Zygote

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Advances in the study of egg activation of higher plants

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Summary

Fertilization in higher plants induces many structural and physiological changes in the fertilized egg, and represents the transition from the haploid female gamete to the diploid zygote, the first cell of a sporophyte. Some changes are induced extremely rapidly following fusion with sperm cells and are the preclusions of egg activation. This review focuses on the early changes that occur in the egg after fusion with sperm cells, but before nuclear fusion. Reported changes include cell shrinkage, cell wall formation, polarity change, oscillation in Ca^{2+} concentration, and DNA synthesis. In addition, the current understanding of egg activation is summarized and the possible functional relevance of the changes is explored.

Review

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Introduction

The conversion from egg to zygote is one of the most important transitions during plant alternation of generations. After fusion of the male and female gametes, the egg cell is immediately activated, displaying structural, physiological, and biochemical changes, and finally results in zygote activation and division, which initiates embryogenesis. The changes induced in the egg after fertilization are an important focus of research on the reproductive biology of higher plants and review papers on the topic are regularly published to summarize advances (Faure & Dumas, 2001; Lord & Russell, 2002; Raghavan, 2003; Weterings & Russell, 2004; Dumas & Rogowsky, 2008; Berger *et al.*, 2008; Ge *et al.*, 2010; Hamamura *et al.*, 2012; Lau *et al.*, 2012; Shivanna, 2016; Bayer *et al.*, 2017). However, the embryo sac of angiosperms is embedded within maternal tissues in an ovule within an ovary, and is where the process of double fertilization occurs. The concealment of the egg cell renders research into the fertilization mechanism technically difficult. Given the difficulty of isolating a fertilized egg, our understanding of the fertilization mechanism in higher plants is limited and less advanced than that for animals.

The final step of fertilization is egg fusion with a sperm cell. During this process, the plasma membranes of the egg and sperm first fuse, and then the nuclei fuse, to form the zygote with a fused nucleus. In *in vitro* fertilization of maize, the time interval between plasma membrane fusion and nucleus fusion is about 35–60 min (Kranz *et al.*, 1995). After plasma membrane fusion, the fused egg cell undergoes structural changes before nucleus fusion occurs. Does the fused egg cell before nuclear fusion become a zygote? Kranz and Lörz (1993) and Kranz *et al.*, (1995) termed the fused egg a 'fusion product' following *in vitro* fertilization of maize, suggesting that it differs from the egg and the zygote. Fusion of the plasma membranes of the egg and sperm cells may induce structural and physiological changes in the fused egg cell, and these changes differ from those observed after nuclear fusion (which mainly induces changes in gene expression in the zygote). However, use of the terms 'egg activation' and 'zygote activation' in the literature is very confusing. To distinguish the two processes occurring before and after nucleus fusion of the egg and sperm, we refer to the changes occurring after plasma membrane fusion but before nucleus fusion of the sperm and egg cell as egg activation, and the changes occurring after nucleus fusion (which mainly induces the molecular biological processes of the zygote) as zygotic activation. In this review, we discuss and analyze the structural and physiological changes that occur in the fused egg that are triggered by sperm entry, and subsequent initiation of egg activation in angiosperms.

Change in the size of the fused egg

It has been observed previously that in cotton the egg cell decreased in size after fertilization (Jensen, 1968). In *Hibiscus* hybrids, the egg of emasculated and bagged (non-fertilized) flowers

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does not shrink, whereas the fertilized egg shows shrinkage of up to 50% (Ashley, 1972). However, some researchers were suspicious of this phenomenon, which might be an artefact caused by dehydration during sample preparation. Using an *in vitro* fertilization technique, which enabled monitoring of egg cells before and after fusion with the sperm cell, shrinkage of the egg cell after fusion with the sperm was observed in maize (Kranz *et al*., 1995; Antoine *et al*., 2000) and wheat (Kumlehn *et al*., 1998). This confirmed that the shrinkage phenomenon is a natural process in the fused egg cell induced by fertilization. The embryo sac of *Torenia fournieri* partially protrudes out of the ovule and enables easier observation of changes in the egg during fertilization. After fusion of the egg and sperm, the size of the fertilized egg is smaller than that of the egg before fusion. The isolated egg cells have large vacuoles and most cytoplasm is concentrated surrounding the nucleus. In isolated zygotes, the zygotic volume decreases because the large vacuole declines in volume and disintegrates, and the cytoplasm shows uniform distribution (Chen *et al*., 2008). Therefore, shrinkage of the fused egg is a natural phenomenon induced by fertilization. This change is due to cytoplasm reconstruction and vacuole metabolism in the fused egg and is the beginning of egg activation after fusion. The importance of egg shrinkage is not well understood.

