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REVIEW

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In vitro fertilization as a tool for investigating sexual reproduction of angiosperms

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Abstract In vitro fertilization (IVF) of isolated male and female gametes of flowering plants was first accomplished in the last decade. Successful isolation of male and female gametes, and culturing of in vitro zygotes to form new plants, is a prelude to the use of IVF for research into the cellular and molecular control of fertilization in higher plants and its application as a tool in biotechnology. Genes unique to male and female gametes and zygotes of higher plants, although currently incompletely characterized, are expected to permit direct molecular dissection of fertilization. By applying IVF and microculture to zygotes and endosperm obtained by both in vivo and in vitro methods, newly activated fusion products may be observed and manipulated in media where they are directly accessible to the techniques of molecular cell biology. IVF and zygote culture may also offer potential for creating new hybrid plants by fusing isolated gametes from different species to produce unique zygotes and ultimately plants that would be impossible to obtain using typical crossing techniques. Transformation and regeneration frequencies using IVF may also be high enough to avoid the necessity of adding controversial antibiotic and herbicide resistant genes to screen transformed products. This review describes advances using IVF in plant sexual reproduction and discusses its potential in the genetic improvement of flowering plants.

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Introduction

Fertilization is a critical event involving the combination of male and female gamete cells and nuclei during sexual reproduction of angiosperms. This union occurs through the fusion of sperm and egg cells, contributing to genetic diversity and providing a basis for crop improvement during plant breeding. However, the protected location of the gametes, particularly the female egg and central cells, restricts our ability to explore the fertilization process of higher plants, and has thus led to a reasonably limited understanding of fertilization in these plants. The technique of in vitro fertilization (IVF), in which isolated sperm and egg cells are induced to fuse under controlled conditions, removes much of the interfering presence of somatic tissues, as well as interference of immediately surrounding maternal gametophytic cells. Thus, IVF provides opportunities to directly test hypotheses about gamete recognition and zygote activation. The use of IVF in higher plants is therefore an important contemporary research area in plant developmental and reproductive biology with potentially significant scientific applications.

The first isolations of living sperm cells were reported by Cass (1973), in which he described cellular characteristics of male gametes of barley (*Hordeum vulgare*) isolated by bursting anthesis pollen grains in a 20% sucrose solution. Following the first mass isolation of sperm cells in 1986, success in male gamete isolation was repeated with modification in numerous angiosperms (reviewed in Russell 1991). Figure 1 shows isolated sperm cells of two flowering plants representative of the size and morphology of male gametes. Isolation of living egg and central cells was first reported in tobacco by Hu et al. (1985) and in *Plumbago* by Huang and Russell (1989). An often-employed method of female gamete isolation is to use a dilute enzyme treatment to loosen

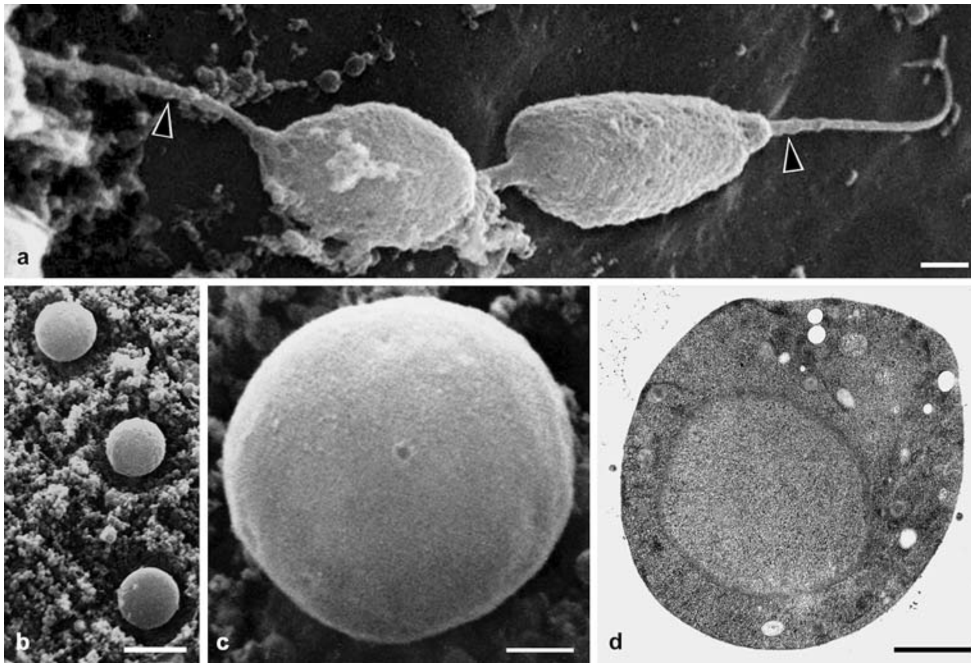

Fig. 1 Sperm cell isolation viewed using scanning (**a**–**c**) and transmission electron microscopy (**d**). **a** *Nicotiana tabacum* sperm cells initially possess cellular projections (*arrowheads*) following isolation. **b, c** Isolated sperm cells rapidly become spheroidal, as in these images of male gametes of *Plumbago zeylanica*. **d** TEM view of isolated rapidly frozen freeze substituted sperm cells of *P. zeylanica*. *Bars* in **a**, **b** = 5 μm; *bars* in **c**, **d** = 1 μm. Images **a**–**c** are courtesy of Zhaojie Zhang; image **d** is courtesy of Strout

surrounding cell walls, followed or accompanied by micromanipulation to separate remaining ovule cells from embryo sacs. Cells of the embryo sac—consisting of the egg cell, two synergids, central cell and three variably present antipodals—can then be isolated as free cells. Kranz et al. (1991) reported the first successful IVF experiments by using electrofusion to combine sperm and egg cells of maize, producing an in vitro zygote (Fig. 2). Such products were cultured to form multicellular structures. Within 2 years, this research group was able to use in vitro-produced zygotes to achieve regeneration of fertile plants by modifying this procedure (Kranz and Lörz 1993).

To date, IVF of angiosperms has had limited success. Tobacco would seem to be an obvious model for IVF given its potential to regenerate plants; however tobacco gametes did not fuse easily and did not produce embryos. Sperm and egg cells exposed to a polyethylene glycol (PEG) solution stimulated fusion, but fused products did not continue their development (Tian and Russell 1997a, b; Sun et al. 2000a, b). Instead, the IVF zygote arrested. The grasses continue to be the most successful group, but success has still been limited. Kovács et al. (1995) were able to fuse sperm and egg cells of wheat using the same IVF technique, but in vitro zygotes in this plant aborted, producing a multicellular structure.

