Pollen Tube Discharge Completes the Process of Synergid Degeneration That Is Initiated by Pollen Tube-Synergid Interaction in Arabidopsis[1][OPEN]

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In flowering plant reproduction, pollen tube reception is the signaling system that results in pollen tube discharge, synergid degeneration, and successful delivery of male gametes (two sperm cells) to the site where they can fuse with female gametes (egg cell and central cell). Some molecules required for this complex and essential signaling exchange have been identified; however, fundamental questions about the nature of the interactions between the pollen tube and the synergid cells remain to be clarified. Here, we monitor pollen tube arrival, pollen tube discharge, and synergid degeneration in Arabidopsis (*Arabidopsis thaliana*) wild type and in male and female gametophytic mutants that disrupt development and function of the gametophytes. By combining assays used previously to study these interactions and an assay that facilitates simultaneous analysis of pollen tube discharge and synergid degeneration, we find that synergid degeneration could be initiated without pollen tube discharge. Our data support the hypothesis that pollen tube-synergid contact, or signaling via secreted molecules, initiates receptive synergid degeneration. We also find that when pollen tubes successfully burst, they always discharge into a degenerated synergid. In addition to this pollen tube-dependent promotion of synergid degeneration, we also show that a basal developmental pathway mediates synergid degeneration in the absence of pollination. Our results are consistent with the model that a complex set of interactions between the pollen tube and synergid cells promote receptive synergid degeneration.

Flowering plant reproduction requires that development of the male and female gametophytes is coordinated so that gametes can be brought together to fuse and initiate seed development. A critical phase of this coordination occurs when the two gametophytes encounter each other to complete double fertilization: each gametophyte directly influences the development and function of the other. The male gametophyte develops in the anther and differentiates into a pollen grain with two sperm cells in its cytoplasm (Berger and Twell, 2011). The female gametophyte develops within the ovule and is more complex, comprising seven cells in Arabidopsis (*Arabidopsis thaliana*), six of which are haploid (two synergids, an egg, and three antipodals) and one homodiploid central cell (Drews and Yadegari, 2002). When a pollen grain lands on a receptive stigma, it germinates a highly polar pollen tube that extends without cell division through stigma and stylar tissue before entering an ovule micropyle (Palanivelu and Tsukamoto, 2012). The first female gametophytic cell encountered by the pollen tube in the ovule is one of the two synergids, and it is clear that synergids directly control pollen tube functions: they secrete attractant proteins that guide the pollen tube into the micropyle (Takeuchi and Higashiyama, 2012) and induce pollen tube discharge (Kessler and Grossniklaus, 2011). After pollen tube arrival, one of the two synergid cells degenerates (called the receptive synergid) and receives the pollen tube contents to allow sperm to access the female gametes, the egg, and the central cell (Christensen et al., 1997; Drews and Yadegari, 2002; Sandaklie-Nikolova et al., 2007; Hamamura et al., 2011, 2014).

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Live imaging of Arabidopsis pollen tubes interacting with female gametophytes in a semi-in vivo system (Palanivelu and Preuss, 2006) has increased our resolution of the timing of these events (Hamamura et al., 2011) and showed that cytoplasmic calcium ($[Ca^{2+}]_{cyto}$) is a key second messenger that can be used to track communication between the gametophytes (Iwano et al., 2012; Denninger et al., 2014; Hamamura et al., 2014; Ngo et al., 2014). Although these studies present slightly different views of interaction kinetics, there is clear agreement that synergids and pollen tubes sense their mutual proximity, and their responses can be observed in the form of changes in $[Ca^{2+}]_{cyto}$. The interaction phase (phase I; Ngo et al., 2014) begins when the pollen tube enters the micropyle; it is prolonged (approximately 40 ± 15 min) and characterized by a slow rate of pollen tube extension, a steady increase in pollen tube $[Ca^{2+}]_{cyto}$, and commencement of $[Ca^{2+}]_{cyto}$ oscillations in both synergids (Denninger et al., 2014; Ngo et al., 2014). The rapid pollen tube growth phase, which begins with a transient increase in pollen tube $[Ca^{2+}]_{cyto}$, is shorter (approximately 20 ± 10 min) and accompanied by a steady increase in $[Ca^{2+}]_{cyto}$ by the receptive synergid (phase II; Ngo et al., 2014). The next phase, which likely includes pollen tube discharge, comprises a final spike in pollen tube $[Ca^{2+}]_{cyto}$ and a decrease in receptive synergid $[Ca^{2+}]_{cyto}$ (phase III; Ngo et al., 2014). The final pollen tube $[Ca^{2+}]_{cyto}$ spike could represent discharge itself, which would expose the fluorescent protein-based pollen tube calcium sensor to the extracellular environment, or it could represent an intracellular signal that leads to pollen tube discharge soon thereafter. It is not clear when the receptive synergid degenerates; most likely, this occurs at or soon after the observed final decrease in receptive synergid $[Ca^{2+}]_{cyto}$ (Ngo et al., 2014). Although the mechanistic relationships between $[Ca^{2+}]_{cyto}$ oscillations, pollen tube discharge, and synergid degeneration remain unknown, these studies nevertheless provide a strong basis for future efforts to understand the molecular nature of the molecules that signal pollen tube arrival to the synergid and trigger the pollen tube to discharge its cargo.

Completion of double fertilization triggers rapid elimination of the remaining synergid cell (persistent synergid cell) by two pathways: fusion between one sperm with the egg cell activates an ethylene response cascade that results in synergid nuclear disorganization (Völz et al., 2013), and the fusion of other sperm with the central cell leads to synergid-endosperm fusion, thereby removing the persistent synergid (Maruyama et al., 2015). Degeneration of the persistent synergid cell removes a source of pollen tube attractants and prevents polytubey (additional pollen tubes accessing a fertilized ovule), thereby reducing the chances of polyspermy (Beale et al., 2012; Kasahara et al., 2012).

In Arabidopsis, a signaling system that coordinates sperm release, termed pollen tube reception, was first appreciated when a female gametophyte mutant (*feronia*) was identified that failed to induce the pollen tube to discharge its cargo of two sperm cells (Huck et al., 2003; Rotman et al., 2003). Subsequent studies identified *lorelei* (*lre*) and *nortia* (*nta*) female gametophyte mutants with a similar loss-of-function phenotype (Capron et al., 2008; Kessler et al., 2010; Tsukamoto et al., 2010). It has been proposed that these genes, which encode a receptor-like kinase (FERONIA [FER]; Escobar-Restrepo et al., 2007), a membrane-anchored protein (LRE; Capron et al., 2008; Tsukamoto et al., 2010), and a 7-pass membrane protein in the Mildew Resistance Locus O family (NTA; Kessler et al., 2010), are central to a signaling mechanism that senses pollen tube arrival and is required for pollen tube discharge. Recent work suggests that reactive oxygen species are a critical component of this signaling system that is required to induce pollen tube discharge (Duan et al., 2014). When pollen tubes enter ovules lacking this signaling system, they continue to grow, forming a coil. A triple mutant lacking three *Myeloblastosis* (*MYB*) transcription factors (*MYB97*, *MYB101*, and *MYB120*) is the only pollen mutant described with the complementary loss of function phenotype (failure to stop growing and discharge sperm upon contact with the female gametophyte; Leydon et al., 2013; Liang et al., 2013). It has been proposed that *myb* triple mutant pollen tubes fail to produce signaling molecules sensed by the female *FER*, *LRE*, *NTA* system; however, this has not yet been demonstrated. Many questions remain about pollen tube reception signaling and how the final acts of pollen tube and synergid cell development, their synchronized destruction, are coordinated to ensure that sperm will be released into a female gametophyte that has been primed to complete double fertilization.

