Review

Double fertilization – caught in the act

Frédéric Berger[1], Yuki Hamamura[2], Mathieu Ingouff[1] and Tetsuya Higashiyama[2]

[1] Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604, Singapore
[2] Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8602, Japan

In flowering plants, fertilization is unique because it involves two pairs of male and female gametes, a process known as double fertilization. Here, we provide an overview of the field and a detailed review of the outstanding recent advances, including *in vivo* imaging of double fertilization and the identification of a signaling pathway controlling the release of the male gametes and of a protein involved in gamete membrane fusion. These recent results are stepping stones for further research; our knowledge of double fertilization is expanding as newly discovered molecular pathways are explored and new mutants are characterized. Controlling plant fertilization is essential for seed production, and molecular understanding of double fertilization will provide the tools to improve crops and breeding programs.

The long-standing mystery of double fertilization

Flowering plants (angiosperms) have evolved a complex fertilization mechanism involving two sperm cells and two female gametes, the egg cell and the central cell (see Glossary for all terms concerning double fertilization). Two fertilization events take place in a coordinated manner and initiate seed development (Figure 1). The fertilized egg cell produces the embryo, and the fertilized central cell develops as the endosperm, which protects the embryo and regulates trophic interactions between the embryo and the mother [1]. This peculiar mode of reproduction was termed 'double fertilization' and was first identified as a hallmark of angiosperm biology at the end of the nineteenth century by S. Nawaschin [2] and L. Guignard [3]. Since its discovery, the cellular and molecular mechanisms involved in this unique reproductive process have remained rather enigmatic. Direct cytological observations have been difficult because the dynamic interactions between the gametes take place deep in the maternal tissues. It was only in the mid-1960s that electron microscopy studies in cotton (*Gossypium hirsutum*) showed that gamete interaction and fusion take place at a location where both male and female gametes are devoid of a cell wall [4,5]. These cytological descriptions were repeated in various species [6]. The design of methodologies allowing *in vitro* fertilization in maize led to physiological studies [7–12], suggesting the conservation of calcium signaling during fertilization in plants and in animals [7]. Species from the genus *Torenia* produce protruding female gametophytes, allowing direct observation of the double fertilization process [13,14]. Laser dissections of ovules from *Torenia* demonstrated the major role played by synergids in short-range attraction of the pollen tube [15]. However molecular mechanisms directly involved in double fertilization have not been identified using these early experimental systems [16].

During the past couple of years, molecular and genetic approaches using the model plant *Arabidopsis thaliana* have triggered a considerable renewal of interest in double fertilization. We review below these new findings and outline how the field might develop in future years.

Glossary

Central cell: the female gamete, which gives rise to the endosperm. Because the endosperm does not initiate a new life cycle, the central cell is not a true gamete. The central cell can be considered as the somatic part of the female gametophyte, which reinitiates its development after fertilization.
Double fertilization: the complex process leading to the production of the endosperm and the embryo. The major steps of double fertilization consist successively of (i) the attraction of the pollen tube toward the embryo sac containing the female gametes, (ii) the release of the two male gametes into the degenerated synergid, (iii) the migration of the male gametes by unknown mechanisms to the two female gametes, (iv) gamete recognition and fusion, (v) the fusion of the parental genetic material during karyogamy and (vi) reinitiation of the cell cycle and the transcription and translation that leads to the onset of the zygotic life.
Egg cell: the female gamete, which produces the embryo. Because the product of the fertilized egg cell reinitiates the plant life cycle, the egg cell can be considered as the true female gamete.
Embryo sac: the female gametophyte, which contains four cell types three antipodals, the two synergids and the two female gametes, namely the central cell and the egg cell.
Endosperm: the product of the fertilized central cell. The endosperm protects the embryo, controls the transfer of nutrients from the mother and, in some species, stores seed reserves. The role of the endosperm can be compared to that of the placenta in mammals.
Filiform apparatus: a structure composed of elaborate membrane folds and extracellular matrix protruding from the synergids in the micropyle. The filiform apparatus is probably the source of attractants for pollen tube guidance.
Gametophyte: the organism produced by the development of the spore. The gametophyte is haploid. It generally comprises a small number of cells with a size of the order of mm or cm, except in mosses, the gametophyte of which constitutes the major part of the life cycle. The germ line is defined in the gametophytic tissues. In many species there are male and female gametophytes. In flowering plants the gametophytic life is extremely reduced to a few cell divisions producing the embryo sac (female gametophyte) and the pollen (male gametophyte).
Integuments: maternal sporophytic tissues of the ovule surrounding the embryo sac.
Karyogamy: the fusion of the male and female nuclei.
Micropyle: the area of the ovule where the pollen tube enters and abuts on the synergid filiform apparatus.
Plasmogamy: the fusion of the plasma membrane of the male and female gamete.
Sperm cells: the two male gametes produced in the pollen.
Sporophyte: the diploid organism where meiosis takes place, producing the haploid spores.
Synergids: the two gametophytic cells on either the side of the egg cell that attract the pollen tube and control the release of the sperm cells.

