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Gamete fusion is required to block multiple pollen tubes from entering an Arabidopsis ovule

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Summary

In double fertilization, a reproductive system unique to flowering plants, two immotile sperm are delivered to an ovule by a pollen tube. One sperm fuses with the egg to generate a zygote, the other with the central cell to produce endosperm[1]. A mechanism preventing multiple pollen tubes from entering an ovule would ensure that only two sperm are delivered to female gametes. We use live-cell imaging[1, 2] and a novel mixed-pollination assay that can detect multiple pollen tubes and multiple sets of sperm within a single ovule to show that Arabidopsis efficiently prevents multiple pollen tubes from entering an ovule. However, when gamete-fusion defective *hap2(gcs1)* or *duo1* sperm are delivered to ovules as many as three additional pollen tubes are attracted. When gamete fusion fails, one of two pollen tube-attracting synergid cells persists, enabling the ovule to attract more pollen tubes for successful fertilization. This mechanism prevents the delivery of more than one pair of sperm to an ovule, provides a means of salvaging fertilization in ovules that have received defective sperm, and ensures maximum reproductive success by distributing pollen tubes to all ovules.

Keywords

fertilization; gamete fusion; supernumerary pollen tubes; GCS1; HAP2; sperm; pollen tube reception; polytubey; polyspermy

Results and Discussion

‘Polytubey’ increases when sperm incapable of gamete fusion are delivered to ovules

We use the term ‘polytubey’ (many pollen tubes) to describe the attraction of multiple pollen tubes to a single ovule. We chose this term to facilitate comparison with ‘polyspermy’ (the fusion of more than one sperm with a female gamete), which is distinct and also needs to be prevented to maximize reproductive success[3]. We developed a mixed pollination assay that unambiguously detects polytubey (Figure 1A). Half of a *male sterile1-1* (*ms1-1*) stigma was hand-pollinated with pollen expressing GFP from the pollen-specific LAT52 promoter[4] *(LAT52:GFP)*; the other half with pollen expressing *DsRed*[5] *(LAT52:DsRed)*. These markers define two nearly isogenic populations of pollen tubes and allow pollen tube growth, guidance, and burst to be monitored in the pistil. Twenty-one hours after pollination 48% of ovules had been targeted by a *LAT52:DsRed* pollen tube (Figure 1A, left) and 51% by a *LAT52:GFP* pollen tube (Figure 1A, middle). This slight

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discrepancy from equality reflects the difficulty of placing an equal load of pollen from each genotype on the stigma. Nonetheless, the ~1% of ovules with both the GFP and DsRed signal were obvious and unambiguously indicate that these ovules had been targeted by two pollen tubes (Figure 1A, right).

Analysis of pollen tube growth to ovules in vitro showed that late-arriving additional pollen tubes were actively repelled[6]. Furthermore, analysis of several female gametophyte mutants (e.g. *feronia*) that fail to signal to the pollen tube to stop growing, burst, and release sperm indicated that these all attract multiple pollen tubes[7–11]. These studies suggested an active mechanism that blocks polytubey and indicate that any of the steps in double fertilization that occur after the pollen tube stops growing and burst could trigger the block. We tested whether gamete fusion is a potential trigger using Arabidopsis *hap2-1* mutants[12, 13]. Arabidopsis *hap2(gcs1)* mutant pollen tubes target ovules, burst, and release sperm, but mutant sperm do not fuse with female gametes [2, 14]. We performed mixed pollination assays using pollen homozygous for *LAT52:GFP* and heterozygous for *hap2-1/ HAP2(GCS1)* and pollen homozygous for *LAT52:DsRed* and *HAP2(GCS1).* In this experiment, half of the GFP-expressing pollen tubes carry wild-type sperm and half carry *hap2-1* mutant sperm. All DsRed-expressing pollen tubes carry wild-type sperm. The rate of polytubey increased 10 fold over wild-type (Figure 1A) even though only ¼ of the pollen on the stigma carried *hap2-1* mutant sperm.

We found that like *hap2(gcs1)* mutants, *duo1-3* (Figure S1) mutants enhance polytubey ~10 fold over levels observed in the No-0 accession (wild type, genetic background of *duo1-3*, Figure 1B). *duo1* mutant pollen tubes carry a single sperm-like cell that is incapable of fertilization[15, 16]. These data support the hypothesis that gamete fusion, or an event shortly thereafter is the trigger that prevents multiple pollen tubes from entering an ovule.

