The evolution and patterning of male gametophyte development

Dieter Hackenberg* and David Twell*

Department of Genetics and Genome Biology, University of Leicester, University Road,
Leicester LE1 7RH

*Authors for correspondence: Dieter Hackenberg (dh253@le.ac.uk) and David Twell (twe@le.ac.uk)

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Abstract: The reproductive adaptations of land plants have played a key role in their terrestrial colonization and radiation. These encompass mechanisms used for the production, dispersal and union of gametes to support sexual reproduction. The production of small motile male gametes and larger immotile female gametes (oogamy) in specialized multicellular gametangia evolved in the charophyte algae, the closest extant relatives of land plants. Reliance on water and motile male gametes for sexual reproduction was retained by bryophytes and basal vascular plants, but was overcome in seed plants by the dispersal of pollen and the guided delivery of non-motile sperm to the female gametes. Here we discuss the evolutionary history of male gametogenesis in streptophytes (green plants) and the underlying developmental biology, including recent advances in bryophyte and angiosperm models. We conclude with a perspective on research trends that promise to deliver a deeper understanding of the evolutionary and developmental mechanisms of male gametogenesis in plants.

1. The evolutionary history of the male gametophyte

1.1 The origins of spermatogenesis in green plants

The ancestral mode of sexual reproduction in plants is isogamy (gametes of similar size and morphology) and is described in basal chlorophyte and charophyte algae. Oogamy (sperm and egg mating systems) evolved independently in both algal lineages, and the transition from isogamy to oogamy is associated with increasing complexity and colonisation (reviewed in Mori et al., 2015; Umen, 2014).

The Volvocine algae have been developed as a model for the evolution of reproductive traits among chlorophytes. The unicellular species *Chlamydomonas reinhardtii* is isogamous and one of the most basal species (Nozaki et al., 2000), whereas colonial and multicellular genera can be isogamous, anisogamous or oogamous (Mori et al., 2015). The differentiation of *plus* and *minus* gametes in *C. reinhardtii* is determined by a 200-300 kb multigenic mating type (MT) locus. Gametes are *MT*⁻, if the *MID* (*minus* dominance) gene is present or *MT*⁺, if *MID* is absent (Ferris et al., 2002; Ferris & Goodenough, 1994). *MID* encodes an RWP-RK domain transcription factor and acts as a master regulator of sexual differentiation (Ferris & Goodenough, 1997). From its ancestral role as a mating-type factor in *C. reinhardtii*, *MID* has evolved to become a determinant of sperm and egg development in oogamous *Volvox carteri* (Geng et al., 2014). Although orthologs of *MID* are present in land plant genomes, their functions are currently unknown (Rövekamp et al., 2016).

In charophyte algae the phylogenetic transition from isogamy to anisogamy (Figure 1) and the genes responsible for sex determination remain unclear. With the exception of the unicellular isogamous *Mesostigma viride*, sexual reproduction in most charophyte lineages is unknown (Delwiche, 2016; McCourt et al., 2004; Mori et al., 2015). However, late divergent lineages, such as Charales and Coleochaetales, developed oogamous reproduction with bi-flagellated sperm (Renzaglia & Garbary, 2001). The stoneworts of the genus *Chara* produce gametes in morphologically complex gametangia, known as globules (antheridia) (Figure 2A) or nucules (oogonia), that are speculated to be homologous to gametangia of basal land plants (Niklas & Kutschera, 2009). Interestingly, the Zygnematales, an extant charophyte order sister to land plants (Wodniok et al., 2011), secondarily lost flagella and reproduce through isogamous conjugation (Mccourt et al., 2004).

1.2 The sporophyte was key for terrestrial plant evolution

Charophyte algae developed a haplontic life cycle, in which the haploid stage is multicellular and the only diploid stage is the unicellular zygote (Shaw et al., 2011). The haplodiplontic life

cycle, which involves the alternation of multicellular haploid and diploid generations, evolved with the colonization of terrestrial habitats (Figure 1). This transition requires a delay of meiosis and the intercalation of mitotic cell divisions; the resulting multicellular sporophyte thereby amplifying haploid spore production from each zygote. The limited capacity of charophyte algae for dispersal via motile sperm, therefore gave way to terrestrial colonisation via dissemination of durable spores shed by increasingly complex and dominant sporophytes (reviewed in Niklas & Kutschera, 2009).

The production of desiccation-tolerant spores by elevated sporophytes promoted aerial dispersal of gametophytes (Strother et al., 1996; Wellman, 2010). This key innovation was enabled by synthesis of the resistant biopolymer sporopollenin in spore walls (Niklas & Kutschera, 2009). The ability to produce sporopollenin is shared by chlorophyte and charophyte algae (Brooks & Shaw, 1978) and is considered pre-adaptive for terrestrial colonization (Wallace et al., 2011). The homology between ‘lower’ plant spores and the pollen of seed plants is bolstered by the presence of homologues of pollen wall associated genes in moss (Wallace et al., 2011). Further, the GAMYB family of transcription factors, which have been shown to regulate sporopollenin biosynthesis genes, are required for the formation of functional spores in moss and for pollen wall formation in angiosperms (Aya et al., 2011, 2009).

The molecular control of the haploid-to-diploid transition seems to share common ancestry in green algae and land plants. In *C. reinhardtii*, *plus* and *minus* gametes express either BEL1-LIKE (BELL) or KNOTTED1-LIKE HOMEODOMAIN (KNOX) class homeodomain proteins, respectively. After gamete fusion, these proteins heterodimerize to initiate zygote development and meiosis (Lee et al., 2008). Similarly, KNOX/BELL heterodimers pattern sporophyte development in moss (*Physcomitrella patens*) and in flowering plants, but are neither functional nor expressed during the gametophytic phase (Hay & Tsiantis, 2010; Keiko et al., 2008). While antagonistic roles are proposed for the duplicated KNOX1 and KNOX2 gene classes in sporophytic development in angiosperms, KNOX2 genes actively represses

the gametophytic body plan in sporophytes of *P. patens* (Furumizu et al., 2015; Sakakibara et al., 2013).