Corresponding authors: Berger, F. (fred@tll.org.sg); Higashiyama, T. (higashi@bio.nagoya-u.ac.jp).

1360-1385/$ – see front matter © 2008 Published by Elsevier Ltd. doi:10.1016/j.tplants.2008.05.011 Available online 21 July 2008

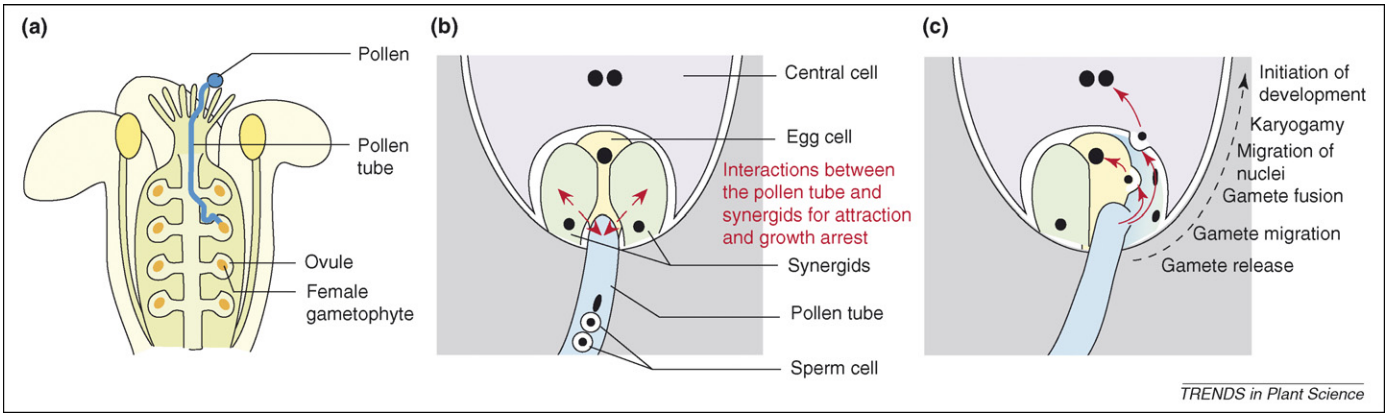

Figure 1. Schematic model of double fertilization. **(a)** Pollen tube guidance after pollination of the pollen to the pistil. When a compatible pollen grain is deposited on the pistil, the pollen hydrates and germinates. A pollen tube, emerging from the pollen, grows inside the pistil to reach the target female gametophyte inside the ovule. Female tissues control directional growth of the pollen tube. **(b)** Attraction and growth arrest of the pollen tube by the synergids. When the pollen tube reaches the vicinity of the female gametophyte (up to a few hundred micrometers), two synergids positioned on either side of the egg cell attract the pollen tube by an unidentified attractant(s). After pollen tube arrival, direct interaction between the pollen tube and the synergids causes growth arrest of the pollen tube. **(c)** The process of double fertilization after pollen tube discharge. One of two sperm cells fuses with the egg cell to form the embryo, and the other fuses with the central cell to form the endosperm. Many steps, including gamete migration and fusion, nuclei migration, karyogamy,and initiation of active transcription, are required to achieve double fertilization.

Imaging double fertilization

The small and simple structures of the pistil and ovule of *Arabidopsis* are suitable for imaging of double fertilization. Major technical hurdles were overcome recently, enabling live imaging of double fertilization. First, identification of gametophytic genes has led to the design of specific fluorescent protein markers for the male and female gametes (Table 1). The other breakthrough was the development of experimental set-ups for *in vivo* imaging. Pollen tubes labeled with *LAT52::GFP* were visualized growing inside the intact pistil using two-photon microscopy [17]. High-resolution confocal microscopic observation of the discharge of the pollen tube content in the female gametophyte [18,19] was achieved by removing the ovary wall using an experimental set-up adapted from the method developed with *Torenia* [20,21]. It is now clear that the pollen tube arrival causes synergid death, but it is still unclear whether synergid death takes place after the pollen tube discharge leading to male gamete delivery between the synergid and the egg cell.