Cell wall changes in the fused egg

The last step of fertilization is egg fusion with a sperm. Ultrastructural observations show that the mature egg cell is partially naked in most angiosperms and no cell wall is present at the chalazal end of the egg, where only a plasma membrane separates the egg and the central cell. The chalazal region of the egg is the target area for sperm entry and fusion with the egg during fertilization (Jensen, 1968; Van Went, 1970). This fusion area also is where the male and female gametes interact during double fertilization (Berger *et al*., 2008; Sprunck, 2010). The fertilization target area of the egg is a specific structure, especially for egg fertilization, that is pivotal for male and female gamete fusion. The area shows two features associated with fertilization: absence of a cell wall in the area before fusion, and formation of a new cell wall after fusion.

It is unclear how the fusion area is formed in the mature egg cell. In the young egg cell of tobacco, a continuous cell wall is formed during embryo sac cellularization. However, following egg cell maturity, the cell wall at the chalazal end disappears (Tian & Russell, 1997a). These observations indicate that the cell wall in the fusion area of the egg cell is digested following egg maturation. Cellulase may be secreted to dissolve the wall in a specific region to form the fertilization target area. However, the identity of the cell in the embryo sac that secretes the cellulase and the regulatory mechanism by which cellulase activity is localized is unknown. Many studies have investigated cellulose synthesis and cell wall formation (Doblin *et al*., 2002; Somerville, 2006; Miart *et al*., 2014), but to the best of our knowledge no information on endogenous partial cell wall dissolution, especially in the fusion area of the egg cell, is available. The experimental degradation of cellulose in the plant cell wall generally uses cellulases that are extracted from fungi that secrete cellulase to degrade plant cell walls during invasion of higher-plant tissues.

After fusion with a sperm, a new wall is formed in the fusion area of the fused egg. The formation of the new wall by the fused egg cell is a result of fertilization, after which the egg is activated. After fusion, the fused egg will not accept other sperm cells to maintain offspring stability. Therefore, the egg cell not only requires a structure to guarantee highly efficient recognition and respective fusion with one of the two sperm cells of a pollen tube, but also requires a mechanism to prevent superfluous sperm from entering the egg (polyspermy). Genes involved in preventing polyspermy have been identified (Spielman & Scott, 2008; Sprunck & Gross-Hardt, 2011; Maruyama *et al*., 2013), but the regulatory mechanism of preventing polyspermy is unclear. After fertilization, a new wall forms over the fusion area of the fused egg, and prevents additional cell fusion. In maize *in vitro* fertilization, weak cellulosic wall material is deposited on the surface of the isolated egg cell. After egg fusion with the sperm, deposition of the cellulose material increases and, at 10 min after fusion, the fused egg has formed an almost continuous cell wall that obstructs fusion with a second sperm cell. At 20 min after fusion, a second sperm could not fuse completely (Kranz *et al*., 1995). These results indicate that new wall formation on the fusion area of the egg cell is regulated by the egg cell. The rapid deposition of the cell wall of the fused egg to prevent multiple sperm from entering the egg is also a process of egg activation. Antoine *et al*. (2001) treated unfertilized maize egg cells with two Ca^{2+} ionophores in a solution containing 5 mM $CaCl_2$. A cell wall was detected 40 min after treatment, suggesting that cell wall formation in the egg is an active process and Ca^{2+} influx is required to induce the establishment of a cellulosic cell wall on the unfertilized egg cell of maize. In the polyspermic tetraspore (*tes*) mutant of Arabidopsis, the egg cell, in contrast with the central cell, displayed resistance to multiple fertilization events, and indicated the existence of an additional *in vivo* polyspermy barrier in the egg (Scott *et al*., 2008).