Analysis of pollen tube reception in wild-type ovules targeted by *myb* triple mutant pollen tubes revealed a complex relationship between pollen tube arrival, pollen tube discharge, and synergid degeneration (Leydon et al., 2013). Ten percent of ovules were targeted by *myb* triple mutant pollen tubes that entered the micropyle and discharged like wild type. These ovules all had at least one degenerated synergid, suggesting that the receptive synergid had always degenerated, and that in many cases, the persistent synergid had degenerated as well. However, in 90% of ovules targeted by *myb* triple mutant pollen tubes, the pollen tube failed to discharge and formed a coil within the female gametophyte. Fifty-eight percent of ovules containing a coiling *myb* triple mutant pollen tube had at least one degenerated synergid. These data suggested that synergid degeneration could be initiated without pollen tube discharge. Conversely, the remaining 42% of ovules containing coiling *myb* triple mutant pollen tubes had two intact synergids. Targeted ovules with intact synergids could indicate that this subset of *myb* triple mutant pollen tubes is deficient in a signaling process that normally promotes synergid degeneration. Taken together, these findings suggest that a combination of signaling and physical interactions can result in synergid degeneration without pollen tube discharge, but that when pollen tube discharge is successful, the receptive synergid had always degenerated.

Here, we explore the spatiotemporal relationship between pollen tube discharge and synergid degeneration using male (*autoinhibited Ca^{2+}-atpase9* [*aca9*]; Schiøtt et al.,

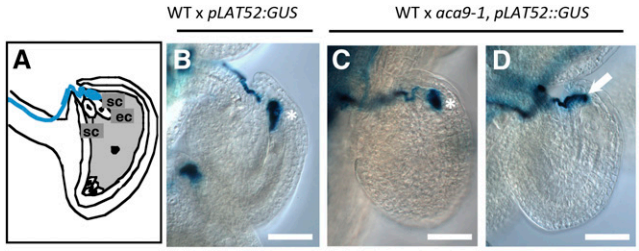
2004) and female (*gametophytic factor2* [*gfa2*] and *lre*; Christensen et al., 2002; Capron et al., 2008; Tsukamoto et al., 2010) gametophyte mutants that disrupt pollen tube-synergid interactions. We find that (1) pollen tube arrival without discharge triggers synergid degeneration above levels observed in unpollinated pistils; (2) pollen tube overgrowth is associated with higher levels of synergid degeneration compared with arrested or unpollinated ovules, but does not cause synergid degeneration at levels found in ovules with successful pollen tube discharge; and (3) pollen tube discharge is always associated with synergid degeneration. Our findings are consistent with the model showing that pollen tube arrival and interaction with the receptive synergid cell initiates synergid degeneration through signaling via direct contact between pollen tube and synergid cells or by exchange of secreted factors. This interaction is completed when the pollen tube discharges, as pollen tube discharge is always associated with synergid degeneration.

RESULTS

Two Patterns of Synergid Cell Degeneration Are Observed in *aca9-1* Pollinations

To further understand the relationship between pollen tube discharge and synergid degeneration in the female gametophyte, we analyzed pollen tube arrival and synergid degeneration in ovules targeted by *aca9-1* mutant pollen tubes. *aca9-1* pollen tubes are only able to target the ovules proximal to the stigma and frequently fail to discharge in the ovules they reach; consequently, seed set is dramatically reduced (Schiøtt et al., 2004; Supplemental Table S1). We analyzed *aca9-1* mutant pollen tube growth by pollinating wild-type pistils with *aca9-1* mutant pollen carrying the *Late Anther Tomato52* (*LAT52*):*GUS* transgene and scoring pollen tube behavior in wild-type ovules (Twell et al., 1989; Johnson et al., 2004). As expected, but unlike wild-type pollen tubes (Fig. 1, A, B, and E), the *aca9-1* GUS$^+$ pollen tubes showed two types of behaviors. Approximately 32% of wild-type ovules contained *aca9-1* pollen tubes that exhibited wild-type behavior as evident from the normal pollen tube discharge (Fig. 1, C and E). In the remainder (67%), *aca9* GUS$^+$ pollen tubes ceased growth in the region of the ovule that contained the synergid cell (Fig. 1, D and E); however, these tubes did not show the characteristic discharge pattern found in wild-type ovules accessed by wild-type pollen tubes (note the lack of a bolus of GUS staining in Fig. 1D compared with Fig. 1C). These results are consistent with the previous report that *aca9* pollen tubes fail to discharge (Schiøtt et al., 2004).

Since a notable fraction of *aca9-1* pollen tubes enter the micropyle, but do not discharge, we analyzed how pollen tube arrival without discharge affects synergid degeneration. For this analysis, we used a CLSM assay that takes advantage of autofluorescence from fixed tissues to image female gametophyte cells and nuclei. This assay has been used extensively to analyze



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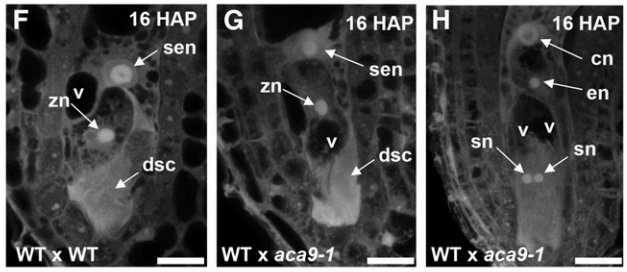
Cross (female x male)	Targeted Ovules: Normal PT discharge #PT	(%)	Panel	No PT discharge #PT	(%)	Panel	Untargeted ovules (%) #	(%)
WT x *pLAT52:GUS*	88	(98.9)	1B	0	(0.0)	-	1	(1.1)
WT x *aca9-1, pLAT52:GUS*	38	(32.2)	1C	79	(67.0)	1D	1	(0.8)

PT, pollen tube; *pLAT52:GUS*, *LAT52* promoter driving expression of *GUS* reporter



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Cross*	Zygote + DSC	(%)	Figure panel	No zygote, No DSC	(%)	Figure panel
WT x WT	151	(94.4)	1F	9	(5.6)	N/A
WT x *aca9-1***	21	(37.5)	1G	35	(62.5)	1H

DSC = Degenerated synergid cell. *Pollinated pistils were observed 16 hours after pollination. **Only female gametophytes in ovules within the first 10 rows from the stigma of a pistil were scored.

Figure 1. Two patterns of synergid degeneration are observed in wild-type (WT) ovules accessed by *aca9-1* pollen tubes. A to E, Pollen tube discharge is defective in wild-type ovules accessed by *aca9-1* pollen tubes. A, Diagram depicting pollen tube discharge in the receptive synergid cell (sc) flanking an egg cell (ec) in a female gametophyte. B to D, Micrographs of *LAT52*:*GUS* pollen tube interaction with ovules. B, Normal discharge (white asterisk) of *LAT52*:*GUS* pollen tube in a wild-type ovule. C, Normal discharge (white asterisk) of *aca9-1, LAT52*:*GUS* pollen tube in a wild-type ovule. D, Failure of pollen tube discharge (white arrow) by an *aca9-1, LAT52*:*GUS* pollen tube in a wild-type ovule. E, Quantification of pollen tube discharge in wild-type ovules accessed by wild-type and *aca9-1* pollen tubes shown in B to D. Panel refers to sections B to D in this figure. Scale bars = 50 μm. F–H, Confocal laser scanning microscopy (CLSM) images of embryo sacs of wild-type ovules isolated from pistils 16 h after pollinating them with either wild-type or *aca9-1* pollen. Only the micropylar end of an ovule is shown, and each image is a projection of several 1-μm optical sections. F, Wild-type ovule from pistils pollinated with a wild-type pollen tube containing an embryo sac with a highly autofluorescent region denoted as degenerating synergid cell (dsc). G and H, Wild-type ovules from pistils pollinated with *aca9-1* pollen containing an embryo sac with (G) or without (H) a highly autofluorescent region denoted as dsc. I, Quantification of ovules from pollinated pistils and analyzed by CLSM. Panel refers to panels F to H in this figure. cn, Central cell nucleus; en, egg cell nucleus; HAP, hours after pollination; N/A, not applicable; sen, secondary endosperm nucleus; sn, synergid cell nucleus; V, vacuole; zn, zygote nucleus. Scale bars = 10 μm.

synergid development and degeneration (Christensen et al., 1997, 1998, Faure et al., 2002; Sandaklie-Nikolova et al., 2007). We pollinated wild-type pistils with wild-type or *aca9-1* pollen and scored synergid degeneration in approximately 20 stigma-proximal ovules/pistil. Unlike in wild-type ovules targeted by wild-type pollen tubes, 94% of which showed a highly autofluorescent region in the portion of the ovule where a synergid cell is located (Fig. 1F), we observed two types of ovules in *aca9-1* pollinations. Of the ovules, 37.5% appeared similar to the wild type and had a highly autofluorescent region in the portion of the ovule where a synergid cell is located (Fig. 1, G and I). The remaining 62.5% of ovules contained what appeared to be two intact synergid cells, suggesting that synergid cells in these ovules did not undergo degeneration (Fig. 1, H and I). The ratio of ovules with and without synergid degeneration is similar to the ratio of *aca9*-1-pollinated ovules with and without pollen tube discharge (Fig. 1, E and I), indicating that lack of discharge by *aca9-1* pollen tubes is correlated with failure of synergid degeneration.

