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Report

Fertilization Recovery after Defective Sperm Cell Release in *Arabidopsis*

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Summary

In animal fertilization, multiple sperms typically arrive at an egg cell to "win the race" for fertilization. However, in flowering plants, only one of many pollen tubes, conveying plant sperm cells, usually arrives at each ovule that harbors an egg cell [1, 2]. Plant fertilization has thus been thought to depend on the fertility of a single pollen tube [1]. Here we report a fertilization recovery phenomenon in flowering plants that actively rescues the failure of fertilization of the first mutant pollen tube by attracting a second, functional pollen tube. Wild-type (WT) ovules of *Arabidopsis thaliana* frequently (~80%) accepted two pollen tubes when entered by mutant pollen defective in gamete fertility. In typical flowering plants, two synergid cells on the side of the egg cell attract pollen tubes [3–5], one of which degenerates upon pollen tube discharge [3, 6]. By semi-in vitro live-cell imaging [7, 8] we observed that fertilization was rescued when the second synergid cell accepted a WT pollen tube. Our results suggest that flowering plants precisely control the number of pollen tubes that arrive at each ovule and employ a fertilization recovery mechanism to maximize the likelihood of successful seed set.

Results and Discussion

Identification of the *g21* Mutant Showing a Higher Rate of Successful Fertilization than Expected

The unique mode of sexual reproduction in angiosperms involves the production of two sperm cells and their delivery by a pollen tube to the female gametophyte (FG; egg producing tissue) within the ovule where double fertilization takes place [1]. Due to the limited number of studies of male-female interactions in vivo and their molecular mechanisms, we performed genetic screening in *Arabidopsis thaliana* plants for reduced fertility mutants. The screening was carried out in plants harboring the synergid-specific *MYB98::GFP* [9] marker by observing GFP signal from the synergid cells. As a primary screen, siliques of T-DNA mutagenized plants were opened and visually screened for reduced seed set. The secondary screen involved observation of the GFP signal from synergid cells when the siliques of T1 mutants showing reduced seed set were opened. This effectively allowed us to exclude mutants with genome rearrangements or early female gametophytic lethal mutants showing up to 50% of ovules that fail to show GFP signals from their synergid cells. The *g21* mutant was isolated as a candidate gametophytic fertility mutant because it showed reduced seed set and all ovules had a GFP signal. Heterozygous *g21* plants had a single sperm-like cell in 49.0% (n = 298) of the pollen population (Figures 1A and 1B). In theory, fully penetrant male gamete defective mutations are expected to show 50% fertility, but the fertility of such mutants has been a controversial issue [10, 11]. Curiously, +/*g21* plants showed 64.6% ± 6.8% (mean ± SD; n = 22 pistils) fertility, a higher rate of successful fertilization than expected (50%) (Figures 1C and 1D).

We considered two possibilities for this enhanced-fertility phenotype in the *g21* mutant. One, that a proportion (~30%) of *g21* pollen tubes would not fail and so be able to fertilize and develop into seed. The other was that due to guidance defects in *g21* pollen tubes, wild-type (WT) pollen tubes would preferentially increase the percentage seed set. Based on reciprocal testcross analysis (see Table S1 available online), the *g21* allele showed no male transmission and most pollen tubes behaved normally when stained with aniline blue [10, 12] compared with the WT. We concluded that the enhanced fertility phenotype neither resulted from successful fertilization by a proportion of *g21* tubes nor pollen tube guidance defects in *g21* pollen tubes.