There are two possible types of polyspermy: one is egg fusion with two sperm cells from the same pollen tube; the second is egg fusion with sperms of multiple pollen tubes (Beale *et al*., 2012; Kasahara *et al*., 2012). The mechanism for prevention of each polyspermy type is different. In the former type, a pollen tube enters a degenerated synergid and releases two sperm cells. To prevent polyspermy from the two sperms of the same pollen tube, a structure within the synergid forms before fusion. In tobacco (Huang & Russell, 1994), two prominent actin bands or 'coronas' appear at the chalazal end in the degenerated synergid where the two sperms are discharged and disappear before zygote division. In maize (Huang & Sheridan, 1998), *T. fournieri* (Huang *et al*., 1999; Fu *et al*., 2000), and *Phaius tankervilliae* (Ye *et al*., 2002), the two actin coronas are also present in the degenerated synergid before egg fusion. The directional movements of organelles in cells depend on the interaction between myosin on the organelles' surface and microfilaments composed of polymers of actin. The actin coronas in degenerated synergids perform a physiological function but which is yet to be confirmed. The two actin coronas may provide routes for the two sperm separately to reach the fertilization target area of the egg and the central cells, and may prevent simultaneous fusion of both sperms of a pollen tube with the egg.

To prevent polyspermy from multiple pollen tubes, a regulatory mechanism has developed to prohibit multiple pollen tubes from entering an ovule. A tobacco fruit may contain more than 3700 fertile seeds (not including some sterile ovules), therefore an ovary contains more than 4000 ovules. In reproduction, each ovule receives a pollen tube for double fertilization. A mature ovule accumulates a large amount of Ca^{2+} in the micropylar cells, especially in the cells at the opening of the

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micropyle, and therefore may attract a pollen tube to enter the micropyle in accordance with pollen-tube chemotropism for Ca^{2+}. After a pollen tube enters the ovule, the Ca^{2+} concentration declines sharply in the micropylar cells, and reduces attraction for other pollen tubes and prevents multiple pollen tubes from entering the fertilized ovule (Tian & Russell, 1997a). Multiple pollen tubes rarely enter the ovule. As mentioned above, the egg at 10 min after fusion with a sperm from a pollen tube has formed a continuous cell wall that will prevent polyspermy.

Although changes in the cell wall of an egg cell before and after fusion were observed early in research on egg activation, to the best of our knowledge, the associated regulatory mechanism has not been elucidated. In cell wall research, cellulose metabolism in the egg is an interesting topic and research on the rapid induction of cellulose metabolism in an egg cell after fusion with sperm is worthy of future attention.

Change in polarity of the fused egg

Ovaries and ovules of higher plants are terminal organs and display polarity. The specific location of the egg in an embryo sac confers differences in morphology and physiology, forming a morphological axis between the apical pole (chazalal end) and the basal pole (micropylar end) (i.e. the micropylar–chalazal polarity), which is especially characterized by a morpho-functional polarity. The micropylar–chalazal axis in the egg is transferred to the zygote after fertilization and determines the differences in cytoplasm function, division orientation, and destiny of the two daughter cells. The origin and regulatory mechanism of egg polarity is unclear. In assays of isolated eggs of maize (Kranz & Lörz, 1990), tobacco (Tian & Russell, 1997b), rice (Zhang *et al*., 2010), and *Solanum verbascifolium* (Yang *et al*., 2015), the embryo sac cells displayed higher osmosis than the somatic cells, which indicates that embryo sac cells are at the terminal end of the nutrition stream, and may exploit high osmosis to retain their physiological polarity.

