

· Invited Review ·

Sexual Reproduction in Higher Plants I: Fertilization and the Initiation of Zygotic Program

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Abstract

Sexual plant reproduction is a critical developmental step in the life cycle of higher plants, to allow maternal and paternal genes to be transmitted in a highly regulated manner to the next generation. During evolution, a whole set of signal transduction machinery is developed by plants to ensure an error-free recognition between male and female gametes and initiation of zygotic program. In the past few years, the molecular machineries underlying this biological process have been elucidated, particularly on the importance of synergid cells in pollen tube guidance, the Ca⁺⁺ spike as the immediate response of fertilization and the epigenetic regulation of parental gene expressions in early zygotic embryogenesis. This review outlines the most recent development in this area.

Key words: calcium; fertilization; imprinting; synergid; zygote.

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The life cycle of flowering plants is divided into two phases, a dominant diploid sporophyte phase and a transient haploid gametophyte phase. Plant sexual reproduction starts from the fusion of male and female gametes to form a zygote, followed by embryogenesis. During this process, how two gametes recognize each other and how the embryogenesis program is initiated are two important questions. Although this process has been studied extensively in animals using model organisms such as fly, nematode and mouse (for review, see Zurita et al. 2008), for a long time the knowledge of plants is rather descriptive and preliminary. Owing to development in genetic dissection tools and *in vitro* fertilization technologies, a large body of knowledge has been obtained in the last two decades

in deciphering this key step in sexual reproduction. In two successive review articles, we outline the recent development in this area. This article focuses primarily on the recognition of the gametophytes, the fusion of the gametes and the contribution of maternal and paternal genes; and the next article will outline the activation of the egg cell, polarity setup in the zygote and the initiation of the zygotic embryogenesis. Readers are directed to several review articles published elsewhere for other related processes in sexual plant reproduction, such as pollination (O'Neill 1997), self-incompatibility (Takayama and Isogai 2005), pollen tube growth (Cole and Fowler 2006), gamete recognition (Peng and Sun 2008), endosperm development (Olsen 2001) and embryo patterning (Willemsen and Scheres 2004).

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Morphology and Function of Male and Female Gametes

Dual fertilization, which is ubiquitous among angiosperms, initiates the development of both the diploid embryo (via the fusion of one sperm and one egg cell) and the triploid endosperm (via the fusion of the second sperm and two central nuclei). This phenomenon was discovered independently by Nawaschin (1898) and Guignard (1899). Two short-life haploid gametophytes are

involved in this process. The male gametophyte, or so-called pollen grain, is formed in the anther. The maturation process of the pollen grain occurs either in the anther, or during the germination of the pollen tubes on a pistil, having two sperm cells and one vegetative nucleus. To be exact, the sperm cells, with thin cell walls, are located within the cytoplasm of the vegetative cell. The transcription-active vegetative nucleus and its metabolism-active cytoplasm function together to allow the pollen tube to germinate on the stigma, to go along the style, and to escort two sperm cells to the female gametophyte (or so-called embryo sac).

An embryo sac formed in the ovule typically consists of seven cells (eight nuclei): one egg, two synergid, one central (two nuclei) and three antipodal cells (Figure 1). Similar to the pollen grain, the embryo sac can be considered as one gigantic cell with two nuclei (central nucleus). Within the cytoplasm of this cell (all with cell wall), six specialized cells are located. The egg cell and the synergid cells are located at the micropylar pole of the embryo sac, while the antipodal cells are near the chalazal pole (Figure 1). Variations of the embryo sac structure (15 different types) have been reported (Maheshwari 1950; Willemse and van Went 1984). In many species, such as *Arabidopsis* and *Torenia fourneri*, antipodal cells in the embryo sac are already degenerated before fertilization occurs. In *Peperomia*, there is

only one synergid per embryo sac, and in *Plumbago*, there is no synergid.