In bryophytes, the gametophytic phase is dominant and the mature gametophyte is either a thallus or a leafy shoot with indeterminate growth (Ligrone et al., 2012). Male (antheridia) and female (archegonia) gametangia are commonly produced by differentiation of the growing tip of the shoot (gametophore), producing either sperm or eggs cells by mitotic division. In the liverwort *Marchantia polymorpha*, antheridia consist of spermatogenous cells surrounded by a single layer of sterile jacket cells (Figure 2B; Durand, 1908; Shimamura, 2016). The spermatogenous cells undergo proliferative mitotic divisions, eventually dividing diagonally to form a pair of triangular sperm cells (Durand, 1908). Similar to their charophycean ancestors, the bryophytes (liverworts, hornworts and mosses) release motile sperm into environmental water (Figure 3A). During spermatogenesis the cells undergo complex morphological changes, including extreme cytoplasmic reduction, nuclear condensation, and flagella assembly (see section 3.1; Renzaglia & Garbary, 2001).

[insert Figure 1 here]

Figure 1. Evolution of the gametophyte and sporophyte in the land plant lineage. The upper panel illustrates the trend from a dominant haploid gametophyte to that of the diploid sporophyte. Charophyte algae have a haplontic life cycle and possess a multicellular gametophyte, but only the zygote is diploid. The haplodiplontic life cycle and multicellular sporophyte evolved in bryophytes, while complex sporophytes are dominant in vascular plants (eg. monilophytes and spermatophytes). In spermatophytes (ie. gymnosperms and angiosperms), the gametophyte is reduced to a few cells and is entirely dependent on the sporophyte. The lower panel shows a cladogram of streptophyte (green plant) taxa indicating their estimated times of divergence and associated reproductive adaptations. Geologic eras are colored; Cenozoic (white), Mesozoic (blue), Paleozoic (green), Neoproterozoic (brown).

1.3 Heterospory and the origin of microgametophytes

The increase in size and complexity of the sporophytes of vascular plants is matched by corresponding reductions in their gametophytes (Figure 1). Basal extant lycophytes and monilophytes produce free-living exosporic gametophytes (prothallia) with flattened thalloid anatomy, or subterranean tuber-like structures (Ligrone et al., 2012). These taxa retain an oogamous life style and prothallia produce gametes in separate male and female gametangia, similar to those of bryophytes.

The spores of extant bryophytes and basal vascular plants are uniform in size, a condition known as homospority. Heterospority, defined as the production of smaller male microspores and larger female megaspores, arose independently in all major vascular plant lineages (lycophytes, monilophytes and spermatophytes) and is considered a key innovation for terrestrial colonization (Bateman & DiMichele, 1994; Petersen & Burd, 2016). Endospority, the development of gametophytes within the spore wall, is tightly coupled to unisexuality and is proposed as an innovation that allowed the evolution of heterospority (Bateman & DiMichele, 1994). The transition to endospority increased the influence of the diploid parent on gametogenesis, and allowed spore production and release to be synchronized with favourable environmental conditions.

The reduction of the gametophyte and transition to endospority is exemplified by comparison of the male gametophytes of the dioicous liverwort *M. polymorpha* and the heterosporous water fern *Marsilea vestita*. In *M. polymorpha*, antheridial initial cells arise from epidermal cells of the receptacle on a reproductive branch called the antheridiophore (Durand, 1908). Initial cells divide several times prior to a formative asymmetric division, which gives rise to a spermatogenous mother cell and a sterile jacket cell. Each mother cell undergoes multiple rounds of division to produce several thousand spermatozooids within each antheridium (Figure 2B). In contrast, microspores of *M. vestita* complete one asymmetric and one symmetric division before spermatogenous cells are produced by a formative asymmetric division. The spermatogenous cells undergo four rounds of symmetric division to produce a total of 32 spermatozooids within each microspore (Figure 2C; Sharp, 1914).

This trend towards fewer antheridial divisions prior to spermatogenesis culminates in the highly reduced male (micro)gametophytes of seed plants (Figure 2D - F). In gymnosperms, microspores undergo asymmetric cell divisions to produce one or more prothallial cells, prior to segregation of a spermatogenous cell lineage (Figure 2D and E). Reduction of the microgametophyte is most extreme in angiosperms, which require only two cell divisions to produce two non-motile sperm cells from the microspore (Figure 2F). This indicates that the asymmetric cell divisions which produce one or more prothallial cells in gymnosperms are absent, and the germline is directly segregated by asymmetric division of the microspore (see Rudall & Bateman 2007).

[insert Figure 2 here]

Figure 2: Overview of cell division patterns during male gametogenesis in representative streptophytes. Sperm cell lineage nuclei are green; primary microspore nucleus (C to F) is blue; basal antheridial/stalk (A and B) and prothallial cell nuclei (C to E) are purple; non-reproductive cell nuclei, derived from the male reproductive lineage, are orange. (A) *Chara spp.* globule (antheridium) development starts with division of an apical initial cell to form apical (green) and basal (purple) antheridial cells. Apical cells form inner primary capitular cells (green) and outer shield cells (orange). Capitular cells give rise to antheridial filament initial cells (green). Antheridial filament cells divide and then differentiate to form biflagellate spermatozoids (green), whereas filament initial cells become non-reproductive (orange). (B) *Marchantia polymorpha* antheridia are formed by division of an antheridial initial to form outer primary antheridial cells (green) and inner stalk cells (purple). Primary antheridial cells divide asymmetrically to generate sterile jacket cells (orange) and spermatogenous cells (green), which divide repeatedly. Each spermatogenous mother cell divides diagonally before differentiating into bi-flagellated spermatozoids. (C) *Marsilea vestita* microspores (blue) divide asymmetrically to form an antheridial initial cell (green) and a prothallial cell (purple), which no longer divides. The antheridial initial divides symmetrically and both daughter cells (green) complete three rounds of asymmetric division to produce three sterile jacket cells

