REVIEW

Insights into the molecular evolution of fertilization mechanism in land plants

Vijyesh Sharma[1] · Anthony J. Clark[1] · Tomokazu Kawashima[1]

Abstract

Key message **Comparative genetics and genomics among green plants, including algae, provide deep insights into the evolution of land plant sexual reproduction.**

Abstract Land plants have evolved successive changes during their conquest of the land and innovations in sexual reproduction have played a major role in their terrestrialization. Recent years have seen many revealing dissections of the molecular mechanisms of sexual reproduction and much new genomics data from the land plant lineage, including early diverging land plants, as well as algae. This new knowledge is being integrated to further understand how sexual reproduction in land plants evolved, identifying highly conserved factors and pathways, but also molecular changes that underpinned the emergence of new modes of sexual reproduction. Here, we review recent advances in the knowledge of land plant sexual reproduction from an evolutionary perspective and also revisit the evolution of angiosperm double fertilization.

Keywords Evolution · Sexual reproduction · Angiosperms · Land plants · Sperm morphogenesis

Introduction

The land is covered by green plants such as bryophytes, ferns, and seed-bearing plants. They have improved the environment for other organisms (e.g., more oxygen, temperate climates, and balanced O_2–CO_2 levels), and animals, including humans, can live on land due to its successful conquest by plants (Dahl and Arens 2020). The terrestrialization of plants was a long process, during which plants have undergone many innovations, including evolution of land plant-type cell walls with phragmoplasts, more control over plastids by the nucleus, the haplodiplontic life cycle (both gametophytic and sporophytic stages are multicellular), and new tissue/organ systems such as vasculature, the leaf, root, pollen, seed and flowers (Pires and Dolan 2012; Rensing 2018). The vascular system functions as the means to transport resources such as water and nutrients to different parts of the plant, including leaves that provide more surface area for photosynthesis.

In particular, features associated with sexual reproduction like pollen, seeds, and flowers have helped terrestrialization. Evolution of the pollen grain and the formation of flowers have increased the range of environments over which fertilization can occur efficiently. The evolution of seeds extended the scope for dispersing offspring in time and space as they could survive for longer periods, until conditions were favorable for germination and were able to spread in novel ways. In seed-bearing plants such as gymnosperms and angiosperms (flowering plants), sperm cells are encapsuled in the pollen grain that protects them until they reach the female. After pollination, an extruded tube from the pollen grain conveys male gametes to female gametes, thus allowing fertilization without environmental water. Compared to gymnosperms, a further innovation occurred in angiosperms; an additional fertilization besides that of the egg cell occurs to initiate the development of an embryo nourishing tissue, called the endosperm. This phenomenon is generally termed "double fertilization" and this simultaneous development of the endosperm alongside the embryo ensures the proper allocation of nutrients for the successful generation of offspring.

✉ Tomokazu Kawashima
tomo.k@uky.edu

[1] Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546, USA

Rapid collection of genomic resources from many species spanning not only land plants but also green algae (Wong et al. 2019) enable us to revisit the relationship between reproductive phenotype and genotype from an evolutionary perspective. Furthermore, the emergence of a new dioecious model plant, *Marchantia polymorpha*, for molecular dissection (Bowman et al. 2017) has accelerated our understanding of sexual reproduction in early diverging land plants. These new data bridge accumulated knowledge in different species and have further elucidated land plant evolution. In this review, we introduce our current understanding of land plant fertilization from an evolutionary perspective. Especially, recent updates on regulation of sperm morphogenesis, fertilization processes, and the mystery of the evolution of double fertilization in angiosperms, are highlighted. The evolution of gametogenesis in land plants (Hackenberg and Twell 2019; Hisanaga et al. 2019) and flowers (Simonini and Østergaard 2019; Woźniak and Sicard 2018) has been reviewed elsewhere.