These results showed that synergid degeneration occurs at a reduced frequency in *aca9-1*-pollinated ovules. However, the CLSM assay for synergid degeneration has two shortcomings. First, it is not clear whether the highly autofluorescent signal that occupies the position of one of the two synergid cells is from the synergid alone, or whether it represents a combination of cell contents from the discharged pollen tube and the degenerated synergid. If the autofluorescent signal is due to the latter, it would not be possible to use the CLSM assay to determine whether synergid degeneration could occur in the absence of pollen tube discharge. Second, the CLSM assay does not allow one to determine whether a given ovule has been targeted by a pollen tube or whether the pollen tube has discharged. An assay that simultaneously analyzes the state of the pollen tube and the synergid cell in individual ovules would be preferable.

Simultaneous Analysis of Pollen Tube and Synergid Cells Shows That Synergid Degeneration Occurs without Pollination, Is Promoted by *aca9-1* Pollen Tube Arrival, and Is Always Associated with *aca9-1* Pollen Tube Discharge

We used a previously developed assay (Leydon et al., 2013) that simultaneously determines the state of the pollen tube and the synergid using the *LAT52*:*DsRED* (for *Discosoma* sp. Red Fluorescent Protein; Francis et al., 2007) pollen tube cytoplasm marker and the *Actin11* (*ACT11*) promoter:*Multicopy Suppressor of IRA1* (*MSI1*):*GFP* nuclear marker (Ingouff et al., 2007; Beale et al., 2012). Analysis of nuclear morphology using 4′,6-diamidino-2-phenylindole staining of *Triticum aestivum* (An and You, 2004) was used previously as a marker for synergid degeneration. More recently, distortion and dissipation of synergid nuclear GFP fluorescence has been used as a marker for synergid cell degeneration (Ingouff et al., 2007; Hamamura et al., 2011; Beale et al., 2012; Leydon et al., 2013). When pistils expressing the *ACT11*:*MSI1*:*GFP* marker, which accumulates in the nuclei of female gametophyte cells, are pollinated with *LAT52*:*DsRED*, confocal microscopy can be used to determine in each ovule whether synergid degeneration has occurred and whether a pollen tube has arrived and discharged.

To establish the time frame for synergid degeneration in the absence of pollen tube arrival, we first analyzed synergid degeneration in unpollinated pistils at 12, 16, 24, and 48 h after the time when we perform hand pollinations (methods and experiments below). In unpollinated pistils, synergid degeneration is rare 12 h after when hand pollinations would have been performed, and nearly 95% of ovules have two intact synergid nuclei (Fig. 2, A and J). At 16 h after the time when hand pollinations would have been performed, synergid degeneration is still rare (5% of ovules have one degenerated synergid and 5% have two degenerated synergids; Fig. 2J). However, synergid degeneration, as indicated by distortion and dissipation of nuclear GFP signal specific to synergids, is progressively observed in unpollinated pistils (Fig. 2, B, C, and J), and roughly 60% of ovules have either one or two degenerated synergids by 48 h after the time when pistils would have been hand pollinated (Fig. 2J). Nuclear distortion was specific to the synergid cells; the nuclei of other female gametophyte cells and the ovule remained intact at all time points analyzed.

Wild-type pistils were pollinated with wild-type *LAT52*:*DsRED* pollen and analyzed 12 and 16 h after the time when pistils were hand pollinated. At either of these two time points, all pollen tubes that had entered ovules had discharged, and all of the targeted ovules had at least one degenerated synergid (Fig. 2, D–J). The fraction of ovules with two degenerated synergids increased from 66% at 12 h to 86% at 16 h after pollination (Fig. 2J). In ovules with one synergid remaining (Fig. 2, D–F), the receptive synergid had degenerated and received DsRED from the pollen tube; the persistent synergid (1 synergid nucleus; Fig. 2) remained with an intact nucleus.

These data show that synergid degeneration can occur in the absence of pollination. However, it does not occur at appreciable levels during the time frame when pollen tubes arrive at ovules following self or hand pollination (8–16 h; see 12 and 16 h unpollinated, Fig. 2J). In contrast, synergid degeneration always occurred in ovules in which a pollen tube discharged (see 12 and 16 h discharged pollen tube, Fig. 2J). We next used this simultaneous assay to determine whether pollen tube arrival, without discharge, is associated with synergid degeneration using *aca9-1* pollen tubes, and whether ovules with degenerated synergids also contained a successfully discharged pollen tube.

When we pollinated wild-type pistils expressing *ACT11*:*MSI1*:*GFP* with *aca9-1, LAT52*:*DsRED,* we found that 37% (50/135) of the targeted ovules had a discharged pollen tube (Fig. 3E) 16 h after pollination. The remaining 63% (85/135) had one (Fig. 3, B and C) or more (Fig. 3D) pollen tubes that had entered the micropyle, but had not discharged. These fractions are in

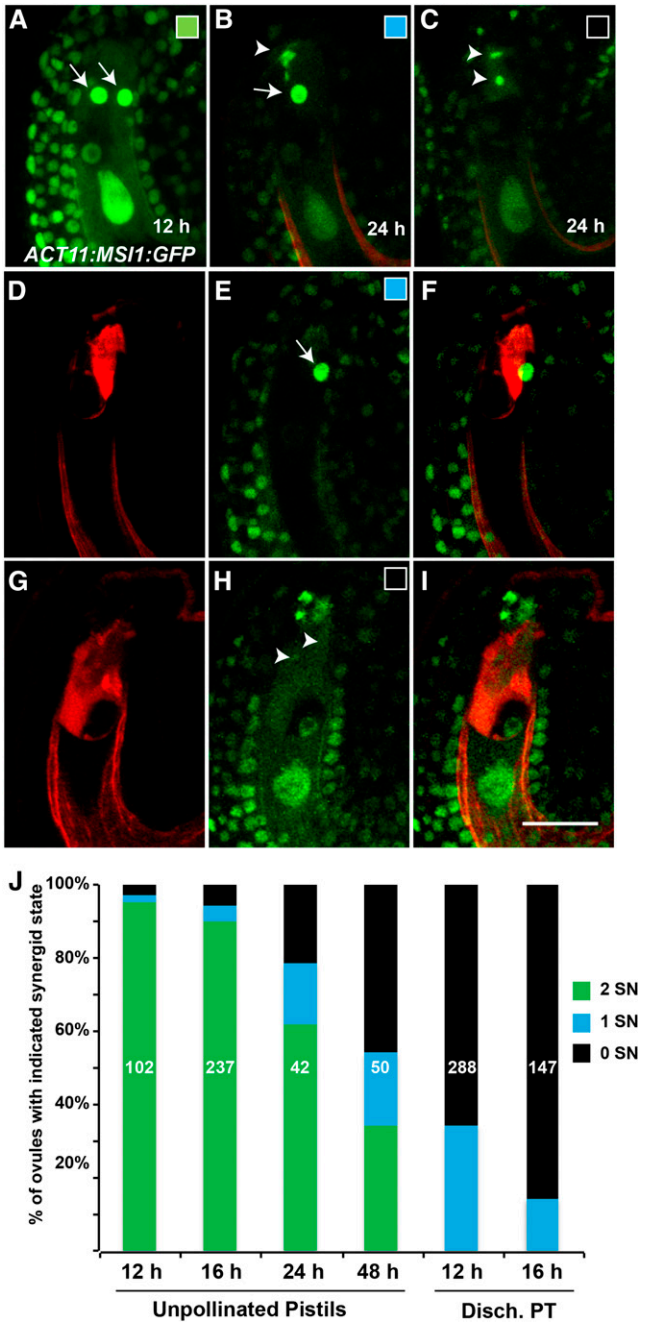


Figure 2. Synergid degeneration is observed in unpollinated ovules, but is enhanced by presence of the pollen tube. A to F, Confocal micrographs of female gametophytes expressing *ACT11:MSI1:GFP* in unpollinated (A–C) or pollinated pistils (D–I). A to C, Unpollinated emasculated pistils were analyzed 12, 16, 24, and 48 h after the time when pistils would have been hand pollinated (see "Materials and Methods" for explanation of time course). D to I, Ovules were imaged 16 h after pollination with *LAT52:DsRED* pollen. The red channel (D and G) indicates that a pollen tube has discharged within a synergid. The green channel reveals the synergid state (E and H). The overlay shows the discharged pollen tube and the synergid nuclei. Images show two intact synergids (A, green square), one intact and one degenerated synergid (B and E, blue square), or two degenerated synergids (C and H, black square). Synergid nuclei are round and brightly fluorescent (arrow); upon degeneration, GFP signal becomes diffuse and/or irregular (arrowhead). J, Quantification of synergid degeneration using the color code described above at indicated time points in ovules in unpollinated pistils compared with ovules that received a pollen tube (PT) that has discharged (Disch. PT). The number of ovules analyzed in each experiment is given in the center of each bar. Scale bar = 20 μm. SN, Synergid cell nucleus.