To closely monitor pollen tube burst and sperm release, we generated *hap2-1* and wild-type pollen tubes expressing *LAT52:GFP* along with the sperm nuclear marker *HTR10:HTR10:mRFP*. Twenty-one hours after pollination, wild-type pollen tubes had burst and sperm nuclei had fused with the egg and central cell nuclei (Figure 1C, left panel). The *HTR10:mRFP* signal diffuses after nuclear fusion as it incorporates into the egg or central cell nucleus[17]. *hap2-1* mutant pollen tubes also burst, but sperm nuclei remained compact at the site where gamete plasma membrane fusion would normally occur (Figure 1C second panel, [2]). In many cases, we observed multiple pairs of sperm nuclei (Figure 1C, third and fourth panels) and as many as four pairs of sperm were detected indicating that four pollen tubes had been attracted to one ovule (1 pair of unfused sperm, n=118; 2 pairs of unfused sperm, n=10; 3 pairs of unfused sperm, n=2; four pairs of unfused sperm, n=1). These data suggest that when gametes do not fuse, the block to polytubey fails and ovules continue to attract multiple pollen tubes.

The block to polytubey enhances reproductive success

To determine whether fertilization can be salvaged in an ovule that has attracted a pollen tube with defective sperm, we performed a mixed pollination experiment to analyze interactions between sperm and female gametes. Two types of differentially marked pollen were placed on the stigma at the same time: 1) *hap2-1/HAP2(GCS1)* pollen marked with *HTR10:HTR10:mRFP*; 2) wild-type pollen marked with *LAT52:GFP*. In addition to the expected cases where a single pollen tube was attracted (Table 1, Figure S2), a significant number of polytubey events were observed (Table 1, 39/432 targeted ovules). These included ovules containing multiple pairs of non-functional sperm (Figure S2). Importantly, these also included ovules that contained a pair of unfused RFP+ sperm nuclei and a wild-type, GFP+ pollen tube; and ovules containing a pair of nonfunctional RFP+ sperm (*hap2-1*) along with a pair of RFP+ wild-type sperm that had fused with female nuclei (Figure S2).

We did not observe ovules that were targeted by two pollen tubes carrying wild-type sperm (e.g. wild-type GFP+ pollen tube and functional RFP+ sperm). As expected, when this mixed pollination experiment was conducted with wild-type pollen, polytubey was very rare (2/411 targeted ovules, Table 1). These data indicate that ovules first targeted by defective sperm can attract additional pollen tubes; but when wild-type sperm are attracted subsequent pollen tubes are blocked.

If gamete fusion is required to trigger the block to polytubey, we would predict that polytubey would by exacerbated if *hap2-1/HAP2(GCS1)* pollen tubes were allowed to begin growth before addition of wild-type (GFP+) pollen to the stigma. In this scenario, the number of ovules that receive defective sperm would be increased. When mixed pollinations were staggered by 1.5 hours and *hap2-1/HAP2(GCS1)* pollen was applied first, the rate of polytubey further increased to 23% (Table 1). However, if wild type pollen was applied first, polytubey decreased to well below 1% and the vast majority of ovules were targeted by a wild-type GFP+ pollen tube.

Pollen tube-attracting synergid cells persist in ovules that have received defective sperm

We observed that ovules receiving defective sperm attracted up to four pollen tubes (Figure 1C). In *Torenia fournieri*, synergid cells secrete pollen tube attractants called LUREs[18] and at least one of the two synergids must be intact for pollen tube attraction[19]. In Arabidopsis, synergids are also required for pollen tube attraction[20] and degeneration of one of two synergids is correlated with arrival of a pollen tube [21]. We propose that in the absence of gamete fusion, ovules are able to continue to attract pollen tubes because one of the synergids persists and continues to secrete attractants. This would potentially explain the adaptive significance for the occurrence of two synergids in many species of flowering plants. We determined the relationship between synergid degeneration, gamete fusion, and pollen tube attraction in ovules receiving wild type or *hap2(gcs1)* mutant sperm.