Changes in the sperm cell

The transition of the mode of sexual reproduction has been intensively studied in the Chlorophyta, the sister group to the Streptophyta (the land plant lineage of green plants) (Fig. 1). The Chlorophyta show a sequential shift from isogamy to anisogamy to oogamy (Mori et al. 2015; Umen and Coelho 2019). Isogamous species reproduce via fusion of two motile gametes of similar size and morphology. Anisogamous species have two motile gametes dissimilar in size, and oogamous species generate a large, sessile female gamete and a smaller, motile male gamete. Transition from isogamy to anisogamy to oogamy is correlated with an increase in size and complexity of the genome as well as a transition from unicellular to multicellular species among the Chlorophyta (Fang et al. 2017; Herron et al. 2010; Nozaki et al. 2014). One important characteristic of oogamous species is the evolution of specialized female organs that provide an environment where the sessile egg cell is maintained and protected
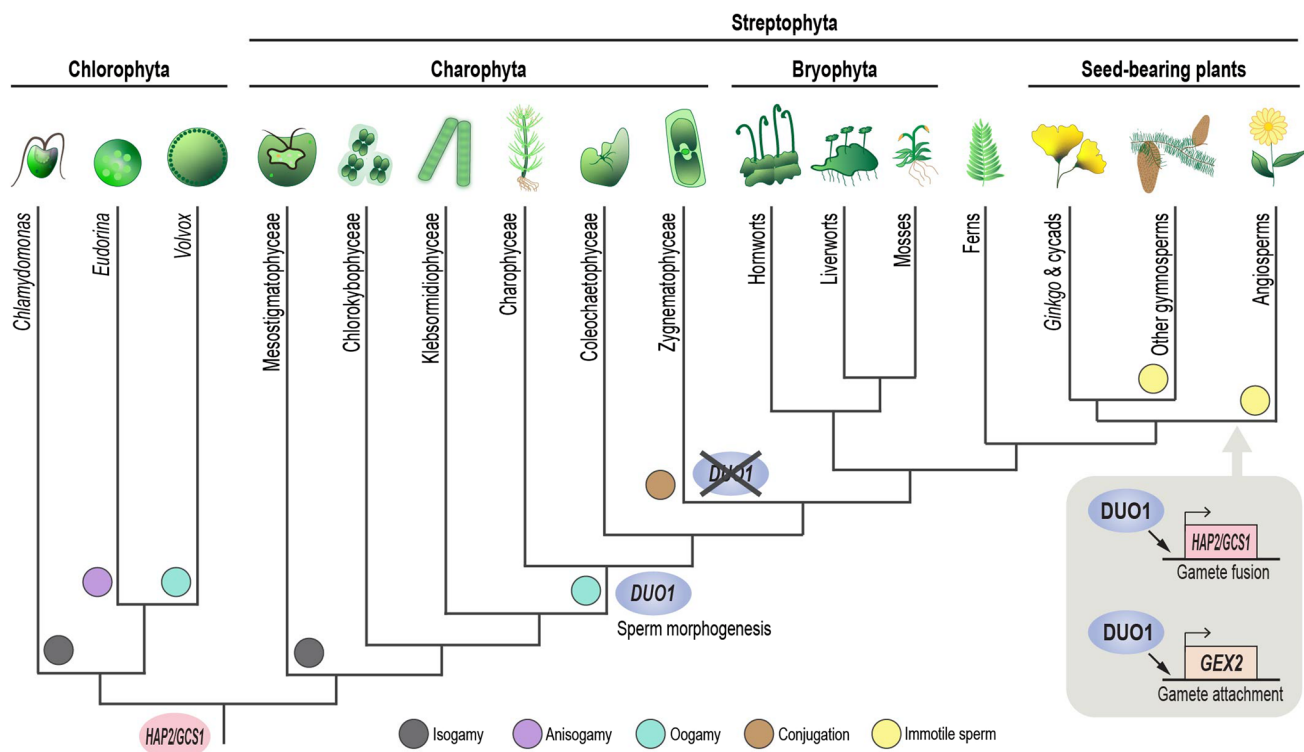

Fig. 1 Phylogeny of green plants and features of male gametes. Transition in male gamete morphology from isogamous to oogamous gametes are observed in both green plant groups: the Chlorophyta and Streptophyta, which includes land plants. The gamete morphology of the Chlorokybophyceae is not known. The DUO1 MYB domain transcription factor in land plants plays a role in sperm morphogenesis and the presence of DUO1 function coincides with anisogamy in streptophyte algae. *DUO1* is also present in conjugating algae, the Zygnematophyceae; however, its function is lost in these

species. In land plants, oogamy further changed to siphonogamy where male gametes are transferred to female gametes by a pollen tube. Male gametes in all angiosperms and many gymnosperms are not motile, whereas early diverging gymnosperms such as *Ginkgo* and cycads produce motile sperm in the pollen (see Fig. 2 for details). During land plant evolution, the *DUO1* gene regulatory network has evolved to control not only sperm morphogenesis, but also fertilization by integrating genes for gamete attachment (*GEX2*) and fusion (HAP2/GCS1) under *DUO1* control

maternally and is fertilized internally. These provide controlled conditions where the development of the zygote can be supported maternally against environmental and natural threats, increasing the chance of survival (Fernando et al. 2010; Gasser and Skinner 2019; Hackenberg and Twell 2019; Nishiyama et al. 2018).

Streptophyte algae also show a transition from isogamy to oogamy (Fig. 1); however, the evolution of this group is not yet clear. One of the most early diverging streptophyte algal groups, the Mesostigmatophyceae, is unicellular (Lemieux et al. 2007) and appears to sexually reproduce via isogamy (Mori et al. 2015). Another early diverging streptophyte algal group, the Chlorokybophyceae, has genes involved in meiosis, suggesting sexual reproduction in its sole species (Wang et al. 2020). It still remains unknown what type of gametes it produces. The Charophyceae, a more complex, multicellular group, reproduce via oogamy with biflagellate sperm fertilizing a sessile egg cell present inside an oogonium, which is more complex than that of Chlorophyceae (Graham and McBride 1979; Nishiyama et al. 2018). Oogamy has been maintained in the Coleochaetophyceae and the early diverging land plants such as bryophytes and ferns (Cove and Knight 1993; Graham and McBride 1979; Lopez-Smith and Renzaglia 2008; Nishiyama et al. 2018).