agreement with data using *LAT52:GUS* as a marker for pollen tube arrival (Fig. 1, C–E).

Simultaneous analysis of synergid degeneration using the *ACT11:MSI1:GFP* marker showed that ovules receiving *aca9-1* pollen tubes that discharged always had at least one degenerated synergid, and the majority had two degenerated synergids (Fig. 3E). Interestingly, we also found that, at 16 h after pollination, 49% of ovules that had attracted an *aca9-1* pollen tube but failed to discharge had at least one degenerated synergid (Fig. 3E, 16 h, arrested pollen tube). This is much higher than the rate of synergid degeneration observed in unpollinated pistils 16 h after the time of pollination (Fig. 2J) and suggests that pollen tube arrival promotes synergid degeneration even when discharge does not occur. In addition, nearly 51% of ovules that had attracted an *aca9-1* pollen tube but failed to discharge had both synergid cells intact (Fig. 3E).

To determine whether additional time for interaction between *aca9-1* pollen tubes and the pistil increased pollen tube discharge or synergid degeneration, as pollen tube growth is affected in *aca9-1* pollen tubes, we also analyzed crosses 24 h after pollination. Interestingly, the fraction of *aca9-1* pollen tubes that discharged did not increase appreciably (Fig. 3E), and as at 16 h, the ovules that received a discharged pollen tube had at least one degenerated synergid. In ovules that received an *aca9-1* pollen tube that arrested without discharge, the fraction of ovules with both synergids intact remained at approximately 50%. The only change observed was an increase in the percentage of ovules with two degenerated synergids from 22% at 16 h to 36% at 24 h after pollination (Fig. 3E). Our results indicate that additional time for interaction between *aca9-1* pollen tubes and the pistil did not substantially increase pollen tube discharge or receptive synergid degeneration.

Taken together, the results of our *aca9-1* experiments (Figs. 1 and 3) corroborate previous analysis of *myb* triple mutants (Leydon et al., 2013) and prompted us to investigate if the same relationships between pollen tube discharge and synergid degeneration hold for female gametophyte mutants that alter pollen tube-synergid interactions.

Pollen Tube Discharge and Synergid Degeneration Occur at Decreased Frequencies in *gfa2* Mutant Ovules

gfa2 is a female gametophytic mutation that blocks polar nuclear fusion and synergid degeneration, resulting in reduced seed set (Christensen et al., 2002; Supplemental Table S1). The role of *GFA2*, which

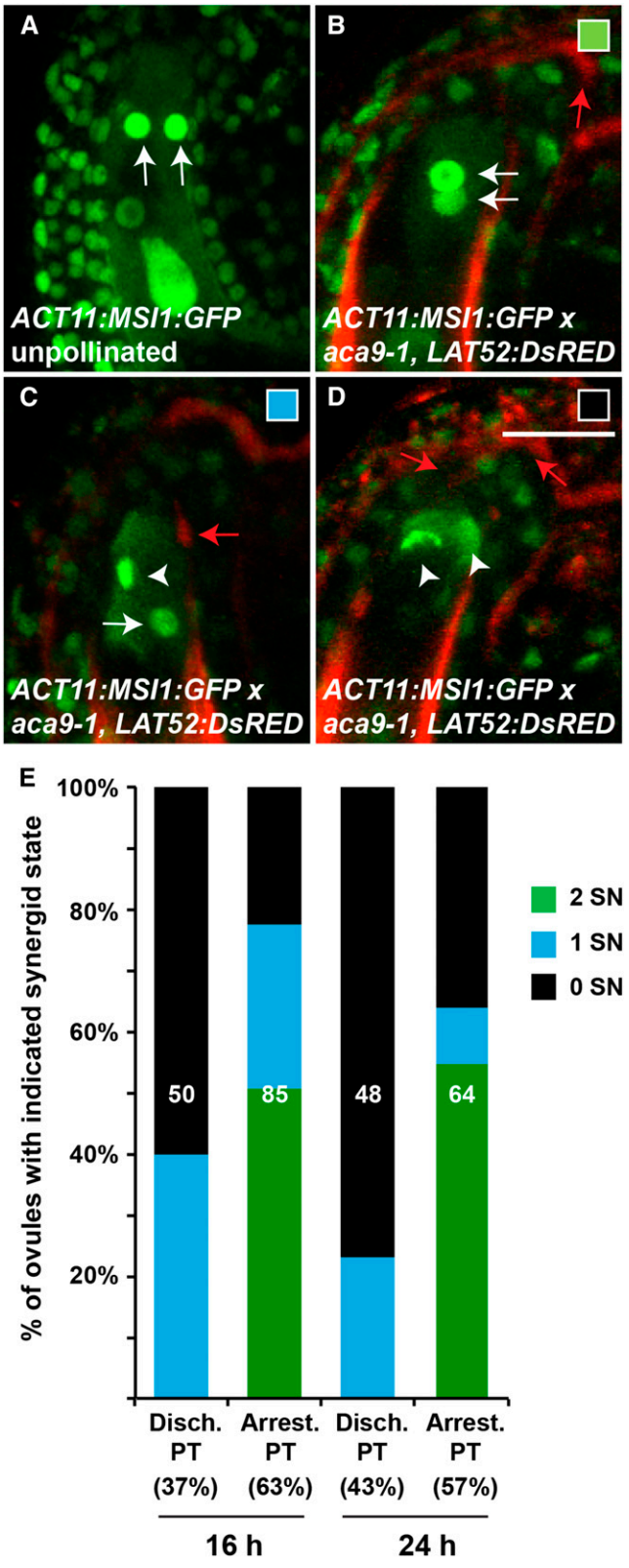


Figure 3. Synergid degeneration is higher in ovules that attract *aca9* pollen tubes that discharge relative to those that enter the micropyle but do not discharge. A to D, Confocal micrographs of wild-type female gametophytes expressing *ACT11:MSI1:GFP* in unpollinated pistils (A) or pistils pollinated with *aca9-1, LAT52:DsRED* pollen and imaged 16 h after pollination (B–D). A, Unpollinated pistils were analyzed 12 h after the time when pistils would have been hand pollinated. Two intact synergid nuclei are present (arrows). B to D, Images show pollen tubes that have arrived in the micropyle but have not discharged. The pollen tube tip is intact (red arrow). Images show two intact synergids (B, green square, white arrows), one intact synergid (C, blue square, arrows; white arrowhead is pointing to the degenerating nuclei), or two degenerated synergids (D, black square, arrowheads). E, Quantification of synergid degeneration using the color code described above in pistils pollinated with *aca9-1, LAT52:DsRED* and observed 16 or 24 h after pollination. The synergid state was determined in all ovules that had attracted a pollen tube; the number with the indicated pollen tube state (discharged, arrested) is given in the center of each bar. Scale bar = 20 μm. SN, Synergid cell nucleus; Disch. PT, discharged pollen tube; Arrest. PT, arrested pollen tube.