In addition to isolation of high quality living gametes, cell cycle appears to be a critical factor in successfully combining gametes. Unlike most eukaryotes, in which fertilization occurs during G_1, fusion, many angiosperms display G_2 fusion and there are a myriad of patterns expressed (Friedman 1999). Pollen may be released prior to sperm cell formation (bicellular pollen species), but even in tricellular pollen, sperm cells may be in G_1, S or G_2 phase and thus not necessarily in synchrony with the female gametophyte. Unfortunately, the condition of the cell cycle during fertilization is described for only a handful of plants but these have displayed remarkable variability (Friedman 1999). According to these studies, grasses are admirably suited to IVF as the male gametes are generally released in G_1 phase and this is the phase of the cell cycle in which they fuse in nature (Mogensen and Holm 1995; Mogensen et al. 1995).

Tobacco in contrast has proven to be a particularly refractory model for IVF. Although sperm cells are formed in the pollen tube at G_1, they ultimately pass through S phase and fuse at G_2 phase in nature (Tian et al. 2005). The exact circumstances of fusion, however, are even more challenging. Sperm cells do not enter S phase until the very last stages of their passage in the pollen tube, and they complete S phase within the synergid, fusing immediately upon entry into G_2; thus the receptive sperm cells are essentially inaccessible (Tian et al. 2005). Egg cell progression through the cell cycle is synchronized with the sperm cells through a short distance communication signal, so obtaining fully receptive egg cells may be equally difficult. Matching the cell cycle of gametes with their normal fusion condition thus represents a potentially grave pitfall in meeting IVF congruity requirements (Friedman 1999).

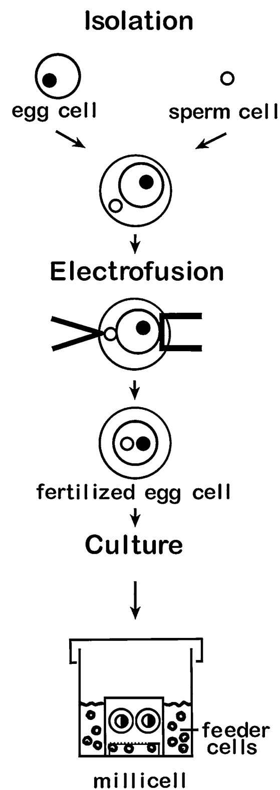

Fig. 2 Schematic diagram of electrically induced in vitro fertilization. Procedure is initiated by gamete isolation, followed by alignment of sperm and egg cell next to electrode, initially dikaryotic artificial zygote after in vitro fusion using electrofusion, and then microculture of artificial zygotes in a millicell with feeder cells. Reproduced with permission from Kranz et al. (1991)

Successes in obtaining products of IVF in grasses, however, are a hopeful sign that this technique is applicable to some of our most important crop plants and may provide unique insight into zygote activation. Since a recently published review by Okamoto and Kranz (2005) focuses on cellular differentiation in IVF products and specifically on cellular determination of higher plant zygotes, we provide little coverage on this topic in our current review. Nonetheless, regeneration of IVF fusion products into fertile plants may be particularly helpful for examining epigenetic effects and nucleo-cytoplasmic interactions inherent to gametes, as well as experimentally separating endosperm, embryo and ovular influences from the post-fusion development of IVF products. Transgenic gametes and fusion products are expected to be particularly valuable for experimental modification of plant development and functional dissection of critical molecular events.

Molecular studies using isolated gametic cells

Molecular biology of the generative cell and sperm

In the 1980s, electron microscopy combined with three-dimensional reconstruction indicated that the two sperm cells in pollen of *Plumbago zeylanica* exhibited structural dimorphism, differing in both morphology (Russell and Cass 1981) and internal cellular organelle complements (Russell 1984). Further observations revealed that the sperm cells from a single pollen tube of *P. zeylanica* exhibited preferential fertilization: one sperm containing mostly mitochondria fused with the central cell, and the other cell containing mostly plastids fused with the egg cell (Russell 1985). Sperm dimorphism and preferential fertilization in *P. zeylanica* suggested the presence of a recognition mechanism between the two gametes, where each had a preferred female target cell. Although preferential fertilization remains best described in *P. zeylanica* and a nuclear-based counterpart in populations of maize, a number of other species display differences in sperm organization that indicate dimorphism in other angiosperms also occurs (Mogensen 1992). These plants with dimorphic sperm cells may also possess preferential fertilization. To the extent that all angiosperms exhibit double fertilization, it also remains plausible that many if not all angiosperms exhibit some preferentiality of fertilization in vivo. Recently, Saito et al. (2002) observed organellar DNA distributions of sperm or generative cells in 115 species (104 genera, 56 families) and found distinctly polarized organellar DNA within generative cells or between sperm cells in six plants (six genera in four families). Since each pollen tube ultimately produces two sperm cells and these cells have two targets, it seems conceivable that there is targeting in a number. Competition for the ability to fuse with the egg cell may have important genetic consequences. Occurrence of dimorphic sperm cells also suggests that, in addition to being dimorphic, the two sperm cells may also differ at the molecular level, and that these differences are likely reflected on the cell surface for successful targeting to occur.

We know from a number of works using isolated male gametes that specific proteins are present on the cell surface. Southworth and Kwiatkowski (1996) tested sperm cells of *Brassica* and *Lilium* using antibodies to arabinogalactan proteins and found two monoclonal antibodies, JIM8 and JIM13, bound to the sperm cell surface. They proposed that antibodies to arabinogalactan proteins may prove useful for discriminating male gametes from other cells. Xu and Tsao (1997) isolated proteins from the plasma membrane of maize sperm cells and found some proteins were different from those of the somatic cell when peroxidase-conjugated Con A was used to label the proteins. Using FITC-conjugated lectins as probes, Sun et al. (2002) localized sugar moieties of glycoconjugates on the cell surface of isolated maize sperm cells, egg cells, central cells, antipodal cells, synergids and in vitro and in vivo fertilized zygotes of maize. The fluorescence signal of FITC-Con A was stronger on egg and central cells but weaker on sperm cells. In contrast, the signal of FITC-WGA was present on egg cells but not on sperm cells; however, the signal for FITC-PHA-E was found on neither egg nor sperm cells (Sun et al. 2002). These

findings reflect surface-based distinctions in glycosylation sites that remain poorly characterized despite their potential importance in cell binding and recognition; this topic remains an attractive target for molecular characterization.

