Current Biology 21, 497–502, March 22, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.02.013

Report

Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in *Arabidopsis thaliana*

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Summary

Flowering plants have evolved a unique reproductive process called double fertilization, whereby two dimorphic female gametes are fertilized by two immotile sperm cells conveyed by the pollen tube [1, 2]. The two sperm cells are arranged in tandem with a leading pollen tube nucleus to form the male germ unit and are placed under the same genetic controls [3]. Genes controlling double fertilization have been identified [4–6], but whether each sperm cell is able to fertilize either female gamete is still unclear [7–9]. The dynamics of individual sperm cells after their release in the female tissue remain largely unknown. In this study, we photolabeled individual isomorphic sperm cells before their release and analyzed their fate during double fertilization in *Arabidopsis thaliana*. We found that sperm delivery was composed of three steps. Sperm cells were projected together to the boundary between the two female gametes. After a long period of immobility, each sperm cell fused with either female gamete in no particular order, and no preference was observed for either female gamete. Our results suggest that the two sperm cells at the front and back of the male germ unit are functionally equivalent and suggest unexpected cell-cell communications required for sperm cells to coordinate double fertilization of the two female gametes.

Results and Discussion

Three-Step Behavior of Two Sperm Cells as Visualized by Time-Lapse Imaging of Double Fertilization

Of the entire fertilization process in flowering plants, very little is known about events in the gametic phase of double fertilization from pollen tube discharge to the initiation of the fusion between sperm nuclei and female gamete nuclei (karyogamy) [1]. We obtained a series of time-lapse recordings from several ovules using a spinning-disk confocal microscope equipped with an automatic stage controller, allowing us to record all events of the gametic phase in wild-type plants (Figures 1B and 1C and Movie S1 and Movie S2 available online represent typical recordings of a total of 107). Ovules fertilized under these conditions initiated endosperm formation and embryogenesis as reported previously [10].

Sperm cells marked with monomeric red fluorescent protein (mRFP) fused to the histone 3.3 variant HTR10 [11] remained stationary at the entrance of the embryo sac before discharge. Pollen tube discharge was monitored by flux of the pollen tube cytosol labeled with GFP and corresponded to the rapid movement of sperm nuclei toward the female gametophyte (Figure 1C; Movie S2). The last video frame before the increase in sperm cell velocity represented the starting point for pollen tube discharge ("0 min") (Figures 1B and 1C). All 107 movies recorded showed that the two sperm cells were rapidly transported between the egg cell and the central cell in less than 1 min after pollen tube discharge was initiated (Figures 1B and 1C; Movie S1; Movie S2), consistent with earlier preliminary reports [12].

After their release, the sperm cells stayed together and were immobile between the egg cell and the central cell for an average time of 7.4 ± 3.3 min (mean ± standard deviation; n = 44). Subsequently, sperm cells fused with each female gamete, and each sperm cell nucleus began to move toward its respective target nucleus in the egg cell and the central cell. Once the male and female nuclei established contact, nuclear fusion (karyogamy) was visualized by sperm cell chromatin decondensation (Figure 1C; Movie S2) [11].

Based on these observations, we distinguished at least three steps between pollen tube discharge and karyogamy (Figure S1): rapid movement of sperm cells from the pollen tube to the female gametes, maintenance of sperm cell positions between the two female gametes, and movement of sperm cell nuclei to the target female nuclei following putative gametic fusion. We further dissected and characterized each step of the gametic phase of double fertilization.

Rapid Transport of Two Sperm Cells upon Pollen Tube Discharge

Imaging the behavior of sperm cells during pollen tube discharge required a temporal resolution of less than 1 s. The best resolution achieved in previous studies was 30 s [12]. We obtained sequential imaging of pollen tube discharge using a time series of single confocal sections or single images acquired with a wide-field microscope equipped with an image intensifier-coupled CCD (ICCD) camera for greater focal length. We recorded the release of sperm cells with a sampling rate of 0.20–0.33 s using either microscopic technique (Figure 2; n = 8).

