Chapter 2

The Regulation of Sperm Cells Delivery to the Embryo Sac

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Additional information is available at the end of the chapter

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Abstract

Pollination, or the first contact between male and female gametophytes, is one of the most important steps in plant reproduction. After pollination, the pollen grains, male gametophytes, are hydrated and then germinate pollen tubes. The pollen tube initially penetrates and grows through the intercellular spaces of the stigma and then grows through the transmitting tract to the placenta connected to an ovule. The pollen tube grows along the surface of the ovule's funiculus, through the micropyle, and into the female gametophyte. After the pollen tube enters the female gametophyte, it ruptures and releases two sperm cells with its contents. The two sperm cells then move toward and fuse with the egg cell and central cell to produce embryo and endosperm, respectively. Multiple sperm cells typically strive to "win the race" and fertilize an egg cell during animal fertilization; however, in flowering plants, each ovule harboring an egg cell generally encounters only one of many pollen tubes conveying plant sperm cells. This chapter mainly addresses reproductive strategies of plants following pollination from the pollen tube extension and the guidance of two sperm cells to the female gametophyte for fertilization in the ovule.

Keywords: plant fertilization, pollen tube guidance, MYB98, LUREs, fertilization recovery system, POEM

1. Male and female gametophytes

Discussing the journey of the pollen tube first requires an introduction to the smallest fertilization units, namely, the male and female gametophytes (**Figure 1**). The male gametophyte (pollen) comprises two sperm cells and one vegetative cell and is found in the stamen of a flower. The two sperm cells fertilize the egg and central cells inside the female gametophyte

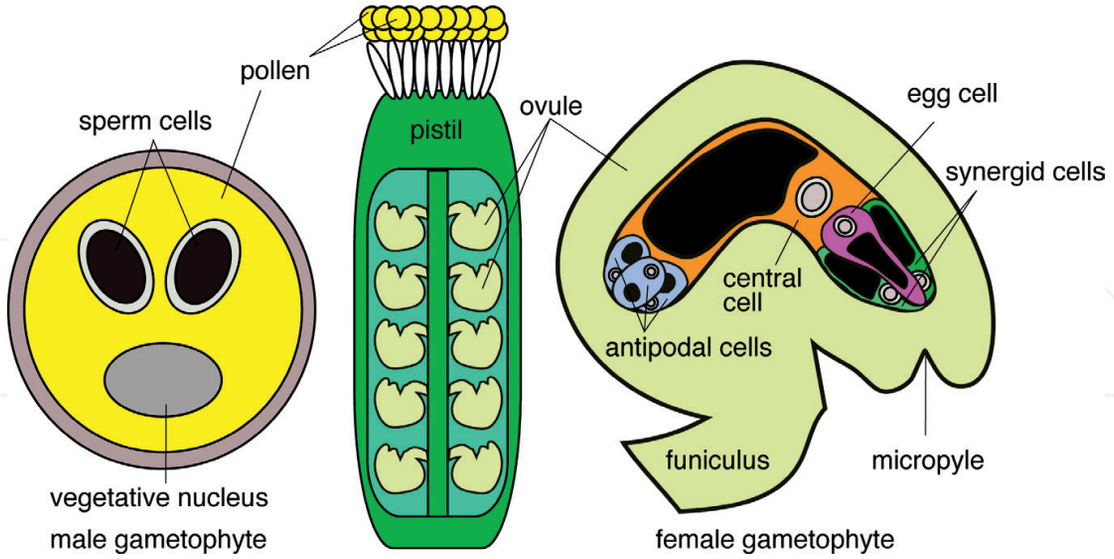

Figure 1. Male gametophyte and female gametophyte. The male gametophyte, also called the pollen grain or microgametophyte, develops within the anther and consists of two sperm cells encased within a vegetative cell (left). The mature female gametophyte (right) inside the pistil (center). The egg and central cells are polarized such that the nuclei of both cells lie very close to each other. This feature is important for double fertilization because these two nuclei are the targets of the two sperm nuclei. After double fertilization, the egg cell forms embryo and the central cell forms endosperm. The synergid cells have at least two functions associated with the fertilization process. First, the synergid cells secrete pollen tube attractants. In addition, the pollen tube enters the synergid cell, suggesting that the synergid cells are important for pollen tube reception. The black areas represent vacuoles of the cells in the female gametophyte.

via a guided pollen tube journey that is described later. The female gametophyte, which is embedded in an ovule within the pistil, contains seven cells of four different types: an egg cell, a central cell, two synergid cells, and three antipodal cells. The egg and central cells are polarized such that their nuclei lie in very close proximity, a feature facilitating double fertilization of these two sperm nuclei targets [1–3]. The synergid cells are extremely essential for the attraction of pollen tubes, as discussed below [4–8].

2. From the stigma to the funiculus

Once a pollen grain reaches the stigma at the top of a carpel, the pollen tubes elongate toward the funiculus to form a bridge-like structure to an ovule, as shown in **Figure 2**. This pollen tube growth through the stigma to the funiculus is controlled via multiple signals from both sporophytic and gametophytic maternal tissues in the carpels. The roles of the female tissues in pollen tube guidance have been focused upon.