Sperm cells are expected to fulfill the functions of recognition, adherence and fusion with the female gamete during fertilization. Each of these functions of the male gamete may be controlled by activation of a unique gene or network of genes. The isolation of such fertilization-related genes in sperm cells seems increasingly likely as more male germ cells are isolated and characterized using molecular biology. Xu et al. (1999a) isolated and characterized two cDNA clones of the lily precursor generative cell that encode generative cell-specific histone genes, *gcH2A* and *gcH3*. This paralleled the prior discovery of specific generative cell-expressed histone proteins (Ueda and Tanaka 1995). In situ hybridization analysis revealed that transcripts of substitution histone genes *gcH2A* and *gcH3* increased in abundance during gametic cell maturation, which was the first report of male germ lineage specific expression in a higher plant. Xu et al. (1999b) also isolated a male gametic cell-specific gene, *LGC1*, and confirmed its expression, demonstrating that the gene product was localized on the surface of male gametic cells. The *LGC1* protein is anticipated to be related to cellular interactions between the male and female gametes, presumably related to recognition or signaling. Ueda et al. (2000) have since isolated genes encoding three histone proteins from *Lilium longiflorum*. The highly heterochromatic appearance of lily male gametic nuclei may link directly to the abundance of these histones, which are suspected to control condensation of chromatin. Heterochromatin is recognized as one of the most conspicuous forms of epigenetic control and is implicated in modeling nuclear organization that affects expression of multiple genes and gene networks.

In the Mariani lab, Xu et al. (2002) constructed a cDNA library of mRNA transcripts isolated from tobacco male germ cells derived from in vitro culture, distinguishing two cDNAs believed to be uniquely expressed in sperm cells. These products, NtS1 and NtS2, apparently represent sperm cell-expressed transcripts that were isolated from an initial screen of 396 clones. The products of these two clones accumulated in both the generative and sperm cells. *NtS1* codes for a polygalacturonase, suggesting a role for this enzyme in wall modification during differentiation of sperm cells in tobacco.

Singh et al. (2002) isolated polyubiquitin-encoding cDNA clones in generative cells of *L. longiflorum* and in sperm cells of *P. zeylanica* that are differentially expressed. They suggested that ubiquitin differentially alters patterns of protein degradation in the germline and is likely involved in determining lifespan of some proteins during male reproductive maturation. In *Plumbago*, the gene-encoding polyubiquitin is up-regulated in one of the two sperm cell types. The sperm cell that is associated with the vegetative nucleus (that which preferentially fuses with the central cell and forms endosperm) displays an 8–16 fold increase in expression. Such differential expression presumably contributes to molecular differences between the two male gametes during development, potentially related to networks of sperm-specific or sperm-enhanced genes. It is suspected that among these genes may be some that are related to male and female gamete recognition during the fertilization process. Early work in this area arose from expressed sequence tag (EST) analysis, which reflects transcript production. Microarray and suppression subtractive hybridization (SSH) may further contribute to understanding gamete gene expression as it approaches the transcriptome and proteome level of analysis.

Since sperm cell dimorphism and preferential fertilization were first reported in *P. zeylanica* (Russell 1985), the phenomena of sperm dimorphism have been reported independently in numerous plants (Mogensen 1992; Hu and Tian 2002). Some dimorphic sperm cells differ in cell size, whereas others differ in organelle content. Relatively few plants have been examined that, when examined in detail, reveal no statistical difference between the two sperm cells. One particularly well-studied example is tobacco, in which many developmental stages from the time of cell division (Yu and Russell 1993) to sperm cell maturation (Yu and Russell 1994) have been examined, up the time of pollen tube entry into the ovary. Examination of later progamic stages (near the time of fertilization), however, indicate that size differences in tobacco become more evident during development (Tian et al. 1998, 2001). Dimorphism between the two sperm cells may play a role in gametic recognition during double fertilization. Interestingly, there is also evidence that the sperm cells differ even more extremely in size within the synergid, just prior to transmission. Although this observation was the result of only one serially reconstructed cell pair (Yu et al. 1994), it is tempting to speculate that this may relate to distinctions in gene expression, which may also reflect molecular differences in the cells. With this goal, the Tian lab has recently isolated populations of over a thousand sperm cells each of the two dimorphic sperm cell types, S_{vn} (vegetative nucleus-associated sperm cells) and S_{ua} (sperm cells unassociated with the vegetative nucleus) from pollen tubes of tobacco using micromanipulator-based collections (Fig. 3), as fluorescence-activated cell sorting is not well adapted to the small number of gametes that can be isolated in most flowering plants (Yang et al. 2005). Interestingly, isolated sperm cells of tobacco display significant differences in electrophoretic mobility that may relate to potential differential expression (Yang et al. 2005). Once dimorphic sperm cells are collected into populations of sufficient quantity (e.g., a thousand or more sperm cells of each type), differences between the two sperm cells may be identified using sensitive molecular methods. The two purified populations of sperm cells can be used to ad-

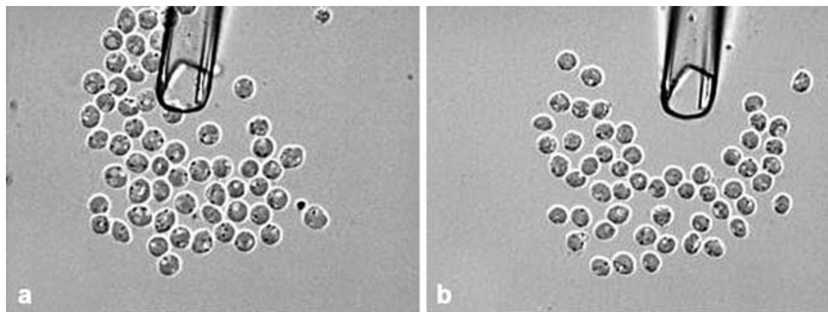

Fig. 3 Isolation of dimorphic sperm cells of *Nicotiana tabacum*. **a** Population of larger sperm cells, representing the S_{ua}. **b** Population of smaller sperm cells representing the S_{vn}. Reproduced with permission from Yang et al. (2005)

vance research on sperm cell biology of higher plants from the cell to the molecular level by using such sensitive techniques as immunoblotting, elicitation of monoclonal antibodies, transcript analysis and work on expressed proteins. The utility of these techniques will enhance our understanding of sperm cell biology in higher plants, allowing greater appreciation of the unique properties of angiosperm male gametes and contributing to our knowledge of double fertilization. Ironically, the sperm cells occupy a diminishing cellular volume during development (Russell and Strout 2005), so their molecular components may represent a diminishing but important resource during maturation. Sperm cells, containing a complex and largely unique network of genes during development that are distinct compared to those of other somatic and reproductive cell types (Engel et al. 2003) remain somewhat of an enigma in light of their outwardly simple appearance and behavioral repertoire.