Upon synergid degeneration, *ACT11:MSI1:GFP* signal is no longer concentrated in the synergid nucleus and becomes diffuse throughout the cytoplasm, providing a clear marker for this important event[17]. We found that all ovules that had received either wild-type sperm (fused *HTR10:HTR10:mRFP* pattern, Figure 2C,D) or *hap2-1* mutant sperm (unfused, Figure 2E,F) had one degenerated synergid (Figure 2C,E), or degeneration of both synergids (Figure 2D,F). Therefore, arrival and burst of a *hap2-1* mutant pollen tube triggers degeneration of one synergid cell as in wild type. Ovules that had attracted multiple pairs of sperm also had either one or two degenerated synergids (Figure 2G,H). This result shows that arrival of a second pollen tube does not trigger degeneration of the persistent synergid and suggests that a downstream event like gamete fusion may be required.

To begin to address this hypothesis, we analyzed synergid degeneration over time. At four hours after pollination, no ovules had been targeted and all ovules contained two intact synergid cells (Figure 2I). At each subsequent time point (8, 12, 16, 20 hours), we found that ovules with unfused sperm were more likely to have an intact second synergid than ovules with fused sperm (Figure 2I U, unfused *hap2-1*, v. F, fused wild type). Twelve hours after pollination, 97% of ovules with unfused sperm had a persistent synergid compared with 48% that had been fertilized by wild-type sperm. By 20 hours after pollination both synergids had degenerated in all ovules with fused sperm, whereas 50% of ovules with unfused sperm still had one intact synergid. These data show that if gamete fusion fails, a pollen tube-attracting synergid cell persists and can attract additional pollen tubes.

Conclusions

We have shown that the attraction of multiple pollen tubes to one ovule is rare in Arabidopsis (Figure 1B), but polytubey increases dramatically when sperm incapable of gamete fusion are deposited (Figure 1, Table 1). In these cases, one synergid persists and can continue to attract multiple pollen tubes until fertile sperm are delivered or the synergid senesces. Interestingly, in wild type, one synergid cell persists well after gamete fusion[21], but multiple pollen tubes are rarely attracted. This suggests that gamete fusion itself, or an event soon thereafter (e.g. nuclear fusion, initiation of development), triggers a mechanism that rapidly blocks additional pollen tubes from entering a fertilized ovule.

The cases of polytubey observed in Arabidopsis mutants can all be accounted for by defects in a block to polytubey that requires gamete fusion. Female mutants defective in pollen tube reception (e.g. *feronia*) fail to instruct pollen tubes to burst, sperm are not released, gamete fusion does not occur, and consequently, they attract multiple pollen tubes [7–11]. We predict that any mutation disrupting gamete fusion will result in polytubey.

The block to polytubey was likely a significant evolutionary innovation allowing flowering plants to maximize reproductive success by promoting fertilization of all ovules and ensuring that only two sperm are delivered to each ovule. Pollen tubes bypassing fertilized ovules can continue to grow to unfertilized ovules. If the block to polytubey were initiated by any earlier step in the process (pollen tube entry, pollen tube contact with the female gametophyte, pollen tube burst), then ovules would go unfertilized in cases where pollen tubes failed to burst or sperm were defective. The block to polytubey is therefore a final opportunity for the flower to scrutinize pollen, reject unworthy suitors and replace them with productive mates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