Attraction of Two Pollen Tubes Results in the Enhanced-Fertility Phenotype

Because techniques for the dissection of fragile plant tissues were required for critical whole-pistil observations, we improved methods for dissection and for microscopic observation. Remarkably, we observed that an ovule attracts two pollen tubes with high frequency in +/*g21* pistils (Figures 1E and 1F), a phenomenon rarely observed in WT plants. We hypothesized that ovules receiving two pollen tubes would increase fertility in +/*g21* mutants. To investigate this hypothesis, we counted the number of ovules that had one or two pollen tubes and scored their fertility when plants of +/*g21* were crossed as the male parent to WT plants. We observed that 50.0% ± 4.9% (mean ± SD, n = 12 pistils) of developing seeds (Figure 1E) were fertilized by single pollen tube insertions, which would result from fertilization by pollen carrying the WT allele. Conversely, 18.2% ± 3.8% of developing seeds (Figure 1F) were fertilized ovules that received two pollen tubes, which likely accounts for the seed increase. Interestingly, similar to the ratio of large seeds with two pollen tubes, 16.6% ± 4.0% were undeveloped seeds penetrated by two pollen tubes (Figures 1G and 1H). Because the +/*g21* mutant showed an obvious male-specific phenotype with a single sperm-like cell in pollen and we mapped the responsible gene to chromosome 1, this indicated that the *g21* mutation may be allelic to *duo3-1*, a loss-of-function mutation in the *DUO POLLEN3* (*DUO3*) gene [13]. DNA sequence analysis and a complementation test confirmed *g21* as a null allele of *DUO3* that was designated *duo3-2* (Figure S1). These data suggest that when the first pollen tube carrying *duo3-2* fails to fertilize, a second WT pollen tube can surprisingly compensate for fertilization. Moreover, our data indicated that undeveloped seeds penetrated by two pollen tubes arise when first and second pollen tubes each carrying *duo3-2* fail to fertilize.

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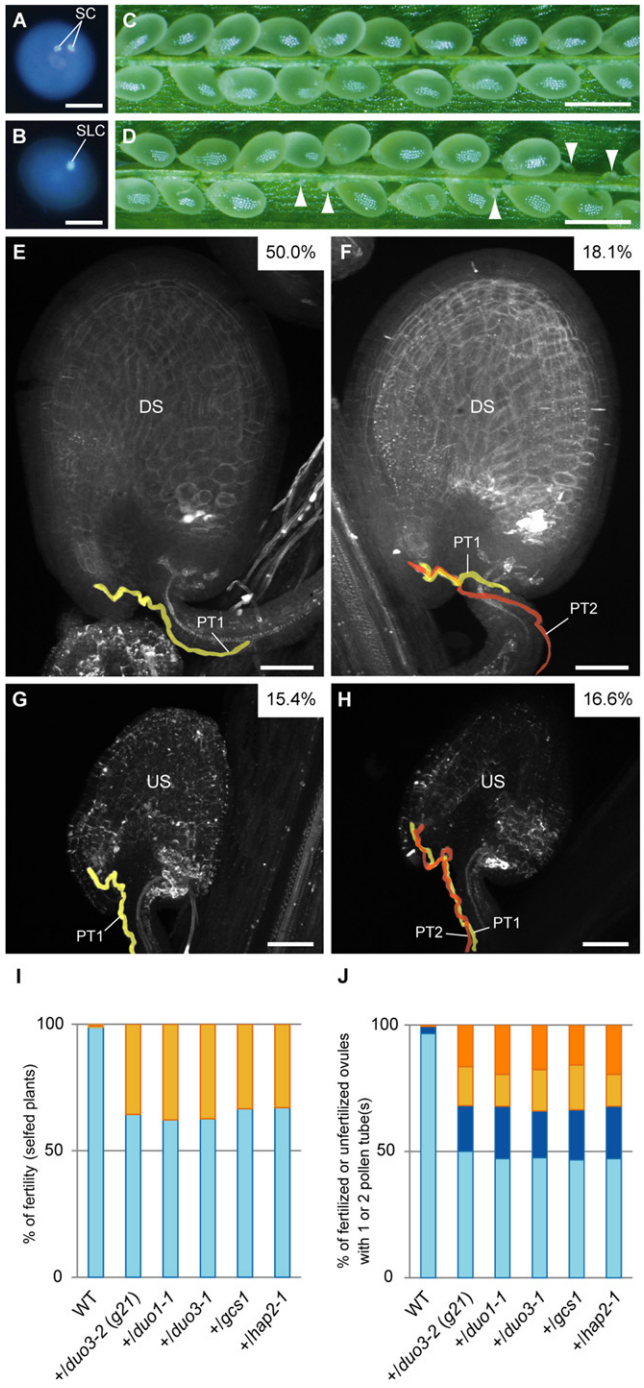

Figure 1. Double Pollen Tubes of *g21* and Other Male Gametophytic Mutants Showing a Fertility Enhancing Phenotype

(A–D) DAPI staining [26] for pollen in WT (A) with two sperm cells (SC) and *g21* (B) with one sperm-like cell (SLC). Dissected siliques for self-pollinated WT (C) and +/*g21* (D). Arrowheads indicate undeveloped seeds. +/*g21* (D) showed ~70% fertility.