Due to land plant-like features such as three-dimensional growth, and the presence of plasmodesmata and oogamy, the Charophyceae was thought to be the sister group to land plants (Evert and Eichhorn 2013). With the accumulation of molecular and genomic information, the Zygnematophyceae is now considered as the closest lineage to land plants (Cheng et al. 2019; Wickett et al. 2014; Wodniok et al. 2011). Although the loss of flagellated gametes in the Zygnematophyceae (Fig. 1) is still puzzling, the study of the *DUO POLLEN 1 (DUO1)* gene shed light on this enigma. DUO1 is a MYB-domain transcription factor (Rotman et al. 2005) that controls sperm morphogenesis in land plants (Borg et al. 2011; Higo et al. 2018). Gene duplication and neo-functionalization of an ancestral MYB transcription factor occurred in the common ancestor to the Charophyceae, Zygnematophyceae, and land plants, and led to the innovation of *DUO1* (Fig. 1). *DUO1* orthologs are present in land plants (Brownfield et al. 2009) as well as oogamous Charophyceae species, but not in early diverging streptophyte algae *Mesostigma viride* and *Klebsormidium flaccidum* (Higo et al. 2018). Although the precise function of *DUO1* in these algae is still not clear, *DUO1* coincides with sperm morphogenesis (i.e., spermatid formation) and *Chara* DUO1 can partly function as DUO1 in *Marchantia* (Higo et al. 2018). Interestingly, *DUO1* is also present in the Zygnematophyceae; however, its expression is minimized and the key amino acid residues of the MYB DNA binding domain have been intensively altered. These changes in *DUO1* in the Zygnematophyceae may account for their loss

of sperm morphogenesis and may have led to conjugation reproduction (Higo et al. 2018; Hisanaga et al. 2019). In contrast to these insights into sperm morphogenesis, how female gametes in the Streptophyta became sessile is still unclear.

It is estimated that the conquest of land by ancient green plants was achieved ~ 470–515 MYA (Harris et al. 2020). Early diverging land plants show oogamy with motile sperm which require conditions wet with environmental water for sperm to swim for fertilization. The number of flagella on sperm in ferns has increased (> 40) compared to (biflagellate) sperm in bryophytes. This increased number of flagella in ferns aids fertilization by helping sperm penetrate venter canal cells of the archegonium and thus reach the egg cells (Lopez-Smith and Renzaglia 2008; Southworth and Cresti 1997). During the evolution of seed-bearing plants, a game-changing innovation occurred: the generation of the pollen grain and tube (Fernando et al. 2010; Friedman 1993). In seed-bearing plants, sperm cells are encapsulated in the pollen grain, which is conveyed to a female organ via wind, insects, or self-pollination. Once the pollen grain lands on the female organ, a pollen tube extrudes from the grain and delivers sperm cells to the female gametophyte for fertilization; thus, environmental water is no longer required. Early diverging gymnosperms such as *Ginkgo biloba* and cycads still produce flagellated sperm in the pollen (Southworth and Cresti 1997). In these species, the extended pollen tube discharges sperm into secreted fluid near the egg cell and sperm, with numbers of flagella ranging from up to 1,000 in *Ginkgo* and 40,000 in cycads, swim a short distance within the fluid for fertilization (Southworth and Cresti 1997). Sperm cells in later-diverging gymnosperms such as *Gnetum gnemon*, *Ephedra nevadensis*, and conifers are not motile, possibly displaying an evolutionary change in sperm from motile to non-motile (Fig. 2).

The terrestrial conquest by angiosperms has covered many different environments around the globe with a vast number of species. This success was possible in part due to advances in reproductive mechanisms including the innovation of flowers and the internalization of ovules into maternal tissues (Baum and Hileman 2018; Specht and Bartlett 2009). Formation of flowers has led to evolution of flower-pollinator interactions, which greatly enhanced sexual reproduction efficiency. The harboring of ovules in carpels provides them further protection from the environment and predators (Simonini and Østergaard 2019). Sperm cells in all angiosperms are immotile and are transported directly to the female gametophyte by the pollen tube. In *Arabidopsis thaliana,* two sperm cells along with the vegetative nucleus in the pollen tube form the male germ unit that is conveyed to the female gametophyte for fertilization.

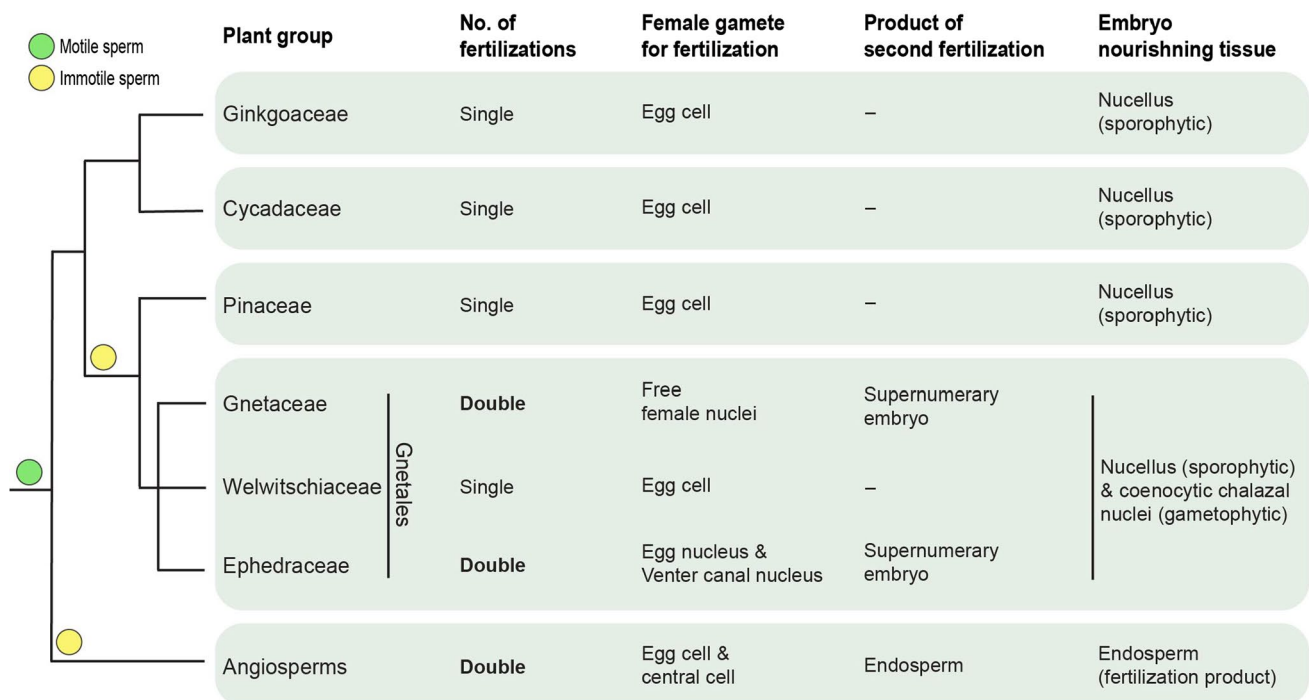Although DUO1 in *Marchantia* clearly has essential functions in sperm morphogenic processes such as