Recently, the entire process of double fertilization was observed *in vivo* in *Arabidopsis* [22]. Gametes were visualized using a combination of markers for sperm cells and female gametes and a state-of-the-art disk-scan confocal scanning laser microscope equipped with a highly sensitive camera, a high-speed piezo Z-axis drive (which allowed rapid recoding of z-stacks) and a prism to monitor two colors at the same time. With this set-up, it was possible to observe the delivery of sperm cells, followed by the observation that after gamete fusion, male nuclei migrate inside the female gametes to reach the female nucleus (Figure 2; see Figure S1 and Movie S1 in the online supplementary material). Karyogamy was observed through the labeling of the nuclei of the male and female gamete with proteins fused to fluorescent reporters (Figure 2). The development of markers for the plasma membrane and the cytoskeleton will allow further investigation of the cellular events of double fertilization, which are still largely uncharacterized.

Table 1. Markers of gametes suitable for imaging double fertilization[a,b]

Gene	Male gametes	Female gametes		Refs
	Sperm cells	Central cell	Egg cell	
CDKA;1	X	-	-	[74]
DUO1	XX	-	-	[51]
GEX1	X	-	-	[52]
GEX2	X	-	-	[52]
GCS1	X	-	-	[43]
HTR10	XXXX	-	-	[22]
HTR12	XXX	-	-	[22]
MSI1	X	-	-	[54]
AGL80	-	XXX	-	[75]
DME	-	X	-	[76]
FIE	-	XXX	-	[77,78]
FIS2	-	X	-	[79]
FWA	-	XXX	-	[80]
MEA	-	X	-	[79]
RB	-	XXX	-	[58]
DD45[c]	-	-	XXX	[81]
SWN	-	X	X	[79]

[a]X refers to the intensity of the fluorescent signal: -, not expressed; X, low intensity; XX, medium intensity; XXX, high intensity (which is compatible with live imaging).
[b]The evaluation of the strength of the signal and practical use of the reporter lines for each gene was obtained from our own observations.
[c]Potential egg-cell marker.

Short-range pollen tube guidance by the synergids

Multiple steps of guidance by the female sporophytic tissues and the female gametophyte enable targeted directional growth of the pollen tube to the ovule [23]. Various mechanisms appear to be involved in pollen tube guidance, including chemo-attraction, mechanical guidance, growth stimulation, adhesion, re-orientation and competence control that enables the pollen tube to respond to the attraction signal [24].

The major player still unidentified is a chemo-attractant responsible for pollen tube guidance in the pistil [24,25]. Studies in this decade have shown that attractants definitely exist in the final phase of pollen tube guidance by the female gametophyte. In *Torenia*, pollen tubes are attracted by a diffusible signal derived from synergids [15,21,24]. In *Arabidopsis*, the synergids might be primarily responsible for the micropylar guidance from the