(E–H) Confocal laser scanning microscopy images [27, 28] 3 days after self-pollination. Developing seeds (DS) with one (E) or two (F) pollen tube(s) and undeveloped seeds (US) with one (G) or two (H) pollen tube(s) are shown. Percentages indicate the ratio of each type of ovule within a silique.

(I) Percentage fertility 3 days after self-pollination of WT, +/*duo3-2* (*g21*), +/*duo1-1*, +/*duo3-1*, +/*gcs1*, and +/*hap2-1*. Developing seeds (blue) and undeveloped seeds (orange) are indicated. Male gametophyte (MG) mutants showed 60%–70% fertility similar to each other.

(J) Percentages of developing seeds with one (blue) or two (dark blue) pollen tube(s) and undeveloped seeds with one (orange) or two (dark orange) pollen tube(s) in 3-day-old out-crossed plants (female, +/+; male, +/*duo3-2*). Percentages of developing seeds in (I) correspond to the sum of developing seeds in (J), suggesting that second pollen tubes increase the fertility in MG mutants. PT1, pollen tube 1; PT2, pollen tube 2. Scale bars in (A) and (B) represent 10 μm, in (C) and (D) represent 500 μm, and in (E)–(H) represent 20 μm.

Because a previous report mentioned that the male gamete defective mutant *hapless 2-1* (*hap2-1*) also had enhanced fertility [11], we carried out a similar analysis for *hap2-1*. We also investigated *duo pollen 1* (*duo1-1*) [14], *duo3-1* [13], and *generative cell specific 1* (*gcs1*) [10] (allelic to *hap2-1*) mutants because they also have defective male gametes leading to failure of fertilization. All mutants showed an increased fertility phenotype similar to *duo3-2* (Figure 1I). Furthermore, the percentage of developing seeds penetrated by two pollen tubes (Figure 1J; dark blue) corresponded to that of undeveloped seeds penetrated by two pollen tubes (Figure 1J; dark orange) as observed in *duo3-2*, indicating that two types of second pollen tube carrying either WT or mutant allele proportionally enter ovules, such that only half of the ovules with two pollen tubes develop into seeds. These data suggested that the enhanced-fertility phenomenon that is common to all three male gamete defective mutants may be explained by the same mechanism—failure of fertilization by a first mutant pollen tube that is rescued by a second WT pollen tube. In angiosperms, a pollen tube delivers nonmotile sperm cells accurately to the FG and completes fertilization. This is called "siphonogamy," a mechanism that is thought to have evolved from zooidogamy (fertilization by motile sperm) [15]. Here we define the term "polysiphonogamy" for cases in which an ovule accepts multiple pollen tubes.

Visualization of the Fertilization Recovery Phenomenon by a Semi-In Vitro Assay

To confirm that the fertilization recovery phenomenon is accomplished by polysiphonogamy and to visualize the moment of the recovery event, we crossed pollen from +/*duo3-2* plants to WT stigmas and observed the destiny of the sperm cells using a semi-in vitro fertilization assay [7, 8, 16] In WT, the two sperm cells successfully fertilized the egg cell and the central cell (Figures 2A–2F; Movie S1). However in +/*duo3-2*, the mutated sperm cell was successfully released into the FG but the cell remained arrested near the degenerated synergid cell without fertilization (Figures 2G–2L; Movie S1). Next, we observed that the first pollen tube failed to fertilize, but remarkably, two sperm cells of the second pollen tube were discharged to the FG and their nuclei migrated to the nuclei of the egg cell and the central cell, respectively (Figures 2M–2R; Movie S2). We also confirmed these phenomena in the +/*gcs1* mutant [10] (Figure S2; Movie S3). In total, we observed six examples in +/*duo3-2* and +/*gcs1* mutants, all of which showed that the first mutant pollen tube failed but the WT second pollen tube succeeded in double fertilization. These data indicate that the second pollen tube and the remaining synergid cell allow fertilization even though the first pollen tube fails to fertilize, thereby rescuing the defect in seed set.