Fig. 2 Phylogeny of seed-bearing plants and their fertilization processes. The common ancestor of seed-bearing plants is conjectured to have had motile sperm, which has been retained in *Gingko* and cycads. Sperm motility has been lost in late-diverging gymnosperms, such as gnetales, the Pinaceae, and angiosperms. The basal form of double fertilization has been observed in Gnetaceae and Ephedraceae, which form two zygotes through two sperm delivered by a pollen tube, only one of which survives. Similarly, polyembryony is observed in the Pinaceae where multiple proembryos are formed by fertilization of sperm cells from multiple pollen tubes. Again, only one embryo survives. There is no sign of multiple fertilization events occurring in *Gingko* and cycads. Angiosperms show double fertilization, forming an embryo and embryo-nourishing endosperm. Similar embryo-nourishing tissues have also been observed in gymnosperms; however, their development is not linked with fertilization

flagella formation and chromatin condensation (Higo et al. 2018), angiosperm sperm cells do not generate flagella, raising the question of how *DUO1* is involved in sperm morphogenesis in angiosperms. The analysis of DUO1 downstream target genes (Borg et al. 2011) as well as investigation of DUO1s binding to *cis*-elements (Higo et al. 2018) showed that the *DUO1* gene regulatory network evolved concomitantly with changes in the mode of sperm delivery in land plants. In *Marchantia*, genes encoding components of the flagella and motility apparatus, as well as protamine-like proteins for chromatin condensation, are under *DUO1* control. Flowering plants have lost these genes and, instead, have incorporated new targets involved in gamete attachment (*GEX2*) and fusion (*HAP2/GCS1*) (see Plasmogamy section for details) (Higo et al. 2018). A sperm-specific histone (H3.10) (Ingouff et al. 2010), important for epigenetic memory reset (Borg et al. 2020), has also been integrated into the *DUO1* network.

Evolution of double fertilization

Double fertilization in angiosperms was first discovered by Guignard (1899), Nawaschin (1898). One sperm cell fuses with the egg cell to form an embryo while the other fuses with the central cell forming the endosperm, which provides nourishment to the developing embryo (Shin et al. 2020). Thus, in angiosperms, nutrient supply becomes dependent on the second fertilization that happens only after the egg cell has been successfully fertilized, allowing plants to focus nutrient/energy allocation only on viable offspring. Interestingly, orchids in general produce tiny seeds compared to other angiosperms, and a significant reduction of the endosperm has been observed. In some orchid species, there is no sign of endosperm development from the fertilized central cell (Kolomeitseva et al. 2020). These species likely underwent a different evolutionary direction in sexual reproduction and further molecular

characterizations will provide a better insight into their embryo and seed development.

Differing ploidy levels have been observed among angiosperm endosperms. Diploid (maternal to paternal = 1:1) endosperm is seen in the Nymphaeaceae (basal angiosperm) and Onagraceae (eudicot) (Baroux and Grossniklaus 2019; Williams and Friedman 2002). Species of the basal angiosperm Amborellaceae and also *Arabidopsis thaliana* are triploid (maternal to paternal = 2:1) (Baroux and Grossniklaus 2019; Kordyum and Mosyakin 2020). Maternal to paternal genomic content of the endosperm guides how nourishment is provided and used. Seeds with higher paternal to maternal ratios in their endosperms selfishly and aggressively derive more nutrition from the maternal sporophyte, causing defects in other developing embryos (Friedman and Ryerson 2009). While endosperm ploidy could be thought to have contributed to the direction of endosperm development, there is no obvious correlation with angiosperm evolution. Endosperm ploidy differences among different taxa could simply be attributed to the difference in embryo sac development (i.e., the number of polar nuclei) (Baroux et al. 2002; Kordyum and Mosyakin 2020). Further analyses are needed to further understand endosperm ploidy from an evolutionary perspective.