Figure 2A shows the migration velocity of sperm cells before and after the start of pollen tube discharge (Movie S3). Before pollen tube discharge, sperm cells carried by the pollen tube moved slowly, at 8.6 × 10^{-2} μm/s, consistent with the

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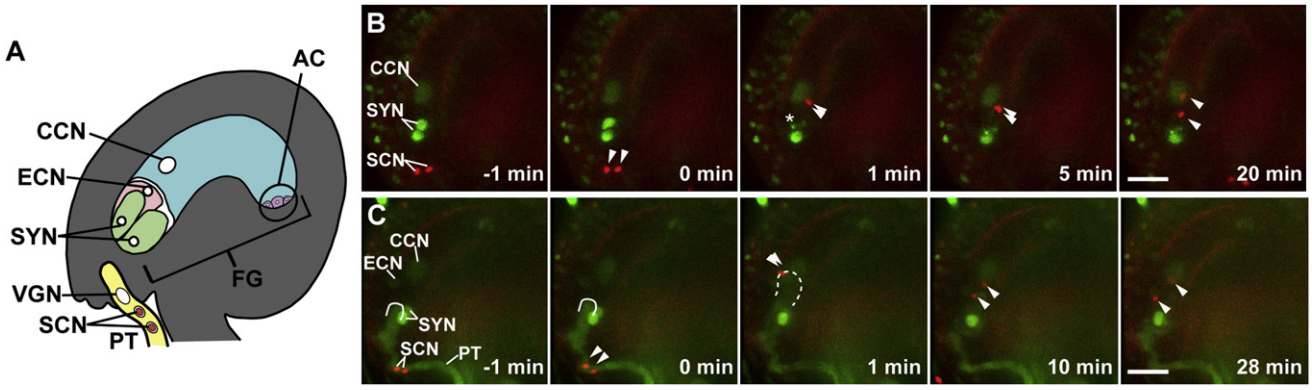

Figure 1. Time-Lapse Imaging of the Entire Double-Fertilization Process

(A) Schematic representation of pollen tube arrival at the ovule. The following abbreviations are used: central cell nucleus (CCN), egg cell nucleus (ECN), synergid cell nucleus (SYN), vegetative nucleus (VGN), sperm cell nuclei (SCN), pollen tube (PT), antipodal cell (AC), female gametophyte (FG).

(B and C) Sperm cell nuclei (arrowheads) were labeled with *pHTR10:HTR10:mRFP*. Nuclei of female gametophyte and ovules were labeled with *pACT11:H2B:GFP*. Pollen tube cytosol was labeled with *pLAT52:GFP*. Asterisk in (B) represents the nucleus of a broken-down synergid cell. The pollen tube tip and the cellular content of the pollen tube after pollen tube discharge are shown by the dotted line in (C).

Numbers indicate time; the last frame just before the rapid movement of sperm cells was designated the onset of the pollen tube discharge, i.e., 0 min. Scale bars represent 20 μm. See also Movie S1 and Movie S2.

time-lapse imaging in Figure 1. Once pollen tube discharge was initiated, sperm cell velocity increased dramatically, and their maximum velocity was 10 μm/s. The duration of the rapid migration phase of sperm cells was only 8.8 ± 5.5 s (n = 8). Much longer durations were reported in previous studies, possibly either because fixed samples were used [13–15] or because the image sampling rate was much lower [12].