Molecular biology of the egg cell and zygote

Egg cell isolation has been considerably more difficult, yielding several orders of magnitude fewer cells compared to sperm cells. Major factors limiting isolation of female gametophytes include that they occur singly within ovules, the egg and central cells are deeply embedded, and female gametophyte cells are integral to the ovules, displaying extensive common interfaces between central cell, synergids and egg cells. Despite these difficulties, female gametophytic cells have been isolated through enzyme maceration, micromanipulation and a combination of these techniques. Their larger size compared with male gametes allows greater yield per cell (Fig. 4). With the successful isolation of female gametic cells, a comparison of expressed genes in egg and somatic cells through cDNA library characterization is possible, which may be useful in examining expressional differences of specific egg cell and zygote genes. The first molecular biology report was that of Dresselhaus et al. (1994), who constructed a cDNA library from 128 isolated maize egg cells using a reverse transcriptase/polymerase chain reaction technique. This first egg cDNA library was then compared with similarly constructed somatic cDNA libraries. This early screen revealed some up-regulated cDNA library members in maize egg cells and some differentially expressed genes.

To uncover expressional differences relating to zygote activation, Dresselhaus et al. (1996) constructed another library from 104 maize IVF-generated zygotes at 18 h after IVF to distinguish and isolate newly expressed and highly up-regulated genes in zygotes. Among the products, they isolated a full-sized cDNA clone encoding calreticulin from a differential screening of egg cell and zygote cDNA libraries. The expression of this isolated gene is enhanced after fertilization and strongly correlated with cell division, constituting the first report of an isolated zygote gene. From further differential screening of cDNA libraries of unfertilized egg cells and in vitro fertilized zygotes of maize, 50 additional transcripts were sequenced and reported (Dresselhaus et al. 1999a). From these products, a cDNA library member encoding the eukaryotic translation initiation factor eIF-5A was isolated—a protein thought to be necessary for selective mRNA stabilization and translation (Dresselhaus et al. 1999b). This protein is stored in the egg cell in large amounts, but is relatively quickly inactivated. After fertilization, selective mRNA translation was quickly triggered by specific developmental changes of the zygote. In addition, among the isolated cDNAs they found, seven encoded proteins related to translation and two related to DNA replication, both of which are strongly induced upon fertilization. Four other novel genes (S21A, S21B, L39, P0) involved in ribosome biosynthesis and translation were also identified. The expression of all four genes was correlated with cell division activity and was strongly induced during the G_1 phase of the zygotic cell cycle (Dresselhaus et al. 1999a). Sauter et al. (1998) examined the expression of cell cycle regulatory genes to explore the mechanisms controlling zygotic cell division of maize. After analyzing transcripts of *cdc2* (*cdc2*ZmA/B) and cyclin (*Zeama;CycB1;2, Zeama;CycA1;1, Zeama;CycB2;1*) genes of sperm cells, egg cells, synergids, the central cell and antipodal cells, they found that the *cdc2* gene is expressed constitutively in all cells prior to fertilization and throughout zygote development, whereas the cyclin genes showed cell-specific expression in the cells of the embryo sac and differential expression during zygote development.

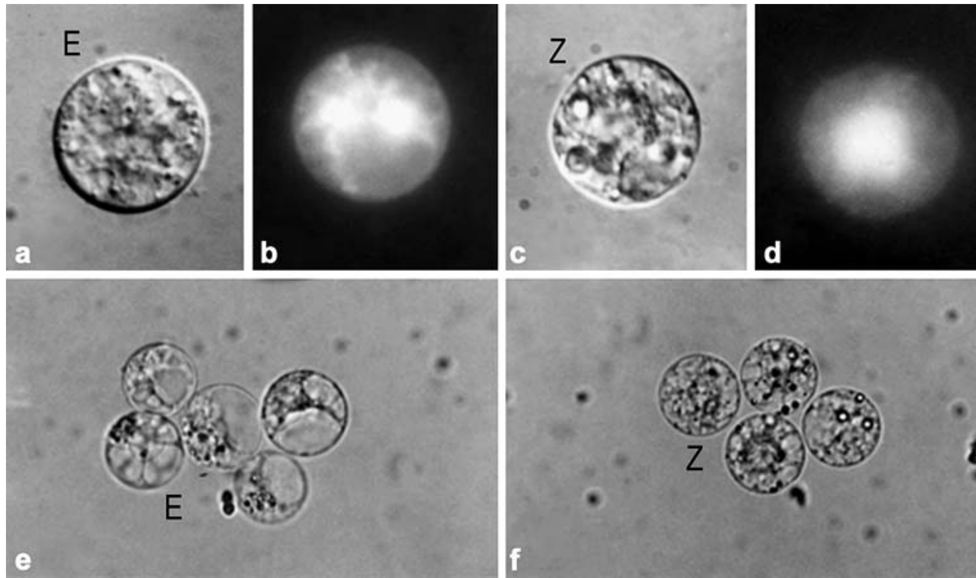

Fig. 4 Isolated egg cells and zygotes of *Torenia fournieri*. **a** Egg cell isolated from an embryo sac 2 days after anthesis. **b** Viability of isolated egg cell evaluated by fluorescein diacetate (FDA) fluorescence. **c** Isolated zygote from an ovule 14 h after pollination. **d** Viability of isolated zygote evaluated by FDA fluorescence. **e** Collection of five egg cells. **f** Collection of zygotes

Recently, Okamoto et al. (2004) identified major protein components expressed in maize egg cells, which included three cytosolic enzymes, two mitochondrial proteins and annexin p35. They identified some genes that are up- or down-regulated in the apical or basal cell of maize two-celled embryos. Genes were categorized into six groups: (1) up-regulated only in the apical cell, (2) up-regulated only in the basal cell, (3) up-regulated in both the apical and basal cells after gamete fusion, (4) down-regulated only in the apical cell, (5) down-regulated only in basal cell and (6) constitutively expressed in the egg cell and embryos. The genes up-regulated in the apical or basal cell were already expressed in the early zygote (Okamoto et al. 2005).