(orange) and a primary spermatogenous cell (green). Each spermatogenous cell divides to form four spermatocyte mother cells (green), which divide into eight spermatocytes. These cells divide to form 16 spermatids which differentiate into multiflagellated spermatozooids. (D) *Gingko biloba* microspores (blue) divide twice to form one antheridial (green) cell and two prothallial (purple) cell that do not divide further. The antheridial cell forms the generative cell (green) and tube cell (orange) by asymmetric division. The generative cell divides again to form a sterile stalk cell (orange) and a body cell (green), which divides and differentiates to produce multiflagellated sperm (see Figure 3C). (E) *Gnetum gnemon* microspores (blue) divide asymmetrically to form an antheridial cell (green) and a single prothallial cell (purple). The antheridial cell undergoes asymmetric division to produce generative cell (green) and a tube cell (orange). The generative cell divides to form two non-motile sperm nuclei. (F) *Arabidopsis thaliana* microspores (blue) divide asymmetrically to form the generative cell (green) and the vegetative cell (orange). The generative cell undergoes a second symmetric division to form two non-motile two sperm cells (green). The figure is adapted and redrawn from (A), Kwiatkowska et al. (2002), (B), Durand (1908), (C), Tsai et al., (2004), and (E-F), Rudall & Bateman (2007).

1.4 Adaptations of seed plant microgametophytes

The development of male and female gametophytes of seed plants (spermatophytes) take place in micro- and megasporangia formed on modified leaves (sporophylls) in cones or in flowers. In contrast to most free-sporing plants, immature microgametophytes of seed plants are dispersed as desiccation-tolerant pollen. The evolution of pollen is a milestone in the adaptation of seed plants to terrestrial habitats. By contrast, the endosporic female megagametophyte remains partially or completely enclosed by sporophytic tissues, the nucellus and integument (see Gasser & Skinner, this issue; Erbasol Serbes et al., this issue). Thus, sperm cells must either swim to the megagametophyte enclosed by the integument or be delivered via the pollen tube.

With some important exceptions, extant gymnosperms and angiosperms transitioned from zooidogamy (swimming sperm) to siphonogamy (conduction of non-motile sperm via a pollen tube) (Friedman, 1993; Gifford & Foster, 1989). In cycads and in *Ginkgo biloba*, pollen tubes have a primarily haustorial role to support the development of large flagellated spermatozoids (Figure 3C; Friedman & Gifford, 1997). Flagellated spermatozoids of these taxa develop slowly after pollination and are released proximally into a pollen chamber. Spermatozoids subsequently migrate to the egg cells at the micropylar end of the ovule (Rudall & Bateman, 2007).

In gymnosperms, pollen reception must be relatively near to the egg as pollen tubes are relatively slow growing. Furthermore, the time between pollination and fertilisation can take days to months. In angiosperms, pollen tube growth is significantly faster, shortening the progamic phase to hours or minutes, thereby facilitating the evolution of rapid reproductive cycles (Williams, 2008). The evolution of fast-growing angiosperm pollen tubes is linked with modifications to pollen tube walls, including callose-reinforced secondary tube walls, callose plugs, and extensible pectin tips. Rapid pollen tube growth and longer-distance growth are considered to be key features enabling carpel diversification and the multiplicity of flower and fruit form (Williams, 2008).

Double fertilization *sensu stricto* is a synapomorphy (derived trait shared by two or more taxa) of angiosperms and occurs following the delivery of a pair of sperm cells to the ovule, resulting in the formation of embryo and endosperm. In the Gnetales however, a modified form of this process produces a supernumerary embryo (Carmichael & Friedman, 1995; Friedman, 1998, 1990). Double fertilisation enhances the fitness of the embryo, but also contributes to maternal fitness, as resources are only committed to the ovule after the egg and central cell have been fertilized. The two sperm cells can be functionally equivalent as demonstrated for *Arabidopsis thaliana* (Hamamura et al., 2011; Ingouff et al., 2009; Kong et al., 2015), and the majority of species show an equal distribution of DNA-containing

organelles among the two sperm (Saito et al., 2002). However, dimorphic sperm, which show preferential fertilization and differing gene expression profiles, are described for *Plumbago zeylanica* (Gou et al., 2009; Russell, 1985).

An important life history trait for adaptation to particular ecological niches is the length of the life cycle. In time-limited habitats, short lifecycles are commonly associated with an annual habit, shorter bud development time, and reduced flower longevity (i.e. reduced time between flower maturation and fertilization) among other reproductive traits (Snell & Aarssen, 2005). Although gymnosperms disperse sexually immature pollen (Fernando et al., 2010; Friedman, 1999), angiosperms have evolved mechanisms to minimize the length of the reproductive phase. This involves the advancement of pollen maturity before dispersal. In about 70% of angiosperms, pollen is dispersed in a bicellular state and division of the generative cell takes place during pollen tube growth. The remainder undergo precocious division of the generative cell and disperse tricellular pollen containing two sperm cells (Brewbaker, 1967; Williams et al., 2014). Tricellular pollen is considered advanced and is associated with shorter reproductive cycles, reduced longevity, rapid germination, and faster pollen tube growth (Brewbaker, 1967; Rowe et al., 2012; Williams, 2012). Although less common, tricellularity does not appear to be irreversible and multiple instances of secondary shifts from tricellular to bicellular pollen are postulated (Williams et al., 2014). In rare cases, as in *Annona cherimola*, a mixture of bicellular and tricellular pollen is dispersed and the onset of generative cell division is advanced by increases in air temperature (Lora et al., 2009). This may be adaptive, potentially providing plasticity in response to dispersal conditions.