Figure 2B shows sequential images of an example of pollen tube discharge (Movie S4). In 4 of 8 cases of pollen tube discharge, we used a double marker line in which the pollen vegetative nucleus, and to a lesser extent the pollen tube cytoplasm, was labeled with *pACT11:H2B:GFP* in addition to the sperm cell nuclei labeled with *pHTR10:HTR10:mRFP* (Figure 2B). As shown in the 0.20 s frame after the start of discharge, the two sperm cells began to move together with the vegetative nucleus. Notably, the two sperm cells always overtook the vegetative nucleus, as shown in the 7.60 and 9.60 s frames. Sperm cells passed smoothly through the contents of the pollen tube to reach the chalazal edge of the degenerated synergid cell. In various plant species, actin cables, called "corona" [16] in the degenerated synergid cell, have been proposed to possibly provide rails for the gradual migration of sperm cells toward the female cells by actomyosin-based movement [13, 14, 17, 18]. However, our direct observations, as shown in Movie S3, suggested that the movement of sperm cells from the pollen tube to the chalazal edge of the degenerated synergid cell was a rapid and continuous movement, possibly propelled by cytoplasmic flow ejected from the pollen tube, as observed in *Torenia fournieri* [19]. Once two sperm cells reached the chalazal edge of the degenerated synergid cell, they did not migrate further for 7.4 min on average, suggesting that the cytoplasmic flow of the tube content was sufficient to discharge the two sperm cells in the close proximity of the female gamete membrane.

Kinetics of Fusion between the Male and Female Gametes

After a relatively long period of apparent immobility between the two female gametes, the two sperm cell nuclei resumed their movement inside each female gamete. To establish the role played by the fusion of male and female gametes (plasmogamy) in the initiation of male nuclei mobilization, we used mutant sperm cells of a null mutant allele of *GENERATIVE CELL SPECIFIC 1* (*gcs1*, also called *hapless 2* [*hap2*]) that is

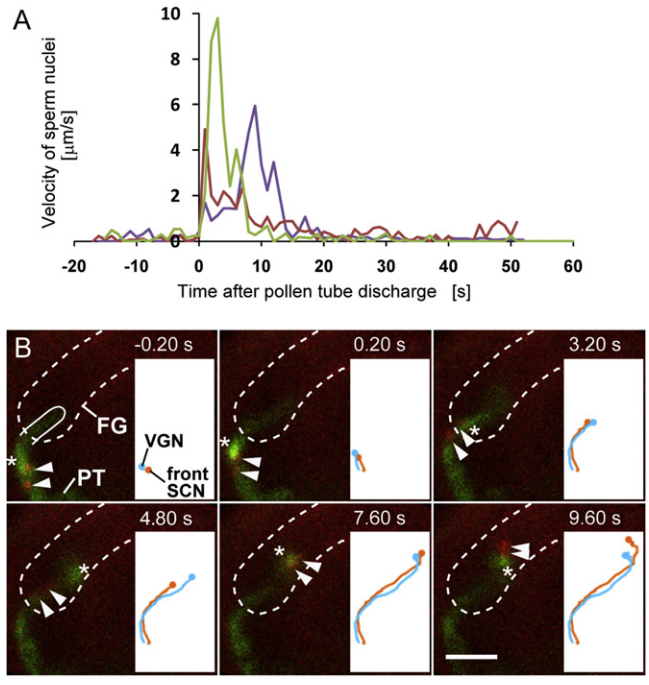

Figure 2. Sequential Imaging of Male Gametic Nuclei after Pollen Tube Discharge

(A) Velocity of the nucleus of the front sperm cell, which is associated with the vegetative cell nucleus. Three different cases of movement are displayed in different colors. See also Movie S3.

(B) Sperm cell nuclei (arrowheads) were labeled with *pHTR10:HTR10:mRFP*. Vegetative nuclei (asterisks) and pollen tube (PT) cytoplasm (solid line) were labeled with *pACT11:MSI1:GFP*. The dashed line outlines the cell wall of the female gametophyte (FG). The trajectory of the front sperm cell nucleus (orange dot and line) and vegetative nucleus (blue dot and line) are shown in the white boxes. Scale bar represents 20 μm. Numbers indicate time as in Figure 1. See also Movie S4.