Synergid Cells Provide Short Range Signal(s) to Attract the Pollen Tube

Pollen grains germinate on the stigma and the pollen tubes enter the embryo sac along the transmitting tract to fertilize the female gametes. During the process, which may take minutes to several hours, pollen tubes are guided first by sporophytic signals and then by gametophytic signals. Several well-studied sporophytic processes from both male and female sides are involved in regulating the polarized growth of the pollen tube, to control the pollen-stigma interaction and to define the self-incompatibility recognition (O'Neill 1997; Johnson and Preuss 2002; Takayama and Isogai 2005; Cole and Fowler 2006). When pollen tubes reach the periphery of the embryo sac, they are guided by signals from the embryo sac, allowing the pollen tube to penetrate one synergid cell to discharge two sperm cells to the embryo sac (Hülkamp et al. 1995; Christensen et al. 2002). Results from genetic analysis have showed that the embryo sac provides diffusible signals to attract the pollen tubes, guiding the pollen tubes towards the egg

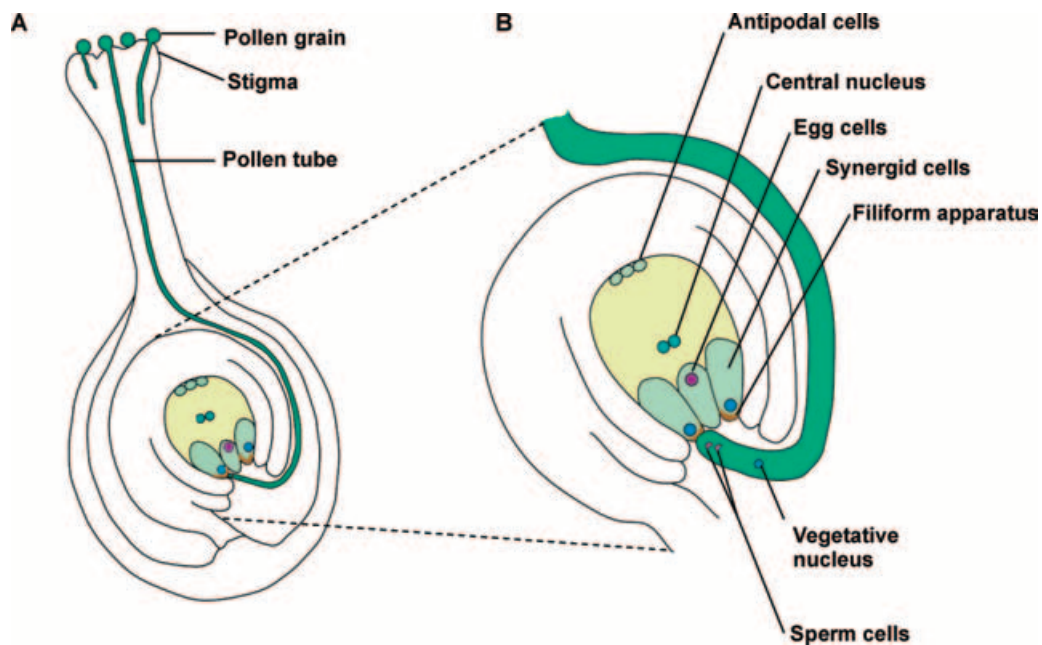


Figure 1. Sexual reproduction in higher plants.

(A) Mature ovary at the time of fertilization, showing the germination and penetration of the male gametophyte (pollen tube) towards the micropylar end of the female gametophyte (embryo sac) to deliver two sperms.

(B) Fertilization process, showing that two sperms carried by the pollen tube enter the embryo sac. One synergid cell sacrifices to assist the fertilization: one sperm fuses to the egg cell to produce a zygotic embryo and one with two central nuclei to produce the endosperm.

Table 1. Proteins involved in micropylar guidance and gamete recognition

Biological events	Proteins and their localization	Biological roles	References
Micropylar pollen tube guidance	MYB98 transcription factor, synergid cell-specific	Synergid cell differentiation	Kasahara et al. 2005
	CENTRAL CELL GUIDANCE (CCG), transcription regulator, central cell-specific	Micropylar pollen guidance	Chen et al. 2007
	Egg apparatus 1 (EA1), putative membrane-associated small protein, expressed both in egg and synergid	Short-range pollen tube attraction	Márton et al. 2005
Gamete recognition	FER/SRN, membrane-bound receptor kinase, synergid cell-specific	Gamete recognition	Huck et al. 2003; Rotman et al. 2003; Escobar-Restrepo et al. 2007
	GENERATIVE CELL SPECIFIC 1 (GCS1), cell surface protein, sperm-specific	Recognition or fusion between male and female gametes	Xu et al. 1998, 1999; Mori et al. 2005; von Besser et al. 2006
	DUO1, R2R3 MYB transcription factor, sperm nuclear-specific	Final division of the sperm and possibly in gamete recognition	Durberry et al. 2005; Rotman et al. 2005

cell (Table 1; Hülskamp et al. 1995; Weterings and Russell 2004).