Light and transmission electron microscopy studies of *Arabidopsis* have led to several observations regarding pollen tube growth in the female tissues of carpels [9–12]. Although the morphologic features of the pollen tube journey are well understood, the underlying regulatory molecular mechanisms remain unclear. Accordingly, previous studies used sporophytic mutants to elucidate the relationship between pollen tube growth and ovule/female

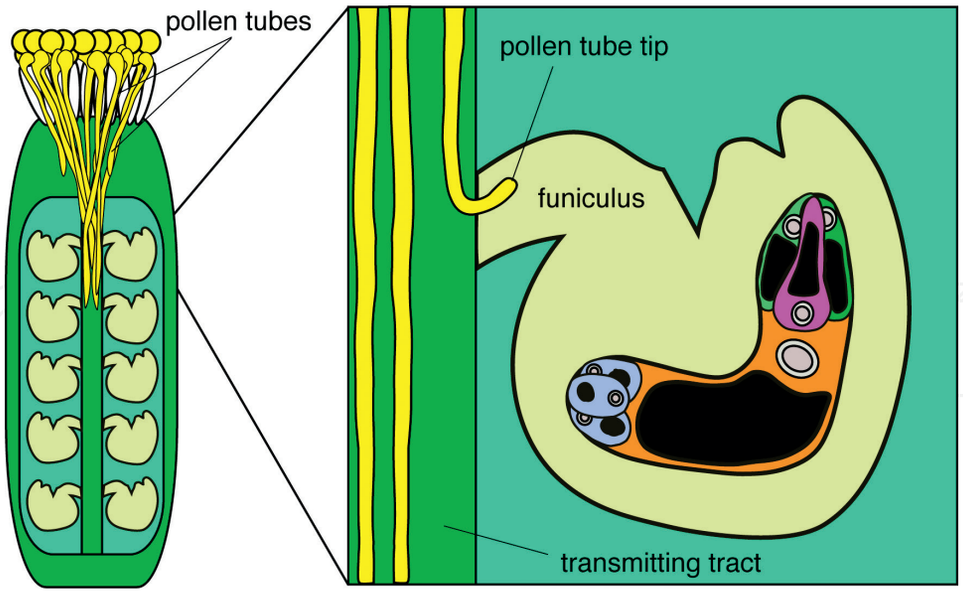

Figure 2. Pollen tube guidance from the stigma to the funiculus. Soon after pollination, the male gametophyte becomes hydrated and then germinates a pollen tube. The pollen tube initially penetrates and grows through the intercellular spaces between the papillar cells of the stigma and then grows through the transmitting tract of the carpel's style and ovary. The pollen tube then emerges from the transmitting tract and grows along the surface of the placenta toward an ovule.

gametophyte development. Particularly, these homozygous mutants mostly produce defective ovules. Although wild-type pollen tubes grow normally during initial phases from pollen hydration to tube emergence from the transmitting tract, they fail to grow toward the mutant ovules, which lack female gametophytes. In other words, although the female gametophyte does not influence early pollen tube growth, it appears to be required for subsequent pollen tube guidance to the ovule [11, 13–15]. These observations suggest that a molecular approach is essential for understanding pollen tube growth from the stigma to the funiculus.

3. From the funiculus to the female gametophyte

The pollen tube is subsequently guided from the funiculus to the female gametophyte. Although the molecular mechanisms underlying this step have been relatively well elucidated, as shown in **Figure 3**, a complete understanding requires a discussion of synergid cell biology (**Figure 1**). Synergid cells within the female gametophyte are essential for reproduction. After the pollen tube grows from the stigma to the funiculus, it enters the female gametophyte by growing into one of the two synergid cells, which typically undergo cell death before or upon pollen tube arrival. Soon after arrival, the pollen tube ceases to grow and subsequently ruptures to release its sperm cells into the receptive synergid's cytoplasm, thus triggering the completion of degeneration. Finally, one sperm cell each migrates to the egg cell and central cell to complete double fertilization of the female gametophyte [3, 16–19].

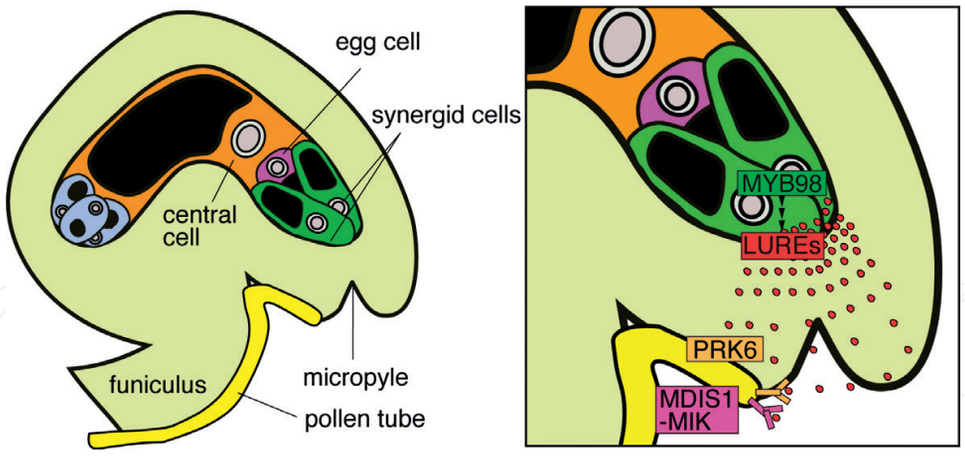

Figure 3. Pollen tube guidance from the funiculus to the micropyle. Synergid cells are required for pollen tube guidance. Several studies using *Arabidopsis thaliana* mutants have reported that pollen tubes fail to grow onto ovules containing abnormal female gametophytes, indicating that the embryo sac provides a guidance cue for the pollen tube. AtLURE1 peptides are attractants that guide pollen tubes to the ovular micropyle. These AtLURE1 peptides are particularly expressed in synergid cells and secreted toward the funicular surface through the micropyle. A transcription factor MYB98 is required for AtLURE1 since myb98 mutants do not express AtLURE1 peptides. PRK6 and MDIS1-MIK receptors are AtLURE1 pollen tube attractant counterparts.