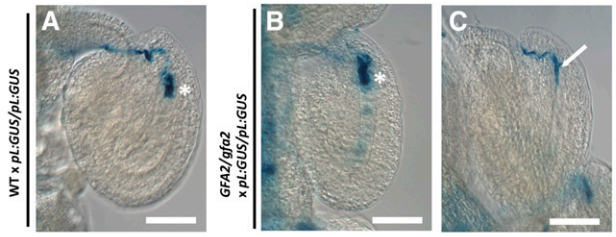
encodes a mitochondrial matrix chaperone protein, in synergid degeneration is not known, and it is possible that defects in synergid degeneration are due to loss of *GFA2* in synergids, or through an indirect effect on synergid function due to a lack of polar nuclei fusion. Although it was reported that *gfa2* female gametophytes attracted pollen tubes at wild-type levels (Christensen et al., 2002), the effect on pollen tube discharge was not reported. Therefore, we used *gfa2* mutants as a tool to gain additional insights into the relationship between the synergid degeneration and pollen tube discharge.

Using the autofluorescence-based CLSM assay, it was shown that, as expected for a fully penetrant gametophytic mutation, approximately 50% of ovules in unpollinated *gfa2/GFA2* pistils contain a female gametophyte with two polar nuclei that fail to fuse and two intact synergid cell nuclei (Christensen et al., 2002); mature wild-type female gametophytes have a single fused central cell nucleus and two synergid nuclei. Our analysis of *gfa2/GFA2* using this CLSM assay (Supplemental Fig. S1) yielded similar results. Also consistent with a previous report (Christensen et al., 2002), analysis of wild-type female gametophytes 16 h after pollination using this technique showed that approximately 94% of ovules have a brightly autofluorescent signal in the position of one of the synergids (denoted as degenerated, Supplemental Fig. S1), whereas approximately 50% of *gfa2/GFA2* female gametophytes contained unfused polar nuclei and two synergids with intact synergid nuclei (Supplemental Fig. S1).

We analyzed pollen tube discharge in *gfa2* ovules using two approaches. First, we imaged pollen tube arrival in real time using a semi-in vivo pollen tube guidance assay that recapitulates much of the in vivo pollen tube behavior in ovules (Palanivelu and Preuss, 2006). For this assay, pollen tubes were marked with *LAT52:DsRED* (Francis et al., 2007). Similar to the wild-type behavior (Supplemental Movie S1; $n = 69$), in *gfa2* ovules, *LAT52:DsRED* pollen tubes always navigated to the synergids (Supplemental Movies S2 and S3). After reaching the synergids in *gfa2/GFA2* ovules, some *LAT52:DsRED* pollen tubes discharged normally (Supplemental Movie S2; $n = 110$), whereas others

arrested growth in a synergid but did not discharge (Supplemental Movie S3; $n = 23$). In the latter, the pollen tube appears to arrest its growth but not discharge. These results showed that pollen tube discharge is defective in *gfa2* female gametophytes despite normal entry into the ovule micropyle and successful navigation to the synergid cells.

Second, we examined pollen tube interaction with a female gametophyte in vivo by pollinating pistils with wild-type *LAT52*:*GUS* pollen and scoring pollen tube behavior in ovules of fixed pistils (Twell et al., 1989; Johnson et al., 2004). In the wild type, the GUS^+ pollen tubes entered an ovule, stopped growth, and discharged in the region of the ovule that contained the synergid cell (Fig. 4A). In contrast, in *gfa2/GFA2* pistils, we observed two types of ovules. Approximately 48% of ovules contained pollen tubes that exhibited wild-type behavior (Fig. 4B). In the remainder, GUS^+ pollen tubes ceased growth in the region of the ovule that contained the synergid cell; however, these tubes did not show the characteristic discharge observed in wild-type ovules (note lack of bolus of GUS staining in Fig. 2C compared with Fig. 2, A or B). The ratio of ovules with and without pollen tube discharge in *gfa2* (Fig. 2G) is consistent with the ratio of pollinated *gfa2* ovules with and without synergid autofluorescence in the position of one of the two synergids (Supplemental Fig. S1), indicating that pollen tube discharge and synergid degeneration are disrupted in *gfa2* ovules, and that lack of discharge by wild-type pollen tube is correlated with failure of synergid degeneration in *gfa2* ovules.



D

Cross (female x male)	Targeted Ovules						Untargeted ovules (%)	
	Normal PT discharge			No PT discharge				
	# PT	(%)	Panel	# PT	(%)	Panel	#	(%)
WT x *pL:GUS/pL:GUS*	88	(98.9)	4A	0	(0.0)	-	1	(1.1)
GFA2/gfa2 x *pL:GUS/pL:GUS*	114	(47.9)	4B	118	(49.6)	4C	6	(2.5)

PT, pollen tube; *pL:GUS*, *GUS* reporter gene expressed the from *LAT52* promoter

Figure 4. Pollen tube discharge is defective in *gfa2* female gametophytes. A to C, Micrographs of *LAT52*:*GUS* pollen tube growth in ovules. A, Normal discharge (white asterisk) of a *LAT52*:*GUS* pollen tube in a wild-type ovule. B, Normal discharge (white asterisk) of a *LAT52*:*GUS* pollen tube in a *gfa2/GFA2* ovule. C, Failure of pollen tube discharge (white arrow) by a *LAT52*:*GUS* pollen tube in a *gfa2/GFA2* ovule. D, Quantification of pollen tube discharge in wild-type and *gfa2/GFA2* ovules accessed by wild-type pollen tubes shown in A to C. Panel refers to A to C in this figure. Scale bars = 50 μm.

Simultaneous Analysis of Synergid Degeneration and Pollen Tube Discharge in *gfa2* Ovules Indicates That Pollen Tube Arrival Promotes Synergid Degeneration

Analysis of pollen tube-synergid interactions in *gfa2* female gametophytes has relied on assays that separately analyze either pollen tube discharge (e.g. Fig. 4) or synergid state (e.g. Supplemental Fig. S1). Consequently, it has not been possible to analyze the state of pollen tube and synergid degeneration simultaneously in individual female gametophytes. We therefore pollinated *gfa2/GFA2* pistils expressing *ACT11*:*MSI1*:*GFP* with wild-type *LAT52*:*DsRED* pollen to analyze pollen tube and synergid states simultaneously. In *gfa2/GFA2* pistils, we can distinguish the wild type from *gfa2* mutant female gametophytes by determining whether there is a single central cell nucleus (*GFA2*; Fig. 5A) or two unfused polar nuclei (Fig. 5, B–E).

Synergid degeneration was similar between *gfa2* and *GFA2* (wild type in Fig. 5) female gametophytes in unpollinated pistils (Fig. 5F) and was comparable with the wild type (Fig. 2J). A total of 224 ovules were analyzed in pollinated *gfa2/GFA2* pistils. The vast majority of *gfa2/GFA2* ovules that attracted a pollen tube were wild type (*GFA2*, polar nuclei fused), and all of these had at least one degenerated synergid (Fig. 5F). We also observed a fraction of targeted ovules that were *gfa2* mutant (polar nuclei not fused), but that contained a discharged pollen tube (Fig. 5, C, D, and F). As in wild-type ovules, synergid degeneration always accompanied pollen tube discharge in *gfa2* ovules (Fig. 5F). We also observed a notable fraction of targeted *gfa2* ovules that attracted a pollen tube that had failed to discharge (as observed in Fig. 4C by GUS assay). Synergid degeneration was reduced in these ovules, but increased over unpollinated pistils (compare Fig. 5F *gfa2* arrested pollen tube with Fig. 2J unpollinated 16 h). In addition, nearly 55% of *gfa2* ovules that had an arrested pollen tube had both synergid cells intact (Fig. 5F). These data are consistent with those from male mutants that enter ovules without discharging (*myb* triple mutant [Leydon et al., 2013] and *aca9-1* [Fig. 2]), showing two types of ovules: one in which pollen tube arrival promotes synergid degeneration, and another in which both synergid cells remain intact. These findings prompted us to analyze a female mutant, *lre*, in which pollen tubes enter and continue to grow without discharging, so that we could determine the impact on synergid degeneration following prolonged physical interaction with a pollen tube that has failed to discharge.