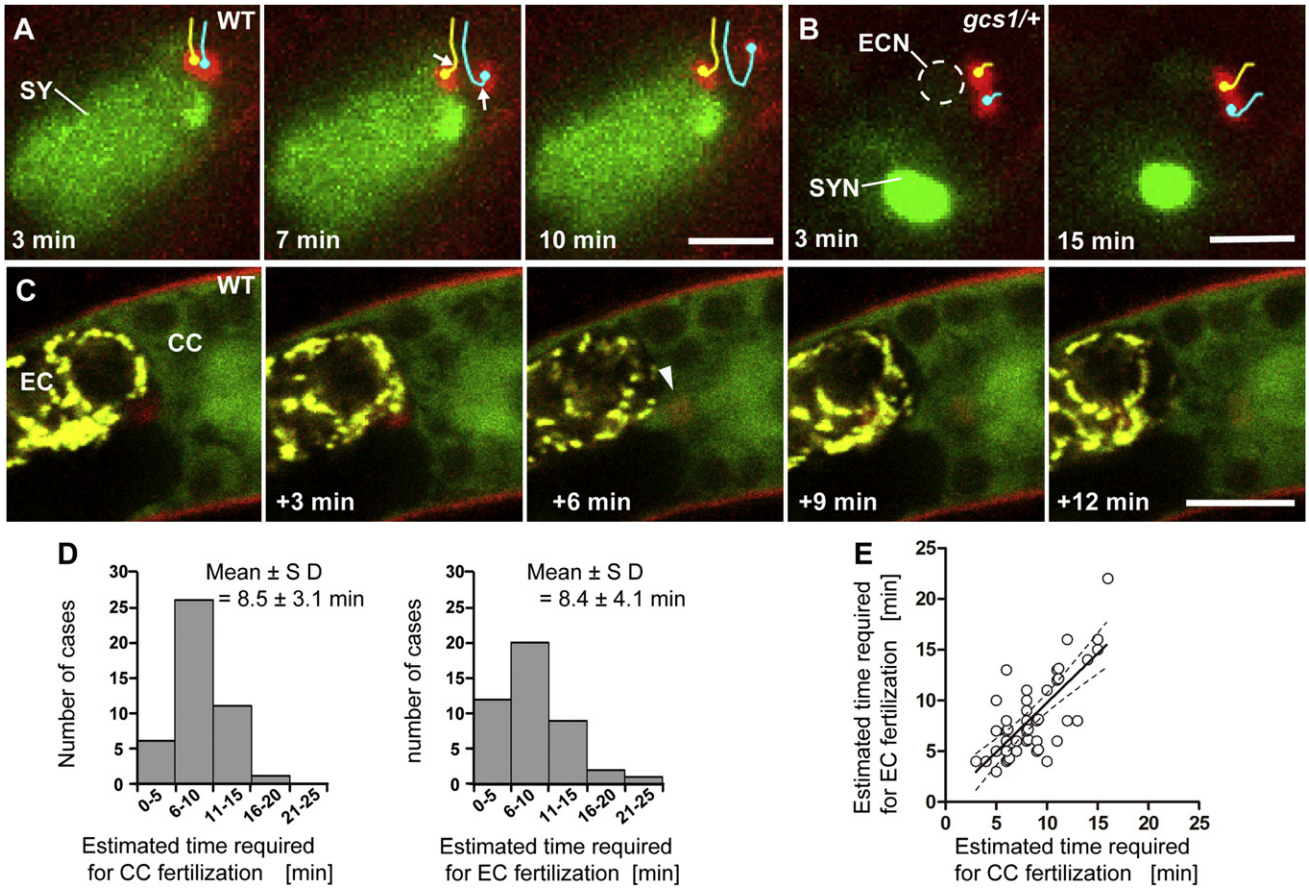

Figure 3. Tracking Analysis of Two Sperm Cell Nuclei during Double Fertilization

(A and B) Movement of sperm cell nuclei of wild-type (A) and *gcs1*/+ (B) was observed with a spinning-disk confocal system. Sperm cell nuclei were labeled with *pHTR10:HTR10:mRFP*. Yellow and blue lines indicate the trajectories of the two sperm cell nuclei. The two sperm cell nuclei were likely pushed back together in parallel, keeping their positions at the apical region of the degenerated synergid cell, possibly as a result of turgor pressure of other female cells. Scale bars in (A) and (B) represent 10 μm. See also Movie S5.