Attraction and Insertion of the Second Pollen Tube by the Second Synergid Cell

We made another significant observation; that is, we never observed a third pollen tube entering the micropyle. As shown

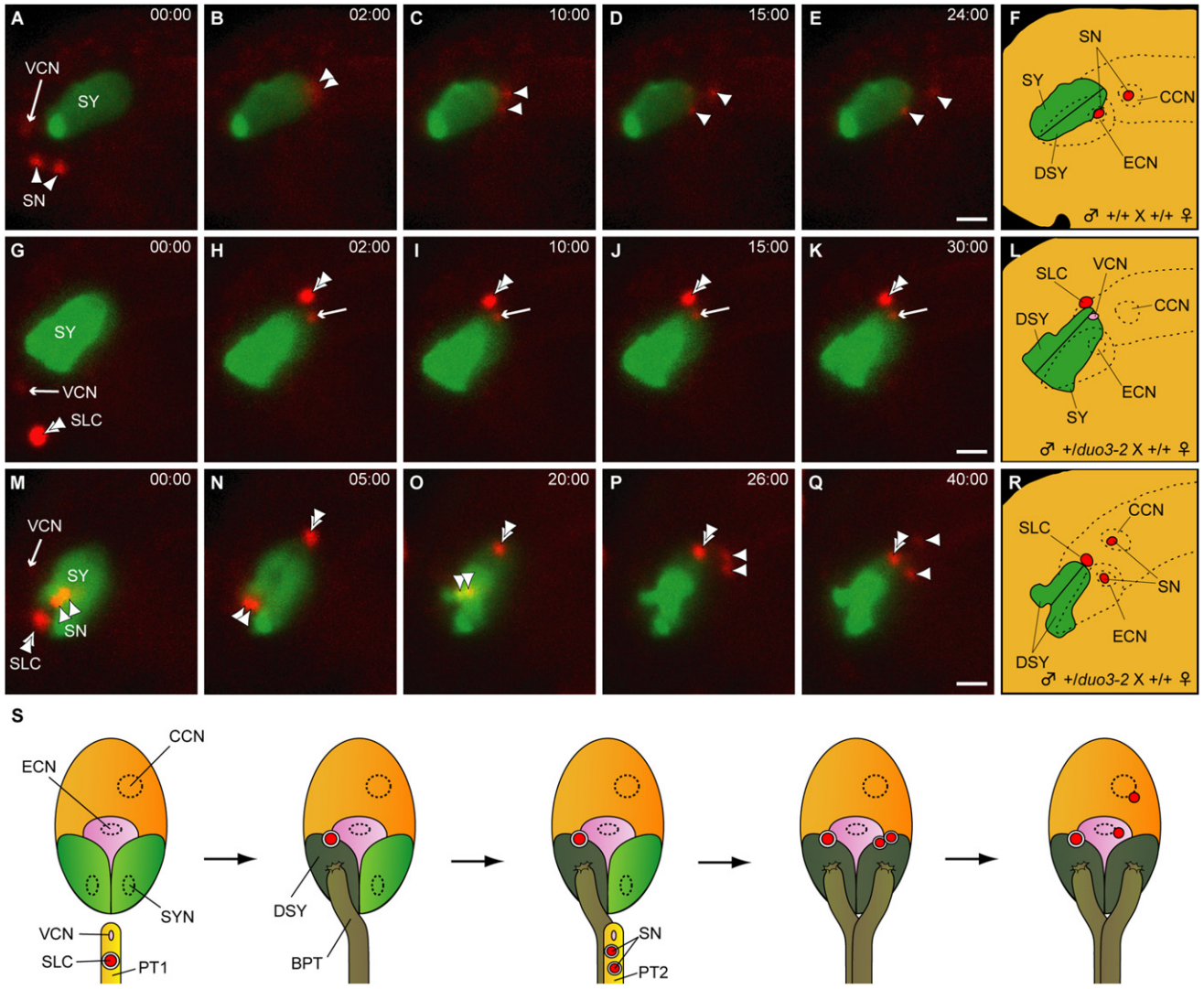

Figure 2. Semi-In Vitro Fertilization Assays for *duo3-2* Showing a Recovery of Fertilization

(A–K) In WT, two sperm cells are released from a pollen tube and fertilize the egg cell and the central cell (A)–(E), Movie S1). In the case of single insertion of a *duo3-2* pollen tube, a single SLC is released from a pollen tube but is arrested in the middle of the FG without fertilization (G–K; Movie S1).