Synergid Degeneration Occurs in *lre* Female Gametophytes Containing an Overgrown (Coiled) Pollen Tube That Has Not Discharged

Prior analysis of pollen tube-synergid interactions in *lre* mutants was based on two assays, one that analyzed synergid degeneration and one that measured pollen tube discharge (Capron et al., 2008; Tsukamoto et al., 2010). These reports revealed that wild-type pollen tubes fail to stop growing and discharge within *lre* ovules, and that pollen tubes often form extended coils

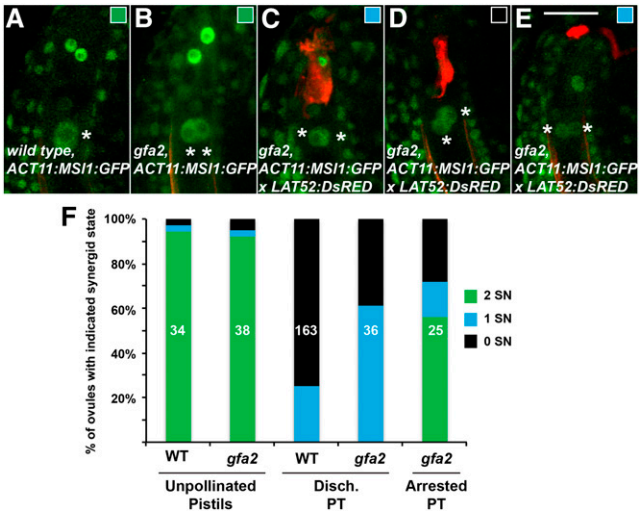


Figure 5. Pollen tube discharge and synergid degeneration are detectable in *gfa2* female gametophytes, and synergid degeneration is increased in *gfa2* female gametophytes containing a discharged pollen tube relative to those containing an arrested pollen tube. A to –E, Confocal micrographs of female gametophytes expressing *ACT11:MSI1:GFP* in unpollinated (A and B) or pollinated pistils (C–E). A and B, Unpollinated *gfa2/GFA2* pistils were analyzed 16 h after the time when pistils would have been hand pollinated. Wild-type (WT; *GFA2)* female gametophytes have a single central cell nucleus (*, 34 of 72 ovules analyzed); *gfa2* mutant female gametophytes have two unfused polar nuclei (**, 38 of 72 ovules analyzed). C to E, Ovules were imaged 16 h after pollination with *LAT52:DsRED* pollen; overlays of the red (*LAT52:DsRED)* and green (*ACT11:MSI1:GFP*) channels are shown. Images show a single pollen tube that has discharged within a *gfa2* mutant ovule (C and D) or a pollen tube that has arrested at the *gfa2* micropyle (E). Images show two intact synergids (A and B, green square), one intact synergid (C and E, blue square), or two degenerated synergids (D, black square). F, Quantification of synergid degeneration using the color code described above in unpollinated pistils compared with ovules that have received a pollen tube that has either discharged or arrested. The synergid state and the state of polar nuclear fusion (**, *gfa2*, two unfused polar nuclei; *, wild-type central cell nucleus, one fused central cell nucleus) was determined in all ovules that had attracted a pollen tube; the number with the indicated pollen tube state (arrested, discharged) is given in the center of each bar. Scale bar = 20 μm. SN, Synergid cell nucleus; Disch. PT, discharged pollen tube; Arrest. PT, arrested pollen tube.

within *lre* ovules. In addition, CLSM autofluorescence patterns suggested that synergid degeneration was dramatically reduced in *lre* ovules (Tsukamoto et al., 2010). We analyzed pollen tube discharge and synergid degeneration simultaneously in *lre* ovules using the assay described in Figures 2, 3, and 5. As expected, *lre-5* female gametophytes displayed wild-type female gametophyte development (Fig. 6A), and the vast majority of ovules in unpollinated pistils contained two intact synergids (Fig. 6, A and E). However, unlike wild-type ovules, which attract pollen tubes that discharge upon entering the micropyle (Figs. 1C, 2D, and 4A), at 16 h after pollination, 88% (147/167) of *lre* ovules contained an overgrown and coiling pollen tube. Interestingly, the majority (123/147, 84%) of *lre-5* ovules containing an overgrown pollen tube that failed to discharge had either one or two degenerated synergids. This is a higher proportion of synergid degeneration than observed in male or female mutants that attracted pollen tubes that arrested without overgrowth in the ovule (compare Fig. 6 coil pollen tube with Fig. 5 arrest pollen tube or Fig. 3 arrest pollen tube). The remaining *lre* ovules with a coiling pollen tube (16%) had two intact synergids, pointing to another instance of a subset of ovules in which synergid cells remain intact when pollen tubes do not discharge. Finally, and consistent with all observations made in wild-type and male or female mutants reported in this study, all *lre-5* ovules that attracted a discharged pollen tube (12% of targeted *lre-5* ovules) had at least one degenerated synergid (Fig. 6E).

DISCUSSION

A Basal Developmental Pathway Mediates Synergid Degeneration in the Absence of Pollination

Previous analyses in Arabidopsis focused on whether the pollen tube induces synergid degeneration and did not define the kinetics of the basal synergid cell death program in the absence of pollination (Christensen et al., 2002; Faure et al., 2002; Hamamura et al., 2011). Our results (Fig. 2) show that synergid degeneration occurs in Arabidopsis ovules in the absence of pollination, as was described previously in barley (*Hordeum vulgare*) and pearl millet (*Pennisetum glaucum*) using electron microscopy (Engell, 1989; Chaubal and Reger, 1993). Our results also point to a basal cell death mechanism in a synergid cell that is gradually activated, but that is much slower than degeneration induced by the pollen tube. For example, at the 12-h time point, >95% of ovules in unpollinated pistils had two intact synergids compared with zero with two intact synergids at the same time point after pollen tubes had discharged (Fig. 2F). Furthermore, even after remaining unpollinated for 48 h (72 h after emasculation), both synergids remained intact in 35% of ovules in unpollinated pistils (Fig. 2F). Thus, our results are consistent with the long-held view that the presence of a pollen tube in the female gametophyte promotes synergid degeneration in Arabidopsis (Christensen et al., 2002; Faure et al., 2002; Sandaklie-Nikolova et al., 2007; Hamamura et al., 2011). However, whether pollen tube discharge is absolutely required for synergid degeneration had not been fully evaluated.

Pollen Tube Discharge Is Not Required for Receptive Synergid Degeneration

The relationship between pollination and synergid degeneration was analyzed in many flowering plant species using electron microscopy (Huang and Russell, 1992 and references therein). In some species, synergids degenerated before pollen tubes entered the ovule, whereas in others it appeared that pollen tube entry was required (Huang and Russell, 1992, and references therein). The temporal sequence of events in pollen tube-female gametophyte interactions was analyzed in multiple