The use of SSH to produce essentially pre-screened gamete libraries has also been exploited to examine ESTs that are uniquely expressed in specific cell types. In a continuation of their early work on maize egg cell ESTs, Le et al. (2005) produced SSH-EST libraries of both isolated egg cells and isolated central cells to further discriminate the unique transcriptional products that may contribute to embryo versus endosperm differentiation. The egg cell displayed increased abundance of transcripts involving cell communication, cell growth/division, DNA synthesis, signal transduction and stress related factors, whereas the central cell displayed increased abundance of transcripts involving metabolism and energy, protein synthesis and protein modification, with many products in the "no hit" category. In each case, these products seemed to reflect functional categories represented in the respective developmental future of the embryo and endosperm (Le et al. 2005).

The above-mentioned results from molecular studies of male and female germ cells are all based on the use of isolated cells. By using isolated sperm, egg cells and zygotes, interference of genes from somatic tissue is avoided, making it possible to locate the genes specifically controlling or contributing to the development of these reproductive cells. Recent research has provided details about expression of female gametophyte-specific elements involved in pollen tube guidance (Marton et al. 2005) and anther maturation (Dresselhaus et al. 2005); both findings are quite promising in extending our knowledge of sperm cell attraction. The *ZmEA1* gene attracts maize pollen tubes to the micropyle and if it is down-regulated, short distance signaling by the egg apparatus to pollen tubes fails and fertilization is unsuccessful. This gene has orthologs in other monocots, including rice, but not in dicots, indicating that such signaling molecules likely diverged after their evolutionary split. The successful isolation of male and female germ cells of higher plants has stimulated considerable research on the molecular biology of sperm and egg cell development and this approach has become a focal point in the study of sexual reproduction of higher plants, with increasingly important papers being published every year.

Exploring egg cell activation

The isolation of sperm and egg cells is considerably easier to achieve in animals and lower plants, so it is not surprising that more is known about the mechanism of egg cell activation in these organisms. Prior to sperm and egg cell isolation in angiosperms, egg cell activation was examined principally through ultrastructural observations; however, physiological understanding of the processes involved was quite limited. Now, using in vitro techniques, direct investigations of living cells

during syngamy and early stages of zygote development are possible using a wealth of microscopic techniques and biochemical manipulations. Egg cell activation of higher plants may also be examined using specialized probes.

The unfertilized mature egg cell is known to be a relatively quiescent cell as is evidenced by ultrastructural observations from many different species, and it requires fusion with the male gamete for reactivation. Since the zygote is the first cell in the ontogeny of a plant, understanding egg and zygote activation is a very interesting research topic. The process of egg reactivation involves the fusion of a sperm cell and an egg cell to form a zygote in vivo. Sperm and egg cells do not fuse spontaneously in nonfusigenic media in vitro; however, an observation that has been reported for a number of plant species (Kranz and Lörz 1993; Tian and Russell 1997b; Cao and Russell 1997). These observations suggest that there are special requirements to ensure male and female gamete fusion in vivo. Kranz and Lörz (1994) fused maize egg and sperm cell in a solution containing 50 mmol/L $CaCl_2$ at pH 11.0, which is a strongly fusigenic solution, and the fusion products of the egg and sperm cell divided and developed into microcalli consisting of approximately 30–50 cells. They observed the early events of cell wall completion, first nuclear division and the unequal division of the zygote following in vitro gametic fusions using both chemical- and electroporation-based methods. They found that newly formed cell wall material began to accumulate within 30 s, and most of the organelles became densely grouped around the nucleus of the zygote within 15 h after gamete fusion. Organelles and the nucleus of the in vitro zygote also displayed a polarized distribution before zygote division (Kranz et al. 1995).

Faure et al. (1994) induced sperm and egg cell fusion of maize using a solution containing 5 mmol/L $CaCl_2$, pH 6–6.5, in which 79.7% of the sperm and egg cells fused after adhering for 4 min. In this medium, the fusion process was completed in less than 10 s. Although this method of fusing sperm and egg cells used a relatively low concentration of calcium solution, there is still the possibility of calcium stimulation, since fusion of isolated gametes in other species, such as rice, also display fusion at these and higher calcium concentrations (Khalequzzaman and Haq 2005; Uchiumi et al. 2006). Using the in vitro procedure described above, Digonnet et al. (1997) used the fluorescent acetoxymethyl ester form of fluo-3 to investigate free calcium change during egg cell activation. They observed a small amount of fluorescence of free calcium in the egg cell prior to fertilization—about the same amount as when the sperm cell adhered to it, and no fluorescence in the sperm cell. When the sperm cell and egg cell began to fuse, however, the adhering sperm cell first displayed some cytosolic Ca^{2+}. The fusion of both cells triggered a transient cytosolic Ca^{2+} increase in the fertilized egg cell, with the increase in fluorescence lasting about 2 min and then subsiding. After 29 min, the calcium level as measured by fluorescence returned to the same level as that observed in the unfertilized egg cell (Fig. 5). This research was the first direct evidence of a transient elevation of free cytosolic Ca^{2+} within in vitro zygotes of flowering plants, opening the way toward identification of early signals induced by fertilization, the role of cytosolic Ca^{2+} during egg activation, and early zygote development in flowering plants.