(L–R) In the case of pollen tubes from +/*duo3-2* heterozygotes, the first pollen tube from a *duo3-2* allele releases a SLC and the cell fails to fertilize (M–Q). However, the second pollen tube from a WT allele is inserted to the same ovule and releases two sperm cells to complete fertilization (Movie S2). At first pollen tube discharge, one of the two synergid cells is collapsed, and upon second pollen tube discharge, the other synergid cell is collapsed. (F), (L), and (R) depict the final destinations of sperm cells or SLC in WT (F), *duo3-2* (L), and double insertion of *duo3-2* and WT pollen tubes (R).

(S) A schematic drawing of the second pollen tube rescuing the fertilization. The synergid cell (SY) was labeled with *MYB98p:GFP:ROP6* [29]. The sperm cell nuclei (SN) and the SLC nucleus were labeled with *RPS5Ap::H2B-tdTomato* [30]. Numbers indicate time (min) after the start of pollen tube discharge. VCN, vegetative cell nucleus; ECN, egg cell nucleus; CCN, central cell nucleus; DSY, degenerated synergid cell; BPT, burst pollen tube; PT1, first pollen tube; PT2, second pollen tube. Scale bar represents 10 μm.

in Figures 2M–2R and Movie S2, every time the pollen tube discharges its contents into the FG, the synergid cell degenerates. Loss of both synergid cells would prevent pollen tube attraction [4, 9], possibly due to the loss of attractants [5, 17], explaining why only two, but not three or more pollen tubes, are attracted in male gamete defective mutants. Although female gametophytic mutants defective in pollen tube guidance (*myb98*) [9] and pollen tube reception (*fer/sir* and *lolelei*) [18–20] sometimes attract more than three pollen tubes, the number of pollen tubes appears to be strictly controlled in WT ovules. We conclude that the recovery of fertilization is limited to the second pollen tube, indicating that there is no third chance for fertilization in two-synergid celled plants. In theory, multiple-synergid celled plants as reported in the *ig1* mutant of maize [21] may therefore have a greater capacity to attract multiple pollen tubes. In fact, the three-synergid celled female gametophytes present in *Amborella trichopoda* [22] sometimes attract three pollen tubes [23], suggesting that some plants might have a third chance to compensate for failed fertilization.

Fertilization Recovery by the Second Pollen Tube Is Restricted Only When the First Tube Fails

To confirm that the recovery event takes place only when the first tube fails during fertilization, we double-stained +/*hap2-1* pollen tubes for β-glucuronidase (GUS) activity