(A) Wild-type sperm nuclei began to move toward their respective target nuclei (arrows). The synergid cell (SY) was labeled with *pMYB98:GFP*.

(B) *gcs1*/+ sperm cell nuclei did not move toward their respective targets. Nuclei of the female gametophyte and ovular sporophytic cells were labeled with *pACT11:H2B:GFP*.

(C) A two-photon laser system was used for high-resolution imaging of the boundary of the female gametophytic cells in wild-type. Sperm cell nuclei were labeled with *pHTR10:HTR10:mRFP*. Egg cell (EC) mitochondria were labeled with *pDD45:mtKaede.* The central cell (CC) nucleus and cytosol were labeled with *pFWA:FWA:GFP.* Arrowhead indicates the point when a sperm cell nucleus reinitiated its movement toward the central cell nucleus. Note that the sperm cell nucleus was in the central cell cytosol at the point indicated by the arrowhead. Time elapsed from the first frame is shown. Scale bar represents 10 μm. See also Movie S6.

(D) Histograms of time required for each fertilization from pollen tube discharge (left, central cell [CC]; right, egg cell [EC]). The means ± standard deviation were 8.5 ± 3.1 min for CC (n = 44) and 8.4 ± 4.1 min for EC (n = 44).

(E) Plot of time required for each fertilization. A regression line (solid line) and 95% confidence interval (dashed lines) are shown (n = 44).

defective in plasmogamy [4, 20]. The kinetics of sperm cell discharge and the deposition site were similar between wild-type and *gcs1* plants (Figures 3A and 3B; Movie S5). However, about 7.4 min after deposition, only wild-type sperm cell nuclei reinitiated their movement toward female gamete nuclei. The observation that the sperm cell nucleus was directed toward the central cell nucleus suggested that the sperm cell nucleus left the apical region of the degenerated synergid cell and entered the cytoplasm of the central cell when it changed direction (Figure 3A; Movie S5). Moreover, two-photon microscopy clearly showed that the two sperm cell nuclei entered female gametes when they reinitiated their movement (n = 5; Figure 3C; Movie S6). These results are consistent with our previous finding that mitochondria in the two sperm cells diffuse to female cells approximately 10 min after pollen tube discharge, as shown by 5 min interval time-lapse imaging [21]. We thus conclude that reinitiation of sperm cell nucleus movement marks plasmogamy between male and female gametes.

Circumstantial evidence indicates that fertilization triggers a mechanism that prevents the fusion of both sperm cells with one of the female gametes (polyspermy) [22]. The *Arabidopsis tetraspore* (*tes*) mutant produces supernumerary sperm cells in a pollen tube. Higher frequency of putative polyspermy involving *tes* sperm cells in the central cell than in the egg cell suggests that prevention of polyspermy is likely to be strict in the egg cell but not in the central cell [23]. Data from another mutant, *retinoblastoma related 1* (*rbr1*), also support that the egg cell has a polyspermy block following fertilization [8]. The ovule of the *rbr1* mutant has two egg cells, and each can be fertilized by only one wild-type sperm cell.

The idea of a stricter polyspermy block in a fertilized egg cell suggested that the egg cell might fuse with a sperm cell before the other sperm cell fuses with the central cell. However, the time required for gamete fusion with the central cell was 8.5 ± 3.1 min, and a similar duration (8.4 ± 4.1 min) was measured for the egg cell (Figure 3C; n = 44). Our observation is comparable to previous estimates in *Arabidopsis* [24] and other species [25, 26]. The time lag between the first and second fertilization was only 2.5 ± 1.7 min (n = 44). In 6 of 44 examples observed using our 1 min time-lapse imaging, both plasmogamy events occurred simultaneously within 1 min. No preference was observed for the order of fertilization of the egg and central cells with wild-type sperm cells (Figure 3D). Note that our observations suggested a temporal correlation between the two plasmogamies involving the egg cell and the central cell (Figure 3D; Pearson's coefficient $r = 0.73$, $p < 0.01$). This correlation implies various possibilities including correlation of timing of adhesion (tethering) of the two sperm cells to their target cells, intercellular communication between the two sperm cells, and intercellular communication between the egg cell and the central cell.