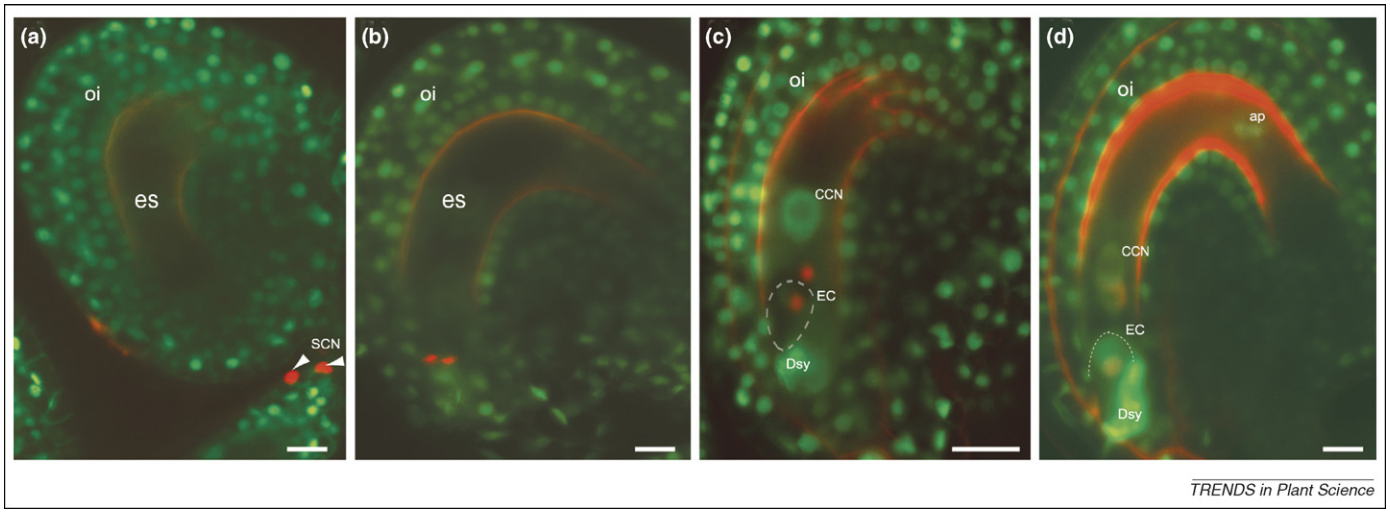

Figure 2. Sequential series of confocal images showing the major steps of the double fertilization process. The two sperm cell nuclei are labeled with prom*HTR10::HTR10:mRFP1* (red fluorescence, arrow heads). The different cell types of the embryo sac and the cells surrounding the integuments are labeled with prom*ACT11::MSI1:GFP* (green fluorescence) [22]. **(a)** The pollen tube (not visible) transports the male gametes towards the embryo sac that contains the female gametes. **(b)** Following discharge of the pollen tube content after growth into one of the two synergids, the two male gametes are released. **(c)** The male gametes migrate further into the embryo sac towards the female gametes. **(d)** Karyogamy (merging of the male and female chromatins). Note that dispersion of the male chromatin begins in panel (d). Scale bars represent 20 μm. Abbreviations: ap, antipodal cells; CCN, central cell nucleus; Dsy, degenerated synergid; EC, egg cell; es, embryo sac; oi, ovule integuments; SCN, sperm cell nuclei.

entrance of the micropyle to the female gametophyte [26]. Even when only a single synergid is produced, as in the mutant *eostre* [27], pollen tube attraction takes place and sperm cells are released properly. The transcription factor MYB98, expressed in the synergids, is required for formation of the filiform apparatus and micropylar pollen tube guidance [26,28]. It is thus likely that synergids produce an attractant secreted by the filiform apparatus. Interestingly, it was shown that the central cell plays a role in the guidance process because mutants for the gene *CENTRAL CELL GUIDANCE* expressed in the central cell are defective in pollen tube attraction [29]. It is thus possible that the central cell either signals the synergids to produce the attractant or produces an attractant precursor that is processed further by the synergids.

In *Arabidopsis*, pollen tube attractant candidates might be proteins encoded by genes expressed in the synergids and secreted into the filiform apparatus [24,28,30,31]. The *Zea mays* EGG APPARATUS 1 (ZmEA1), a small membrane protein of 94 amino acids, is a potential pollen tube attractant [32,33]. Knockdown of *ZmEA1* resulted in impaired guidance at the entrance of the micropyle, although its ability to attract the pollen tube has not yet been demonstrated. This could be tested directly by local application of the potential attractant in an *in vitro* pollen culture system. Even if a pollen tube attractant was identified, it might be difficult to find homologues in other species because attractants have evolved rapidly, as shown by the high species preferentiality for the attraction signal produced by the synergids [34]. Therefore, the rapid evolution of attractants might contribute to reproductive isolation barriers [20,35].

The control of sperm cell release

After entering the embryo sac, the pollen tube ceases growth and discharges its contents through interaction with the synergids (Figure 1b). The delivery of the sperm cell was originally hypothesized to take place inside one synergid. According to this hypothesis the contact between the egg cell membrane and the sperm cell membrane could only take place if the synergid was removed. Microscopic observations using fixed tissue led to the proposal that degeneration of one of the two synergids occurs before pollen tube arrival, allowing the sperm cells to migrate to the membranes of the female gametes [36]. However, *in vivo* observations suggested that the degeneration of one synergid is triggered only after pollen tube arrival [18,19] or pollen tube discharge [14]. This implies that sperm cells are released between the two synergids and that a dialogue between the synergids and the pollen tube regulates sperm cell release.