Synergid cells are required for pollen tube guidance. Several studies using *Arabidopsis thaliana* mutants have reported that pollen tubes fail to grow onto ovules containing abnormal female gametophytes, indicating that the embryo sac provides a guidance cue for the pollen tube [11, 20, 21]. Higashiyama *et al*. used laser ablation in an *in vitro Torenia* pollen germination system to demonstrate that synergid cells, but not other female gametophyte cells, produce a pollen tube attractant [4]. Early in 2005, the requirement of a small protein, maize EA1, for pollen tube guidance was reported; however, maize EA1 has no homolog in *Arabidopsis* or other dicots and is unlikely to be a universal attractant [5].

MYB98, which is exclusively expressed in synergid cells (**Figure 4**), provides the first molecular evidence of pollen tube guidance in *Arabidopsis* [6]. Laser ablation studies have demonstrated that synergid cells secrete attractants that guide the pollen tube to the female gametophyte [4], suggesting that defects in pollen tube guidance should be observed in the *myb98* mutant. Accordingly, Kasahara *et al*. observed the pollen tubes of *myb98* mutant pistils pollinated with wild-type pollen. In the wild-type plant, the pollen tube grew along the funiculus of the ovule and through the micropyle to the female gametophyte; however, the wild-type pollen tubes grew abnormally on ovules containing *myb98* female gametophytes, specifically, the pollen tubes grew from the placenta to the funiculus but failed to grow into the micropyle (**Figure 4**).

MYB98 is expressed during the very early stage of synergid cellularization during female gametocyte development, consistent with the *myb98* mutant phenotype, in which pollen tube guidance and filiform apparatus structure are affected. However, several observations indicate that other synergid cell development aspects, including cell specification, remain normal in *myb98* mutants. Female gametophyte development and overall synergid cell morphology appear to be unaffected in *myb98* mutants. Additionally, the *myb98* mutation does not appear to affect the steps of fertilization process subsequent to pollen tube guidance, including the control of pollen tube growth cessation, pollen tube rupture, and sperm cell migration. These

data suggest that MYB98 functions as a transcription factor within the synergid cell gene regulatory network, where it particularly controls the expression of downstream genes required for pollen tube guidance and filiform apparatus formation.