T. fournieri, which has a naked embryo sac, provides an excellent model for examining and manipulating the fertilization process directly (Higashiyama et al. 2001; Higashiyama 2002). Using this plant, it has been observed in a living situation that, when the pollen tube reaches the micropylar end of the embryo sac, it is received by one of the synergid cells. Synergid cells in different species are similar in function, with a high content of soluble Ca^{++} in the vacuole. A specialized structure called filiform apparatus is formed through in-growth of the cell wall at the micropylar end of the synergid cell to increase the surface area of the cell, possibly to allow high efficiency secretion of the signal molecules. The pollen tube enters the embryo sac through the filiform apparatus, leading to the rupture of the synergid cell. Afterwards, the tip of the pollen tube ruptures to discharge the content through the degenerated synergid cell to the embryo sac (Higashiyama et al. 2001). The remaining synergid cell undergoes apoptosis and degenerates after a few days. In some species, one synergid cell even degenerates before the arrival of the pollen tubes, which is also considered to assist the penetration of the pollen tube.

In vitro laser ablation experiments using *T. fournieri* suggested that the pollen tube attracting signal is emitted from the synergid cell (Higashiyama et al. 2001). When both synergid cells are ablated, the embryo sac is not able to attract the pollen tubes, while ablations of other cells, such as egg, central cells or a single synergid in the embryo sac, do not affect the pollen tube guidance. It is likely that, in most flowering plants, the synergid cell provides the signal of pollen tube attraction. In species such as *Plumbago* with no synergid cells, the egg cell may play the

role of synergid because the egg cell in these species has the common features of the synergid (filiform apparatus and high Ca^{++} content). The signal provided by synergid cell counts for a short distance signal, mainly to attract the pollen tubes that have already reached the periphery of the micropylar region. Long distance pollen tube guidance in the pistil is governed primarily by sporophytic tissues.

Genetic studies have revealed several maternally expressed genes controlling the function of the synergid cells, particularly in the final step of sperm delivery to the embryo sac. A member of MYB family proteins, MYB98, identified in *Arabidopsis* is required for the pollen tube guidance and synergid cell differentiation (Kasahara et al. 2005). In the embryo sac, the putative transcription factor gene is exclusively expressed in the synergid cells. Mutation of *MYB98* leads to defects in synergid cell development, in particular filiform apparatus formation. In *sm* and *feronia* (*fer*) mutants, pollen tubes are correctly guided towards and are able to penetrate the embryo sac, but cannot release the sperms. More interestingly, it has been observed that no degeneration of synergid cells occurs when the pollen tubes enter the *sm* embryo sac, and multiple pollen tubes penetrate the embryo sac (Huck et al. 2003; Rotman et al. 2003). Most likely, the burst of synergid cell provides a signal to trigger the release of the sperms and to block the arrival of multiple pollen tubes. Lately, it has been shown that *SRN* and *FER* are allelic, encoding a synergid cell-expressed plasma membrane-bound receptor-like kinase (Escobar-Restrepo et al. 2007). The *FER/SRN* protein accumulates asymmetrically in the synergid membrane, specifically targeted to the filiform apparatus. Therefore, *FER/SRN* represents a signal perception pathway provided by the synergid cells to mediate the recognition between male

and female gametes during fertilization. It will be interesting to examine if *FER/SRN* is the direct target of MYB98 transcription factor.

Roles of Egg and Central Cells in Pollen Tube Guidance

Although the major signals attracting the pollen tubes are from synergid cells, molecular genetic analyses have shown that the egg and central cells are also involved in guiding the pollen tube for executing fertilization (Table 1). The *central cell guidance (ccg)* mutant identified in *Arabidopsis* from a transferred DNA insertion population showed 44% ovule abortion, suggesting a female gametophytic defect (Chen et al. 2007). Detailed morphological analysis shows that the *ccg* mutant ovule is defective in micropylar pollen guidance. Pollen tubes can reach the vicinity of funiculus, but fail to find the micropylar opening of the ovule. *CCG* encodes a nuclear protein with an N-terminal conserved zinc-ribbon domain that is functionally interchangeable with that of TFIIIB in yeast. This suggests that *CCG* might act as a transcription regulator for pollen tube guidance. In the female gametophyte, *CCG* is exclusively expressed in the central cell, while outside the embryo sac, *CCG* is expressed in the root tips and shoot apical meristems. Expression of *CCG* under the control of central cell-specific *FIS2* promoter of the *ccg* plant is sufficient to rescue the mutant phenotype (Chen et al. 2007). It is interesting to know what kind of downstream signal is induced by *CCG* to provide the mobile signal for attracting the pollen tube to the embryo sac.