In summary, the advanced microgametophytes of seed plants evolved from their embryophyte ancestors by the acquisition of several key features. These are, endospory and an extreme reduction of mitotic cell divisions prior to segregation of the male germline *sensu stricto*, increased sporophytic dependency, and adaptation to a siphonogamous gamete delivery

system involving the loss of gamete motility. Subsequent diversification in the onset and pace of gametogenesis has enabled the microgametophytes of angiosperms to adapt to a diversity of niches.

[insert Figure 3 here]

Figure 3: Micrographs of motile spermatozooids of plants. Scanning electron micrographs of (A), bi-flagellated spermatozoid of the liverwort *Conocephalum conicum* (reprinted with permission from Renzaglia & Garbary, 2001), (B), multi-flagellated spermatozoid of the leptosporangiate fern *Ceratopteris richardii* (reprinted with permission from Lopez-Smith & Renzaglia, 2008) and (C), light micrograph of motile sperm of the gymnosperm *Ginkgo biloba* (reprinted with permission from Suzuki et al., 2000). Scale bars, (A, B), 2.0 μm , (C), 80 μm .

2. Male germline development in angiosperms

Molecular and genetic studies of male germline development documented in earlier reviews are dominated by those of angiosperms (Berger & Twell, 2011; Russell & Jones, 2015; Twell, 2011). Other recent reviews focus on omics (Fíla et al., 2017; Rutley & Twell, 2015), chromatin remodelling (Borg & Berger, 2015), post-transcriptional processes (Guan et al., 2013; Hafidh et al., 2016; Michard et al., 2017), or wider aspects of reproductive cell lineages (Schmidt et al., 2015). Here we focus on regulatory aspects of male gametogenesis including comparative studies and advances in pre-angiosperm models. We discuss the role of asymmetric division and cell cycle control in patterning, and the roles and regulation of transcription factors in cell specification; we also highlight chromatin remodelling and communication between germline and non-germ cells.

2.1 Asymmetric microspore division and cell fate determination

Studies in angiosperms have shown that asymmetric microspore division is formative and essential for the establishment of germ cell fate. The induction of symmetric microspore divisions by environmental or chemical agents, such as the microtubule poison colchicine,

results in daughter cells with vegetative cell-like nuclei (Tanaka & Ito, 1980; Terasaka & Niitsu, 1987) and vegetative cell marker expression (Eady et al., 1995). Mutations which disturb microspore polarity or division have also been informative. In *gemini pollen1* (*gem1*) mutants of *A. thaliana*, microspores can undergo equal divisions and both daughter cells adopt vegetative cell fate, similar to the products of chemically-induced symmetric divisions (Park et al., 1998). The pollen phenotypes of *gem1* result from loss of microspore polarity and cytokinesis defects, arising from mutations affecting the MICROTUBULE-ASSOCIATED PROTEIN215 (MAP215)-family protein MICROTUBULE ORGANIZATION1 (MOR1)/GEM1 (Figure 4; Park et al., 1998; Twell et al., 2002; Whittington et al., 2001). Vegetative cell fate is also adopted in single-celled pollen resulting from colchicine treatment of tobacco microspores (Eady et al., 1995), or from cytokinesis defects (Park et al., 1998; Twell et al., 1998). Recent studies of additional single-celled pollen mutants confirm vegetative cell fate as the default programme, and show that pollen tubes are guided and discharge their contents in the absence of gametes (Glöckle et al., 2018; Zhang et al., 2017).

A novel class of cytokinesis mutants, which fail to compartmentalize newly-formed vegetative and generative cell nuclei, provides insight into the mechanisms underlying germ cell fate determination. Microspores polarize in *two-in-one* (*tio*) (Oh et al., 2005) and *Kinesin-12A/B* double mutants (Lee et al., 2007), but failure of cytokinesis results in binucleate pollen unable to differentiate sperm. The TIO *Fused* kinase and KINESIN-12 interact to form a signalling module (Figure 4), which is thought to act via the canonical NACK-PQR MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) pathway (Soyano et al., 2003) to regulate phragmoplast expansion (Oh et al., 2008, 2012). Collectively, mutants of this class form a presumptive generative cell nucleus, but this fails to divide and nuclear chromatin is gradually dispersed, consistent with the default vegetative cell fate. Thus, germ cell fate cannot be established without confinement of daughter nuclei to unequal compartments.

The correct developmental timing of mitosis and spindle orientation are essential requirements for asymmetric microspore division and cell fate determination. Mutations in *SIDECAR POLLEN (SCP)* disturb the orientation of microspore division (Figure 4), resulting in tricellular pollen containing an extra vegetative cell (Chen & McCormick, 1996). Defects arise following delayed microspore division, indicating close coupling of cell cycle progression and spindle orientation (Oh et al., 2010). SCP is a microspore-specific LATERAL ORGAN BOUNDARIES-DOMAIN/ASYMMETRIC LEAVES2-LIKE (LBD/ASL) domain family protein, whose expression overlaps with that of other family members in developing pollen. SCP (LBD27) and LBD10 form nuclear-localised heterodimers and act synergistically to control pollen development (Kim et al., 2015). Thus, combinatorial interactions among LBD proteins are thought to be important for their roles in the male gametophyte (Kim et al., 2016).

In summary, the asymmetric division of angiosperm microspores is critical for the formation and specification of the male germline *sensu stricto*. Studies of a variety of mutants strengthen the hypothesis that germ cell fate is specified by the segregation and confinement of yet unknown determinants by a new cell wall (Twell, 2011; Twell et al., 1998).