After fertilization, the egg polarity is retained in the zygote, which shows differences in polarity in various plants. In the majority of monocotyledons, the egg contains many small vacuoles but no large vacuole, and the morphological polarity of the egg and zygote is not evident. However, physiological polarity still exists in the plants. In *in vitro* fertilization of maize, the free round zygote still divides asymmetrically to form a small cell and a large cell (Kranz *et al*., 1995), which confirms that physiological polarity exists in the zygote. In the majority of dicotyledons, the egg contains a large vacuole located at the micropylar end that results in concentration of the nucleus and most cytoplasm at the chalazal end, thus displaying evident micropylar–chalazal polarity. After fertilization, the polarity of the fused egg changes in some plants. In *Arabidopsis thaliana*, after egg fusion with a sperm, the large vacuole disintegrates into many small vacuoles that are uniformly distributed within the cell, and the nucleus moves to the centre of the fused egg, which shows weakened polarity (Sprunck and Gross-Hardt, 2011; Lau *et al*., 2012). The fused egg then elongates two-fold to three-fold before a new large vacuole is re-assembled (Faure *et al*., 2002). After the large vacuole forms at the basal end and the nucleus moves to the chalazal end, the polarity of the zygote is established (Ueda & Laux, 2012; Zhao & Sun, 2015). In *T. fournieri*, the egg cell is pear-shaped with several large vacuoles at the micropylar end and the nucleus is located in the chalazal region, thus displaying evident polarity. At 18 h after pollination, the fertilized egg cell begins to elongate to become rod-shaped in the embryo sac. The nucleus moves further towards the chalazal end of the cell, resulting in enhanced polarity (Chen *et al*., 2008). In rice zygotes, the vacuoles and nucleus, which are localized at the apical (chalazal end) and basal regions (micropylar end), respectively, are repositioned to opposite poles in the cell (Sato *et al*., 2010). These descriptions demonstrate dissimilarities in the polarity remodelling process in zygotes of different species; each creates a cytologically polarized cell, with the nucleus positioned at the apical end and the vacuoles at the basal region. The polarity of the zygote is derived from that of the egg and is transferred to the young embryo, which develops root and stem polarities. Some changes in the polarity caused by sperm entrance in the fused egg are expressions of egg activation. However, the micropylar–chalazal polarity of the egg and zygote is persistent throughout their development; the changes in polarity are an adaption for the egg-to-zygote transition.

The large vacuole in the egg that pushes the nucleus to the chalazal end to establish polarity is only an external appearance because the nucleus may be pushed to any peripheral position. The cytoskeleton in the egg is the main structure that fixes the nucleus in a specific position (the chalazal end). Therefore, it is the cytoskeleton that determines egg polarity. Fertilization also causes rearrangement of the cytoskeleton of the fused egg, which induces the change in polarity. In Arabidopsis, microtubules are concentrated in the chalazal region of the egg, thus displaying a polar distribution, which anchors the nucleus in the chalazal area, in accordance with egg polarity (Webb & Gunning, 1994). After fertilization, the cytoskeleton determines the division orientation of the zygote and the developmental destiny of the two daughter cells. Therefore, the regulatory mechanism of the cytoskeleton in the egg and zygote functions differently. Even in the zygote, the arrangement of microtubules will vary at different developmental stages. During zygote development, re-orientation of microtubules to a transverse cortical distribution occurs, predominantly in a subapical band, which accompanies a phase of apical extension. These cortical arrays coincide with elongation of the zygote. During zygote division, the microtubules are confined to a spindle array (Webb & Gunning, 1991). In maize egg cells, a few cortical microtubules can be detected and well organized microtubules are rarely observed. In contrast, distinct cortical microtubules and strands of cytoplasmic microtubules radiating from the nucleus to the cell periphery are observable in developing zygotes (Huang & Sheridan, 1994). Pónya and Barnabás (2001) observed that filamentous actin in the fused egg shows rapid, dynamic reorganization upon sperm–egg cell fusion. The cortical actin filaments are distributed uniformly in *in vitro* fertilized egg cells after characteristic accumulation of an actin patch at the site of sperm entry. Throughout mitosis and cytokinesis, rearrangement of the interphase actin cytoskeleton results in transverse cortical filamentous actin becoming concentrated in a widening band that predicts the future division plane. Hoshino *et al*. (2004) examined fertilization-induced changes in the microtubular architecture of the maize egg and zygote. A few cortical microtubules were detected in the egg cell. However, in the zygote, cortical microtubules increased in abundance and remained visible up to 7 h after *in vitro* fertilization. Then, strands of microtubules radiating from the nucleus to the cell periphery were formed and persisted during zygote development. Based on the above-mentioned results, we have a preliminary understanding of polarity change between the egg and the zygote, which

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is controlled by the cytoskeleton system during egg activation. However, the regulatory mechanism of the cytoskeleton system during fused egg activation is unclear and will be an active research focus.

With regard to egg polarity, most attention has been paid to the changes in nucleus position. An additional polar structure is the polar distribution of the large vacuole, which is always located in the basal position (micropylar end). The polar distribution of the egg nucleus will result in asymmetric zygotic division, but what is the function of polar distribution of the large vacuole in the egg and the zygote? Why is the large vacuole always anchored in the basal position? The nuclear membrane and nucleus-associated proteins can attach to microtubules to anchor in a specific position in the egg. How is the vacuolar membrane attached to the cytoskeleton? In addition to its polar distribution, the large vacuole in the fused egg also undergoes changes during egg activation. In Arabidopsis, the large vacuole becomes fragmented after fertilization, and the zygote elongates two-fold to three-fold before a large vacuole is re-assembled (Faure *et al.*, 2002). The significance of the remodelling process for the large vacuole is unclear, and the polar distribution and changes in the large vacuole during egg activation require further investigation.