Recently, molecular insights into this dialogue between the pollen tube and the female gametophyte have been obtained. Cessation of pollen tube growth in the female gametophyte requires the receptor-like kinase FERONIA, which accumulates on the plasma membrane in the filiform appartus of synergids [37]. In absence of FERONIA in the embryo sacs of the mutant alleles *sirène* and *feronia*, the pollen tube grows and coils in the female gametophyte without releasing its content [18,38]. Sequence divergence in the extracellular domain of FERONIA correlates with a *feronia*-like phenotype in interspecific crosses. [37]. FERONIA probably responds to a ligand provided by the pollen tube. Unexpectedly, peroxisomes in both male and female gametophytes also play a key role in this process, as suggested by the phenocopy of the *sirène* (*srn*) and *feronia* phenotypes observed in the mutant *abstinence by mutual consent*, which is defective in a peroxin essential for protein import into peroxisomes [39]. The triggering of pollen tube discharge also requires ACA9, a calcium pump on the plasma membrane of the pollen tube, suggesting a contribution of calcium signaling to this process [40]. Two other mutants, *lorelei* (F. Berger, unpublished) and *scylla* [41] show a phenotype similar to that of *srn*-class mutants.

Surprisingly, *scylla* and *srn* produce autonomous endosperm in absence of fertilization [41], a phenotype typical of mutants impaired in the FERTILIZATION INDEPENDENT SEED (FIS) Polycomb group complex activity in the central cell [42]. Polycomb group complexes methylate Lys27 of Histone 3, and this marker is associated with transcriptional repression. The *FIS* and *SRN* pathways have synergistic interactions [41]. These results suggest an interaction between the synergids and the central cell in the control of the delivery of the male gametes. Currently it is not possible to predict the origin of the signal binding to the FERONIA receptor. The signaling by FERONIA could either (i) send a feedback signal to the pollen tube, causing its arrest, or (ii) ensure the proper maturation of the synergids controlling the release of male gametes.

Gamete recognition and fusion

Once the two sperm cells reach the female gamete, specific recognition signals are likely to take place, leading to plasmogamy (gamete membrane fusion) (Figure 1c). The protein GENERATIVE CELL SPECIFIC 1 (GCS1), which is expressed specifically on the surface of sperm cells, was identified in *Arabidopsis* from homologies to sperm cell proteins isolated from *Lilium* (lily) generative cells [43]. The loss-of-function mutations in *GCS1* prevent fertilization, most probably because the *gcs1* mutant sperm cells are unable to be recognized by, or to fuse with, the female gametes. The GCS1 protein is present at the sperm cell membrane [43,44]. Although GCS1 is conserved across a large range of plant species, it does not contain any known functional domains, and its function as a recognition signal remains unclear. The analysis of other alleles of *gcs1* (*hap2-1* and *hap2-2*) has also indicated that the sperm cells might play a role in short-range pollen tube guidance [44]. Proteins with lower homology to GCS1 have been reported in algae and in even more distantly related unicellular slime molds, suggesting conservation of an ancient recognition system in flowering plants [37]. Loss of function of GCS1 homologs impairs gamete fusion in the unicellular green alga *Chlamydomonas reinhardtii* and in the malaria parasite *Plasmodium berghei* [45,46]. Although possessing animal-like features, *Plasmodium* belongs to the phylum Apicomplexa and is a distant relative of plants. Interestingly, knockdown of the gene encoding *Plasmodium* GCS1 prevents fertilization and thereby mosquito transmission of malaria, suggesting novel strategies for controlling this life-threatening parasite. Further studies on GCS1 and its homologs will provide insights into the mechanism of membrane fusion during fertilization.

In most species the two female gametes are functionally very dissimilar, whereas the two sperm cells are morphologically identical. There has been a long-standing controversy regarding the potential specialization of each of the two sperm cells to fertilize either the central cell or the egg cell [16]. In the rare case of *Plumbago zeylanica*, sperm cells are dimorphic, and one type of sperm cell fuses preferentially with one type of female gamete [47]. In most species, sperm cells are isomorphic but, nevertheless, could differ in their capacity to fuse either with the egg cell or with the central cell [48].

