATIVE CELL SPECIFIC 1* (*gcs1*, also called *hapless 2*) that produces fertilization-defective sperm cells (Mori et al., 2006; von Besser et al., 2006). To visualize the fertilization state, we used different nuclear markers expressed from a constitutively active *Ribosomal Protein Subunit 5A* (*RPS5A*) promoter: GFP-fused Histone 2B

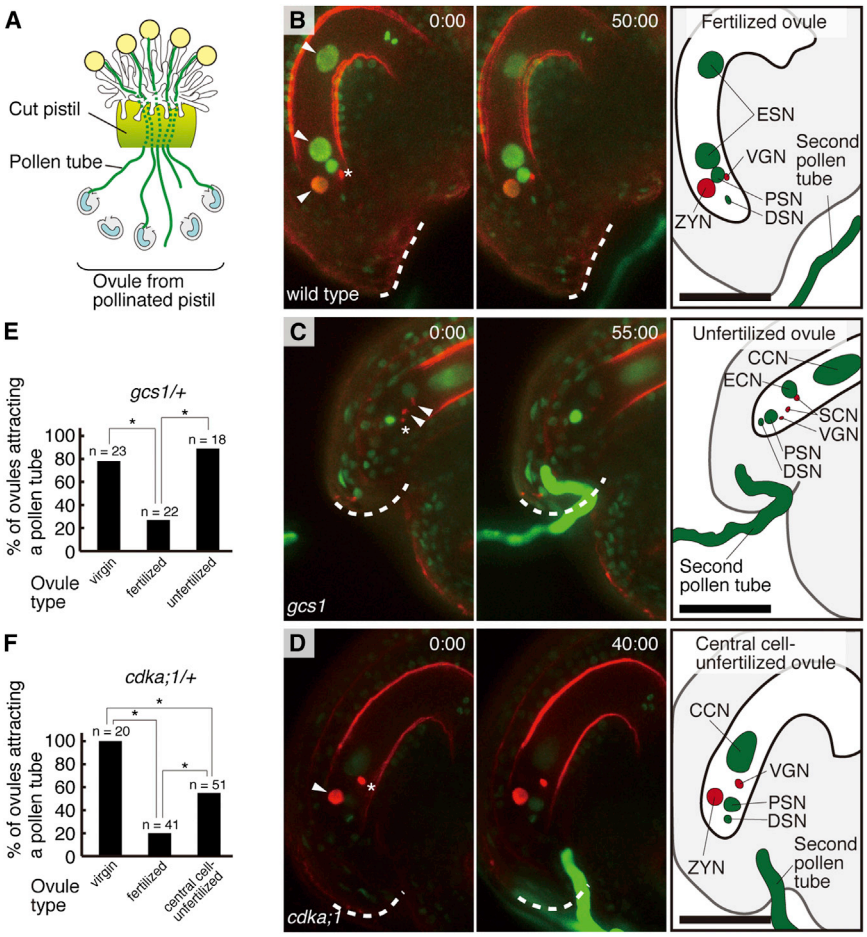

Figure 2. Dynamic Regulation of the Second-Pollen-Tube Guidance

(A) Diagram of the semi-in-vitro pollen-tube guidance assay. Targeting of GFP-labeled pollen tubes was analyzed in the ovules prepared from cross-pollination between wild-type female and mutant male plants that carried the nuclear markers *pRPS5A::H2B-GFP* and *pRPS5A::H2B-tdTomato*, respectively.

(B–D) Micrographs of the semi-in-vitro pollen-tube guidance. Pollen tubes failed to penetrate fertilized ovules without any symptom of degeneration of the persistent synergid cell (B). However, the second pollen tube succeeded in targeting unfertilized ovules that had accepted a *gcs1* pollen tube (C) and the central-cell-unfertilized ovule produced by the *cdka;1* pollen tube perception (D). Numbers stamped in each frame indicate time (min:s) from the start of the observation. Final destinations of the pollen tube are depicted on the right-hand side of each image. Dashed lines represent the boundary of the micropyle region. Arrowheads indicate the nucleus showing the male-gamete-derived tdTomato signal, including zygote nucleus (ZYN), endosperm nucleus (ESN), and sperm cell nucleus (SCN). Asterisks represent the vegetative nucleus (VGN). PSN, nucleus of the persistent synergid cell; DSN, nucleus of the degenerated synergid cell; ECN, egg cell nucleus; and CCN, central cell nucleus. Scale bars, 50 μm.