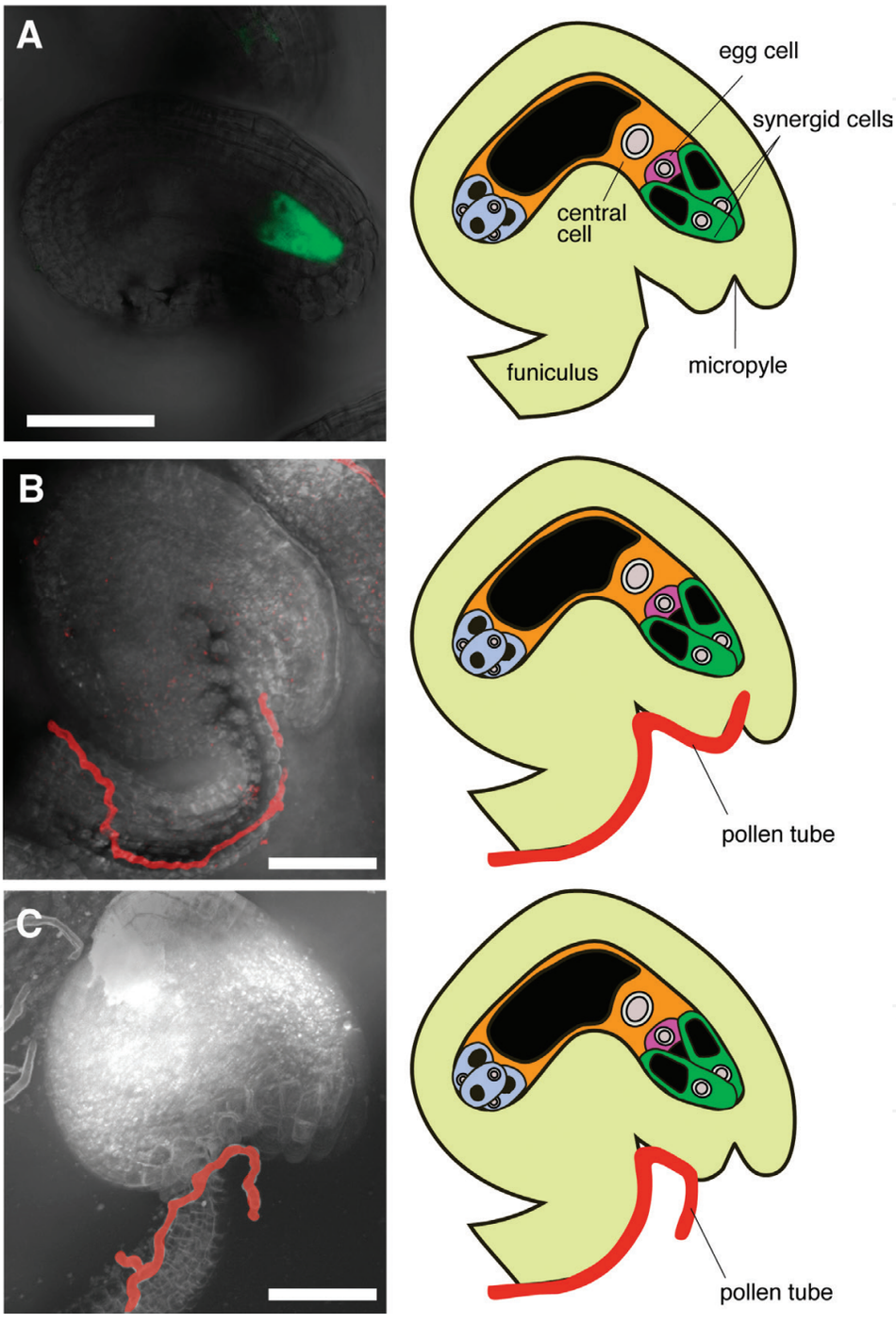

Figure 4. pMYB98::GFP expression and *myb98* phenotype. (A) MYB98 is expressed predominantly in the synergid cells (*pMYB98::GFP* photograph captured by Liyang Xie, HBMC, FAFU). *myb98* female gametophytes are defective in pollen tube guidance. (B) In the wild type, the pollen tube grew along the ovule's funiculus, through the ovule's micropyle, and into the female gametophyte. (C) By contrast, wild-type pollen tubes grew abnormally on *myb98* ovules. Pollen tubes grew from the placenta to the funiculus but then failed to grow into the micropyle. These data suggest that MYB98 functions as a transcription factor within the synergid cells to regulate the expression of genes required for pollen tube guidance. Bars = 30 μm.

The female gametophyte pollen tube attractants LURE1 and LURE2 have also been identified in *Torenia* [7]. LUREs are cysteine-rich proteins (CRPs) within the defensin-like (DEFL) family. *LURE* genes are expressed in synergid cells, which secrete the encoded proteins into the filiform apparatus. Accordingly, LURE downregulation reduces pollen tube attraction, and recombinant mature proteins attract pollen tubes *in vitro* and in a species-specific manner [7]. As discussed above, *myb98* mutation affects the filiform apparatus within synergid cells. However, MYB98 is also required for the expression of at least 83 genes encoding CRPs similar to LURE1 and LURE2 [22, 23]. Many of these CRPs exhibit localization and diffusion patterns similar to those of ZmEA1 [5, 24]; particularly, the CRPs are secreted into the filiform apparatus and subsequently diffuse into the micropylar region [22]. In 2012, Takeuchi and Higashiyama [8] finally identified a recently evolved DEFL gene cluster in *Arabidopsis* and demonstrated that these DEFL [cysteine-rich peptide (CRP810_1)] peptides, or AtLURE1 peptides, are attractants that guide pollen tubes to the ovular micropyle. These AtLURE1 peptides are particularly expressed in synergid cells and secreted toward the funicular surface through the micropyle. Genetic analyses have revealed that gametophytic mutants defective in micropylar guidance *myb98* [6], *magatama3* [21], and *central cell guidance* [25] do not express AtLURE1 peptides and that recombinant AtLURE1 peptides were found to preferentially attract *A. thaliana* pollen tubes vs. *A. lyrata* pollen tubes, indicating that these peptides act as species-preferential attractants in micropylar guidance [8]. Several female-secreted peptides have been identified as species-specific attractants directly controlling pollen tube growth direction. However, the method by which the pollen tubes precisely and promptly respond to guidance signals from their own species remains unknown. In 2016, two research groups reported AtLURE1 pollen tube attractant counterparts [26, 27]. Takeuchi and Higashiyama [26] reported that the tip-localized pollen-specific receptor-like kinase 6 (PRK6), featuring an extracellular leucine-rich repeat domain, serves as an essential sensor of LURE1 [8] in *Arabidopsis* under semi-*in vivo* conditions and is important for ovule targeting in the pistil. PRK6 interacts with pollen-expressed ROPGEFs (Rho of plant guanine nucleotide-exchange factors), which facilitates pollen tube growth by activating the Rho GTPase ROP1 [28, 29]. Particularly, PRK6 acts as a key membrane receptor for external AtLURE1 attractants and recruits core tip-growth machinery, including ROP signaling proteins. Furthermore, Wang *et al*. [27] identified that a cell-surface receptor heteromer, MDIS1-MIK, perceives the female attractant AtLURE1 on the pollen tube of *Arabidopsis*. MDIS1, MIK1, and MIK2 are plasma-membrane-localized receptor-like kinases containing extracellular leucine-rich repeats and an intracellular kinase domain. AtLURE1 particularly binds the extracellular domains of MDIS1, MIK1, and MIK2, whereas *mdis1* and *mik1 mik2* mutant pollen tubes respond less sensitively to AtLURE1.

4. Discharge of sperm cells from the pollen tube tip to fertilization

Immediately after growth cessation, the pollen tube ruptures at or near its tip, leading to release of the pollen tube's contents, including the two sperm cells. In *Arabidopsis* and *Torenia*, rupture occurs within 1 min after entry of the pollen tube into the female gametophyte [16, 17]. Regarding the molecular mechanisms underlying this step, two proteins localized in the sperm cells have been reported (**Figure 5**): GCS1 [30] and GEX2 [31]. Mori *et al*. [30] identified