Taken together, our data suggest the possibility that plasmogamy might trigger a rapid event leading to a polyspermy block in both the egg cell and the central cell. Fast electrical events following gamete fusion leading to a polyspermy block have been reported in the fucoid algae, including *Fucus* [27, 28], but not from in vitro fertilization studies using isolated plant gametes [29]. Alternatively, other rapid signaling events might be required to prevent multiple sperm entries into the egg cell and the central cell, including the rapid initiation of cell wall formation [30].

Nonpreferential Fertilization by Two Sperm Cells

The two sperm cells are isomorphic in most flowering plants, including *Arabidopsis*. However, in some plant species, such as *Plumbago zeylanica*, generative cell division is exceptionally asymmetrical, leading to two dimorphic sperm cells [31–33]. The larger *P. zeylanica* sperm cell contains more mitochondria; it is associated with the vegetative nucleus and preferentially fuses with the central cell [34]. Because the association of one of two sperm cells with the vegetative nucleus (the male germ unit) is generally observed in flowering plants, whether such a preferential fertilization occurs in plants having isomorphic sperm cells, such as *Arabidopsis*, has been an issue of debate.

A series of data obtained with mutants producing additional egg cells [11, 35] or fewer sperm cells [9, 36] supported the idea that *Arabidopsis* sperm cells are functionally identical. However, a single sperm-like cell formed by translational inhibition in male germ cells likely causes single fertilization preferentially with the central cell [37]. Reaching a definitive conclusion about preferentiality of fertilization targets was difficult because none of these studies involved both wild-type sperm cells and female gametes under physiological conditions. Thus, we tried to address whether *Plumbago*-type preferential fertilization occurs in *Arabidopsis* by labeling two wild-type sperm cells differently and imaging wild-type fertilization with our live-cell imaging system.

Figure 4A shows the experimental scheme. Two sperm cell nuclei were labeled with a photoconvertible fluorescent protein, a monomeric Kikume Green-Red (mKikGR) [38] variant, fused to HTR10. We converted the fluorescence spectra of only one sperm cell nucleus from green to red by focusing an ultraviolet spotlight 2.5 μm in diameter on the nucleus. This enabled us to distinguish between the two isomorphic *Arabidopsis* sperm cells (Figure 4B). Using this technique, we examined which of the two sperm cells, identified by their position relative to the vegetative nucleus, preferentially fertilized the egg or central cell. During pollen tube growth, the two sperm cells did not exchange positions over an extended time period (>95 min) (Figure 4C; Movie S7). After photoconversion, we observed that the "back" sperm cell (the sperm cell unassociated with the vegetative nucleus) and the "front" sperm cell (the sperm cell associated with the vegetative nucleus) fertilized the egg cell and the central cell with a similar probability (Figures 4D and 4E; Table 1). We concluded that the isomorphic sperm cells of *Arabidopsis* do not show a preference with regard to fertilization targets based on their position in the male germ unit.

Our results explain why no preference for fusion was observed in previous studies involving mutants and why no gene was identified as preferentially expressed in one of the two gametes [4, 11, 39]. For example, the *SHORT SUSPENSOR* gene, which is necessary for normal embryogenesis, is transcribed in both sperm cells [40]. A sperm-specific *cis*-nat-siRNA is also involved in fertilization of the egg cell and the central cell [41]. Homologs of genes distributed unequally in two *P. zeylanica* sperm cells are expressed equally in both sperm cells of *Arabidopsis* [42]. It might be possible to assume that preferential fertilization in species with dimorphic sperms cells such as *P. zeylanica* is the exceptional case and that most species bear isomorphic functionally identical sperm cells.