Antoine et al. (2000) used an extracellular Ca^{2+}-selective vibrating probe to measure influx of extracellular Ca^{2+} induced by gamete fusion during maize IVF and found small and stable Ca^{2+} fluxes in egg cells before gamete fusion. Adhesion of a male gamete to the egg cell did not induce flux changes. After male and female gametes fused, however, a long-lasting Ca^{2+} influx took place at the site of fusion with a peak average of 14.9 ± 1.83 pmol cm^{-2} s^{-1} and an average duration of 24.4 ± 3.13 min. The Ca^{2+} influx spread from the position of fusion throughout the egg cell plasma membrane as a wavefront with an average rate of 1.13 μm s^{-1}. They also found that the calcium influx is a necessary condition for cell wall deposition and is mediated by gadolinium-sensitive channels. This result also indicates that similar Ca^{2+} signaling events occur in higher plants, as reported in animals and lower plants, and that the sperm triggers a transient rise of intracellular free Ca^{2+} levels in the fertilized egg. An effect seen

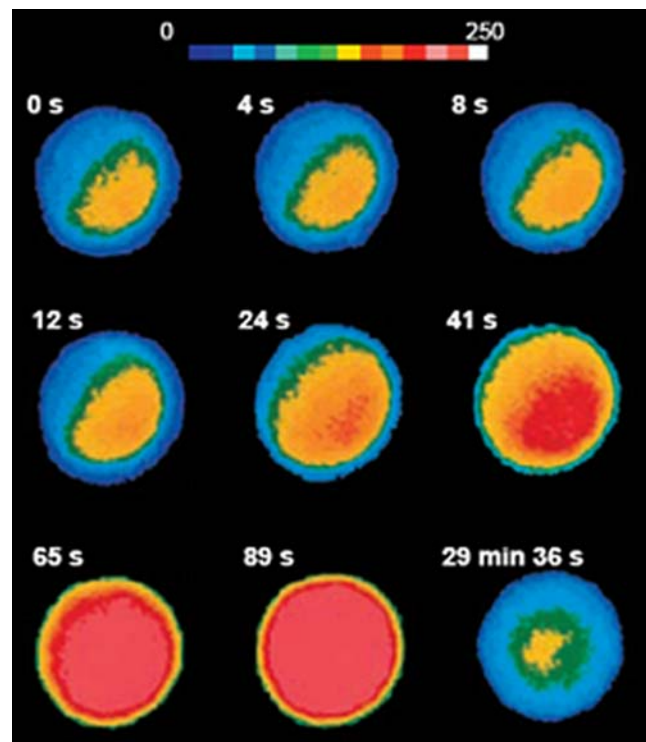

Fig. 5 Evidence for intracellular Ca^{2+} elevation triggered by IVF of *Zea mays* egg cell. Time sequence of pseudo-color images illustrating temporal and spatial distribution of the Ca^{2+} transient triggered by sperm–egg fusion. Reproduced from Digonnet et al. (1997) with permission of Dumas

in plants but not in other systems is the requirement of cell wall completion for further development, under a requirement of calcium oscillations, as well. Recently, Pónya et al. (2004) fused isolated sperm and egg cells of wheat and found that within 3 min of in vitro sperm–egg fusion, a transient fragmentation of polar accumulations of endoplasmic reticulum occurred. They hypothesized that this ER fragmentation serves as a calcium reservoir that contributes to increased capacity of fertilized eggs to release calcium by increasing the curvature of the membranes, from which calcium transport is facilitated by calcium transport proteins.

Han et al. (2002) examined Ca^{2+} changes in the central cell of *Torenia fournieri* using another method. They microinjected a soluble sperm extract, along with calcium green-1 conjugated 10 kDa-dextran (CG-1) into the mature central cell, which induced a significant rise in cytosolic free calcium concentration ($[Ca^{2+}]_i$). The rise reached a maximum 20 min after injection and then steadily declined. A relatively high level of $[Ca^{2+}]_i$ was maintained even 40 min after injection. They also injected caged inositol 1,4,5-triphosphate ($InsP_3$) into *Torenia* central cells to compare the pattern of Ca^{2+} rise induced by the sperm extract. The Ca^{2+} elevation triggered by the release of $InsP_3$ was much faster than that induced by sperm extract, but the increase in $[Ca^{2+}]_i$ reached a maximum at 70–80 s and then declined to resting levels within 300 s. The authors hypothesized that sperm extract might contain factors triggering the release of Ca^{2+} into central cell. There are some differences in the results reported by Han et al. (2002), Digonnet et al. (1997) and Antoine et al. (2000). First, the cells used in their assays are different—Han et al. (2002) used the central cell and Digonnet et al. (1997) and Antoine et al. (2000) used egg cells; the former study also used a different trigger, sperm extract or IP_3, rather than sperm cells as in the latter studies. Thus, it is necessary to determine how reactions may differ between these two cells. Second, the origin of increased calcium is different—in the Antoine et al. (2000) study the calcium influx came from external calcium in the medium, but in the Han et al. (2002) study, the Ca^{2+} rise is dependent upon internal Ca^{2+} released from internal calcium stores. Third, Digonnet et al. (1997) and Antoine et al. (2000) demonstrated that sperm-induced transient Ca^{2+} elevation generated signals on the surface of the female gamete, but injected soluble sperm extract did not induce signals on the plasma membrane of the cell. These differences are interesting and worthy of further investigation. Based on their observations, Antoine et al. (2000) proposed a process of initial fertilization where (1) membrane fusion of both gametes starts a calcium influx close to the fusion site; (2) a wave of contraction, presumably of actomyosin nature, is triggered; and (3) the calcium triggered opening of more stretch-activated calcium channels. The rise of calcium contributes to a positive calcium feedback loop of oscillation that is related to zygote activation.

Recently, Hoshino et al. (2004) examined fertilization-induced changes in the microtubular architecture of the maize egg and zygote and described that relatively few cortical microtubules occur in the unfertilized, mature egg cell. However, in the zygote, cortical microtubules clearly increased, and microtubules remained visible up to 7 h after IVF. Following this phase, strands of microtubules radiating from the nucleus into the cell periphery were formed and persisted throughout the remainder of zygote development. Based on the abovementioned results, an understanding of egg cell activation is emerging. We believe that continuing research on egg cell activation in higher plants using IVF and modern techniques could provide crucial insights into understanding the unique biology of the flowering plant zygote.

Patterns of paternal contribution to transcription between eggs, sperm cells and zygotes have been further explored by Ning et al. (2006), providing evidence of (1) clusters of genes present in egg cells persistent in zygotes, (2) clusters of genes represented in sperm cells and persistent in zygotes, as well as (3) a large cluster of genes produced by apparently new transcripts within the zygote. Their report includes one histone gene present in both the egg cell and zygote, which may represent one potential mechanism by which gene networks are controlled. Activation of other histone modeling genes in the male gamete has also been reported that could potentially be involved in male germ line gene expression, as well (Okada et al. 2005a). Cell-specific promoters acting in sperm cells represent further evidence of gametic control of expressional networks (Okada et al. 2005b). Taken together, these reports contribute to an emerging recognition that cellular identity and expression during early embryogenesis in angiosperms may be controlled through a number of multigenic control mechanisms.