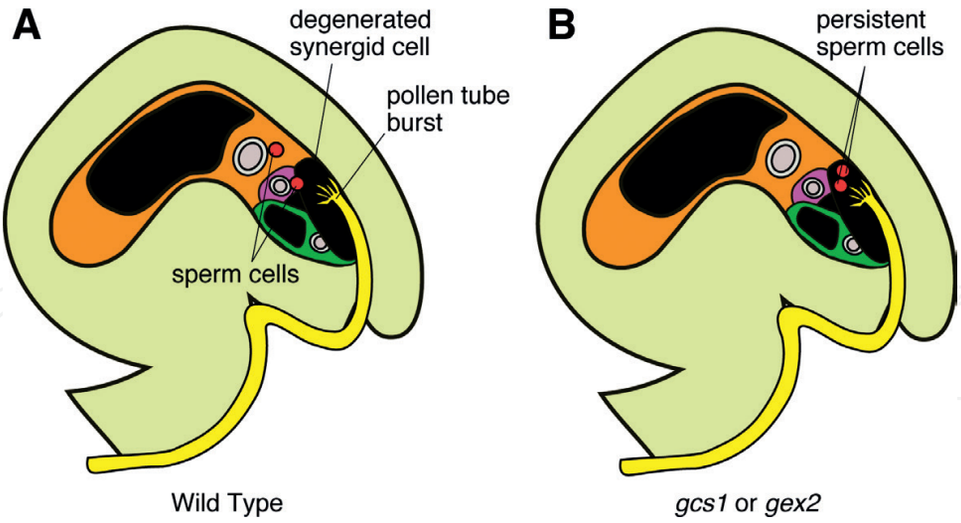

Figure 5. Discharge of sperm cells from the pollen tube tip to double fertilization. (A) Upon reaching an ovule, the pollen tube grows along the surface of the ovule's funiculus, through the micropyle, and into the female gametophyte. The pollen tube enters the female gametophyte by growing through the synergid cells. The pollen tube then comes in contact with the synergid cells and ceases growth. One of the synergid cells then undergoes cell death. Finally, soon after synergid degeneration is initiated, the pollen tube ruptures and releases two sperm cells into the degenerating synergid cytoplasm. The two sperm cells then move toward and fuse with the egg cell and central cell to complete double fertilization. (B) The male gametes of *gcs1* mutant fail to fuse with the egg or central cell. GCS1 accumulates during late gametogenesis and localizes on the plasma membranes of generative cells. The male gametes of *gex2* mutant also fail to fuse with the egg or central cell but the frequency of failure is lower than the *gcs1* mutant.

a protein, GCS1 (generative cell specific 1), using generative cells isolated from *Lilium longiflorum* pollen. Homologs of GCS1, possessing a carboxy-terminal transmembrane domain, are present in various species, including non-angiosperms. Immunological assays have indicated that GCS1 accumulates during late gametogenesis and localizes on the plasma membranes of generative cells. Notably, *Arabidopsis gcs1* mutants exhibit male sterility because the male gametes fail to fuse with the egg or central cell (**Figure 5**). Mori *et al*. [31] identified another important male factor, GEX2 (gamete expressed 2), which encodes a sperm-expressed protein of unknown function that localizes to the sperm membrane and contains extracellular immunoglobulin-like domains, similar to the gamete interaction factors in algae and mammals. Using a novel *in vivo* assay, Mori *et al*. demonstrated the requirement of GEX2 for gamete attachment, as double fertilization is compromised in its absence.

5. Fertilization recovery system

In angiosperms, double fertilization within the ovule occurs with the entry of two sperm cells, which are usually delivered by a single pollen tube. In 1904, Wylie [32] observed the insertion of two pollen tubes in an *Elodea canadensis* ovule and concluded, "It often happens that two pollen tubes pass into one ovule; in such cases both synergids disappear." Since this discovery, the reception of two pollen tubes in an embryo sac, although rare, has been reported in at least 12 species [33]. Similarly, the reception of two pollen tubes has been reported in several *Arabidopsis* mutants, including the *gcs1* mutant [30]. Although this phenomenon is interest-

ing, it has long been considered anomalous. However, Kasahara *et al*. [34] investigated the mechanisms underlying this phenomenon in higher plants upon frequently observing ovules that accepted two pollen tubes in the fertilization-defective *hap2-1* (allelic to *gcs1*) mutant [35], as shown in **Figure 6**. As the *hap2-1* mutant pollen tubes were marked by the pollen tube-specific reporter gene *LAT52:GUS* [36], Kasahara *et al*. traced the tube behaviors *in vivo* by staining for GUS activity, followed by aniline blue staining, to trace the behaviors of the first and second pollen tubes. Accordingly, most ovules contained one pollen tube at 10 hours

