1 **Title** **The end of temptation: the elimination of persistent synergid cell identity**

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1 **Abstract**

2 In flowering plants, sexual reproduction culminates in double fertilization, which occurs
3 after an ovule receives two sperm cells from a single pollen tube. Recent progress in
4 pollen tube guidance, as well as analyses of fertilization-defective mutants, have
5 highlighted a post-fertilization event that rapidly terminates pollen tube attraction. This
6 event plays a crucial role in ensuring a [one-to-one](#) fertilization system between males
7 and females. This phenomenon is controlled by the activity of persistent synergid cells,
8 which secrete peptides that attract and thus guide the pollen tube. This review briefly
9 introduces new findings on cell biology and signaling pathways that regulate the unique
10 [inactivation mechanism](#) of persistent synergid cells.

11

1 Introduction

2 During sexual reproduction in flowering plants, the pollen tube grows rapidly into the
3 pistil to transport sperm cells into the ovule (Fig. 1). Each ovule has a female
4 gametophyte, consisting of an egg cell, a central cell, two synergid cells, and three
5 antipodal cells [1]. The synergid cells secrete attractant peptides that control the growth
6 direction of the pollen tube via counterpart receptor-like kinases [2–7]. Then, the
7 attracted pollen tube discharges two sperm cells into one of the two synergid cells.
8 These sperm cells independently fertilize two female gametes: the egg cell and the
9 central cell. As a result of double fertilization, the egg cell gives rise to an embryo and
10 the central cell develops into the embryo-nourishing endosperm (Fig. 1).

11 *Arabidopsis thaliana* has an efficient fertilization system, in which each ovule
12 usually completes fertilization after penetration of a single pollen tube. Interestingly,
13 this one-to-one relationship is disturbed in various fertilization-defective mutants. For
14 example, in *Arabidopsis* mutants such as *feronia* and *lorelai*, multiple pollen tubes are
15 attracted if the ovule does not induce the initial pollen tube to discharge [8–10]; this is
16 called polytubey [11]. Polytubey is also induced by a mutation in the *GENERATIVE*
17 *CELL SPECIFIC 1* (*GCSI*)/*HAPLESS2* (*HAP2*) gene, which encodes a sperm
18 cell-specific transmembrane protein that is essential for plasma membrane fusion
19 between male and female gametes [11–13]. Expression of *HAP2/GCSI* is controlled by
20 *DUO POLLEN 1* (*DUO1*) and *DUO POLLEN 3* (*DUO3*) [14,15]. The egg cell-secreted
21 Egg Cell 1 (EC1) peptides are responsible for relocation of the *GCSI/HAP2* protein
22 onto the sperm cell surface [16,17]. Mutants of these genes exhibit the polytubey
23 phenotype [11,13,16,17]. There are up to two pollen tube insertions into mutants of the
24 *HAP2/GCSI* pathway, because each synergid cell is destroyed by the reception of a
25 single pollen tube [11, 16,17].

26 These polytubey phenotypes are very informative when considering the
27 one-to-one relationship between ovules and pollen tubes in wild-type crosses. After the
28 first pollen tube destroys the receptive synergid cell, the other synergid cell (then called
29 the persistent synergid cell) has the potential to attract a second pollen tube. However,
30 this occurs only when the first set of sperm cells fails fertilization [12]. In other words,
31 persistent synergid cells are inactivated in fertilized ovules to block polytubey. An
32 analysis of persistent synergid cells will provide new insight into the evolution of the
33 polytubey block and fertilization mechanisms in flowering plants. Moreover, the study

1 of persistent synergid inactivation may promote crop science because the polytubey
2 block can maximize plant fertility under conditions of limited pollen. Here, we review
3 recent findings on persistent synergid inactivation in *A. thaliana*.

5 **Fertilizations and polytubey block**

6 A polytubey block is induced by double fertilization. However, the role of each female
7 gamete in the initiation of the block is difficult to analyze, as fertilization of two female
8 gametes occurs almost simultaneously. To study the fertilization process, previous
9 studies have induced single fertilizations of either egg cells or central cells using pollen
10 from mutants of *CYCLINE DEPENDENT KINASE A1* (*CDKA;1*), which produces
11 single sperm-like cells in mature pollen grains [18–20]. In such experiments, polytubey
12 has been observed in ~30% of ovules with developing endosperms but without embryos
13 [21]. This rate is higher than that reported for ovules that completed double fertilization
14 (~5%), but the phenotype was weaker than for ovules that failed to fertilize both female
15 gametes (~80%) [12,22]. Weak polytubey has also been observed in ovules with
16 opposite-type single fertilization containing developing embryos but no endosperms
17 [21]. These data suggest that the egg and central cells independently generate signals
18 that coordinately activate the polytubey block after their fertilization.