In gymnosperms, different forms of double fertilization have been observed (Fig. 2). In the order Gnetales, the female gametophyte of *Ephedra* contains two egg cells. Each egg cell is binucleate with a venter canal nucleus and egg nucleus. After the release of the binucleate sperm cell from the pollen tube, the two sperm nuclei enter the egg cell. One sperm nucleus fuses with the venter canal nucleus to form a supernumerary zygote while the other fuses with the egg nucleus. Both fused nuclei start to develop an embryo; thus, two embryos are formed per egg cell (polyembryony). Only one of the developing embryo matures while the other degenerates (Carmichael and Friedman 1996; Friedman and Carmichael 1996). A rudimentary polyembryony type of double fertilization has also been observed in *Gnetum gnemon* where the functional female gametophyte does not contain an egg cell per se but has a cell with free nuclei that act as individual gamete nuclei. During fertilization, each of the two sperm nuclei released from the pollen tube can fertilize any two free nuclei in their vicinity to form two zygotes. Ultimately, only one embryo survives at the end of seed maturation (Carmichael and Friedman 1996; Friedman and Carmichael 1996). In *Welwitschia mirabilis,* the micropylemost cells form tubular projections called prothalial tubes. The egg nuclei are carried in the prothalial tubes, which protrude into the nucellus, to meet the incoming pollen tube. Each prothalial tube carries two to three egg nuclei and one of the two sperm released from the pollen tube enters the prothalial cell and fertilizes an egg nucleus. In some rare conditions, double fertilization happens from a single pollen tube but generally only one sperm nucleus fuses with the egg nucleus while the other remains outside the prothalial cell (Friedman 2015).

In the early diverging gymnosperms, such as *Ginkgo* and cycads, two motile flagellated sperm are released from the pollen tube into the archegonium but only one sperm fertilizes the egg to form a zygote. Like in *Ginkgo* and cycads, double fertilization is not observed in the Pinaceae. In the Pinaceae, one ovule contains many archegonia and each archegonium harbors an egg cell that contains the egg nucleus and venter canal cells. A pollen tube reaches each archegonium, grows through the venter canal cell, ruptures it and releases two sperm nuclei. The leading sperm nucleus fuses with the egg nucleus, forming a proembryo. The other sperm nucleus deteriorates (Runions and Owens 1999). More than one embryo starts to develop from each fertilized egg cell in each ovule, but only one survives and matures as the others undergo programmed cell death (Filonova et al. 2002).

Although the end products are supernumerary zygotes/embryos, the second fertilization does therefore exist in gymnosperms (Fig. 2). Based on the current understanding of gymnosperm evolution (Fig. 2), the presence of "double fertilization" events in Gnetales and angiosperms is likely the result of convergent evolution and several hypotheses have been made to relate them to the origin of angiosperm double fertilization. One is that formation of the supernumerary embryo could be the key to the origin of the endosperm in the ancestral angiosperm and that a supernumerary embryo took on the function of embryo nourishing tissue. As it otherwise degenerates, like in *Ephedra*, one of the embryos instead might have become altruistic and switched its role to support its twin embryo's development. Another hypothesis is that the origin of the angiosperm endosperm was not the supernumerary embryo and the fertilization process happened to be incorporated into an already existing embryo nourishing tissue. In the case of *Gnetum* and *Welwitschia,* free nuclei are present at the chalazal end of the developing female gametophyte and provide nourishment to the embryo (Baroux and Grossniklaus 2019; Friedman 2015; Friedman and Carmichael 1996; Wang et al. 2014). This chalazal tissue with free nuclei becomes cellularized later, resembling the development of the endosperm in angiosperms. In *Arabidopsis*, the initiation of endosperm development can be independent of the fertilization event (Hands et al. 2016). Many different types of cells accept sperm at the time of fertilization in extant gymnosperms (Carmichael and Friedman 1996; Friedman 2015; Friedman and Carmichael 1996), thus incorporation of the fertilization process into a non-gametic cell appears not so uncommon. There are several key factors essential for fertilization processes such as gamete attachment and fusion, that underwent regulatory network rewiring during land plant evolution (see

Changes in Sperm Cells section for details). In either scenario, such rewiring of gene regulatory networks (Halfon 2017; Lai et al. 2020) should play a major role in the origin of angiosperm double fertilization. Comparing gene regulatory networks that control egg cell and central cell fertilization will further elucidate the evolutionary events that determined the angiosperm central cell's fate to be sexual.

One of the key pre-requisites for the innovation of double fertilization may be the presence of two male gametes in one pollen grain. Two sperm are also present in one pollen grain in gymnosperms and the incorporation of an additional mitotic division prior to sperm morphogenesis likely occurred before the divergence and gymnosperms and angiosperms. In early diverging land plants such as *Marchantia* and ferns, sperm morphogenesis coincides with the mitotic division of spermatid mother cells, and in *Arabidopsis*, major gene regulatory pathways controlling sperm differentiation and mitotic division are interlinked (Hackenberg and Twell 2019). More elucidation on the male side of reproductive evolution including the relationship between divisions and morphogenesis should shed light on the evolution of angiosperm double fertilization.