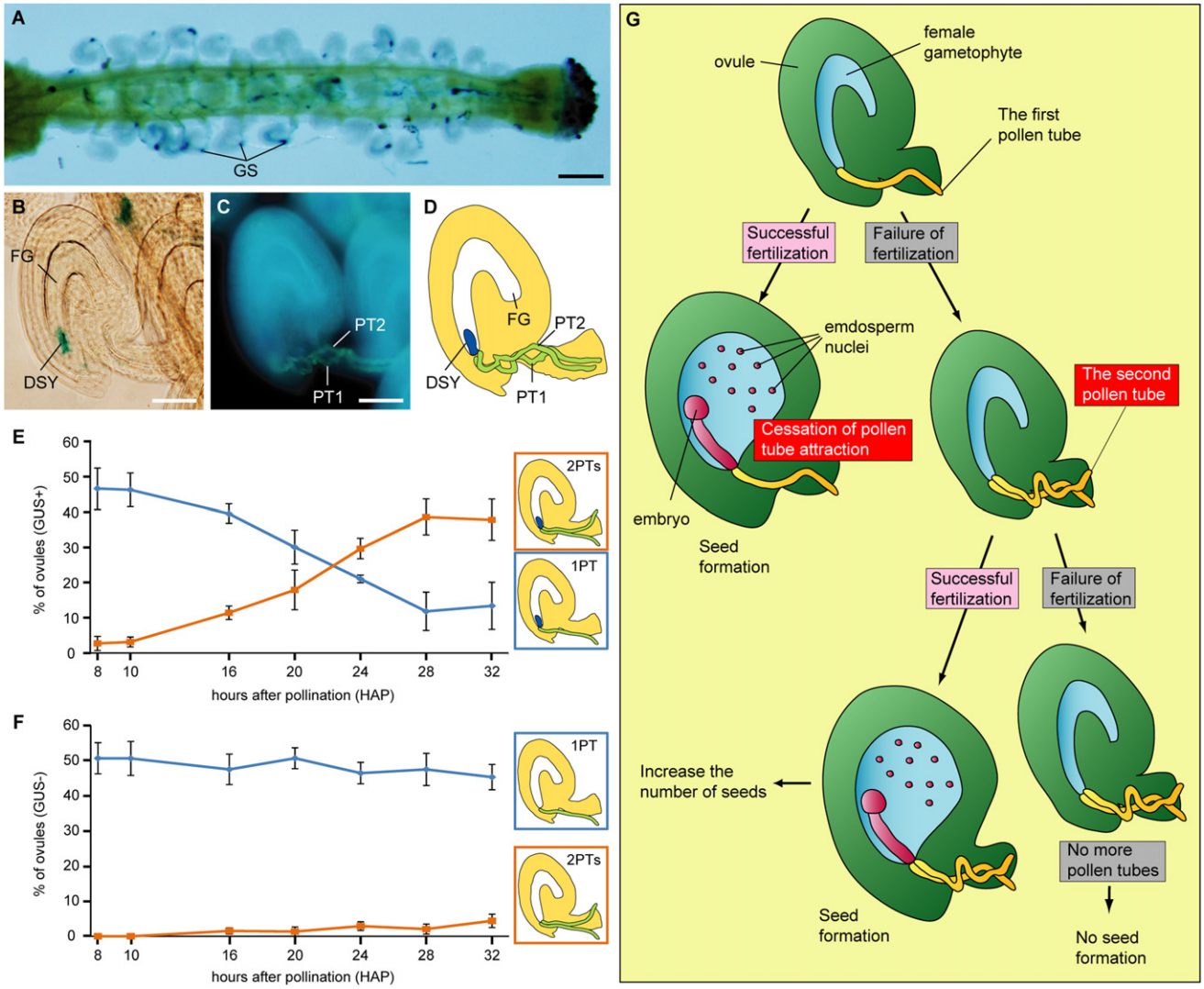

Figure 3. GUS Staining of *hap2-1* Showing the Second Pollen Tube Systematically Recovering Fertility

(A) A typical pistil (female, +/+; male, +/*hap2-1*) 10 HAP. About 50% of ovules have a blue GUS spot (GS), suggesting that WT and *hap2-1* pollen tubes are proportionally inserted to each ovule.

(B–D) An ovule with blue degenerated synergid cell (DSY), (B) 20 HAP (female, +/+; male, +/*hap2-1*). Aniline blue staining of two pollen tubes (PT1, PT2) inserted to an ovule (C) as illustrated in (D).

(E) Percentage of GUS+ ovules with one (blue) or two (orange) pollen tubes. At 8 or 10 HAP, only one pollen tube was inserted to almost all GUS+ ovules but at 28 HAP or later, the ratio of two pollen tubes reached a maximum, suggesting that the second pollen tube was positively inserted to rescue fertilization.

(F) Percentage of GUS− ovules. One pollen tube was inserted to most ovules, and the second pollen tube was scarcely observed. FG, female gametophyte. Error bars indicate SD from the means of at least four independent experiments.

(G) Schematic drawing of the fertilization recovery system. Once a single pollen tube is inserted to an ovule, the pollen tube bursts and releases two sperm cells. When the sperm cells complete fertilization, the ovule blocks the entry of the other pollen tubes and develops into a seed by forming embryo and endosperm. When the sperm cells fail to fertilize, the ovule attracts the second pollen tube to rescue fertilization. The rescued ovule develops into a seed, resulting in increased fertility. In the case of failure of fertilization by the second pollen tube, the ovule does not attract a third pollen tube possibly due to depletion of pollen tube attractant from synergid cells because both synergid cells are collapsed upon double entry of pollen tubes. Scale bar in (A) represents 200 μm and in (B) and (C) represent 40 μm.