2.2 Cell cycle control and male gametophyte patterning

Correct patterning of the male gametophyte requires differential control of the cell cycle in vegetative and generative cells. Failure of generative cell division, resulting in bicellular pollen with a single germ cell, has been described for several *A. thaliana* mutants. Among the genes affected are those encoding the core cell cycle machinery, like the A-type cyclin-dependent kinase (CDK), CDKA;1. In *cdka;1* mutants, S-phase is retarded and generative cells are only able to divide during pollen tube growth (Figure 4; Aw et al., 2010; Iwakawa et al., 2006; Nowack et al., 2005). CDKA;1 activity and cell cycle progression are maintained in the generative cell by the proteasome-dependent degradation of the KIP-RELATED PROTEIN (KRP) CDK inhibitors. KRPs are targeted for degradation by the SCF^{FBL17} (SKP-

CULLIN-F-BOX) E3 ubiquitin protein ligase complex, which contains the F-box protein FBL17 (Figure 4; Kim et al., 2008). In *fb17* mutants, generative cells fail to divide as CDK inhibitors are not degraded (Gusti et al., 2009; Kim et al., 2008). The transient expression of *FBL17* only in the generative cell soon after microspore division is thought to license proliferation of the germline, while KRPs are maintained in the vegetative cell (Figure 4; Kim et al., 2008). Importantly, the *FBL17-KRPs-CDKA;1* cascade is linked to the *RBR1-E2F* pathway to form a general G1/S regulatory module that also functions in microspores of *A. thaliana*, but is likely to control entry into S-phase in all cells (Zhao et al., 2012).

Patterning of angiosperm microgametophytes is also determined by the retinoblastoma (*Rb*)-*E2F* pathway, which regulates cell cycle progression in a variety of organisms (Figure 4; Gutzat et al., 2012; Harbour & Dean, 2000). *Rb* function is dependent on its phosphorylation by CDK in late G1-phase. This alleviates Rb-mediated repression of the E2F/DP family transcription factors which commit cells to S-phase (Weinberg, 1995). The *A. thaliana* *Rb* homologue *RETINOBLASTOMA-RELATED1* (*RBR1*) is highly expressed in the vegetative cell along with *KRP* genes encoding CDK inhibitors (Kim et al., 2008). Loss of *RBR1* results in hyperproliferation of the vegetative cell and to a lesser extent the generative cell (Chen et al., 2009; Johnston et al., 2008). Hyperproliferation in *rbr1* mutants is caused by *CDKA;1* activity, since introduction of a *cdka;1* mutant allele suppresses this phenotype in *rbr1* pollen (Chen et al., 2009). Interestingly, cell fate is incorrectly specified in a fraction of *rbr1* vegetative cells, but this phenotype is completely reversed in the absence of *CDKA;1*. These findings point to *RBR1* primarily controlling cell cycle progression, while having a secondary impact on cell fate commitment. This places RBR1-mediated repression of E2F/DP downstream of KRP-dependent inhibition of CDKA;1, with both mechanisms enforcing cell-cycle exit, thereby restricting cell fate (Figure 4). In addition, E2F and RBR1 can both bind to the *FBL17* promoter and are thought to act antagonistically to stimulate or repress *FBL17* expression, respectively (Zhao et al., 2012). Thus, increased RBR1 protein in the vegetative

cell would result in less FBL17 and reduced degradation of KRPs, thereby reinforcing cell vegetative cell cycle exit.

2.3 Transcriptional control of male gametophyte patterning

Regulators that integrate the control of cell division and germline fate

The male germline-specific R2R3 Myb-domain protein DUO POLLEN1 (DUO1) was the first transcription factor to be identified with a role in generative cell division (Rotman et al., 2005). Mutant generative cells of *duo1* pollen complete S-phase, but fail to enter mitosis due to reduced accumulation of the mitotic cyclin, CYCB1;1 (Brownfield et al., 2009a; Durbarry et al., 2005). *DUO1* also regulates a network of genes required for sperm cell differentiation (Borg et al., 2011; Brownfield et al., 2009a). Direct targets of DUO1 include genes encoding the sperm-egg adhesion protein, GAMETE EXPRESSED2 (GEX2) (Engel et al., 2005; Mori et al., 2014), and the sperm cell-surface fusogen, GENERATIVE CELL-SPECIFIC1/HAPLESS2 (GCS1/HAP2) (Takahashi et al., 2017; von Besser et al., 2006). Thus, *DUO1* has an integrative role, controlling G2 to M-phase transition and shaping the germline transcriptome to promote spermatogenesis (Borg et al., 2011; Brownfield et al., 2009a). DUO1 forms a regulatory module with two of its direct targets, the C₂H₂ zinc finger repressor protein encoding genes *DUO1-ACTIVATED ZINC FINGER PROTEIN1 (DAZ1)* and *DAZ2*. Current data support a *DUO1-DAZ1/2* network model in which the *DAZ1/2* node controls G2- to M-phase transition and *DUO1*-dependent gamete differentiation through a negative regulatory circuit involving physical interaction of DUO1 with the corepressor TOPLESS (TPL) (Figure 4; Borg et al., 2014).

The expression of a subset of germline genes under *DUO1* control, including *GCS1/HAP2* and *GEX2*, also requires the homeodomain-like protein DUO3 (Brownfield et al., 2009b). DUO1 and DUO3 have overlapping roles in gametogenesis, but unlike in *duo1* mutants, generative cell division cannot be rescued by *CYCB1;1* expression in *duo3* mutants. Moreover, *DUO3* is expressed in sporophytic tissues and has an essential role in

embryogenesis. DUO3 is a functionally conserved protein in land plants, as the *A. thaliana* *duo3* mutant is rescued by the moss *DUO3* orthologue (Brownfield et al., 2009b). The ancestry of DUO3 also extends to protein domains conserved with cell lineage regulators including GON-4 (GONADLESS-4) of *Caenorhabditis elegans* and vertebrate Gon4l (Gon4-like), which are required for gonadogenesis (Liu et al., 2007; Williams et al., 2018) and haematopoiesis (Friedman et al., 2000) respectively. DUO3, in common with Gon4l, is proposed to act as a scaffold, enabling the assembly of distinct transcriptional complexes in restricted cell types (Brownfield et al., 2009b; Lu et al., 2011; Williams et al., 2018).