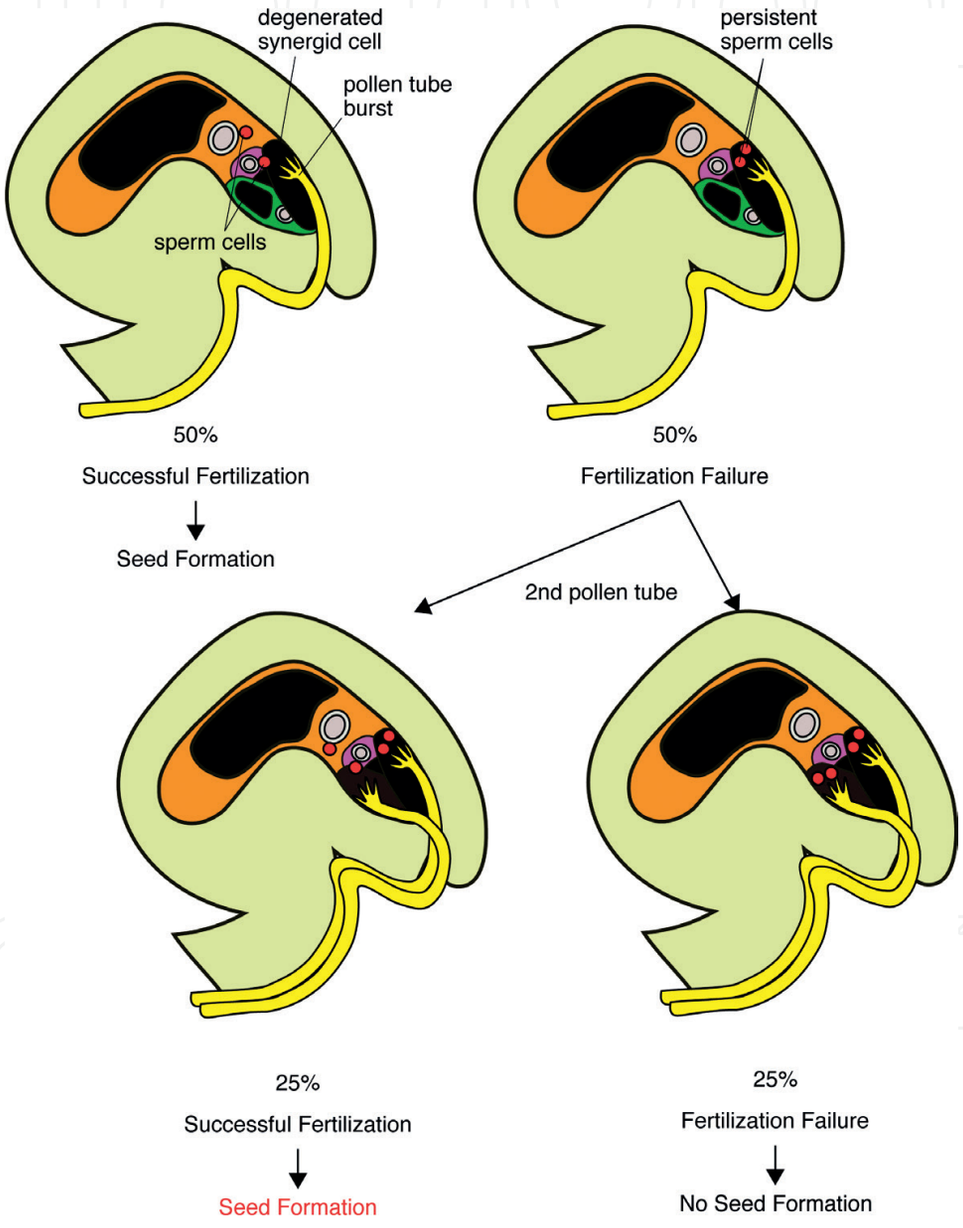

Figure 6. Fertilization recovery system. Upon insertion of a single pollen tube into an ovule, the pollen tube bursts and releases two sperm cells. When the sperm cells complete fertilization, the ovule blocks the entry of the other pollen tubes and develops into a seed by forming an embryo and endosperm. When fertilization fails, the ovule attracts a 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 the pollen tube attractant from synergid cells, since both synergid cells collapse after entry of two pollen tubes.

after pollination (HAP), indicating that the reception of a second pollen tube is independent of sperm cell fertility until several hours after the arrival of the first pollen tube. This delay may represent a blocking system by which ovules avoid polysiphonogamy [34, 37]. However, after 10 HAP, ovules that failed to be fertilized by the first *hap2-1* pollen tube began to attract a second tube. In this case, the persistent synergid cell, which would degenerate upon successful fertilization, continued to attract pollen tubes, leading to a second pollen tube acceptance rate of ~80% among failed ovules by 28 HAP. Although no particular role has been proposed for synergid cell persistence after the arrival of the first pollen tube, Kasahara *et al.* demonstrated that the second synergid cell could retain its function and thus attract and accept a second tube to rescue fertilization. This might explain the presence of two synergid cells in many higher plants (**Figure 6**).

Previously, several research groups [35, 38, 39] studied why several sperm cell–defective mutants exhibited an enhanced fertility phenotype (60–70% fertility); particularly, the frequency ratio of double pollen tube reception was almost completely consistent with the frequency of enhanced fertility (**Figure 6**). Additionally, the GUS staining experiment revealed that by 10 HAP, ~50% ovules had accepted a mutant allele, indicating that the mutant and wild-type pollen tubes were similarly competent to enter the embryo sac and release their contents. von Besser *et al*. [35] suggested that *hap2-1* sperm cells affect pollen tube guidance. However, our data led us to conclude that sperm cells are passive pollen tube cargo and do not influence pollen tube guidance in *hap2-1* mutants, consistent with the observation that sperm cell–defective mutants (i.e., no transmission via the male germline) exhibit only 30–35% sterility, instead of the expected 50%. Very recently, Zhang *et al*. [40] demonstrated that in the absence of two bHLH transcription factors, *Arabidopsis* produces an abnormal, sperm cell-free pollen exhibiting behavior similar to its wild-type counterpart, thus indicating that sperm cells are dispensable for normal pollen tube development. This result reinforced our concept of sperm cells as passive cargo, with no control over pollen tube growth and behavior.