Fertilization mechanism

Plasmogamy

Discovery of *GAMETE EXPRESSED 2 (GEX2)* in *Arabidopsis* sperm cells localized on the plasma membrane (Mori et al. 2014) provided insights into the adhesion of the sperm cell membrane with that of the egg cell. GEX2 protein contains a filamin-like domain. A filamin-like domain is also present in FUS1 protein of *Chlamydomonas* where it helps in adhesion of two mating types to complete fertilization (Mori et al. 2014). A gene encoding a protein with a filamin-like domain is also found in *Selaginella* (Maruyama et al. 2016), and the *GEX2* homolog in *Zea mays* is important for fertilization (Mori et al. 2014; Warman et al. 2020). In *Arabidopsis, DUO1* controls the expression of *GEX2* and GEX2 is specifically found on the male germ cells (Maruyama et al. 2016; Sprunck 2020). Sperm cell specific expression of *GEX2* was also observed in rice (Sharma et al. 2011) and GEX2 promoters in angiosperms contain several DUO1 binding motifs (Higo et al. 2018), implying the incorporation of *GEX2* in the *DUO1* regulatory network predates the divergence of monocots and eudicots.

HAPLESS 2 (HAP2)/GENERATIVE CELL SPECIFIC 1 (GCS1) encodes a transmembrane protein that is specifically expressed in the gametes and essential for the gamete membrane fusion process. The mutant phenotype of *HAP2* was first discovered in a genetic screen of *Arabidopsis* that identified haploid disrupting (hapless) mutations of pollen

development and function (Johnson et al. 2004). Independently, a strongly expressed sperm-specific *GCS1* transcript was identified and shown to accumulate in the plasma membrane of generative cells of *Lilium longiflorum* (Mori et al. 2006). In their seminal study, Mori et al. identified that *gcs1* mutant sperm cells failed to fuse with the female gametes in *Arabidopsis* and also recognized that homologs are found in rice and, further afield, in green and red algae, social amoeba and protists including *Plasmodium falciparum* (Mori et al. 2006). von Besser et al. (2006) identified that *HAP2* is allelic with *GCS1*.

HAP2/GCS1 is redistributed from the sperm cell endomembrane system to the cell surface in response to the release of small Egg Cell 1 (EC1) proteins by the egg cell in *Arabidopsis* (Sprunck et al. 2012). EC1-like proteins are found in flowering plants, including basal angiosperm *Amborella trichopoda*, but not in gymnosperms, ferns or bryophytes (Sprunck et al. 2012). Mutant analyses confirmed that, once on the sperm cell membrane, HAP2/GCS1 has a role in the fusion of the gamete membranes rather than attachment to them as *hap2/gcs1* sperm cells were able to adhere to *Arabidopsis* female gametes but membrane fusion did not occur (Mori et al. 2014). Attachment of gametes, bringing membranes within 10 nm of each other, is a prerequisite for fusion (Sprunck 2020). HAP2's function as a fusogen was confirmed by its ability to cause fusion in heterologous systems (Valansi et al. 2017).

The ancient origins of *HAP2/GCS1* became even clearer following X-ray crystallographic structural studies. HAP2 of *Chlamydomonas* has a conserved fold similar to viral class II fusogens (Fédry et al. 2017). Based on well-studied viral fusogens (Fedry et al. 2018), HAP2 is proposed to trimerize and undergo a conformational shift driving membrane bending and pore formation during plasmogamy. HAP2s in various eukaryotes have a fusion loop or loops that is/are inserted into the lipid bilayer during membrane fusion. In the protozoan *Trypanosoma cruzi,* the three loops that are suggested to contact the female gamete contain nonpolar residues and are expected to interact together. In *Arabidopsis,* while the same three loops are present, one of them, containing an amphipathic helix, protrudes beyond the others and would be expected to enter the female gamete membrane (Fedry et al. 2018). While the overall fold of HAP2 is thought to be well conserved, based on amino acid alignment further differences are seen in the residues of the prominent surface loops and their helices among different flowering plants. Larger differences are found among *Selaginella moellendorffii*, *Physcomitrium patens,* and *Marchantia* (Fedry et al. 2018). These structural differences have been suggested to have evolved as those organisms diverged (Fedry et al. 2018) and, as well as those found in *Chlamydomonas* (Baquero et al. 2019; Feng et al. 2018), could relate to differences in the membranes and proteins they interact with.

Further, detailed investigations of the changes in interactions of HAP2, if any, that have occurred during evolution and speciation are awaited. Genes involved in reproduction and responsible for speciation evolve relatively quickly (Swanson and Vacquier 2002). Interestingly, comparing the relative divergence of *Arabidopsis* and the closely related *A. lyrata*, *HAP2/GCS1* evolved more slowly than the *GEX2* gene for gamete attachment (Mori et al. 2014). Maybe attachment genes evolve faster than those involved in fusion and are more usually responsible for speciation (Mori et al. 2014). Even if so, how the structural changes that have evolved in HAP2 may account for speciation remains to be explored in detail.

Sperm membrane proteins DUF679 DOMAIN MEMBRANE PROTEIN 8 and 9 (DMP8/9) are other key regulators of sperm-egg cell fusion in *Arabidopsis* (Cyprys et al. 2019; Takahashi et al. 2018). Like *HAP2/GCS1* and *GEX2*, at least *DMP9* is regulated by *DUO1* (Borg et al. 2011), and *dmp8/dmp9* mutants show a defect in fertilization, yet with normal sperm-egg cell adhesion, indicating their functions particularly in the egg-sperm fusion. *DMP9* orthologs are present in *Chlamydomonas* (Ning et al. 2013), which suggests that there are conserved DMP8/9 sperm-egg cell interaction pathways among green plants. Interestingly, *dmp8/9* mutants show no or minimal effect on sperm-central cell fusion (Cyprys et al. 2019; Takahashi et al. 2018). These findings indicate that the fertilization process between the egg and central cells are not exactly the same, and these differences might be contributing to the stronger polyspermy block observed in the egg cell compared to the central cell (Nagahara et al. 2021; Scott et al. 2008).