(E and F) Percentages of second-pollen-tube attraction in the *gcs1*/+ cross (E) and *cdka;1*/+ cross (F). Asterisks represent statistical significance ($p < 0.01$, chi-square test).

See also Figure S1 and Movie S1.

(H2B-GFP) for the female nuclei and H2B-tdTomato for the male nuclei. After perception of the first pollen tube, we collected ovules and evaluated the attraction of the GFP-labeled second pollen tube on growth medium (Figure 2A). After fertilization by *GCS1* wild-type pollen, sperm-nucleus-derived tdTomato signals diffused into the egg cell and central cell. Although these ovules were analyzed before the degeneration of the persistent synergid cell, only 27% (n = 22) displayed second-pollen-tube attraction (Figures 2B and 2E; Movie S1). In contrast, unfertilized ovules that received unfertilized *gcs1* sperm cells attracted a second pollen tube as efficiently as did virgin ovules that showed no sign of pollen tube penetration (89%, n = 18, Figures 2C and 2E; Movie S1). To examine the effects of single fertilization, we analyzed ovules that had completed fertilization only in the egg cell, because this type was easily recognized in our *cdka;1*/+ cross. They, too, showed frequent attraction of a pollen tube; however, the frequency (55%, n = 51) was significantly lower than in the virgin ovules (Figures 2D and 2F; Movie S1). These results strongly support the existence of a dual-control system, which shows a rapid effect on pollen tube guidance prior to degeneration of the persistent synergid cell.

FIS-PRC2 Is Required for the Polytubey Block

To gain insight into the molecular mechanism of the independent control, we searched for other gametophytic mutants and found a defect in the blockade of polytubey in the *mea* mutant (Grossniklaus et al., 1998); 41.0% ± 5.2% of seeds showed two-pollen-tube insertions in *mea*/*mea* pistils pollinated by wild-type pollen (mean ± standard deviation; Figures 3A–3C). *MEA* encodes a SET domain protein incorporated into FIS-PRC2 (Hennig and Derkacheva, 2009). Thus, we analyzed mutants of other FIS-PRC2 components, including FERTILIZATION-INDEPENDENT SEED2 (FIS2) (Chaudhury et al., 1997) and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) (Ohad et al., 1999). Approximately 20% of the seeds received two pollen tubes in the *mea*/+, *fis2*/+, and *fie*/+ mutants, with twice the number in the *mea*/*mea* and *fis2*/*fis2* mutants (Figure 3A). FIS-PRC2 is a chromatin-modifying complex involved in gene silencing via trimethylation of lysine 27 on histone H3 (H3K27me) (Köhler et al., 2012). These results suggested a role of chromatin remodeling in the polytubey blockade in the *Arabidopsis* female gametophyte.

After identifying a role for FIS-PRC2 in the blockade of polytubey, we examined the relevance of other regulators of chromatin-remodeling pathways and DNA methylation/demethylation enzymes (Brodersen and Voinnet, 2006). We analyzed the following mutants: the *dme* mutant for DNA demethylation (Choi et al., 2002); *met1* mutant for the maintenance of CG DNA methylation (Ronemus et al., 1996); single or multiple mutants of *drm1*, *drm2*, and *cmt3* for non-CG-DNA methylation (Chan et al., 2006); *nrpd1a*, *nrpd1b*, and *rdr2* mutants for the