Creating distant hybrids using IVF

Another advantage of using IVF is the ability to fuse sperm and egg cells of phylogenetically separated plants using their respective gametes to produce hybrid zygotes in vitro. Since gametes are naturally recombinant cells, it is presumed that barriers to wide hybridization may be reduced at the zygotic level. Such zygotic hybridization through IVF would avoid the effects or influence of somatic tissues of the stigma, style and ovule and may also avoid ploidy problems occurring during somatic fusion. To date, the technique of somatic hybridization can successfully fuse living cells of any plant species, but hybrid cells are difficult to culture, usually polyploid and often unstable in generation of desirable regenerated hybrids. During somatic hybridization, the volume of sporophytic parent cells differs little and thus mixing of the two cells may result in an equal contribution of cytoplasmic components, including mitochondria and plastids, which have their own semi-autonomous

heredity. After the two somatic cells fuse, the cytoplasm of the hybrid cell is often physiologically incompatible; this may be particularly conspicuous immediately after fusion, with consequences that may persist for a variable amount of time thereafter. One phenomenon often observed in hybrid cells of distant species is that the chromosomes of one parent are eliminated. There are many other observed problems associated with distant somatic cell hybridization such as absence of cell division in hybrid cells, lack of differentiation in cells of the hybrid callus, genetic instability, refractory ability to regenerate plants and a high level of sterility in regenerated hybrid plants. These problems have interfered with success in somatic hybridization of higher plants, resulting in only limited success, such as production of potato–tomato hybrids (Gleba and Sytnik 1984), but no wider crosses. Given the complexity of nuclear-cytoplasmic, inter-cytoplasmic and ploidy level conflict in the offspring, it is not surprising that somatic hybridization has not had widespread success in plant genetic engineering.

Cultured in vitro zygotes overcome a number of the intrinsic limitations of somatic cell hybridization and provide a number of advantages for wide hybridization. First, sperm cells contain little cytoplasm, with most of the cytoplasm in the zygote contributed by the egg cell. The early development of the zygote may be regulated by the resources stored in the egg cytoplasm (Dresselhaus et al. 1999a), and there is reduced conflict in organellar DNA. Secondly, some genes of the sperm cells are activated only after the zygote undergoes several divisions to form a proembryo of 32–46 cells, suggesting that genes from the egg cell regulate early development of the zygote (Vielle-Calzada et al. 2000). Recently, Okamoto et al. (2005) reported that the genes up-regulated in the apical or basal cell of two-celled embryos of maize were expressed in the early zygote, proving zygotically predetermined expression during early embryogenesis. Therefore, although the zygote is a product of the fusion of male and female gamete, the early development of the zygote may be controlled by the predominate volume of the maternal cytoplasm, which reduces cytoplasmic conflict and thus promotes successful cell signaling, progression through the cell cycle, mitotic division and cytokinesis.

Kranz et al. (1995) induced maize egg cell fusion with sperm cells of such genetically remote species as *Coix*, *Sorghum*, *Hordeum* and *Triticum*. The hybrid zygotes divided at a ratio of 78 (21/27), 50 (17/34), 43 (13/30) and 24% (9/38), respectively, to form microcalli. The high frequency of hybrid zygotes dividing to form microcalli makes the fusion of egg cells with sperm cells of different species or genera a potentially viable method to obtain distant hybridization in angiosperms. Successful division of the in vitro zygotes resulting from distant hybridization appears to be related to the disproportionately greater abundance of egg cell cytoplasm. The typical volume discrepancies between the sperm and egg cells can be illustrated by comparing a sperm cell of a diameter of approximately 3 μm to a diameter of the egg cell of 30 μm. Although their relative difference in diameter is 1:10, the volume difference is 1:1,000, meaning that cytoplasmic components of the male gamete are exceeded by three orders of magnitude over the egg cell. In nature, however, the relative diameter ratios of many male:female gamete pairings in angiosperms, however, may exceed a 1:10 ratio, and be 1:20 or more, resulting in a dramatic dilution of any paternal or dual parent effect during early zygote development.

Culturing in vitro hybrid zygotes, however, does not always overcome the problem of the remote genetic relationships. Fusion products of maize egg cells with the sperm cells of *Brassica* did not result in zygotes capable of division. Although cultured maize eggs remain viable for about 3 weeks after isolation, cell death occurred 18–42 h after fusion with *Brassica* sperm cells, demonstrating the possible influence of the distant relationship (Kranz et al. 1995). Another equally plausible explanation is simply that the gametes are not in the same phase of the cell cycle. Whereas grass gametes tend to be in G_1 phase at anthesis and gametic fusion, *Arabidopsis* is in S phase at anthesis and G_2 phase at fusion (Friedman 1999). There is no known mechanism by which such cell cycle conflicts can be easily resolved in a hybrid cytoplasmic background. Thus, both underlying physiological and cell cycle-based differences may need to be carefully considered when selecting suitable parents, so that difficulties encountered in subsequent culture of the hybrid zygote are minimized.

Improving plant transformation using the zygote as a recipient cell

Transgenic tobacco plants were among the first obtained transgenic plants in the early 1980s. Regeneration of other such plants, however, has become increasingly common and is a vigorous research area in modern molecular biology. To date, the most prevalent techniques for introducing foreign genes into higher plants include electrofusion, electroporation, PEG-mediated fusion and microinjection, with incorporation of transgenes mediated by *Agrobacterium*. Techniques for transformation and regeneration present different advantages and disadvantages, with myriad protocols proving to be optimal for different species and specific applications. This combinatorial resource of techniques for introducing and incorporating genes assures a wealth of potential techniques to ensure success in transgenic research. A major limitation to introducing foreign genes into cells of higher plants is therefore one of time to achieve optimal incorporation and rapid regeneration.

After introduction of foreign genes into the target cell, DNA must be incorporated meaningfully into a chromosome under the direction of appropriate con-

trolling elements, and in order to be transmitted, the cell must divide, thus greatly decreasing transformation frequency and it should be stable. The recipient cells, regardless of whether they are protoplasts, young embryos or calli, would also need to retain the ability to differentiate, form organs and photosynthesize in order to regenerate into differentiated plantlets. During the process of cell growth and differentiation in culture, the genes of recipient cultured cells may undergo additional genetic or epigenetic changes during callus differentiation that inhibit regeneration or result in sterile plants.