Regulation of DUO1 expression

The *DUO1-DAZI* regulatory module is an essential switch in angiosperm spermatogenesis. Strict spatial control of *DUO1* is vital as ectopic expression results in vegetative growth defects or pollen cell death (Brownfield et al., 2009a; Palatnik et al., 2007). Initial efforts to understand this tight control, identified a 198 bp *DUO1* promoter fragment sufficient for generative and sperm cell-specific transcription (Brownfield et al., 2009a). Phylogenomic studies have delimited important sequences in this region to an ordered *cis*-regulatory module (CRM), termed *regulatory region of DUO1 (RODI)*. *RODI* has a distinct architecture in eudicots; three DNGTGGV motifs are required to initiate germline expression and two YAACYGY motifs bound by DUO1, enhance expression in a positive feedback loop (Peters et al., 2017). *RODI* is functionally conserved in *Medicago trunculata*, but absent from pre-angiosperm *DUO1* promoters, indicating a shift in the transcriptional control of *DUO1* in pollen after the divergence of gymnosperms and angiosperms 300-350 Mya (Peters et al., 2017).

The control of *DUO1* expression is fine-tuned by several mechanisms. A negative feedback loop is thought to contribute to the developmental phasing of *DUO1* and *DAZI/DAZ2* expression, involving the limitation of *DUO1* accumulation in sperm by *DAZI/DAZ2* (Figure4; Borg et al., 2014). A possible mechanism could involve *miR159*-mediated

regulation, since the *DUO1* transcript contains a functional *miR159* binding site (Palatnik et al., 2007), and increased expression of *DUO1* has been associated with reduced levels of the Anaphase Promoting Complex APC/C and *miR159* (Zheng et al., 2011). There is also evidence for positive regulation of *DUO1* by the pollen-specific AT-RICH INTERACTING DOMAIN1 (ARID1) protein, which binds to a promoter region upstream of *ROD1* (Zheng et al., 2014).

In recent work, mutations in two distinct families of basic helix-loop-helix (bHLH) transcription factors were reported to cause profound defects in *A. thaliana* male gametogenesis. BONOBO1 (BNB1) and BNB2 are pollen-specific members of the bHLH subfamily VIIIa, which are redundantly required for generative cell specification (Yamaoka et al., 2018). DEFECTIVE REGION OF POLLEN1 (DROP1) and DROP2 are members of bHLH subfamily XI (Heim et al., 2003) that were previously shown to function in root hair elongation (Lin et al., 2015). In pollen of *bnb1 bnb2* double mutants, generative cells are not engulfed by vegetative cells (Yamaoka et al., 2018), while generative cell nuclei disintegrate in *drop1 drop2* pollen (Zhang et al., 2017). Generative cells fail to differentiate in both double mutants and sperm cell marker genes like *DUO1* and *GCSI/HAP2* (in *drop1 drop2*) or *HISTONE THREE RELATED10 (HTR10)* (in *bnb1 bnb2*) are not expressed (Yamaoka et al., 2018; Zhang et al., 2017). In conclusion, *BNB1/2* and *DROP1/2* are required for early generative cell development and for germline differentiation, highlighting their potential as upstream regulators of the *DUO1-DAZI* module (Figure 4).

Regulators of pollen maturation that act in the vegetative cell

The MIKC* MADS-domain proteins are an ancient class of transcriptional regulators which play an essential role in pollen maturation and germination in angiosperms (Adamczyk and Fernandez, 2009; Liu et al., 2013; Verelst et al., 2007a, 2007b). Pollen-specific members of the MIKC* gene family in *A. thaliana* and *Oryza sativa* (rice) are restricted to the vegetative cell and form heterodimeric complexes (Liu et al., 2013; Verelst et al., 2007a). In *A. thaliana*,

AtMIKC* complexes regulate pollen development by repressing early pollen genes and activating mature pollen genes. For example, the male gametophyte-specific transcription factor gene *WRKY34* is upregulated, while *MYB97* is downregulated in the absence of MIKC* complexes (Figure 4; Verelst et al., 2007b). Interestingly, MYB97 is a member of a sub-clade of S18 (GAMYB) R2R3 MYB proteins (MYB97, MYB101, MYB120), which act redundantly to promote the discharge of sperm from pollen tubes in the embryo sac (Leydon et al., 2013; Liang et al., 2013). These pollen-specific GAMYB genes are negatively regulated by a ternary complex of WRKY2, WRKY34, and the pollen-specific VQ motif-containing protein VQ20 (Lei et al., 2018, 2017). Moreover, the temporal control of WRKY2/34-VQ20 ternary complex activity by MITOGEN-ACTIVATED PROTEIN KINASE3 (MPK3)/MPK6-mediated phosphorylation is a compelling example of the post-translational regulation of transcription in developing pollen (Guan et al., 2014).