followed by aniline blue staining to trace the behavior of the first and the second pollen tubes (Figures 3A–3D). Because *hap2-1* mutant pollen tubes are marked by the pollen tube-specific reporter gene, *LAT52:GUS* [11, 24], we could trace the destination of *hap2-1* mutant pollen tubes in vivo. Moreover, the *hap2-1* mutant was generated by an insertion of T-DNA harboring the *LAT52:GUS* reporter gene, so GUS-positive signals originate from *hap2-1* alleles, whereas WT alleles are GUS-negative. We counted the number of the GUS-positive ovules, providing evidence of a burst *hap2-1* pollen tube and gamete release into the FG, by crossing +/*hap2-1* as a male parent to WT flowers. As shown in Figure 3E, 10 hr after pollination, we observed that 49.4% ± 4.8% (n = 5 pistils) of the ovules accepted a *hap2-1* allele, suggesting that *hap2-1* and WT pollen tubes were similarly competent to enter each FG and release their contents successfully. It has been suggested by von Besser et al. [11] that *hap2-1* sperm cells affect pollen tube guidance [11]. However, judging from *duo3-2* (*g21*) data

(Figure S1) and our *hap2-1* data, we conclude that the sperm cells appear to be passive cargo of the pollen tube and do not influence pollen tube guidance.

To explore the temporal sequence of events involved in the rescue of fertilization after failure of the first mutant pollen tube in vivo, we performed a time course experiment by double-staining of pollen tubes. Ovules began to accept pollen tubes from 5 hr after pollination (5 HAP), and at 10 HAP, all ovules accepted at least one pollen tube as reported previously [6]. At 10 HAP, 6.3% ± 2.7% (mean ± SD, n = 5 pistils) of GUS-positive ovules had attracted two pollen tubes (Figure 3E). However, by 20 HAP, 38.5% ± 8.7% (n = 7) of GUS-positive ovules had attracted two tubes (note that the frequency of GUS-positive ovules with a single *hap2-1* pollen tube decreased accordingly). In contrast, at 10 HAP, no ovules (n = 5 pistils) accepting a WT allele had attracted two pollen tubes (Figure 3F) and by 20 HAP, only 2.7% ± 2.7% (n = 7) of ovules had attracted two WT tubes, indicating that second pollen tubes were attracted at a much higher frequency by ovules that had first accepted a *hap2-1* pollen tube. We conclude that ovules have an unknown system that senses the completion of fertilization and might prevent other pollen tubes from entering the micropyle. However, if the first pollen tube fails to fertilize, ovules do not sense the completion of fertilization, allowing the attraction of a second pollen tube and its entry into the micropyle (Figure 3G).

It has long been suggested that only one pollen tube principally enters and releases sperm cells into an ovule [1, 7]. Although classical studies [1], in at least 13 species, have reported rare cases of two pollen tubes in an embryo sac and Mori et al. showed that an ovule rarely had two sets of sperm cell pairs after pollination with +/*gcs1* pollen [10], these have been regarded as anomalous events [1]. We showed that most ovules have one pollen tube at 10 HAP, indicating that until several hr after the arrival of the first pollen tube, the one tube—one ovule system might be maintained by a blocking system to avoid polysiphonogamy [2]. Then, the second pollen tube starts to be attracted again by ovules that failed at fertilization in *hap2-1*. In this case, the persistent synergid cell, which may begin to degenerate in fertilized ovules [1], continued to attract pollen tubes, resulting in 76.7% ± 2.7% (n = 5) of failed ovules accepting the second pollen tube at 28 HAP. No particular role has been proposed for the persistent synergid cell after the arrival of the first pollen tube [1, 3, 7]. However, we demonstrate that the second synergid cell retains its function and is able to attract and accept a second tube to rescue fertilization. This could be one of the reasons why two synergid cells are present in many higher plants. Further, we observed that it takes 28 hr for all second tubes to complete their entrance into the ovules. This result indicates that some pollen tubes are maintained inside the pistil for an extended time providing an opportunity to compensate for the failure of other pollen tubes.

Conclusions

Overall, we have unveiled a fertilization recovery system that allows plants to restore ovule fertility after initial failure of fertilization within the ovule. Further investigation of this phenomenon could advance the understanding of how natural populations of sympatric species are maintained. For example, heterospecific pollen tubes interfere with the fertilization of ovules by conspecific pollen by various mechanisms [25]. When heterospecific pollen tubes are able to enter the ovules but fail at steps leading to gamete fusion, fertilization recovery by conspecific pollen tubes could diminish the detrimental effect of pollen competition, contributing to the maintenance of reproductively isolated populations of sympatric species.

Supplemental Information

Supplemental Information includes two figures, one table, Supplemental Experimental Procedures, and three movies and can be found with this article online at doi:10.1016/j.cub.2012.03.069.

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