20 **Discovery of synergid-endosperm fusion**

21 Single fertilization experiments have revealed independent and additive control of
22 polytubey block by two female gametes; however, they have not explained the
23 inactivation mechanism of persistent synergid cells. In the history of embryology, the
24 degeneration process of persistent synergid cells has been studied less than synergid
25 cells receiving a pollen tube. However, it has been reported that cotton (*Gossypium* spp.
26 L.) forms thick cell walls around persistent synergid cells in an ovule that has been
27 penetrated by a pollen tube [23], whereas in *Capsella bursa-pastoris*, persistent
28 synergid cells lack cell walls and appear to be absorbed by the developing endosperm
29 [24]. Engell [25] observed developing seeds in barley (*Hordeum vulgare* L.) and
30 presented an image of disintegrated cell walls that resemble those observed in *Capsella*,
31 but only mentioned the longevity of persistent synergid cells.

32 To elucidate dynamic cellular changes in persistent synergid cells, time-lapse
33 imaging was performed in *A. thaliana* [26]. Dissected ovules exhibited the migration of

1 cytosolic GREEN FLUORESCENT PROTEIN (GFP) from persistent cells to the
2 endosperm within a few hours of double fertilization. Electron micrographs of fertilized
3 ovules displayed the absence of a cell wall. Rapid migration of synergid contents is not
4 only evidence of cell absorption, but also suggests novel cell fusion prior to absorption,
5 which has recently been called synergid-endosperm (SE) fusion [26]. Time-lapse
6 imaging of the pollen tube attractant peptide AtLURE1.2-GFP has demonstrated that
7 pre-secreted AtLURE1.2 in persistent synergid cells is rapidly diluted after SE fusion.
8 Theoretically, it is likely that the pollen tube targets synergid cells as a source of
9 attractant peptides. Thus, SE fusion may act as an initial polytubey block system, by
10 disrupting the secretion of pollen tube attractants. Immunostaining has highlighted the
11 absence of AtLURE1.2 on the surface of ovules soon after fertilization, supporting the
12 idea of rapid inactivation of persistent synergid cells through SE fusion.

13

14 **Nuclear disorganization**

15 The degeneration of persistent synergid cells has also been examined via nuclear
16 disorganization. In disorganized nuclei, the nuclear envelope does not enclose the entire
17 nucleoplasm and sometimes shows an invagination [24,26], where GFP-labeled nuclear
18 proteins no longer accumulate [11,26,27]. Time-lapse imaging has shown how abrupt
19 chromosomal condensation in the persistent synergid nucleus occurs during mitosis of
20 the endosperm, particularly after SE fusion [26]. It is likely that SE fusion forces a
21 synchronized cell cycle between persistent synergid cells and endosperm because
22 persistent synergid nuclei and endosperm nuclei share the same cytosol. Nuclear
23 elimination could be caused by premature entry of the persistent synergid nucleus into
24 mitosis propelled by endosperm proliferation. Indeed, a persistent synergid nucleus
25 abruptly increases the accumulation of a mitotic marker (cyclin B1;2-YFP) as the signal
26 increases gradually in endosperm nuclei [26]. This strategy would be useful to eliminate
27 a particular nucleus in a multinucleate cell [28].

28 Three factors have been shown to be involved in nuclear disorganization in
29 persistent synergid cells. The first factor is ethylene signaling [27]. Degeneration of
30 synergid nuclei can be induced by treatment with the ethylene precursor
31 1-aminocyclopropane-1-carboxylic acid (ACC) in unfertilized ovules. In addition,
32 persistent synergid nuclei in a double mutant (*ein3 eil1*) of major transcription factors
33 that regulate ethylene signaling exhibit longevity and frequently attract a second pollen

1 tube. The second factor is *FERTILIZATION INDEPENDENT SEED (FIS)*-class
2 *polycomb repressive complex 2 (PRC2)* [29]. *FIS* class genes encode a central
3 cell/endosperm-specific PRC2 that negatively regulates gene expression via
4 trimethylation of lysine 27 on histone H3. The FIS-PRC2 consists of four proteins:
5 MEDEA (MEA) [30], FERTILIZATION INDEPENDENT SEED 2 (FIS2) [31],
6 FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) [32], and MULTICOPY
7 SUPPRESSOR OF IRA1 (MSI1) [33]. Polyubey has been reported in the *mea*, *fis2*, and
8 *fie* genes [21], and *mea* and *fis2* have been confirmed to have defective nuclear
9 disorganization in persistent synergid cells [26]. In ethylene signaling and FIS-PRC2
10 mutants of these factors, polyubey has been observed in ~20% of heterozygous ovules
11 and about 40% in homozygotes, suggesting that the phenotype is a gametophytic defect.
12 The third factor required for persistent synergid nuclear disorganization is
13 *JAGGER/AGP4*. Polyubey becomes evident only in homozygous mutants, suggesting a
14 sporophytic defect [34]. *JAGGER* encodes an arabinogalactan protein (AGP) expressed
15 in the entire pistil and ovule integuments around synergid cells [34]. The AGPs are
16 heavily glycosylated by type II groups of the three-step sugar chain, and they function
17 as intercellular signaling molecules that play pivotal roles in various developmental
18 processes in plants [35–37]. Some AGPs affect the pollen tube and alter its tip growth,
19 self-incompatibility, and ability to respond to attractant peptides [38–41]. In *Torenia*
20 *fournieri*, a specific structure of a terminal disaccharide in the arabinogalactan sugar
21 chain is sufficient to change pollen tube behavior [41]. *JAGGER/AGP4* may control
22 nuclear disorganization of the persistent synergid through a similar signaling
23 mechanism mediated by terminal saccharides of the arabinogalactan sugar chain.