Recent studies in *Arabidopsis* have brought forward arguments to support each hypothesis. A difference in sperm cell fate was suggested from studies of mutants deficient in CYCLIN DEPENDENT KINASE A1 (CDKA;1); this deficiency was shown to cause cell-cycle arrest of the generative cell and production of a single sperm cell [49,50]. The *cdka1* single sperm cell appears to be able to fertilize only the egg cell. This indicates that mechanisms leading to female-gamete-specific recognition might exist in *Arabidopsis*. However, a distinct body of data supports an equivalent fate for the two sperm cells; (i) to date, the genes specifically expressed in sperm cells have all been reported to be expressed in both sperm cells [22,43,44,49,51–53]; (ii) mutants affecting the function of CHROMATIN ASSEMBLY FACTOR 1 produce pollen that contains a single sperm cell, which is able to fertilize equally the central cell or the egg cell [54]. It thus remains unclear whether the two wild-type male gametes have an identical capacity to fuse with both female gametes. Such a specific identity could be essential for preventing repeated fusion of the sperm cells with the egg or the central cell, causing polyspermy. Alternatively, sperm cell specificity might be dispensable because there might be other mechanisms preventing polyspermy [55]. Live imaging of sperm cell release with increased time-resolution will be required to determine whether or not both sperm cells reach the female gametes simultaneously. Additional experiments involving mutants producing multiple female gametes [27] will also help to solve this long-standing question.

Coordination of the cell cycle

Gametic fusion brings together the cytoplasm and nuclei from both gametes. The cellular components of the gametes must synchronize their cell-cycle status to initiate harmoniously the developmental programs of the embryo and the endosperm.

The male gametes reinitiate S phase at the end of pollen maturation and are likely to arrest at the G2/M transition before their release in the female gametophyte [56]. The phase of cell-cycle arrest of the female gametes has still not been determined. Measurements of DNA contents in *Nicotiana tabacum* (tobacco) have suggested that both the egg cell and the central cell are arrested at the G1/S transition [57]. However, in *Arabidopsis*, several observations suggest that each female gamete is arrested at distinct stages of the cell cycle: (i) the retinoblastoma homologue RBR1, which controls the G1/S transition, is expressed in the mature central cell but not in the egg cell [58]; (ii) the initiation of the cell cycle is nearly immediate in the endosperm, whereas the first embryonic division takes place 16 h after fertilization in the embryo [59]; (iii) the division of the zygote but not of the endosperm strictly requires the expression of the thymidylate kinase expressed at the G1/S transition [60]; and (iv) in the absence of fertilization, distinct mechanisms prevent unwanted onset of development of each female gamete. A Polycomb group complex controls this arrest in the central cell [61], whereas the arrest of the egg cell is dominated by another pathway involving the retinoblastoma-associated protein MULTICOPY SUPPRESSOR OF IRA1 [62]. We propose that the egg cell is arrested at the

G1/S transition, whereas the central cell arrests at the G2/M transition. This hypothesis can be addressed by additional studies of the cell cycle status in the female gametes based on patterns of phase-specific cell-cycle markers.

Coordination of the chromatin conformation and activity of the parental genomes

In sexually reproducing animals and plants, the chromatin of the male gamete undergoes an extensive compaction, in contrast with the chromatin in female gametes (Figure 2). After fusion of the two gametes, the male chromatin must regain a composition compatible with karyogamy. The decondensation of the paternal chromatin inside the fertilized egg notably involves the histone (H) variant H3.3 [63]. In eukaryotes, constitutive H3 is composed of two types of variants that differ by three or four amino acid residues [64]. The H3.1 variant is incorporated at the DNA replication fork, but H3.3 can be incorporated in the absence of DNA replication in the nucleosome [65].

In *Arabidopsis*, *HISTONE THREE RELATED 10* (*HTR10*) encodes a H3.3 variant expressed specifically in sperm cells [66]. The fate of the paternally provided H3.3 variant is different in the two fertilization products [22]. In the endosperm, HTR10 is passively diluted during the first syncytial divisions, whereas in the zygote, HTR10 is actively removed before the first mitosis. The mechanism responsible for paternal HTR10 removal in *Arabidopsis* remains to be established.

A H3.3 variant expressed in the egg cell might participate in the replacement of HTR10 from the zygote nucleus. Establishing which of the fifteen HISTONE3 variants [66] (http://www.chromdb.org) are expressed in the female gametes and which histone chaperone is involved will be essential to determine the degree of conservation of the H3.3 dynamics during fertilization in plants and in animals [63].