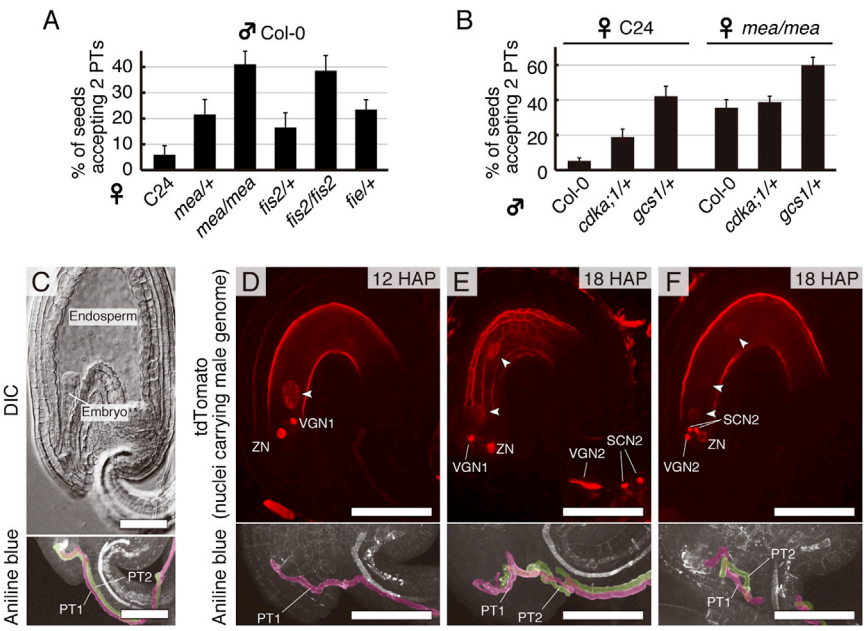

Figure 3. FIS-PRC2 Disruptions Induce Second-Pollen-Tube Attraction after Successful Double Fertilization

(A) Polytubey in *mea*, *fis2*, and *fie* mutants.

(B) Analysis of genetic interactions between *mea* and other mutants (*cdka;1* and *gcs1*) in the polytubey block. Bars and error bars in (A) and (B) represent the mean and standard deviation (n = 5 or more pistils), respectively.

(C) Insertions of two pollen tubes in the *mea* seed with the embryo and endosperm at 3 days after pollination.

(D–F) Fertilization of the *mea* mutant visualized by crosspollinations with the *pRPS5A::H2B-tdTomato* line. At 12 HAP, double fertilization was accomplished by the first pollen tube (D). At 18 HAP, the second pollen tube entered into the ovule (E) and discharged its content (F).

Lower images in (C)–(F) indicate aniline blue fluorescence of micropyler area of the same ovules shown in the upper images. Arrowheads indicate endosperm nuclei. ZYN, zygote nucleus; SCN1/SCN2, sperm cell nucleus 1/sperm cell nucleus 2; VGN1/VGN2, vegetative nucleus 1/vegetative nucleus 2. PT1/PT2, pollen tube 1/pollen tube 2. Scale bars, 50 μm. See also Figure S2 and Table S1.

RNA-directed DNA methylation pathway (Xie et al., 2004; Herr et al., 2005; Pontes et al., 2006; Pontier et al., 2005); and the *kyp* mutant for the methylation of lysine 9 on histone H3 (Jackson et al., 2002) (Table S1). These mutants were pollinated with wild-type pollen, and the percentages of seeds with two inserted pollen tubes were examined, but none showed significant second-pollen-tube attraction (Figure S2). This suggests that the blockade of polytubey in *Arabidopsis* is specifically regulated by FIS-PRC2-mediated H3K27me modification. However, the fact that the *dme/dme* mutant showed a normal polytubey block was unexpected, because the *MEA* and *FIS2* loci are silenced by CG DNA methylation, and that methylation is removed by DME in the central cell (Choi et al., 2002; Jullien et al., 2006). Recently, Ikeda et al. (2011) demonstrated that *FIS2* expression is not completely abolished in the *dme/dme* mutant. Presumably, leaky expression of *MEA* and *FIS2* is sufficient for the blockade of polytubey in the *dme/dme* mutant.