According to previous report by Kasahara *et al*. [37], all hand pollination experiments were performed using large numbers of pollen grains. Particularly, *Arabidopsis* pistils, usually containing 50–60 ovules, were pollinated with approximately 20, 40, 80, 120, and 700 grains. Two days after pollination, the insertion of few second pollen tubes into ovules were observed among the pistils pollinated with 20 and 40 grains, indicating that under restricted conditions (ovules > pollen tubes), the pollen tubes selectively inserted into ovules that had not previously accepted any pollen tube. Conversely, when a wild-type pistil was pollinated with 80 (ovules ≤ pollen tubes) and 120 (ovules < pollen tubes) grains, approximately 12 and 25% of the ovules accepted second pollen tubes, respectively, suggesting that ovules accept second pollen tubes while under saturated conditions (ovules < pollen tubes) [37]. In other words, excess pollen is required to saturate the fertilization recovery system, and approximately 80% (not 100%) of the failed ovules can accept a second pollen tube to complete recovery, consistent with a previous report [34] that a substantial period of ~28 h is required for ovules to complete the fertilization recovery system. This delay in secondary guidance may be attributable to the functional synergid cell numbers; a previously penetrated ovule contains only one persistent synergid cell (the other would have been disrupted by a burst pollen tube) to provide guidance. Higashiyama *et al*. [4] reported that an ovule containing two synergid cells

attracts more pollen tubes than does an ovule with one synergid cell, suggesting that the latter produces insufficient levels of attractant. This may explain why only approximately 80% ovules with one synergid cell will attract a second pollen tube.

6. Pollen tube-dependent ovule enlargement morphology (POEM)

In angiosperms, the pollen tube releases its contents (including sperm cells) into the embryo sac upon insertion into the ovule, thus completing double fertilization. Recently, Kasahara *et al*. [41, 42] reported that the expansion and initiation of seed coat formation occurred even in ovules wherein fertilization failed after pollen tube insertion. This phenomenon was designated as pollen tube–dependent ovule enlargement morphology (POEM), which occurs only when the ovule accepts the pollen tube content (PTC). POEM was the first report addressing the paternal functions of PTC in facilitating the ovule's maternal development without fertilization in plants.

In animals, once semen is discharged into the uterus, the seminal plasma carries the sperm to the egg [43, 44], whereas in plants, PTC, which transports sperm cells to the ovules, has an analogous function. In mice, fertilization requires seminal vesicle secretory protein 2, which localizes only in the seminal plasma [45]. As seminal plasma is essential for fertilization in animals, Kasahara *et al*. proposed that PTC should also be important for fertilization in plants. To understand the function of PTC, a *gcs1* mutant [30] was used, which fails to accomplish fertilization despite releasing PTC to evaluate transcriptional variations after PTC release into the embryo sac and compared the transcriptomes between two ovule RNA types: one after normal fertilization and the other after PTC release without fertilization. At 12 and 24 HAP, the observation of similar expression profiles for both RNAs was unexpected because early events after pollen tube insertion were thought to be fertilization-dependent. However, these events were instead found to depend on PTC. Notably, between 24 and 48 HAP, multiple genes associated with cell expansion, cell division, and seed coat formation were upregulated regardless of fertilization, suggesting that PTC can affect ovule shape. Hence, the ovule phenotype was investigated. Interestingly, ovules that accepted PTC expanded without fertilization because of cell expansion and division and the production of a partial seed coat, consistent with the results of the transcriptome analysis. Using data from the successful transcriptome analysis, Kasahara *et al*. identified that the novel plant phenomenon POEM occurs only when the ovule accepts PTC, irrespective of fertilization (**Figure 7**) [41].

In angiosperms, pollination is the first step toward fertilization. Once the pollen reaches the stigma, the grains elongate to form pollen tubes and move toward the synergid cells observed within the female gametophyte. Fertilization occurs when the pollen tubes pierce the female gametophyte; this action terminates pollen tube growth and induces bursting, resulting in the deposition of the two sperm cells inside the female gametophyte. The phenomenon represents a new reproductive phase between pollen tube guidance and fertilization because PTC release itself could induce POEM. Roszak and Köhler [46] demonstrated failure of seed coat synthesis in *agl62* mutant ovules (Kang *et al*. [47]), which exhibited early endosperm cellularization, resulting in abnormal endosperm formation. Roszak and Köhler suggested that central cell

fertilization and normal endosperm formation was required for the initiation of the seed coat formation. Contrarily, our observation of vanillin staining in *agl62* mutant ovules indicated that the central cell fertilization is not required for the initiation of the seed coat formation (**Figure 7**).