Ca^{2+} changes in the fused egg

In animals, increase in Ca^{2+} concentration in the egg cell is confirmed to be the earliest action in egg activation. Following sperm and egg fusion, the Ca^{2+} concentration in the egg cell increases and forms a Ca^{2+} oscillation (Stricker, 1999; Ciapa & Chiri, 2000). Nakasaka *et al.* (2000) treated unfertilized mouse egg cells with medium containing Ca^{2+} ionophore A23187, which induced division of almost half of the egg cells, suggesting that Ca^{2+} elevation is involved in egg activation. In the alga *Fucus serratus*, fertilization of the egg cell is accompanied by uptake of Ca^{2+} from the external medium, which appears to be necessary for egg activation (Roberts & Brownlee, 1995). However, in higher plants, the egg is embedded in an ovule within an ovary and Ca^{2+} changes occurring in the egg cannot be observed simultaneously as is possible for eggs of animals and lower plants. In higher plants, using ultrastructural localization of Ca^{2+}, positioning of the synergids in the embryo sac is consistent with the highest concentration of Ca^{2+}, which has been confirmed in many plants. But the egg, which is a close neighbour of the synergids, persistently contains a lower concentration of Ca^{2+} (Chaubal & Reger, 1990, 1992a, 1992b; He & Yang, 1992; Tian & Russell, 1997a; Tian *et al.*, 2000). Recent studies have revealed that, in Arabidopsis, upon arrival of a pollen tube at the synergid, Ca^{2+} oscillations occur at the micropylar pole of the synergid and spread towards the chalazal pole. Ca^{2+} concentrates as the synergid peaks upon pollen tube rupture (Iwano *et al.*, 2012; Denninger *et al.*, 2014; Hamamura *et al.*, 2014; Ngo *et al.*, 2014), which is consistent with Ca^{2+} function for attraction of the pollen tube to the synergid (Ge *et al.*, 2007).

Faure *et al.* (1994) established an experimental system for fusion of an egg and sperm of maize in a Ca^{2+} medium. Using this system, images of 4′,6-diamidino-2-phenylindole (DAPI) fluorescence and changes in Ca^{2+} concentration in the egg during fusion with the sperm can be simultaneously observed, as in animals and lower plants. The maize egg contained a low concentration of Ca^{2+} before fusion, even when a sperm adhered to it. Upon initiation of egg fusion with a sperm, the Ca^{2+} fluorescence intensity increased at 4 s and attained maximum intensity at 89 s. Thereafter the Ca^{2+} fluorescence intensity in the fused egg declined and returned to that of the egg before fusion (Digonnet *et al.*, 1997). This is the first evidence for Ca^{2+} oscillation in the egg of higher plants induced by fertilization, and also is the earliest event of egg activation detected. Antoine *et al.* (2000) measured an influx of extracellular Ca^{2+} induced by gamete fusion using a Ca^{2+}-selective vibrating probe. Before fusion of an isolated maize egg, Ca^{2+} influx, with or without adhesion to a sperm cell, was close to zero and stable over time. Gamete fusion, however, triggered a Ca^{2+} influx in the vicinity of the sperm entry site with a delay of 1.8 ± 0.6 s. The Ca^{2+} influx spread subsequently throughout the whole egg cell plasma membrane as a wave front, progressing at an estimated rate of $1.13\ \mu m \cdot s^{-1}$ (Antoine *et al.*, 2000). Antoine *et al.* (2001) further confirmed a small and stable Ca^{2+} flux in the egg between an efflux of 1.29 and an influx of 6.02 $pmol \cdot cm^{-2} \cdot s^{-1}$ over more than 60 min. Adhesion of one sperm to the egg cell did not change this situation. These authors analyzed the relationship between Ca^{2+} influx and cytoplasmic Ca^{2+} level during egg fusion with a sperm, and observed that the Ca^{2+} influx preceded cytoplasmic Ca^{2+} elevation by 40–120 s. When the Ca^{2+}-channel inhibitor gadolinium ($GdCl_3$) was included in the fusion solution, cytoplasmic Ca^{2+} concentration still increased in the fused egg cell but no Ca^{2+} influx was measured (Antoine *et al.*, 2001). The results indicated that the sperm triggers a transient elevation in intracellular free Ca^{2+} concentration in the fused egg, and that the Ca^{2+} signalling events reported in animals and lower plants also operated in higher plants.