Conclusions

We present the dynamics of two sperm cell nuclei during the entire process of double fertilization in *A. thaliana* at high temporal resolution (Figure S1). The initial delivery of sperm cells is faster than anticipated from previous studies. Once delivered to the chalazal edge of the degenerated synergid cell between the two female gametes, the two sperm cells remain immobile together for approximately 7.4 min. They then fuse with the egg cell and the central cell in no particular order. The long stationary phase of the two sperm cells before fusion with female gametes suggests that uncharacterized cell-cell communication mechanisms exist between male and female gametes in flowering plants.

Fluorescent conversion experiments in wild-type plants demonstrate that either sperm cell is able to fertilize either female gamete. Two models have been proposed to explain the origin of double fertilization [11, 36, 37, 41]. One is based on the idea that the two sperm cells develop differently depending on their position in the male germ unit to fertilize predetermined targets. The other is based on the idea that the two sperm cells are equal but that a polyspermy block ensures the second fertilization between the remaining cells. Our results are consistent with the second model, where fast blocking must be required in both the egg and central cells. Ancestral flowering plants, as well as gymnosperms, likely had isomorphic male gametes, and our results suggest that flowering plants might evolve mechanisms causing a rapid polyspermy block in both the egg cell and the central cell to achieve double fertilization.

Supplemental Information

Supplemental Information includes Supplemental Results and Discussion, one figure, one table, Supplemental Experimental Procedures, and seven movies and can be found with this article online at doi:10.1016/j.cub.2011.02.013.