[Insert Figure 4 here]

Figure 4. Overview of the morphological stages and genetic pathways regulating male gametophyte development in *Arabidopsis thaliana*. Polarised microspores (light blue) undergo a highly asymmetric division to produce a bicellular pollen grain with a small generative cell (pink), engulfed within the vegetative cell (dark blue). The generative cell undergoes mitotic division to produce two differentiated sperm cells (red). Genes and regulatory pathways with important functions are shown with direct interactions indicated by solid arrows. The major regulatory pathways indicated are (1), the *WRKY2/34-MIKC** network in the vegetative cell, (2), the *DUO1-DAZI* differentiation network in germline cells, and (3), the *FBL17-KRPs-CDKA;1-RBR1-E2F* G1/S-phase regulatory module. Physical interactions between proteins are denoted by double dashes. Indirect regulatory pathways are indicated with dotted lines. Cell cycle phases (G1, S, G2) are labelled. Pollination and fertilization events indicated with grey dotted lines. (1) AtMIKC* MADS-domain protein (AGL65, AGL66 and AGL104) complexes repress early genes and activate late pollen genes in the vegetative cell. Pollen-specific GAMYB genes (*MYB97*, *MYB101* and *MYB120*) are

negatively regulated by WRKY2 and WRK34 via interaction with the pollen-specific VQ motif-protein VQ20 (Lei et al., 2018, 2017). WRKY2/34-VQ20 complex activity is regulated by MPK3/MPK6-mediated phosphorylation (Guan et al., 2014). (2) DUO1 controls gamete specification genes, including *DAZ1* and *DAZ2* (*DAZ1/2*), which counteract repressive mechanisms (R) via EAR-dependent interaction with the corepressor TPL. Through this mechanism, *DAZ1/2* promote the G2/M-phase accumulation of mitotic cyclins, generative cell division, and the *DUO1*-dependent activation of gamete differentiation genes and DUO1 turnover (Borg et al., 2014). (3) *CDKA* activity and generative cell cycle progression are maintained by the proteasome-dependent degradation of CDK inhibitors (KRPs) by the SCF^{FBL17} (Gusti et al., 2009; Kim et al., 2008). The transient expression of *FBL17* in the generative cell promotes G1/S transition, while KRPs persist in the vegetative cell (Kim et al., 2008). The *FBL17-KRPs-CDKA;1* cascade is linked to the *RBR1-E2F* pathway to form a general G1/S regulatory module that also functions in microspores (Zhao et al., 2012).

2.4 Chromatin remodelling in male gametophyte development

Chromatin organisation differs dramatically between the two different cell types of angiosperm pollen. The vegetative cell has diffuse nuclear chromatin, whereas sperm cell nuclei possess highly compact chromatin, and these differences can be traced to asymmetric microspore division. After formation of the vegetative cell, centromeric heterchromatin is gradually decondensed, coincident with the loss of the histone histone H3-like variant CENH3 from centromeres and repressive histone H3 lysine 9 di-methylation (H3K9me2) marks (Schoft et al., 2009). The loss of CENH3 is consistent with terminal differentiation of the vegetative cell, while germ cells maintain centromeric CENH3 and their ability to divide (Ingouff et al., 2006; Schoft et al., 2009). CENH3 removal from centromeres was shown to require the CDC48A AAA-ATPase molecular chaperone and a pair of interacting small ubiquitin-like modifier (SUMO) adaptors, NPL4A and NPL4B (Méraï et al., 2014). The

CDC48A^{NPL4} complex is proposed to bind and promote active unloading of sumoylated CENH3, while CDC48A^{NPL4}-mediated decondensed centromeric heterochromatin was also proposed to “euchromatinize” rDNA loci to facilitate rRNA gene transcription to support the high metabolic needs of pollen tubes (Mérai et al., 2014). In this regard, it is interesting that CENH3 is not excluded from centromeres in vegetative cell nuclei of rye and barley where repressive H3K9me2 marks persist (Houben et al., 2011; Pandey et al., 2013). In a survey of immunodetected chromatin marks reported for pollen of different species, there was otherwise general agreement on their prevalence, with notable differences between eudicots and monocots (Borg & Berger, 2015). Although only monocots were studied, repressive H3K27me3 marks were enriched in subtelomeric chromatin and specific for the vegetative cell (Houben et al., 2011; Pandey et al., 2013; Sano & Tanaka, 2010).

The chromatin of male germ cells shows dynamic changes in histone composition which varies significantly among angiosperms (reviewed in Borg & Berger, 2015). Generative cells of lily incorporate distinct histone H2A, H2B, and H3 variants compared with those of somatic tissues (Ueda et al., 2000; Ueda & Tanaka, 1994; Xu et al., 1999). While chromatin of *A. thaliana* microspores is mainly composed of H3.1 and H3.3 histone variants, sperm cell chromatin is strongly biased towards H3.3 and H3.10 variants (Borg & Berger, 2015; Okada et al., 2005). Consequently, the dynamics of histone variants are thought to be linked to chromatin reprogramming and the compaction of the sperm cell genome (Borg & Berger, 2015). These roles may arise through functional differences exhibited by histone variants. In particular, the abundant germline-specific histone H3 variant H3.10 is proposed to reshape the chromatin landscape by conferring immunity to the repressive chromatin mark H3K27me3 (Borg & Berger, 2015). The absence of immunodetected H3K27me3 marks observed for germline cells of several monocots is in accord with this notion. Unexpectedly, an insertion mutant in the *HTR10* gene, encoding H3.10, did not show an obvious phenotype, suggesting functional compensation among germline histones (Okada et al., 2005). There is also evidence

that rice sperm cells express an unique and diverse combination of histone variants, highlighting the differing chromatin landscapes of sperm among angiosperms (Anderson et al., 2013; Russell et al., 2012). In addition to potential roles in chromatin compaction, germline-specific histones might function to remove somatic chromatin marks to facilitate the specification of germline fate and/or to allow resetting of the chromatin state prior to fertilization (see Borg & Berger, 2015).