Given that sperm fusion with the egg induces transient elevation in the intracellular free Ca^{2+} concentration in the egg, and initiates egg activation, regulatory factors in the sperm may stimulate Ca^{2+} elevation in the egg fusing with a sperm. Han *et al.* (2002) microinjected soluble sperm extract and Ca^{2+} Green-1 10 kDa – dextran conjugate (CG-1) into the mature central cell of *T. fournieri*, which induced a significant rise in cytosolic free Ca^{2+} concentration. The rise attained a maximum 20 min after injection and then steadily declined. These authors also injected caged inositol 1,4,5-triphosphate ($InsP_3$) into the central cells to compare the pattern of Ca^{2+} elevation induced by the sperm extract. The elevation triggered by the release of $InsP_3$ was much faster than that induced by the sperm extract but the increase in Ca^{2+} attained a maximum at 70 – 80 s and then declined to resting levels within 300 s. The authors hypothesized that the sperm extract might contain factors that triggered the Ca^{2+} release in the central cell.

Ca^{2+} elevation induced by male and female gamete fusion is the earliest detected fertilization reaction event. Identification of the mechanism by which Ca^{2+} causes the events of egg activation and to what extent these temporal Ca^{2+} responses encode developmental information is required. Various extracellular stimuli elicit specific Ca^{2+} signatures that can be recognized by different Ca^{2+} sensors. Studies of animals have indicated how these Ca^{2+} signals are interpreted by specific proteins, and how these proteins regulate egg activation responsible for the onset of development. Many of these proteins are protein kinases (CaMKII, PKC, MPF, MAPK, and MLCK) whose activity is directly or indirectly regulated by Ca^{2+}, and whose amount increases during late oocyte maturation (Ducibella & Fissore, 2008). Calmodulin (CaM), the predominant Ca^{2+} receptor, is one of the best-characterized Ca^{2+} sensors in eukaryotes. In plants, similar studies are scarce because of the difficulties associated with isolation

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of the embryo sac. In the mature embryo sacs of *Petunia* the concentration of CaM is almost uniform in all cell types except that one of the synergids and the three antipodal cells show a slightly higher concentration (Tirlapur *et al.*, 1993). In work based on the hypothesis that Ca^{2+} triggers egg activation by a transient cytosolic Ca^{2+} elevation, Tirlapur *et al.* (1995) observed a high concentration of CaM in the vicinity of the nucleus in egg cells and an elevation in cytoplasmic CaM concentration in maize artificial zygotes compared with those of isolated egg cells, suggesting that Ca^{2+} may trigger egg activation by the CaM pathway. Therefore, the next step in the investigation of egg activation is to explore gene expression induced by Ca^{2+} elevation caused by egg fusion with a sperm, especially using molecular biological methods.

Regulation of the cell cycle of the fused egg

Cell-cycle regulation is important for the growth and development of plants. The terminal development of the fertilized egg is division to begin ontogenesis. The regulatory mechanism of DNA synthesis in gamete cells and the fused egg cell is an important event in egg activation. The DNA content of male and female gametes and zygotes was measured by following the development of isolated sperm and egg cells of higher plants using DNA fluorescent dyes (Sherwood, 1995; Mogensen & Holm, 1995; Mogensen *et al.*, 1999; Pónya *et al.*, 1999). Friedman (1991) measured the DNA content of male gametes of *Ephedra trifurca* using DAPI and combined fertilization events with the concept of the cell cycle, introducing the relationship between sperm and egg development and the cell cycle, and proposed a model of the cell cycle during gamete fusion. In addition, DNA synthesis in developing sperm cells of *Gnetum gnemon* and the relationship between the cell cycle and sexual reproduction in this gymnosperm has been investigated (Carmichael & Friedman, 1995). The amount of DNA in an non-replicated gametic chromosome complement is known as the C-value. In *Arabidopsis thaliana*, the DNA content of both newly formed sperm cells begins to increase, meaning that the two sperm cells enter the S phase of the cell cycle. At the time of anthesis the DNA content of both sperm cells is 1.5 C. After pollination both sperm cells continue to synthesize DNA in the pollen tube and attain 1.75 C when they arrive at the ovary. Close to the time of fertilization, the DNA content of the sperm cells can attain 1.98 C (Friedman, 1999). These results indicated that the sperm cells of *A. thaliana* begin DNA synthesis in the pollen grain and attain nearly 2 C prior to fusion with the egg cell. Both male and female gametes may fuse in the G_2 state of the cell cycle, representing the G_2 type of fertilization. However, Friedman was unable to measure egg cell DNA changes because the nuclei of mature egg cells showed no fluorescence. These results lead us to question how the DNA content of eggs and central cells changes when sperm cells attain 2 C DNA before fusion with the egg and central cells, especially for the latter, which contain a second nucleus or two polar nuclei.