A ‘block to polytubey’ is triggered by gamete fusion or an event soon thereafter

If defective sperm are deposited, additional pollen tubes are attracted

Pollen tube-attracting synergid cells persist when defective sperm are delivered

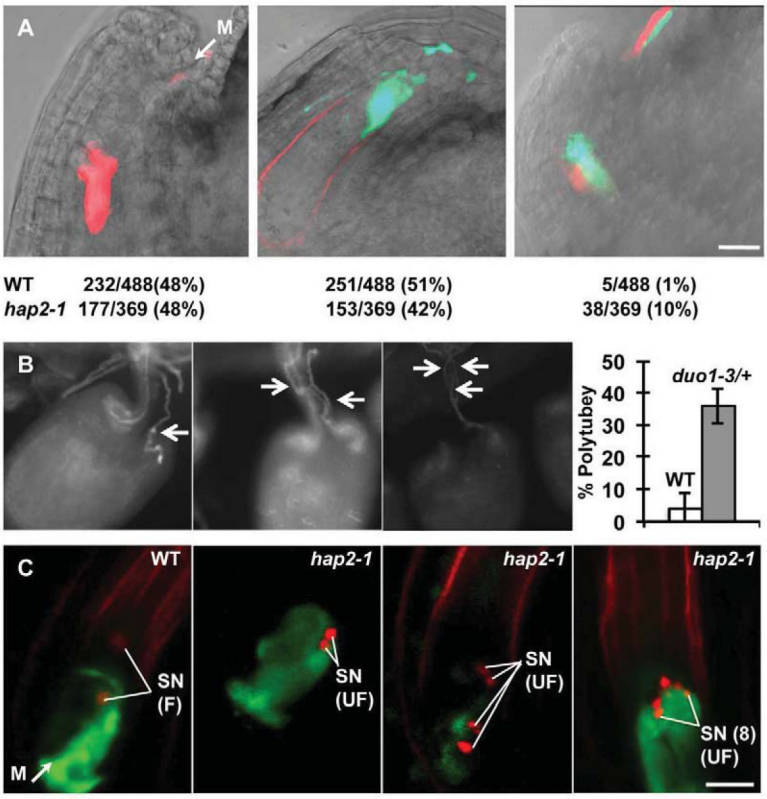

Figure 1.

Polytubey is rare in wild type, but increases significantly when gamete fusion fails. (**A**) *hap2(gcs1)* enhances polytubey. Differentially marked pollen tubes can be tracked as they grow into an ovule at the micropylar end (M). Representative images of ovules targeted by a DsRed+ pollen tube (left), a GFP+ pollen tube (center), or by two different pollen tubes (right). The number of each type of targeting event observed is also shown (bottom). WT, *LAT52:GFP* pollen mixed with *LAT52:DsRed* (both types of pollen are wild type) or *hap2-1* (*hap2-1/HAP2(GCS1), LAT52:GFP* mixed with wild-type, *LAT52:DsRed* pollen). Scale bars, 20μm. (**B**) *duo1* enhances polytubey. *duo1-3/+* heterozygous plants were manually self-pollinated and pollen tubes were analyzed 24 hours later using aniline blue staining[14]. Ovules are shown that have attracted a single pollen tube (left panel), two pollen tubes (center panel), or three pollen tubes (right panel). The percentage of ovules with two or more pollen tubes is plotted for No-0 (wild type, 9 pistils, 260 targeted ovules) and *duo1-3/+* (8 pistils, 275 ovules targeted). Error bars represent standard deviation (each pistil is one trial). Rates of polytubey are higher in the No-0 accession (4%) than in *ms-1* (Landsberg accession, 1%, Figure 1A) and are significantly enhanced by *duo1-3* (36%). (**C**) Multiple *hap2-1* sperm are released into a single ovule. The first panel shows an ovule targeted by a wild-type *HTR10:HTR10:mRFP, LAT52:GFP* pollen tube. Green signal is released from the burst pollen tube; sperm nuclei (SN, red signal) have fused (F) with the egg cell and central cell nuclei. The second panel shows an ovule targeted by a *hap2-1, HTR10:HTR10:mRFP, LAT52:GFP* pollen tube. The pollen tube has burst (green signal), but sperm cell nuclei remain unfused (UF). The third panel shows an ovule containing four unfused *hap2-1, HTR10:HTR10:mRFP* sperm. The last panel shows an ovule containing eight unfused *hap2-1, HTR10:HTR10:mRFP* sperm. Five sperm nuclei are clearly visible, the others are outside the plane of this image. In this ovule, pollen tube cytoplasm (GFP) appears to fill the area occupied by both synergids. Scale bars, 10μm.