FIS-PRC2 Participates in the Blockade of Polytubey via the Central Cell Pathway

We hypothesized that FIS-PRC2 participates in the blockade of polytubey through the central cell pathway for the following reasons. First, *MEA* and *FIS2* are strongly expressed in the central cell and endosperm, but not in the egg cell (Wang et al., 2006). Second, seed abortion of *fis*-class mutants is largely due to severe defects in the endosperm (Köhler et al., 2003; Nowack et al., 2007; Bouyer et al., 2011). Third, the percentage of seeds receiving two pollen tubes in the *mea/mea* plants was not increased by the pollination of *cdka;1/+*, which would in most cases induce second-pollen-tube attraction by the central cell pathway (Figures 1E and 3B). Impaired fertilization of the central cell was also found for *gcs1*. However, the pollination of *gcs1/+* using *mea/mea* pistils resulted in additional attraction of a second pollen tube (59.9% ± 4.7%, Figure 3B), which probably corresponds to a defect in the egg cell pathway.

Previously reported examples of multiple-pollen-tube attraction were induced by defects in the fertilization process (Kasahara et al., 2012; Beale et al., 2012). FIS-PRC2 component mutants have also been reported to exhibit defective pollen tube perception by the synergid cell, especially in aged pistils (Rotman et al., 2008). As we used fresh *mea/mea* pistils for cross-pollination, only 0.6% of the ovules displayed abnormal pollen tube perception (n = 321). To monitor double fertilization, *mea/mea* pistils were pollinated with the *pRPS5A::H2B-tdTomato* line. At 12 hr after pollination (HAP), 88% of the ovules had received a single pollen tube and 2% had accepted a second pollen tube (n = 41; Figure 3D). All of them showed normal double fertilization and nuclear fusion, because male-derived tdTomato signals diffused into the nucleus of both female gametes (Figure 3D). Persistent synergid cells have a round-shaped nucleus in fertilized *mea* ovules cleared by a chloral hydrate solution (data not shown). Although the primary endosperm nucleus started to proliferate by 18 HAP, we observed the entrance and discharge of a second pollen tube in 14.3% of the ovules (n = 126; Figures 3E and 3F; Figure S2). This percentage, less than half of the maximum (~40%, Figure 3A), corresponded to differences of time points after pollination, because it takes 28 hr for second-pollen-tube attraction to complete (Kasahara et al., 2012). Interestingly, none of the second set of sperm cells caused fertilization, probably due to the polyspermy blocking system or onset of the development of fertilized cells, which prevents additional gametic fusion (Scott et al., 2008). We conclude that *mea* is a polytubey-blockade-defective mutant without any defects in fertilization. FIS-PRC2 might participate in the generation of the polytubey blocking signal via gene silencing of an unknown target. FIS-PRC2 mediated the central cell signal, while another signal from the egg cell may coordinately restrict polytubey by a dynamic reduction in pollen tube attractants and subsequent programmed cell death in the persistent synergid cell