Compared with male gametes, data on the DNA content of egg cells are scarce given the difficulties encountered when isolating egg cells from higher plants and because mature egg cells from some plants cannot be dyed. Mogensen and Holm (1995) measured the DNA content of isolated barley egg cells and zygotes using DAPI and reported the DNA content of egg cells to be 1 C and that of zygotes to be 2 C. Mogensen *et al.* (1999) examined the DNA content of isolated maize egg cells and zygotes and confirmed the DNA content of egg cells to be 1 C and zygotes to be 2 C. The male and female gametes in barley and maize contain 1 C DNA during gamete fusion, and both plants show the G_1 type of fertilization. Tian *et al.* (2005) examined the nuclear DNA content of male and female gametes of tobacco using DAPI and quantitative microfluorimetry. Pollen of tobacco is bicellular and the generative cell will divide in the pollen tube to form two sperm cells 8 h after pollination. Pollen tube growth through the 4-cm-long style of tobacco requires approximately 2 days from pollination to fertilization, and the sperm cell DNA content remains at 1 C. When a pollen tube enters the embryo sac and discharges two sperm cells in the degenerated synergid, the two sperm cells begin to synthesize DNA, and the DNA content eventually attains 2 C before fusion with the egg and central cells. These findings suggest that both sperms start the cell cycle and move into the S phase after release in the degenerated synergid. Concomitant with pollen tube arrival, the DNA content of the egg cell also begins to increase and finally attains 2 C. The DNA content in newly formed zygotes is 4 C and remains at 4 C until zygote division. In the absence of pollination, the S phase in egg cells is delayed by up to 36 h, which is suggestive of a signal reaction arising from the pollen tube (Tian *et al.*, 2005). The male and female gametes of tobacco fuse when both conclude the S phase, thus tobacco belongs to the G_2 type of fertilization. In *Lycium barbarum*, the period from pollination to fertilization is 34 h. After pollination, two sperms in the pollen tube begin to synthesize DNA and the content of DNA continues to increase. At 16 h after pollination, the content of sperm DNA attains 1.5 C. When the pollen tube reaches the degenerative synergid and rupture, the DNA content in the two released sperms is 1.92 C. At anthesis, the content of DNA in eggs is about 1.3 C, and at 30 h after anthesis the DNA content in eggs attains 1.63 C. Before fertilization the egg DNA content is 1.83 C. After fertilization, zygotes contain 3.53 C. In emasculated flowers, the eggs also synthesize DNA and attain 1.2 C of DNA content, but DNA synthesis ceases without pollen tube stimulation. Before male and female gamete fusion of *L. barbarum*, both gametes synthesize DNA and pass through the S phase of the cell cycle, and fuse in the G_2 phase of the cell cycle. The fertilization of *L. barbarum* belongs to the G_2 type (Deng *et al.*, 2012). Recently, in *Helleborus bocconei* it was observed that the egg begins DNA synthesis and attains 2 C DNA content prior to fertilization. The central cell attains 4 C DNA content before fertilization. However, the two sperm cells show a large difference in DNA content: one synthesizes DNA and attains 2 C DNA content before fusion with the central cell to form a sextuploid endosperm, and the other sperm does not synthesis DNA before fusion with the egg and shows 3 C DNA content (Bartoli *et al.*, 2016). This result makes it difficult to believe that a zygote with 3C DNA content will undergo development.

From the above results, the G_2 type of fertilization was confirmed in angiosperms, demonstrating the diversity of fertilization from the point of view of the cell cycle. These results also highlight some novel characteristics of fertilization:

(1) The sperm of Arabidopsis begins to synthesize DNA in the pollen grain, that of *L. barbarum* in the pollen tube, and that of tobacco in the degenerative synergid after release. These three species indicate that different mechanisms to initiate DNA synthesis may operate in different plants. Given the diversity of fertilization types, additional higher-plant species require investigation.