Gamete nuclear migration

In most animals, not only the sperm pronucleus, but also the sperm centrioles are delivered to the fertilized egg. The inheritance of paternal centrioles has been also observed in brown algae (Nagasato 2005; Nagasato et al. 1999) and these sperm derived centrioles generate a centrosome, which then establishes a microtubule sperm aster that facilitates pronuclear migration for the completion of fertilization. The importance of microtubules for pronuclear migration has been evident genetically and pharmacologically (Fatema et al. 2019). Likewise, early diverging land plants such as bryophytes and ferns produce flagellated motile sperm, and pharmacological studies using a fern, *Marsilea vestita*, suggested the important role of microtubules in early events of fertilization including gamete nuclear migration (Kuligowski et al. 1987, 1985). Flowering plants, on the other hand, have evolved non-motile sperm cells and have lost components essential to centriole organization (Carvalho-Santos et al. 2011), raising the question of how do flowering plants control gamete nuclear migration for fertilization?

Pharmacological studies in rice, tobacco, and maize show that microtubules are dispensable and, instead, F-actin plays the critical role in sperm nuclear migration (Ohnishi et al. 2014; Peng et al. 2017). This was confirmed by genetics in *Arabidopsis* (Kawashima et al. 2014). Interestingly, F-actin in the female gamete cells generate constant inward movement prior to fertilization: F-actin is assembled at the plasma membrane periphery and migrates toward the center of the cell where the female nucleus resides (Fig. 3a). Upon sperm delivery to the female gamete cell, the sperm nucleus starts moving, together with F-actin, for karyogamy.

In *Arabidopsis*, the small Rho GTPase of plants 8 (ROP8) controls the dynamic F-actin inward movement in the central cell (Fig. 3b) (Kawashima et al. 2014). ROPs are associated with the plasma membrane and activate the WAVE/SCAR signaling pathway that controls cytoskeleton organization (Craddock et al. 2012; Uhrig et al. 2007). For somatic F-actin dynamics, WAVE/SCAR relays to ARP2/3 and activates branching of F-actin in vitro (Frank et al. 2004; Zhang et al. 2008) and in trichomes (Basu et al. 2005; Zhang et al. 2005). Very recently, it has been discovered that SCAR2 plays a role in the F-actin inward movement in the central cell, and surprisingly, that not ARP2/3, but another actin nucleator group, formin, is involved in this process (Fig. 3b) (Ali et al. 2020; Ali and Kawashima 2021). These data show that there is a novel gametophyte-specific ARP2/3-independent WAVE/SCAR signaling pathway, controlling fertilization processes. In addition, the class XI myosin, XI-G was identified to play an important role in the F-actin inward movement in the *Arabidopsis* central cell (Fig. 3b) (Ali et al. 2020). Class XI myosins in plants walk on F-actin and transport molecules and organelles as cargo (Duan and Tominaga 2018). In the central cell, XI-G appears to generate forces to move F-actin per se. This action is independent from the organelle movement function of myosins (Ali et al. 2020), indicating a novel non-canonical class XI myosin function in the female gametophytic cell.

All factors identified for sperm nuclear migration in *Arabidopsis* (i.e., ROP8, SCAR2, and XI-G) are from the central cell (Ali et al. 2020; Kawashima et al. 2014); however, the egg cell also requires proper sperm nuclear migration for successful fertilization. Furthermore, the function of WAVE/SCAR and myosin is also important in the egg cell (Ohnishi et al. 2014; Peng et al. 2017). Because the expression of *ROP8* and *XI-G* are not detected in the egg cell (Ali et al. 2020; Kawashima et al. 2014), the simplest scenario is that other members of ROP, SCAR, and myosin XI are responsible for sperm nuclear migration in the egg cell. If this is the case, another interesting question would arise: what are the biological and evolutionary reasons why the egg and central cells utilize different members of factors for the same function? The fertilization processes of the egg and central cells are also different, like in the case of
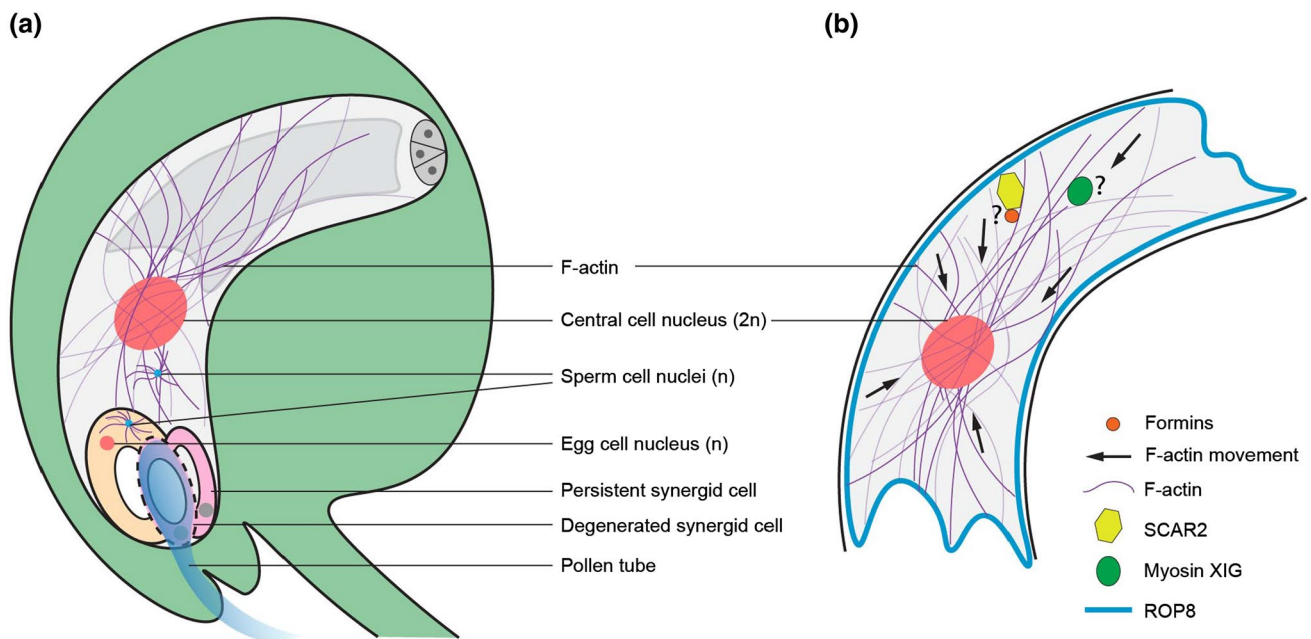
(a)