Egg apparatus 1 (EA1) has been identified as an egg- and synergid-specific gene from maize (Márton et al. 2005). *EA1* encodes a putative membrane-associated small protein with 94 amino acids involved in short range signaling required for pollen tube attraction by the female gametophyte. Downregulation of *EA1* using RNA interference technology in transgenic plants leads to female sterility caused by loss of pollen attraction signal to the micropyle. Because *EA1* is expressed in both egg and synergid, it is not clear if the signal is released from the synergid or egg, or both. Replacing the *EA1* promoter with a synergid or egg cell-specific promoter to perform the complementation analysis will provide a direct answer to this question.

Involvement of Sperm Cells in Gamete Recognition

In higher plants, each of the four cells from the male meiosis enters two mitotic divisions, to produce one pollen grain. The pollen grain tube germinates and carries out polarized growth to deliver two sperm cells within its cytoplasm. The sperm cells are highly compacted, with very little cytoplasm. Previously, it has been generally believed that the sperm cells are transcriptionally

silenced (McCormick 1993). Large scale expressed sequence tag (EST) analysis of the sperm cell-expressed genes (5176 EST deposited in the National Center for Biotechnology Information database) indicates that sufficient mRNAs are present in the sperm cells (Engel et al. 2003). Reverse transcription polymerase chain reaction testing has showed that none of the genes are really sperm-specific. Those transcripts present in sperm cells are also available in unicellular or bicellular microspores, suggesting that the expression of these sperm-carried mRNA are already, if not exclusively, expressed during microspore development. Of course, these data do not exclude the possibility that the transcription continues in the sperm cells.

The first sperm cell-expressed gene identified was *GENERATIVE CELL SPECIFIC 1 (GCS1)* from lily (Xu et al. 1998, 1999). *GCS1* encodes a sperm cell surface protein. Mutation of *GCS1* in *Arabidopsis* does not diminish pollen tube growth *in vitro* or in the pistil, but it reduces ovule targeting twofold. Further, the *gcs1* sperms delivered to the ovules fail to initiate the fertilization, suggesting the role of *GCS1* in the recognition or fusion between male and female gametes (Mori et al. 2006; von Besser et al. 2006).

Genetic analysis of pollen development has identified two loci, *duo1* and *duo2*, that control the final division of the generative cell in *Arabidopsis*. The *duo1* pollen fails to enter mitosis at the G2-M phase, while *duo2* enters PMII but arrests at prometaphase (Durberry et al. 2005). *DUO1* encodes a novel R2R3 MYB transcription factor. *DUO1* proteins accumulate in the nuclei of sperm cells. Mutation in *DUO1* produces a single large diploid sperm cell unable to perform fertilization. This gene seems evolutionarily conserved. It will be interesting to know if the function of *DUO1* is solely one male gametogenesis, or also plays a role in controlling the fusion of the gametes (Rotman et al. 2005).

Ca⁺⁺ Spike is Critical for Pollen Tube Guidance and Zygotic Activation

Although the exact chemical property of the attractant from the synergid cell is still unclear, many experimental observations suggest that Ca⁺⁺ is the candidate molecule. In cotton and tobacco, it has been observed that the synergid cell contains a high concentration of Ca⁺⁺ (Jensen, 1965; Huang and Russell, 1992). *In vitro* assay also showed that a high concentration of Ca⁺⁺ attracts the pollen tubes of snapdragon (Mascarenhas and Machlis 1964). The embryo sac ceases to attract the pollen tube after fertilization, despite the continuous presence of one synergid cell. This suggests that an active mechanism is available in the synergid cell to halt further arrival of the pollen tubes to the embryo sac, preventing polyspermy. Even in the *in vitro* situation, as observed in maize, the isolated egg cell fuses with the first sperm but not the second one (Faure et al. 1994). This barrier is established as early as 45 s after the initial

fusion (Faure 2001), which may be related to the transient Ca^{++} spike occurring right after the fusion of the sperm and egg cells (Digonnet et al. 1997).