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(2) Egg cells in non-pollinated flowers delay DNA synthesis, which indicates that egg cell DNA synthesis is itself an inherent feature, but that the process can be promoted by sperm that synthesize DNA. However, in tobacco, the sperm cells that synthesize DNA induce the beginning of egg DNA synthesis, whereas in *L. barbarum* the DNA-synthesizing sperm cells promote the continuation of egg DNA synthesis.

The cell cycle is a very complex signal network. Sperm cells that synthesize DNA promote egg DNA synthesis, which indicates that a cell cycle signal system exists between the gametes, of which nothing is known. Additional topics to study are whether DNA synthesis in egg cells is a precondition of fusion with a sperm cell and how the DNA content is changed in central cells.

The change in DNA content in the cell is regulated by cell-cyclic genes. Sauter *et al.* (1998) studied the expression of the cell-cycle regulatory genes *cdc2ZmA/B*, Zeama;*CycB1*;*2*, Zeama;*CycA1*;*1*, Zeama;*CycB2*;*1*, and *histone H3* in maize sperm cells, egg cells and in other cells present in the embryo sac and the zygotes produced by *in vitro* fertilization techniques. The *cdc2* and *histone H3* genes are expressed constitutively in all cells, and the cyclin genes display cell-specific expression in embryo sacs and differential expression during zygote development. The expression of the three cyclin genes display evident differences: Zeama;*CycB1*;*2* and Zeama;*CycB2*;*1* are not expressed in all cells of the embryo sac and sperm cells. Zeama;*CycA1*;*1* is expressed in sperm cells and all cells of the embryo sac, except in the antipodals. During zygote development, the three cyclin genes are expressed at different times. *Histone H3* in somatic cells is expressed during DNA replication, but is present in sperm and egg cells, and in zygotes, for up to 24 h before decreasing in concentration. After the zygote divides, *histone H3* mRNA again is detected in the bicellular embryo.

The study of DNA contents in gametes of angiosperms has been ongoing for years. Sperm cell induction of egg synthesis DNA before fusion is also an action of egg activation. After the introduction of the concept of the cell cycle, the study of egg activation has been invigorated and many interesting new questions have arisen, a primary one being: what mechanism controls DNA synthesis in gametes and fused egg cells? Furthermore, what is the difference between both? One obvious next step for this research is to probe cyclin regulation in both gametes and fused egg cells. We anticipate that the cell cycle in both male and female gametes and fused egg cells will shortly be an active field of study in the area of egg activation and early ontogenesis of angiosperms.

Conclusion and prospects

Fertilization as the starting point of ontogeny is an old maxim and an interesting research topic in angiosperms. During the processes of egg and zygote activations, many events are triggered in the fused egg cell in response to sperm entry. Each event is controlled in a complex and strictly programmed manner, displaying a series of structural and physiological changes that constitute egg and zygote activations and finally induce zygote division according to special manner (embryogeny). Structural studies of fused eggs have enhanced our understanding of the structural changes and inferred physiological functions occurring in the fused egg cell. However, plant embryogenesis is extremely diverse; six types are recognizable even at the first two zygotic divisions (Bhojwani & Bhatnagar, 1974). Considering other structural changes occurring in the fused egg, additional types of plant embryogenesis will be documented. From the preceding discussion, it is apparent that the early change in cell wall formation in the fused egg is likely to be a response to fertilization (egg activation). The dynamic changes in Ca^{2+} concentration in the fused egg may indicate the start of molecular biological action of the zygote (zygote activation). The polarity of the fused egg is an heredity feature because the egg is located in the most terminal position of a stem, which may be a regulatory mechanism of the embryo type because some studies report polar transportation of phytohormones associated with embryogenesis. The cell cycle of the egg of plants is a novel research field but a few species have been analyzed, which may show phylogenetic patterns, but additional species require investigation. In addition to the above-mentioned characteristics, other small structural changes may occur in the early fused egg in different plants and may be unique features of plant embryogenesis, and also require attention to thoroughly understand the embryogenesis of angiosperms. Combined analysis of the structural and molecular biological changes induced by egg fusion with the sperm cell will help to elucidate the programmed network of egg activation of higher plants.

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