(b)

Fig. 3 Schematic representation of sperm nuclear migration mechanism in *Arabidopsis*. **a** The pollen tube enters through the micropyle and releases two sperm cells in between the egg and central cells. One of the sperm cells is destined to fertilize the egg cell while the other fertilizes the central cell. Inside the fertilized egg and central cells, the sperm nucleus becomes surrounded by F-actin, generating a sperm F-actin aster. The sperm F-actin aster moves to the female nucleus for karyogamy. **b** There is a continuous F-actin inward movement, from the plasma membrane periphery to the nucleus in the cen- tral cell. This movement is essential for sperm nuclear migration and is controlled by formins and the plasma membrane-anchored plant RHO-GTPase, ROP8, and its downstream signaling factor SCAR2 via a novel ARP2/3-independent manner. The class XI myosin, XI-G, also plays a positive role in the F-actin movement. It remains unknown how these factors are integrated to control the F-actin movement. Factors responsible for the F-actin movement in the egg cell still remain unknown

DMP8/9 (see Plasmogamy section for details). Understanding the evolutionary changes in these factors in the egg and central cells will also elucidate the origin of angiosperm double fertilization.

Karyogamy

After sperm nuclear migration, fusion of gamete nuclei (karyogamy) takes place, ending fertilization and initiating embryo and endosperm development in angiosperms. The nuclear envelope consists of two lipid bilayer membranes (inner and outer nuclear membranes). The immunoglobulin binding protein (BiP), a molecular chaperone of the Hsp70 family of proteins in the endoplasmic reticulum (ER), and its partner, J-domain-containing protein, play roles in each nuclear membrane fusion during karyogamy. The fusion of the outer nuclear membranes is critical for sperm chromatin decondensation in the *Arabidopsis* central cell and the onset of endosperm development (Maruyama et al. 2020). The involvement of BiP and J-proteins in karyogamy has also been evident in yeast (Ng and Walter 1996; Nishikawa and Endo 1997; Rose et al. 1989). Other karyogamy factors highly conserved among organisms are the integral nuclear membrane protein, Kar5 family proteins. *Kar5* was first identified in yeast (Beh et al. 1997) and is required for outer nuclear membrane fusion. The ortholog of *Kar5* has been found in many species including zebrafish, *Chlamydomonas*, and *Arabidopsis* (Abrams et al. 2012; Ning et al. 2013; Nishikawa et al. 2020; Speijer et al. 2015), indicating that, while the mechanism of gamete nuclear migration has diverged, the molecular repertoire for karyogamy is highly conserved and has been maintained during evolution.

Concluding remarks

With the rapid accumulation of omics data in many species, both conserved and altered molecular processes in sexual reproduction in land plants have become gradually evident. In particular, DUO1 functions in the Streptophyta have highlighted how neo-functionalization of transcription factors and rewiring of gene regulatory networks can explain a part of evolution in land plant sexual reproduction (Higo et al. 2018). Further molecular dissections of streptophyte algae will further advance our understanding of land plants. Extant gymnosperms display evolutionary changes in sperm motility and some species show an early form of double fertilization. In orchid species, the endosperm has been largely

reduced and some species do not appear to have endosperm development. Thus, not only model plants like *Marchantia* and *Arabidopsis*, but such species should also be further investigated, and this would provide new insights into the evolution of sexual reproduction in land plants. In addition, it would be useful to know the biological and/or evolutionary meanings of the egg and central cell's utilization of different genes for controlling sperm nuclear migration in *Arabidopsis*. Together with sperm evolution, plasmogamic paternal contributions to fertilization and the onset of embryo/endosperm development (Ohnishi and Kawashima 2020) is also an exciting field to study from an evolutionary perspective. We have now many resources and methods available to address these questions and revealing the remaining evolutionary mystery of angiosperm sexual reproduction could be a big step in the fundamental understanding of overall green plant evolution.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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