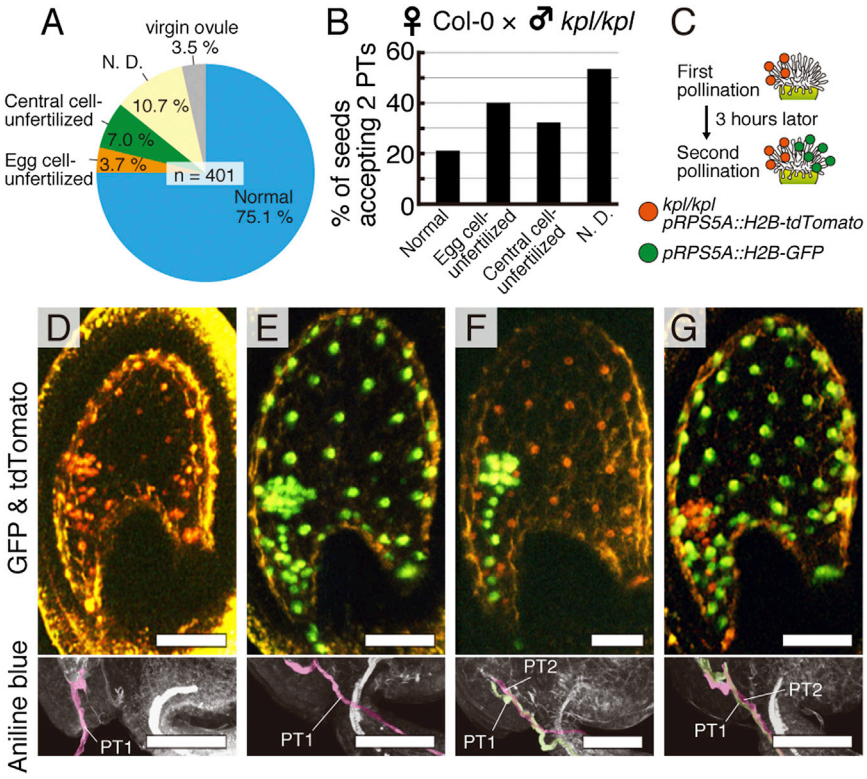

Figure 4. Fertilization Recovery for Incomplete Double Fertilization

(A and B) Frequency of seeds (A) and percentages of second-pollen-tube insertions (B) observed in wild-type pistil pollinated by the *kpl/kpl* plant.

(C) Diagram of the dual pollination experiment.

(D–G) Analyses of seed development and an inserted pollen tube 3 days after dual pollination. Successful double fertilization by a single pollen tube from the first pollination (D) and the second pollination (E) is shown. Heterofertilizations resulting from the recovery of the egg cell fertilization (F) or the central cell fertilization by the second pollen tube (G). Lower images indicate aniline blue fluorescence of the micropyler area of the same ovules shown in the upper images.

ND, not identified (determined) due to seed abortion. PT1/PT2, pollen tube 1/pollen tube 2. Scale bars, 50 μm.

(Figure S2). In this issue, Völz et al. (2013) show an essential role for ethylene signaling in programmed cell death; thus, ethylene could be a signaling molecule originating from either the fertilized egg cell or the fertilized central cell.

The Active Recovery of Half-Successful Fertilization Results in Heterofertilization

Why do flowering plants have a dual-control polytubey blocking system? The most probable reason is for fertilization recovery of the unfertilized egg cell or central cell by the second pollen tube. To investigate fertilization recovery, we used *kokopelli* (*kpl*), a mutant that produces aberrant sperm cells with reduced fertility (Ron et al., 2010), because pollen from *kpl/kpl* homozygous plants induced a higher percentage of single fertilizations than pollen from *cdka;1*/+ (Figures 1E and 4A). Importantly, 15.7% of the seeds were morphologically normal and inserted two pollen tubes; they are likely candidates for fertilization recovery (Figure 4B). To visualize fertilization recovery, wild-type pistils were pollinated by a *kpl/kpl* mutant carrying *pRPS5A::H2B-tdTomato*, and after 3 hr, the same pistils were pollinated by a *pRPS5A::H2B-GFP* transgenic plant (Figure 4C). Under these conditions, the percentage of normal seeds that inserted two pollen tubes was reduced to 8.1% (n = 694). Driven by the constitutively active *RPS5A* promoter, the nuclei in the embryo and endosperm were uniformly labeled by tdTomato (Figure 4D) or GFP (Figure 4E) when double fertilization occurred with a single pollen tube. However, we observed discrepancies between the fertilization products in 1.2% of the seeds (n = 987): four seeds had a GFP-labeled embryo and tdTomato-labeled endosperm (Figure 4F), while eight seeds had a tdTomato-labeled embryo and GFP-labeled endosperm (Figure 4G). These types of seeds were not observed in the negative control, in which a wild-type L*er* plant containing *pRPS5A::H2B-tdTomato* but no *kpl* mutation was used for the first round of pollination (n = 663). Similar peculiar fertilization events, which produced a genetically distinct embryo and endosperm, were previously shown in maize and were termed heterofertilization (Sprague, 1929). A major reason for heterofertilization was expected to be independent fertilization by two genetically distinct pollen tubes (Sprague, 1932; Kato, 2001). Our data provide cytological evidence that directly links polytubey and heterofertilization. Furthermore, we demonstrated a possible advantage of the dual-control polytubey block in fertilization recovery. The identification of heterofertilization in *Arabidopsis* and maize implies wide conservation of the dual-control polytubey block, which might have evolved to maximize reproductive fitness in flowering plants.