A dramatic increase in intracellular Ca^{++} concentration occurring in the egg right after fertilization is common to all animals examined so far. The Ca^{++} elevation in the cytoplasm serves as an essential signal for zygotic activation (for review, see Miyazaki and Ito 2006). In mammalian egg cells, a repetitive Ca^{++} rise (so-called Ca^{++} oscillation) is produced due to the release of Ca^{++} from the endoplasmic reticulum (ER) to the cytoplasm through the type 1 inositol 1,4,5-triphosphate (IP3) receptor. Most likely, the sperm factor leading to the Ca^{++} oscillation is phospholipase C zeta (PLCzeta), because injecting PLCzeta into the egg cell is sufficient to induce Ca^{++} oscillation without fertilization. In plants, a Ca^{++} spike is also associated with fertilization.

The role of Ca^{++} in plant fertilization is first addressed in brown algae *Fucus*. In this lower plant model, a specialized elevation of the cytoplasmic Ca^{++} concentration seems to be required for early fertilization events: the generation of potential fertilization and the secretion of the cell wall (Roberts 1994; Roberts and Brownlee 1995). Using the *in vitro* fertilization of maize, two critical events have been observed: a Ca^{++} influx that spreads as a wave front from the fusion site, and a rise of cytoplasmic Ca^{++} that is sufficient for egg activation (Digonnet et al. 1997). The Ca^{++} influx occurs 1.8 s after the gamete fusion, and for a period of 24.4 min. Furthermore, using a combination method to simultaneously monitor the extracellular Ca^{++} influx with a Ca^{++} vibrating probe and cytoplasmic Ca^{++} by wide-field imaging, it has been observed that the fusion of the gametes is accompanied by a Ca^{++} influx occurring 40–120 s before the transient increase of cytoplasmic Ca^{++} concentration (Antoine et al. 2000, 2001a; 2001b). Studies using a cell surface Ca^{++} channel blocker, Cd^{+++} , have found that the inhibition of the Ca^{++} influx does not affect the Ca^{++} spike and the fusion of the sperm with the egg cell, but prevents the incorporation of their cytoplasm, suggesting that the Ca^{++} spike may come from the release of Ca^{++} from the intracellular compartment, rather than the Ca^{++} influx. The influx of Ca^{++} is required for cytoplasm incorporation and karyogamy (Antoine et al. 2001a, 2001b). Consistent with this observation, it has been observed during the *in vitro* fertilization of maize that calreticulin, a major Ca^{++} storage protein, located in the lumen of ER, is strongly induced after fertilization (Dresselhaus et al. 1996). These observations suggest a common model of zygotic activation through the presence of the Ca^{++} spike during the fertilization in multicellular organisms.

Differential Contribution from Egg and Sperm in Early Zygotic Embryogenesis

Mendelian inheritance predicts that each individual plant receives two equal sets of nuclear DNA materials from two

parents, which is transmitted to their progeny following free segregation law. This is true for most traits studied so far. However, research in the last two decades has discovered differences in epigenetic modification between the male and female genomes, which lead to tremendous variations in expression profiles between the genes' delivery by male and female gametes before and after fertilization. This is the phenomenon that was first discovered in animal systems.

In most animal species, the early zygotic embryo development appears to occur mainly under maternal control and does not require transcription of the zygotic genome. A large population of maternally deposited mRNAs is present in the cytoplasm of the egg cell. For example, approximately 50% and 40% of mRNAs encoded in the genomes of fly and mouse, respectively, are already present in the cytoplasm of the egg through maternal contributions (Wang et al. 2004; Tadros et al. 2007; Zurita et al. 2008). In fly, no transcription is observed during the first eight cycles of synchronic nuclear division. In nematode, the first signal of zygotic transcription is detected at the four-cell stage, while in mouse it is at the late two-cell stage. Some maternal gene products are localized in the egg cell in a polarized manner, defining the body plan well before the zygotic embryo patterning occurs. For instance, the maternal gene *Bicoid* is crucial for specifying distinct domains along the anteroposterior fragmentation in fly, especially for the development of the head and thorax (Berleth et al. 1988). Both *Bicoid* mRNA and protein are localized in a gradient fashion with maximum at the anterior pole of the egg and the embryo.