A recent study has suggested that chromatin conformation in the egg cell and the central cell might be different. The global structure of the chromatin was compared among nuclei from the endosperm, embryo and somatic tissue [67]. The endosperm chromatin is less condensed and is enriched in monomethylated H3 at the Lys9 residue in comparison with the chromatin of the embryonic cells. The unusual chromatin conformation of endosperm nuclei might result in a unique repertoire of genome expression in endosperm. Crosses between diploid pollen and tetraploid females causes an increased maternal genomic dosage paralleled by an increased relative amount of the relaxed fraction of endosperm chromatin. This result suggests that the central cell chromatin might have an unusual composition that plays a crucial role in the architecture of endosperm nuclei. According to this hypothesis, the two female gametes are likely to have distinct chromatin architectures.

The peculiar structure of the central cell chromatin could lead to a segregation of the two parental chromatins after fertilization. This hypothesis is supported by the segregation of the paternal centromeres during the first syncytial division of the endosperm [22]. In-depth immunolocalization of chromatin features in female gametes and after fertilization will be essential to address how rapidly the two parental genomes merge and how expression is initiated from each parental complement.

Concluding remarks and perspectives

For more than a century after the initial discovery of double fertilization, progress towards an understanding of the molecular and cellular mechanisms involved was limited mostly by the lack of a proper model species and imaging technologies. Recent advances in *Arabidopsis* have clarified the distinction between several steps involved in double fertilization and have identified a few genes involved in gamete release and fusion (Figure 1c). Study of the *sirène* class mutants impaired for pollen tube reception and sperm cell delivery now promises to unravel the signaling events initiating double fertilization. GCS1, a candidate for gamete interaction, has been isolated, and its conservation is likely to indicate its participation in gamete membrane fusion. Whether sperm cells are distinguished by specific determinants involved in recognition of the egg cell versus the central cell is still unclear. This will be important to establish because it is directly connected with the potential requirement for a polyspermy prevention mechanism. Mechanisms essential for other steps of double fertilization remain totally unknown. New imaging tools have shown that, after gametic fusion, male gamete nuclei actively migrate towards the immobile female nuclei of the central cell and the egg cell. What directs and enables this migration might be uncovered by the development of cytoskeletal markers and the isolation of mutants defective for this step. What controls karyogamy is unknown, and monitoring histones and other chromosome-associated proteins, such as condensins, might shed light on this process. It is also unclear whether transcription and translation need to be reinitiated after karyogamy is established. Some conflicting reports have debated whether the paternal genome remains transcriptionally silent for some time after fertilization [68–71]. Although we still do not understand how transcription and translation are controlled in the zygote and in the one-cell embryo, it is now clear that both parental genomes are active after fertilization in the early embryo [68]. However, parent-of-origin-specific expression characterizes a few genes expressed in endosperm. This specific transcriptional regulation, referred to as parental genomic imprinting, results from the asymmetrical removal of silencing marks from one of the parental alleles during gametogenesis [72].

Ultimately, molecular knowledge of double fertilization might question the nature of the fertilization of the central cell. The central cell derives from the somatic part of the female gametophyte in the ancestors of angiosperms and gymnosperms (*Ginkgo* and pine trees) [73]. It is possible to speculate that in contrast to the global initiation of a zygotic development in the fertilized egg cell, the sperm cell fusion only reinitiates gametophytic development from the central cell. According to this hypothesis, one might expect to find distinct mechanisms after gamete fusion between the two fertilization products. Hence molecular mechanisms might shed light on the evolution of double fertilization from the single fertilization event in ancestral seed plants.

Further knowledge of plant reproduction will be crucial to increase food supplies because seeds, which are the product of double fertilization, represent 60% to 70% of the human diet. In addition, understanding how epigenetic memory is supplied to the next generation through double fertilization will be the key to gaining knowledge on how plants adapt to the environment. This will be essential for adaptation of crops to the effects of global warming.

Acknowledgements

M.I. is funded by the Singapore Millenium Foundation. F.B. is supported by Temasek Life Sciences Laboratory and the Department of Biological Sciences at the National University of Singapore. Y.H. is funded by the Japan Society for the Promotion of Science. T.H. is supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (No. 18075004 and No. 19370017) and from PRESTO (Precursory Research for Embryonic Science and Technology) project of the Japan Science and Technology Agency.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2008.05.011.

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