24 **Three-step inactivation of persistent synergid cells**

25 Single fertilizations induced by defective sperm cells in the *kokopelli* mutant have
26 elucidated how two female gametes control the inactivation of persistent synergid cells
27 [26,42]. Single fertilization of central cells induces SE fusion, while single fertilization
28 of egg cells predominantly regulates the activation of ethylene signaling. A three-step
29 inactivation mechanism has been proposed from these data (Fig. 2). First, SE fusion is
30 induced by fertilization of the central cell, and this disrupts pollen tube attraction. SE
31 fusion usually occurs 9–11 hr after pollination. Second, ethylene signaling is
32 predominantly activated by the fertilization of the egg cell, and this initiates the
33

1 disorganization of persistent synergid nuclei. Ethylene signaling is activated 14–16 hr
2 after pollination, but the precise timing of the initiation of ethylene signaling has not
3 been investigated. Finally, chromosomal condensation destroys persistent synergid
4 nuclei during mitosis of the endosperm and completely eliminates the identities of
5 synergid cells. Nuclear disorganization is also regulated by FIS-PRC2 in the endosperm
6 and JAGGER signals generated by maternal tissues.

7 **Conclusions and perspectives**

9 Knowledge on the polytubey block mechanism has considerably improved over the past
10 decade. Consideration has been given to the physiological roles of polytubey block,
11 including efficient fertilization with a minimum number of pollen tubes and the
12 prevention of polyspermy by restricting the number of sperm cells conveyed to the egg
13 cell. In the next decade, studies on the block mechanism should shift from a cellular to a
14 molecular perspective. For example, our group is currently studying signaling pathways
15 and plasma membrane fusion related to SE fusion using forward- and reverse-genetics
16 approaches. The elimination of persistent synergid nuclei should be analyzed in terms of
17 interactions among ethylene signaling and the FIS-PRC2 and JAGGER pathways. Such
18 data would help elucidate the significance of polytubey block. Cell wall discontinuity
19 between the persistent synergid cell and endosperm in barley suggests broad
20 conservation of SE fusion [25]. Other mechanisms of persistent synergid elimination
21 may be common as well. A comparison of elimination systems would increase our
22 understanding of the evolution of the fertilization system in flowering plants. Moreover,
23 studying the polytubey block in multiple species might improve crop fertility even
24 under conditions of limited pollen and contribute to agriculture.

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1

2 **Figure legends**

3

4 **Figure 1 Pollen tube attraction and double fertilization.**

5 (a) Side view (left) and front view (right) of an *Arabidopsis* ovule attracting a pollen
6 tube.

7 (b) Arrival of a pollen tube to one of the two synergid cells.

8 (c) Pollen tube discharging two sperm cells onto the egg and central cells.

9 (d) Double fertilization.

10

11 **Figure 2 Inactivation of persistent synergid cells.**

12 (a) An ovule immediately after double fertilization.

13 (b) Synergid-endosperm (SE) fusion is induced by fertilization of the central cell. The
14 contents of persistent synergid cells are diluted by a mixing of cytoplasm, which
15 disrupts the secretion of peptides that attract the pollen tube. Three pathways originating
16 from three different places are shown to be involved in nuclear elimination processes of
17 persistent synergid cells. Ethylene signaling induced by egg cell fertilization (1). The
18 FIS-PRC2 pathway from the central cell and endosperm (2). An arabionogalactan
19 protein JAGGER/AGP4 from the sporophytic tissues (e.g., ovule integument) (3). Note
20 that ethylene signaling is activated after double fertilization, while the remaining two
21 are expressed before fertilization.

22 (c) Elimination of the persistent synergid nucleus during nuclear proliferation in the
23 endosperm.

24

Figure 1

(a)

(b)

(c)

(d)

Figure 2

(a)

(b)

(c)