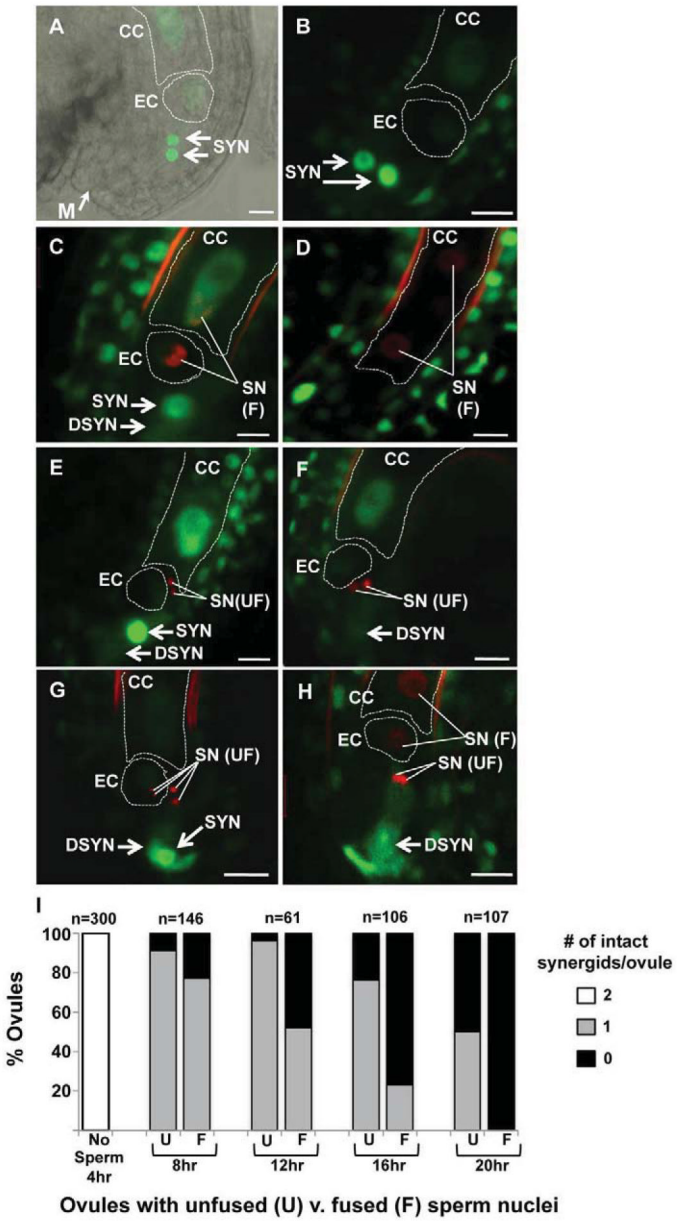

Figure 2.

The remaining synergid cell persists longer in ovules targeted by nonfunctional sperm. (**A**) An unfertilized ovule expressing *ACT11:MSI1:GFP*. DIC and confocal images are overlaid to highlight the position of the micropyle (M, same in all panels) and GFP accumulation in the egg (EC), central (CC) and synergid (SYN) nuclei. (**B–H**) Pistils were pollinated with *hap2-1/HAP2(GCS1), HTR10:HTR10:mRFP*. Representative confocal micrographs are shown of an unfertilized ovule (**B**), ovules that have received sperm and have a persistent synergid (SYN) (**C,E,G**), or ovules that have received sperm and both synergids have degenerated (DSYN) (**D,F,H**). (**D**) *ACT11:MSI1:GFP* signal was not detectable in synergids because this image was obtained later in development (note dividing primary endosperm) and significantly after synergid degeneration. (**I**) Ovules were scored for the number of synergids that remain intact (2, white bar; 1 gray bar; 0 black bar) and whether they had received sperm whose nuclei fused with female nuclei (fused, F) or sperm that remained

unfused (U). Data are reported as the percentage of the total number of ovules analyzed (n) at each time point following pollination (hours, hr). Scale bar, 10μm.

Table 1

Ovules that have attracted defective sperm can attract functional sperm and polytubey increases if pistils are first pollinated with *hap2-1/HAP2* pollen before wild-type pollen is added

		Single pollen tube (signal observed)			Polytubey (signals observed)					
		RFP+F	**RFP+U**	**GFP+**	**RFP+F GFP+**	**RFP+U GFP+**	**RFP+U RFP+U**	**RFP+U RFP+F**		
Simultaneous Mixed Pollination									***N***	**Polytubey**
hap2-1/HAP2 HTR10:RFP	wild type *LAT52:GFP*	34	70	289	0	33	3	3	432	39/432 (9%)
wild type *LAT52:GFP*	wild type *LAT52:GFP*	178	0	231	1	1	0	0	411	2/411 (0.4%)
Staggered Mixed Pollination										
Added first	Added 1.5 hours later									
hap2-1/HAP2 HTR10:RFP	wild type *LAT52:GFP*	87	58	93	0	51	7	14	310	72/310 (23%)
wild type *LAT52:GFP*	*hap2-1/HAP2 HTR10:RFP*	12	4	353	0	1	0	0	370	1/370 (0.3%)

Pollen genotypes for mixed pollinations are given in the first two columns.(h), time in hours of pollination; *HTR10:RFP, HTR10:HTR10:RFP;* RFP+F, ovule contains RFP+ sperm nuclei that have undergone karyogamy; RFP+U, RFP positive sperm that remain unfused; GFP+, GFP positive pollen tube has burst in the ovule; N, total number of ovules targeted.