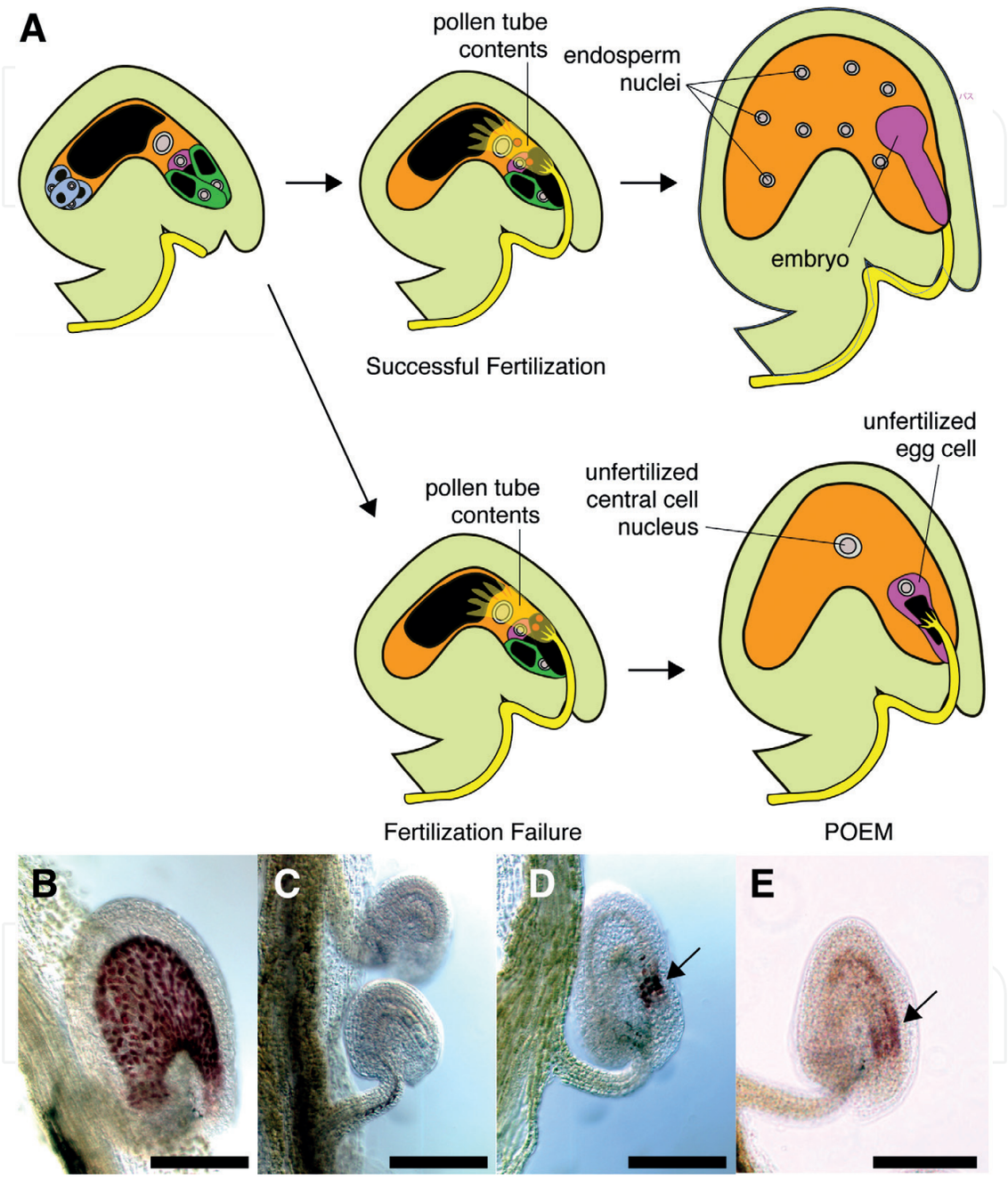

Figure 7. Pollen tube–dependent ovule enlarged morphology (POEM). (A) After the pollen tube is inserted to the female gametophyte, the pollen tube bursts and releases its contents (yellow region) with two sperm cells. Double fertilization is accomplished by these sperm cells fertilizing egg cell and central cell, respectively. *gcs1* mutant sperm cells fail to fertilize and the ovule does not produce a seed. It had long been suggested that the ovule will just die if the ovule fails to fertilize. However, if the pollen tube contents are supplied to the ovule, the ovule will be enlarged and initiate seed coat formation without fertilization. (B) A wild-type seed stained by vanillin at 3DAP. Whole seed coat region is stained. (C) Ovules without pollen tubes. Seed coat is not stained by vanillin. (D) A wild-type ovule crossed by *gcs1*/*gcs1* pollen. The ovule is partially stained. (E) An *agl62* mutant ovule. The ovule is partially stained even though it has abnormal endosperm. Arrows indicate the stained part by vanillin. Bars = 100 μm.

PTC was previously found to initiate central cell/endosperm nuclei division without fertilization when it was released to an autonomous endosperm mutant, *mea* [48, 49]. The finding that segmentation can be induced in a fertilization-independent manner by physical stimuli, leading to the development of some eggs into normal tadpoles, was first reported in 1910 [50]. In plants, Kasahara *et al*. [41] showed that PTC could increase central cell/endosperm nuclei division in the absence of fertilization, suggesting a function parallel to the observed fertilization-independent division of animal germ cells in response to external stimuli. By inducing endosperm nuclear division, PTC facilitates apomixes [51] in important crops when the POEM phenomenon is combined with autonomous endosperm and embryo mutants. In plants, seed formation without fertilization, or apomixis, is agriculturally valuable because important genetic traits can be easily fixed in apomictic crops, which then propagate without interference from unfavorable environmental conditions. POEM could therefore be categorized as "pseudogamy," which is defined as any reproductive process requiring pollination but no inheritance from the male gametophyte [52]. Although Focke [53] first defined pseudogamy as a part of apomixis, the underlying cellular or molecular mechanisms have remained obscure. Given the conceptual similarities, POEM may therefore be a key in understanding pseudogamy, particularly concerning pollen and PTC stimuli.

7. Summary

This chapter discusses the journey of the pollen tube from the stigma to fertilization as well as the POEM phenomenon. Because very few factors related to pollen tube guidance from the stigma to the funiculus of the ovule have been elucidated, additional insights into this step are eagerly awaited. However, the molecular mechanisms underlying pollen tube guidance from the funiculus to the female gametophyte are well known in *Arabidopsis* because the pollen tube attractants AtLURE1 peptides had previously been identified downstream of the master synergid cell regulator MYB98. During the final step after pollen tube bursting, only two proteins, GCS1 and GEX2, have been identified as direct male-related key fertilization factors in the pollen tube. Accordingly, further molecular evidences are required to understand the final step for plant fertilization. Finally, very few factors related to new plant phenomena, fertilization recovery system, and POEM have been identified. New insights into the underlying molecular mechanisms are anticipated.

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