In plants, it seems that the initiation of de novo gene expression occurs immediately after fertilization. Suppression and subtractive hybridization analysis in combination with mirror orientation selection has been used to study differential gene expressions in egg and zygote cells in tobacco, which allows identification of a group of nine genes that are expressed only in the zygote, not in sperm and egg cells (Ning et al. 2006).

Further, it has been reported that maternal genes contribute to both embryo and endosperm development. During *Arabidopsis* endosperm development, maternally expressed genes of *FIE*, *FIS2*, *MEA*, *MSI* and *GLAUGE* are present to repress the endosperm development without fertilization (for review, see Huh et al. 2008; Ngo et al. 2007). It is plausible to believe that similar machinery is present in the egg cell to prevent it entering embryonic development before fertilization takes place. Although such a factor has not been identified from plants yet, some experimental results have showed delayed expression of paternally transmitted allele in several species.

Delayed activation of the paternal genome was first reported by Vielle-Calzada et al. in 2000. Among 20 genes tested for their expression in early zygotic embryogenesis, none of them expressed when transmitted paternally. Most of them became actively expressed 3–4 d after fertilization. Based on this observation, the authors proposed that most, if not all, of the paternally transmitted genes are initially silenced. Similarly, in apomictic

hybrids between maize and its wild relative of *Tripsacum*, it has been shown that, for all 16 genes expressed during early seed development, only maternally inherited alleles are detected during 3 d after fertilization in both the embryo and the endosperm (Grimanelli et al. 2005). The parental gene expression has been further studied using microarray analyses of precocious embryonic development, demonstrating that early embryo development occurs without significant quantitative changes to the transcript population in the ovule before fertilization. Precocious embryo development is also correlated with a higher proportion of polyadenylated mRNA in the ovules. These data suggest that the maternal-to-zygotic transition occurs several days after fertilization.

However, controversial results have been reported. Meyer and Scholten (2007) examined the allele-specific expression of 25 genes after fertilization of the egg in maize and found immediate equivalent parental genomic contribution to the zygote. Paternal transcripts have been found in all examined genes that are expressed before the first zygotic division. Sequence comparisons indicate that these genes are involved in a range of processes and are distributed throughout the genome. This finding confirms that some plant species have evolved a strategy to activate the paternal genome immediately after fertilization, in contrast to the situation in other plants and in animals.

Such an extensive activation of the paternal genome right after fertilization is consistent with many molecular genetic results. Early transcription of paternal alleles has been observed in *Arabidopsis* using reporter construct (Weijers et al. 2001). Also, the phenotypes of numerous embryo lethal mutants segregate with a typical sporophytic 3:1 ratio (Liu and Meinke 1998; Tzafir et al. 2004), suggesting no apparent maternal effects. Among 323 embryo lethal genes identified in *Arabidopsis* so far, only four of them (1.24%) showed 50% embryo lethality instead of 25% (www.seedgenes.org). Mutation in *EMBRYONIC FACTOR 1 (FAC1)* leads to the arrest of 25% of embryos at the zygotic elongation or after the first zygotic division stage, suggesting that the paternally delivered wild-type copy of the *FAC1* gene is sufficient to rescue the mutated *fac1* allele in the embryo sac. Expression analysis was consistent with the genetic data. The paternally transmitted *FAC1* gene is expressed 2–3 h after fertilization (Xu et al. 2005).

We therefore propose the hypothesis that, in higher plants, most of the paternally delivered genes are expressed quickly after fertilization. The biased segregation observed in some cases might represent exceptions in nature or technological artifacts in experiments. Additional studies are needed to elucidate globally the epigenetic regulation in early zygotic embryo development.

Concluding Remarks

Sexual plant reproduction relies on a precise molecular genetic control along multiple steps. In this review, we outlined the

recent development from the point of recognition between male and female gametes, fusion of the gametes (as summarized in Table 1) and the expression of the parental genes before and after fertilization. Although the whole process takes only a few minutes to hours in most plant species, defined machineries are available from male and female sides to guard the process error-free. Behind the curtain of these events, the most important thing is communication. Although major players regulating the fertilization and gamete fusion processes remain to be discovered, powerful molecular, genetic and genomic technologies provide us with tools needed for deciphering them in a timely manner.

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