Zygotes exhibit very strong vigor in vivo and have a proven ability for cell division and differentiation; they serve as the primary founder cell during ''typical'' embryogenesis to form plantlets; and they execute a pre-established template of pattern formation during development. Isolation of sperm and egg cells, in vitro zygote formation through IVF, and cellular differentiation following IVF have been successful in maize and wheat. In vivo zygotes of maize, wheat, and barley have also been successfully isolated and regenerated. These in vitro and in vivo zygotes readily divide and undergo modified embryogenesis to produce fertile plants during culture (Kranz and Lörz 1993; Holm et al. 1994; Kumlehn et al. 1998, 1999). Thus, successful isolation and culture of zygotes provides a strong impetus for using zygotes as recipient cells for forming transgenic higher plants.

Microinjecting foreign genes into zygotes, as recipient cells, is attractive because zygotes lend themselves well to this technique. Leduc et al. (1996) experimentally microinjected two types of reporter genes (the GUS gene and anthocyanin regulatory genes) into 227 maize zygotes isolated 24 h after pollination. About 3.5% (8/227) of the microinjected zygotes showed transient expression of both genes. Pónya et al. (1999) exposed egg and zygote protoplasts of wheat to a high-frequency alternating-current field to immobilize cells and microinjected exogenous DNA. Two constructs were used: (1) a GUS reporter gene under the control of 35S promoter and (2) a GFP reporter gene under the control of ubiquitin promoter. A significantly higher transient expression rate of both injected genes was displayed in their protoplasts (46 and 52% for 98 egg cells and 77 zygotes, respectively). Holm et al. (2000) used a rice actin promoter construct, Act1-gusA-nos, as a gene construct for microinjection into wheat zygotes. Among the hundreds of injected zygotes, 34% continued development into embryo-like structures and eventually developed into plants. PCR screening showed that 21% of the derived structures contained exogenous DNA; however, only two green plants were transgenic. It was proposed that transformation of barley by microinjection of DNA into isolated zygotes was feasible but that gene expression was rare, possibly due to degradation of the introduced DNA (Holm et al. 2000). Scholten and Kranz (2001) attempted transformation of gametes and zygotes using microprojectile bombardment of immature embryos to overcome the problems and time-consuming nature of microinjection experiments. Transgenic techniques using in vivo or in vitro zygote cultures convincingly demonstrate the potential of female gametic cells to greatly enhance transgenic efficiency in plant improvement and experimentally dissect the role of genes that control fertilization and early zygote development.

Current procedures for obtaining transgenic plants use antibiotic or herbicide resistance genes as part of their construct in order to select transformed cells, calli and plants; however, concerns about the safety of these marker genes in crop transformation have caused significant concern for scientists and society. Using zygotes as receptor cells may be attractive for crop plants because gene incorporation may be sufficiently effective that lethal screens using antibiotic and herbicide resistance genes are not needed. When screening tens or hundreds of plants, rather than up to 100,000 plants, the investment of using single gametic cells may be rewarded by eliminating a need for additional reporters (Zhang et al. 1999).

Conclusions and prospects

Clearly, difficulties in isolating gametes of higher plants have impeded our understanding of gamete physiology, activation of development and early embryogenesis in flowering plants. Increasing number of tools, however, are now available to manipulate male and female gametes of higher plants, providing numerous opportunities for scientific and biotechnological progress (Fig. 6). Isolated gametes can be analyzed directly during IVF with modern cellular and physiological probes, while means of regulating sexual reproductive development

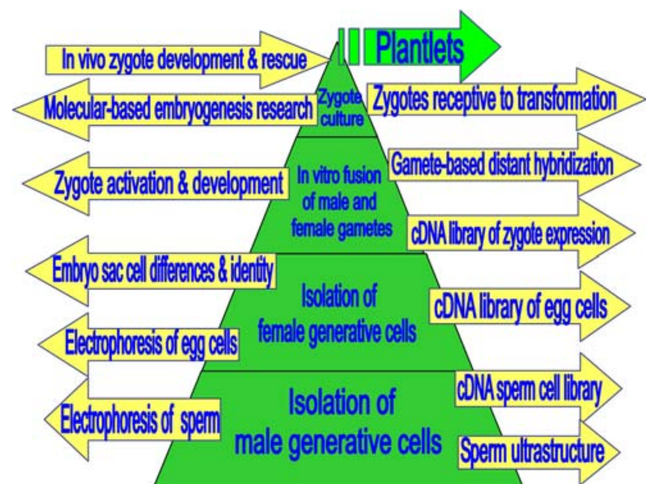

Fig. 6 A schematic summary of techniques that have been applied to deepen our understanding of the events of double fertilization from early microscopic study, to isolation and physiology, to cellular and molecular biology. Each of these represents accomplishments that would have been difficult or impossible to study within the context of in vivo systems

are being refined. Research on reproductive biology of angiosperms has therefore entered a new phase where methods of molecular biology will permit many questions to be answered about fundamental mechanisms of fertilization and early developmental activation. Molecular characterization of male and female gametes and zygotes is expected to become a new focus of plant molecular understanding and bioengineering, which will combine cell hybridization techniques with transformation and regeneration of transgenic plants. Interestingly, parallel techniques are also being applied to gymnosperms (Fernando et al. 2005) that are uncovering the potential advantages of using gametic cells with IVF as fertilization models, and as founder populations for transgenic plants.

Presently, the most significant obstacle to using IVF has been the isolation of male and female gametes, especially the isolation of egg cells, for manipulation of living gametic cells. Although initial successes have been limited to only two species, both are critical crop plants. Findings from these plants may also provide a model for obtaining insights on fertilization and activation of development that apply to numerous other angiosperms, including other molecular and crop models. Developing successful experimental systems for IVF in other plants will be needed to confirm results obtained from maize and wheat using IVF. Both of our current best developed models are grasses, which are highly specialized angiosperms. Perfecting IVF in other plants, particularly dicotyledonous models and crops, may provide critical data allowing better understanding of critical molecular events during fertilization and improving methods for the production of fertile sporophyte plants and new, stable genetic combinations of high efficiency.

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