A recent study demonstrated that heterofertilization between genetically distinct maize inbred lines resulted in smaller embryo production, and that study predicted a kin recognition within a seed (Wu et al., 2013). Other studies have suggested that small RNAs from the endosperm affect embryo development, but small-RNA migration has not yet been demonstrated (Bauer and Fischer, 2011; Ibarra et al., 2012). siRNA migration between plant organs was demonstrated by a grafting experiment that produced chimera plants with a shoot containing an siRNA transgene and a root with the target gene (Molnar et al., 2010). Heterofertilization also generates chimeric seeds and therefore should be useful for investigating the migration of such signaling molecules.

Further analysis of FIS-PRC2 and ethylene signaling will enhance our understanding of the polytubey block at the molecular level and may contribute to an increased heterofertilization rate. Moreover, such studies could uncover relationships among the polytubey block, chromatin-remodeling pathway, zygotic activation, and evolution of double fertilization in flowering plants.

EXPERIMENTAL PROCEDURES

Plant Materials

Col-0, L*er*, and C24 accessions were used as wild-type plants. The mutant alleles used were: *cdka;1-1*, *gcs1*, *kpl-2*, *mea-7*, *fis2-6*, *dme-1*, *drm1-2*, *drm2-2*, *cmt3-11t*, *met1-3*, *rdr2-1*, *nrpd1a-4*, *nrpd1b-11*, and *kyp-6*. All plants were grown on soil at 22°C under continuous light. We also used wild-type plants carrying *pLAT52::GFP* or *pRPS5A::H2B-GFP*, and *gcs1*/+, *cdka;1-1*/+, and *kpl-2*/*kpl-2* lines that were homozygous for *pRPS5A::H2B-tdTomato*. Details are provided in the Supplemental Experimental Procedures.

Histological Studies

Aniline blue staining was performed as follows. Pollinated pistils were fixed with a 9:1 mixture of ethanol and acetic acid (v/v) for several hours and treated with 1 M NaOH for ~16 hr. The samples were directly stained by aniline blue solution (0.1% [w/v] aniline blue, 0.1 M K_3PO_4) and incubated for more than 3 hr. After the samples were dissected on glass slides and mounted in the aniline blue solution, the seeds were scanned using the 405 nm laser line and the images were captured using a CSU10 confocal laser scanning system (Yokogawa Electric, Tokyo, Japan) mounted on a BX60 microscope (Olympus, Tokyo, Japan) combined with 405 nm laser irradiation. To observe the fluorescent signals from the aniline blue and tdTomato in the same seeds, siliques were first dissected and mounted in 10% (v/v) glycerol and imaged using the 568 nm laser line. Aniline blue staining and analysis of the samples were then performed as described above. To examine seed development together with the pattern of pollen tube growth, siliques were fixed with a 9:1 mixture of ethanol and acetic acid (v/v), rehydrated with an ethanol series, and cleared in 70% (w/w) chloral hydrate and 8.8% (w/w) glycerol. After the samples were dissected and viewed with differential interference contrast microscopy (DIC), they were rinsed with Milli-Q (Millipore, Billerica, MA)-purified water and subjected to the aniline blue assay described above. In this study, the thickness of the optical section was 0.5 μm for the 12 or 18 HAP seeds and 2.0 μm for the 3 DAP seeds. The maximum signals of each image were stacked and are shown in the figures.