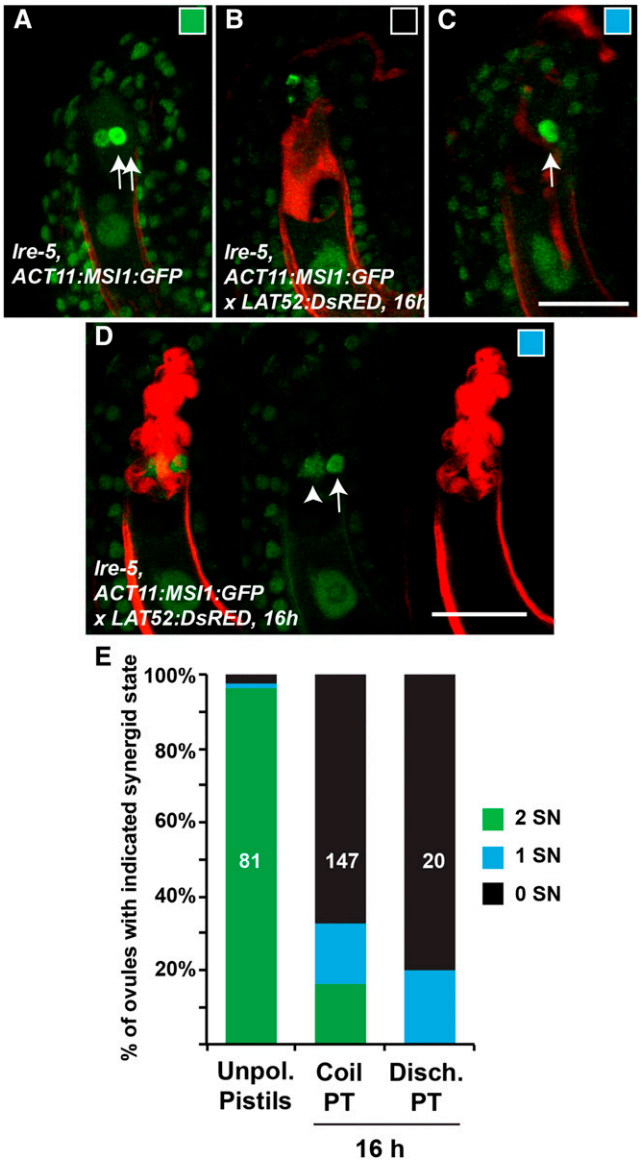


Figure 6. Synergid degeneration occurs more frequently in *lre* female gametophytes containing a discharged pollen tube relative to those containing an arrested or a coiled pollen tube. A to D, Confocal micrographs of *lre-5* female gametophytes expressing *ACT11:MSI1:GFP* in unpollinated pistils (A) or pistils pollinated with *LAT52:DsRED* pollen and observed 16 h after pollination (B–D). A, Unpollinated *lre-5/lre-5* pistils were analyzed 12 h after the time when pistils were hand pollinated. Two intact synergid nuclei are present (arrows). B to D, *lre-5* ovules images show discharged pollen tubes (B) or pollen tubes that are overgrowing (coiling) within the female gametophyte (C and D). Images show two intact synergids (A, green square), one intact synergid (C and D, blue square), or two degenerated synergids (B, black square). All images are overlays of green (nuclei of female cells) and red (pollen tube) channels except for D, which shows an overlay on the left along with green (center) and red (right) channels. Synergid nuclei are round and brightly fluorescent (arrow); upon degeneration, GFP signal becomes diffuse and/or irregular (arrowhead). In D, the middle (green channel) shows that one synergid has degenerated; this is difficult to appreciate in the overlay (left) E, Quantification of synergid degeneration using the color code described above in *lre-5* ovules from unpollinated pistils or pistils pollinated with *LAT52:DsRED* and observed 16 h after hand pollination. The synergid state was determined in all ovules that had attracted a pollen tube; the number with the indicated pollen tube state (coiled, burst) is given in the center of each bar. Scale bar = 20 μm. SN, Synergid cell nucleus; Disch. PT, discharged pollen tube; Unpol., unpollinated.

studies in Arabidopsis (Christensen et al., 1997; Faure et al., 2002; Sandaklie-Nikolova et al., 2007). Using transmission electron microscopy and real-time observation of synergids marked with a cytoplasmic marker in a semi-in vivo assay, it was proposed that receptive synergid cell death initiates after the pollen tube arrives at the female gametophyte but before pollen tube discharge (Sandaklie-Nikolova et al., 2007). A subsequent study using the same semi-in vivo assay and a synergid cell marked with a nuclear marker showed that receptive synergid cell degeneration occurs after pollen tube arrival at the synergid cell, but upon pollen tube discharge (Hamamura et al., 2011). These studies, however, did not address a fundamental question about pollen tube reception: is pollen tube discharge required for synergid degeneration?

Insights into this question first came from a recent study that simultaneously analyzed synergid degeneration and pollen tube discharge in wild-type ovules targeted either by wild-type pollen tubes or *myb* triple mutant pollen tubes, which fail to discharge (Leydon et al., 2013). By directly observing the states of pollen tube discharge and synergid degeneration in an ovule, it was shown that 58% of ovules containing an overgrown (coiling) *myb* triple mutant pollen tube had at least one degenerated synergid. These data suggested that synergid degeneration could be initiated without pollen tube discharge. However, this observation could have been the consequence of this specific set of male mutations, so we tested an additional male gametophytic mutant and two female gametophytic mutants. Consistent with the results obtained for *myb* triple mutant pollinations, we identified ovules that contained at least one degenerated synergid despite having a pollen tube that had not discharged. This was found in a male gametophytic mutant (*aca9*, Fig. 3) and two female gametophytic mutants (*gfa2*, Fig. 5; *lre*, Fig. 6). Additionally, pollen tube arrival without discharge promoted synergid degeneration much more rapidly than the basal pathway (Figs. 3, 5, and 6 compared with Fig. 2J). Our results confirm that pollen tube discharge is not essential for synergid degeneration, and that uncoupling of synergid degeneration from pollen tube discharge is not unique to ovules accessed by *myb* triple mutant tubes.

Pollen Tube Discharge Is Always Associated with Synergid Degeneration, and Synergid Degeneration Rates Are Lower in Ovules That Attract Pollen Tubes That Fail to Discharge

Forty-two percent of ovules containing a coiling *myb* triple mutant pollen tube (failed to discharge) had two intact synergids. Conversely, at least one synergid always degenerated in the 10% of ovules that attracted an

myb triple mutant pollen tube that discharged (Leydon et al., 2013). These results suggest that pollen tube discharge is associated with synergid degeneration. Combined with our findings here, this means that pollen tube arrival can induce synergid degeneration, but at lower rates than pollen tube discharge. This pattern held when additional female gametophytic (*gfa2*, Fig. 5; *lre*, Fig. 6) and male gametophytic (*aca9*, Fig. 3) mutants were analyzed. We found that (1) pollen tube arrival and arrest without pollen tube discharge triggers synergid degeneration above levels observed in unpollinated pistils (Figs. 1, 3, and 5), and (2) pollen tube overgrowth, without pollen tube discharge, is associated with higher levels of synergid degeneration compared with arrested or unpollinated ovules, but does not cause synergid degeneration at levels found in ovules with successful pollen tube discharge (Figs. 2, 3, 5, and 6).

Our results suggest a continuum of synergid degeneration associated with different pollen tube behaviors (from highest to lowest): synergid degeneration was always associated with pollen tube discharge (Figs. 2, 3, 5, and 6), and this was followed by lower rates of degeneration induced by pollen tubes that overgrow in the female gametophyte but do not discharge (*myb* triple mutant [Leydon et al., 2013], *lre* [Fig. 5]), followed by pollen tubes that arrived and ceased growth (*aca9-1* mutant pollen tubes, *gfa2* mutant ovules), followed by basal synergid degeneration that occurs in the absence of pollination (Fig. 2). Among the mutants we examined, the highest level of synergid degeneration was observed in *lre* (Fig. 5) and *myb* triple mutants (Leydon et al., 2013). A common characteristic of these two mutants, lacking in *aca9* and *gfa2*, is their prolonged interaction with the female gametophyte due to pollen tube overgrowth. Pollen tube-synergid interactions involving either mutant are consistent with a threshold model for receptive synergid degeneration, in which a certain level of either physical interaction or signaling from the pollen tube to the synergid cell has to be reached before synergid degeneration is initiated. Thus, in some cases, prolonged interaction between the synergids and the pollen tube in *lre* or *myb* triple mutant perhaps reached the requisite level of physical interaction or signaling to initiate synergid degeneration. Alternatively, a signaling or mechanical threshold for synergid degeneration could be surpassed because of polytubey, a feature common in these two mutants.

Synergid Degeneration Is Mediated by Three Developmental Pathways

In this study, we provided evidence for a basal pathway of synergid degeneration that occurs in the absence of pollination (Fig. 2). Previous studies (Christensen et al., 2002; Faure et al., 2002; Sandaklie-Nikolova et al., 2007; Hamamura et al., 2011) and work reported here (Figs. 1–6) provide evidence for a second pathway that leads to degeneration of the receptive synergid upon pollen tube arrival. Finally, two independent pathways are activated in the egg cell and the central cell upon successful double fertilization that results in degeneration of the persistent synergid (Völz et al., 2013; Maruyama et al., 2015). It is not yet known whether these modes of synergid degeneration share common mechanistic features. We found that *gfa2* mutant female gametophytes, which were reported to have a defect in synergid degeneration (Christensen et al., 2002), do not affect the basal pathway. In unpollinated *gfa2/GFA2* pistils, the frequency of synergid degeneration after 16 h is comparable with the wild type (Fig. 5F). Future experiments will be aimed at extending this observation and determining whether each mode of synergid degeneration is altered by mutations known to affect the other.