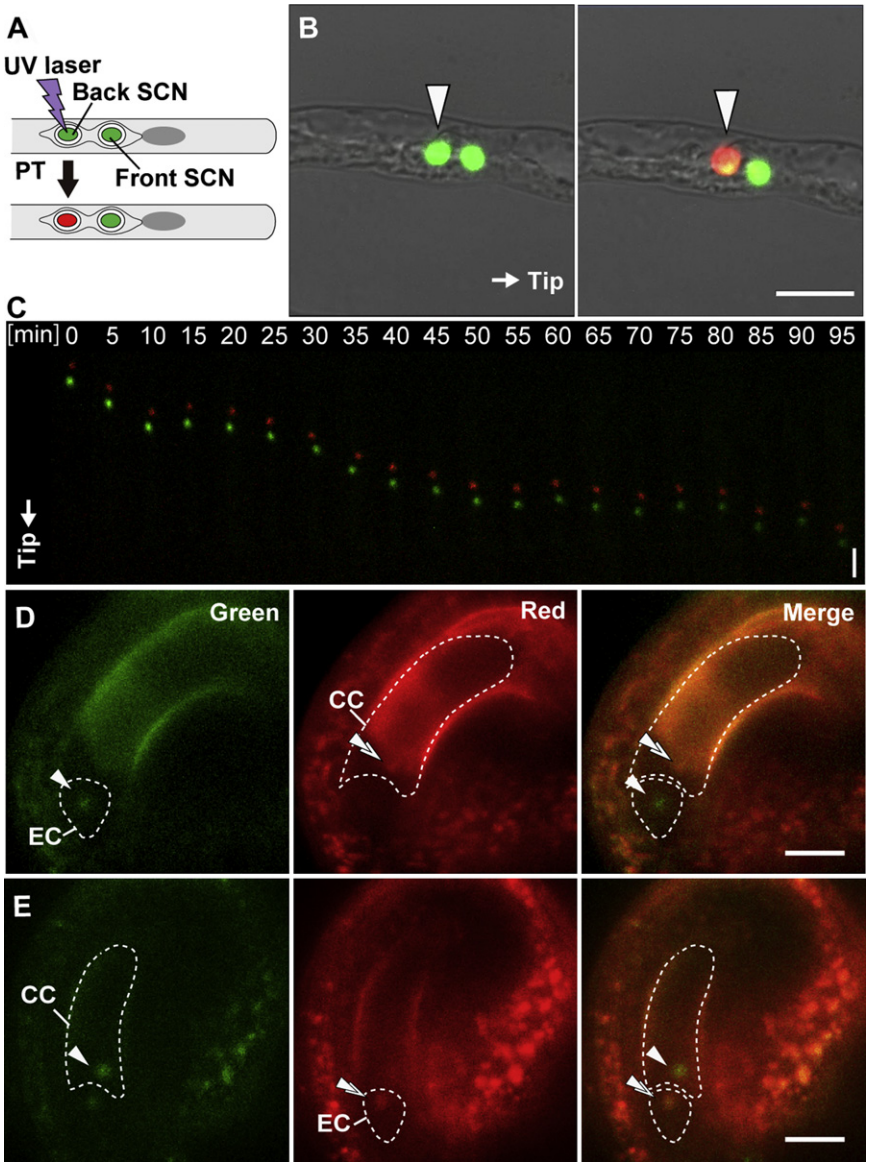

Figure 4. Photoconversion Analysis of the Two Aligned Sperm Cell Targets

(A) The scheme for photoconverting the two sperm cell nuclei (SCN), which were labeled with *pHTR10:HTR10:mKikGR*, is shown. Two sperm cells, internalized by the tube cell, are enclosed by the endocytic membrane and directly associated with the vegetative nucleus to form the male germ unit.

(B) The two sperm cells could be distinguished by photoconverting the mKikGR variant. Arrowheads indicate the photoconverted sperm nuclei. Scale bar represents 10 μm.

(C) Photoconverted sperm cells did not switch their positions in the pollen tube. Time represents elapsed time from the first frame. Scale bar represents 10 μm. See also Movie S7.

(D) When the back sperm cell nucleus at the far side from the pollen tip was photoconverted, the photoconverted sperm cell fertilized the central cell (CC, double arrowheads), whereas the other sperm cell (single arrowheads) fertilized the egg cell (EC). Scale bar represents 20 μm.

(E) Under the same experimental conditions as in (D), the photoconverted sperm cell (double arrowheads) fertilized the egg cell, whereas the other sperm cell fertilized the central cell (single arrowheads). Scale bar represents 20 μm.

Regions of the egg and central cells estimated by autofluorescence of the female gametophyte edge are shown by dotted lines in (D) and (E).

Acknowledgments

We thank Minako Ueda, Daisuke Maruyama, and Toshiyuki Mori for discussions and critical comments on our work; Tetsu Kinoshita for discussions and *pFWA:FWA:GFP* seeds; Masaru Fujimoto for *pDD45:mtKaede* seeds; Fumi Kumagai-Sano for the ARGUS image processor used in ICCD imaging; Masako Ino and Toyofumi Kameoka for technical assistance with two-photon laser microscopy; and Ayako Sawada and Koichiro Ushijima for diagram design. Y.H. was supported by grant number 9138 from the Japan Society for the Promotion of Science Fellowships. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (numbers 18075004 and 19370017 to T.H., number 18GS0314-01 to N.S., and numbers 20870020 and 21770041 to M.M.K.), 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.).

Table 1. Target of Photoconverted Sperm Cells

	Egg Cell	Central Cell	Total
Photoconversion of the back sperm cell	9	11	20
Photoconversion of the front sperm cell	11	10	21

Sperm cell nuclei were labeled with the photoconvertible protein mKikGR. One of the two sperm cells was photoconverted by an ultraviolet laser. Numbers indicate fertilizations of photoconverted sperm cells. No significant difference was observed for the target of the photoconverted sperm cells (chi-square test).

Received: December 31, 2010
Revised: February 4, 2011
Accepted: February 10, 2011
Published online: March 10, 2011

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