Heterofertilization

Wild-type pistils were pollinated with the *kpl-2*/*kpl-2* or wild-type (L*er*) plants carrying the *pRPS5A::H2B-tdTomato* gene, and subsequently pollinated with the *pRPS5A::H2B-GFP* transgenic plant 3 hr after the first pollination. Then, the siliques were dissected on glass slides and mounted in 10% (v/v) glycerol 3 days after pollination. Two-photon images were acquired using a fluorescence microscope (Ti-E; Nikon, Tokyo, Japan) equipped with a spectral detector (32 photomultiplier tubes in the range 460–650 nm with 6 nm resolution; Nikon). Z-stack images were taken using multiple z-planes (5.0-μm intervals) and 12 planes for maximal intensity projections with a 25× objective lens (CFI Apo LWD 25XW; Nikon). A Ti:sapphire laser (Mai Tai HP DeepSee, Spectra-Physics, Tokyo, Japan) tuned to 960 nm was used for excitation. Images were processed with a NIS-Elements 3.2 (Nikon) to create maximum-intensity projection images and to add color. After the two-photon imaging, the samples were fixed and stained using aniline blue as described above. They were scanned using the 405 nm laser line to capture optical sections with 2.0 μm thickness and the maximum signals were stacked.

Semi-In-Vitro Pollen-Tube-Attraction Assay

The *pRPS5A::H2B-GFP* line was pollinated with pollen from the *gcs1*/+ or *cdka;1-1*/+ lines carrying the *pRPS5A::H2B-tdTomato* gene. Ovules from the pistils at 6.5 HAP were collected and placed on the agarose medium in a semi-in-vitro assay chamber as reported previously (Hamamura et al., 2011). In the semi-in-vitro assay chamber, pollen tubes from the *pLAT52::GFP* line emerged from a cut style and were growing on the medium. These pollen tubes were prepared as follows: wild-type pistil was pollinated by the *pLAT52::GFP* pollen and the style was cut by a 27-gauge needle, and the pistil was then incubated in the chamber for 3.5 hr. After a further 1 hr incubation, the pollen tube attraction was analyzed. Confocal images were acquired using an inverted microscope (IX-81, Olympus, Tokyo, Japan) equipped with an automatically programmable XY stage (MD-XY30100T-Meta; Molecular Devices, Sunnyvale, CA), a disk-scan confocal system (CSU-XI, Yokogawa Electric, Tokyo, Japan), 488 nm and 561 nm LD lasers (Sapphire, Coherent, Santa Clara, CA), and EM-CCD camera (Evolve 512, Photometrics, Tucson, AZ). Time-lapse images were acquired every 5 min using multiple z-planes (3.0 μm intervals) and seven planes with a water-immersion objective lens (UApo 40XW3/340; Olympus). Images were processed with Metamorph Ver. 7.7.7.7.0 (Universal Imaging Corp., Downingtown, PA) to create maximum-intensity projection images and to add color. Adobe Photoshop CS4 (Adobe Systems, Inc., San Jose, CA) was used to adjust the images. MacBiophotonics ImageJ software (http://www.macbiophotonics.ca/) was used for editing of the images and movies.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, one table, one movie, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.03.013.

ACKNOWLEDGMENTS

We thank F. Berger, T. Kinoshita, Y. Ikeda, H. Shiba, H. Saze, S. Nishikawa, and R. L. Fischer for the materials, T. Endo for providing support in the Endo Lab confocal microscope facility, H. Takanashi for the statistical analyses, and N. Iwata for assistance in preparing plant materials. D.M. was supported by the Global Center of Excellence program (Nagoya University). D.M., Y.H., H.T., and D.S. were supported by grant numbers 6526, 9138, 5834, and 7811, respectively, from the Japan Society for the Promotion of Science Fellowships. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (numbers 18075004 and 19370017 to T.H.), the Japan Science and Technology Agency (PRESTO project to T.H.), the Yamada Science Foundation (to T.H.), and the Mitsubishi Foundation (to T.H.).

Received: November 17, 2012
Revised: January 24, 2013
Accepted: March 18, 2013
Published: May 13, 2013

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