Opportunities for Further Development of Assays for Imaging of Pollen Tube-Synergid Interactions

Future analysis of pollen tube-synergid signaling will benefit from the development of assays that simultaneously image $[Ca^{2+}]_{cyto}$ and cellular integrity. By analyzing pollen tube rupture (using a pollen tube cytoplasm marker, *LAT52:DsRED*) and synergid degeneration (using a nuclear fluorescent protein marker) simultaneously, we improved on prior methods that monitor these two cellular events in isolation. However, analysis of the relationships between pollen tube arrival, discharge, and synergid degeneration could be improved if calcium signaling dynamics were also monitored. For example, Denninger et al. (2014) used a sperm nuclear marker to monitor pollen tube discharge while tracking $[Ca^{2+}]_{cyto}$ in the synergid cells. These experiments indicated a tight temporal association between pollen tube discharge and changes in synergid $[Ca^{2+}]_{cyto}$. Future studies should be aimed at live imaging of the relationship between changes in receptive synergid $[Ca^{2+}]_{cyto}$ and changes in nuclear, cytoskeletal, and membrane morphology that accompany cellular degeneration in preparation for double fertilization. Live imaging of double fertilization has benefited tremendously from a semi-in vivo system in which ovules are removed from the pistil and placed on the surface of medium (Palanivelu and Preuss, 2006; Hamamura et al., 2011, 2014; Denninger et al., 2014; Ngo et al., 2014). Future work should explore noninvasive live imaging techniques (Rotman et al., 2003; Cheung et al., 2010) and analysis of additional signaling molecules known to be involved in pollen tube reception such as reactive oxygen species (Duan et al., 2014).

CONCLUSION AND FUTURE PERSPECTIVES

A defining feature of sexual reproduction in flowering plants is the interaction between the male and female gametophytes, which results in fusion of a pair of female gametes with a pair of male gametes. Both male and female gametophytes are multicellular, consisting of gametes and accessory cells, which are critical for successful double fertilization. The pollen tube is the

male accessory cell, whereas the female gametophyte has a pair of synergid cells and three antipodal cells. The role of antipodals in double fertilization is not known (Song et al., 2014). However, it is clear that the pollen tube and synergids function to coordinate their mutual demise, and in so doing, facilitate gamete delivery for double fertilization (Kessler and Grossniklaus, 2011).

We show that Arabidopsis pollen tube discharge is associated with synergid degeneration, and that when pollen tube discharge is successful, at least one synergid always degenerates. We propose that, although pollen tube discharge is not required to initiate synergid degeneration, pollen tube discharge ensures that synergid degeneration is completed in all ovules that receive sperm. Live imaging (Hamamura et al., 2011) suggests that degeneration of the receptive synergid is required so that the sperm cells can be projected by the force of pollen tube discharge to the site where they will fuse with female gametes. Synergid degeneration is likely required to make this site available to sperm cells. By promoting completion of synergid degeneration in all ovules, signaling interactions between the pollen tube and synergid coupled with pollen tube discharge ensure successful double fertilization between the sperm cells and the female gametes and increase the likelihood of successful seed formation.

Our findings are consistent with the emerging idea (Boisson-Dernier et al., 2011; Cheung and Wu, 2011; Kessler and Grossniklaus, 2011; Lindner et al., 2012) that a complex set of interactions between the pollen tube and synergid cells result in synergid degeneration. We conclude that signaling between the pollen tube, either by direct physical contact and/or secreted factors, and the receptive synergid induces degeneration, and that these interactions are always completed when the pollen tube discharges. Many interesting questions need to be addressed: How is the receptive synergid selected? What is the nature of the signaling molecules that do so? How do these molecules affect changes in $[Ca^{2+}]_{cyto}$ that accompany pollen tube synergid interactions and mark the receptive synergid (Denninger et al., 2014; Hamamura et al., 2014; Ngo et al., 2014)? Genomic and imaging resources for the study of flowering plant reproduction have improved dramatically in recent years and should enable rapid progress toward addressing these questions.

MATERIALS AND METHODS

Growth Conditions and Plant Materials

The wild-type (Columbia ecotype), *LAT52:DsRED*, *LAT52:GUS*, *gfa2/GFA2*, *aca9-1*, and *lre-5* seeds were plated on Murashige and Skoog medium (Carolina Biological Supply Company, catalog no. 195703) in a growth chamber maintained at 20°C and 24-h illumination. Seven- to 10-d-old seedlings were moved from plates to soil (SUNSHINE MIX no. 1, Crop Production Services, Inc., product no. 1000188678), which was mixed with vermiculite (Vermiculite no. 2, Crop Production Services, product no. 1000190472) in a ratio of 5:1, and were grown in chambers maintained at 20°C and 24-h illumination. *ACT11:MSI:GFP* and *gfa2/GFA2* and *aca9-1* mutations in the *ACT11:MSI1:GFP* background were grown as described (Johnson et al., 2004; von Besser et al., 2006; Wong et al., 2010).

In Vivo Analysis of Pollen Tube Discharge in Ovules

LAT52:GUS or *aca9-1, LAT52:GUS* pollen was crossed to wild-type or *gfa2/GFA2* pistils and harvested 16 h after hand pollination. Ovary walls were removed as previously described (Johnson and Kost, 2010), stained for GUS activity (Johnson et al., 2004), and imaged using a Zeiss Axiovert 200 differential interference contrast (DIC) microscope (Carl Zeiss). For emasculation, stage 12b or 12c buds (Smyth et al., 1990) were emasculated, and then pollinated after 24 h. For each cross reported in this study, at least three emasculated pistils were stained for GUS activity.

CLSM Analysis of Female Gametophytes and Double Fertilization

Stage12b or 12c buds (Smyth et al., 1990) were emasculated, and 24 h later, pistils were either observed directly (unfertilized ovules) or were hand pollinated and analyzed 16 h afterward, and were prepared for CLSM to score unfertilized female gametophytes (Christensen et al., 1997) or double fertilization (Faure et al., 2002). For each genotype, ovules in six pistils were observed using a Leica TCS SP5 confocal microscope.

Simultaneous Assay of Pollen Tube Discharge and Synergid Degeneration

Stage12b or 12c buds (Smyth et al., 1990) were emasculated and hand pollinated 24 h later. Twenty-four hours after emasculation marks time = 0 h in all of the experiments reported here (unpollinated or pollinated). Hand pollinations were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating emasculated *ACT11:MSI1:GFP* stigmas (Smyth et al., 1990) with pollen from male donor plants carrying *LAT52:DsRED* (Francis et al., 2007). Sixteen hours after hand pollinations were performed, pistils were harvested and ovary walls were removed as previously described (Johnson and Kost, 2010). Unpollinated pistils were analyzed 12, 16, 24, and 48 h after time = 0 (the time point when hand pollinations were performed, 24 h after emasculation). Dissected pistil tissues were mounted in 80 mM sorbitol for analysis by CLSM using a 40× objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514 mn at 30 mV) and DsRED/monomeric Red Fluorescent Protein expression (helium neon laser 633 nm at 3 mV). Signal intensities were optimized for each fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (*z* stacks).

Time-Lapse Imaging of Pollen Tube Discharge in Ovules Using a Semi-in Vivo Pollen Tube Guidance Assay

To directly observe pollen tube discharge in ovules, time-lapse imaging of pollen tube-ovule interactions was performed essentially as described in Palanivelu and Preuss (2006). Pistils (*male sterile1*) were pollinated with *LAT52:DsRED* pollen and time lapse imaged using a Zeiss Axiovert 100 DIC microscope equipped with automated X, Y, Z stages and shutter to generate multiple time-lapse movies using bright light plus red fluorescent laser lights. A Retiga CCD digital camera (Q imaging) was used to capture images, and Metamorph (Molecular Devices) and ImageJ (http://imagej.nih.gov/ij/) image software were used to analyze these images.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Confocal analysis of synergid degeneration in *gfa2/GFA2*.

Supplemental Table S1. *gfa2* and *aca9* show reduced seed set phenotype in self-pollinated pistils.

Supplemental Movie S1. A semi-in vivo pollen tube guidance assay to monitor pollen tube discharge in wild-type ovules.

Supplemental Movie S2. A semi-in vivo pollen tube guidance assay showing normal pollen tube discharge in *gfa2/GFA2* ovules.

Supplemental Movie S3. A semi-in vivo pollen tube guidance assay showing no pollen tube discharge in *gfa2/GFA2* ovules.

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