2.5 Communication between non-germ and germ cells

Angiosperm pollen is a functional unit in which the vegetative cell nourishes, protects, and transports the germ cells to the embryo sac via the pollen tube. An intrinsic feature of this ‘cell within a cell’ architecture is the reliance of the germline on the vegetative cell for the transfer of nutrients and biomolecules. This unilateral dependence is illustrated by genetic ablation studies, in which the expression of diphtheria toxin A-chain (DTA) was restricted to each cell-type. When vegetative cell development was arrested with DTA, generative cells were not engulfed and most rapidly lost viability (Twell, 1995), but when DTA was used to block germline development, pollen matured normally and discharged single gametes into the embryo sac (Frank & Johnson, 2009). Similarly, pollen tubes of single-celled pollen mutants are guided to the ovule and are competent to discharge their contents in the absence of gametes (Glöckle et al., 2018; Zhang et al., 2017).

The vegetative nucleus and generative cell or sperm cell pair are also physically linked in an assemblage known as the male germ unit (Dumas et al., 1985; Jensen & Fisher, 1970; Russell & Cass, 1981). This structure is established in bicellular pollen and is maintained until discharge of the pollen tube contents into the embryo sac (reviewed in McCue et al., 2011; Mogensen, 1992). The potential for molecular communication between pollen cell types, via the physical extension linking one of the sperm cell pair to the vegetative nucleus, was first postulated by Mogensen (1992). Plasmodesmata-like channels between the two cells have not been identified using rapid freeze fixation and freeze substitution, which avoids artifacts

caused by conventional methods (Cresti et al., 1987), although the topic remains controversial (see McCue et al., 2011). However, it is evident that mechanisms exist to transport both mRNAs and short interfering RNAs (siRNAs) from the vegetative cell to the developing germline.

While studying the expression of the sperm cell-enriched ABA-related protein phosphatase gene *ABA-HYPERSENSITIVE GERMINATION3* (*AHG3*), Jiang and coworkers (2015) unexpectedly found that the *AHG3* promoter was transcriptionally active in the vegetative cell, whereas a translational fusion protein was localized to sperm (Jiang et al., 2015). The authors provided evidence that *AHG3* transcripts move from the vegetative cell to sperm cells, and that transport requires sequences in both the 5' UTR and coding region. The mechanisms involved and the extent of communication via this route are not yet clear, but these results document an additional role for the vegetative cell in shaping the sperm cell transcriptome (Jiang et al., 2015).

Different classes of small RNAs are present in vegetative cells and sperm cells (reviewed in He et al. 2015). These span most conserved microRNA (miRNA) families and include biogenesis genes such as *DICER-LIKE1* (*DCL1*) (Borges et al., 2011; Creasey et al., 2014; Grant-Downton et al., 2009; Slotkin et al., 2009). Based on the de-repression of transposable elements (TEs) in the vegetative cell, and the appearance of corresponding 21 nt siRNAs in sperm cells, it was proposed that siRNAs could move between the vegetative cell and sperm cells of *A. thaliana* to maintain TE silencing in sperm (Slotkin et al., 2009). Evidence in support of this proposal was that artificial miRNAs expressed under control of the vegetative cell-expressed tomato *LAT52* promoter could repress gene expression in the sperm cells (Slotkin et al., 2009). However, the *LAT52* promoter is first active in the microspores of *A. thaliana* (Eady et al., 1995), raising the possibility of inheritance of miRNAs by the germline. Truly vegetative cell-specific promoters also failed to silence targets in the germline, indicating that miRNA cannot move between cells (Grant-Downton et al., 2009). However,

recent experiments utilising robustly cell-specific promoters demonstrate that siRNAs produced from vegetative cell transcripts can silence TE reporters in sperm cells (Martínez et al., 2016). Thus, siRNAs, but not miRNAs, are trafficked into the germline, although the mechanisms involved in transfer, or which discriminate between these two classes of small RNAs, remain to be discovered.

3. Molecular evolution of the male germline

3.1 Motile plant and animal sperm share common features

The morphology of sperm cells has changed markedly during the evolution of land plants. Excellent reviews by Renzaglia & Garbary (2001) and Southworth & Cresti (1997) discuss the diversity, development, and evolution of land plant sperm. Only selected aspects and recent molecular and genetic data are highlighted here.

Biflagellate spermatozoids with condensed chromatin, packaged into an elongated and coiled nucleus, are produced by advanced charophyte algae and bryophytes (Figure 3A). These biflagellated spermatozoids are not only superficially reminiscent of mammalian spermatozoa, but recent analysis of antheridia-enriched transcripts in *M. polymorpha*, revealed homologs of a number of motility-related genes that are absent from angiosperms (Higo et al., 2016). These include the mammalian Parkin co-regulated gene (Mp*PACRG*), responsible for axoneme formation (Ikeda et al., 2007; Lorenzetti et al., 2004; Yanagimachi et al., 2004), light chains of the inner arm dynein of the axoneme (Mp*CL7*), adenylate cyclase, required for sperm motility (Schmid et al., 2007), and human CHD5, which is required for spermatid chromatin remodelling and condensation (Li et al., 2014; Zhuang et al., 2014).

One of the most striking similarities between spermatozoa of most animals and the spermatozoids of charophytes and basal land plants is their nuclear chromatin compaction. This character is thought to improve the hydrodynamic properties of sperm (Erenpreiss et al., 2006), and to protect the genome against irradiation damage, nuclease degradation and

mechanical forces (Rathke et al., 2010; Wykes and Krawetz, 2003). In animal sperm, histone-based nucleosomes are replaced by highly basic proteins called protamines (Johnson et al., 2011; Meistrich et al., 2003). Protamine-like proteins can be found in spermatozooids of algae (*Chara corallina*), bryophytes (*M. polymorpha*) and ferns (*M. vestitia*), but not in seed plants (reviewed in Borg & Berger 2015). In *M. polymorpha* sperm, histones are replaced by protamines and the exchange mechanism involves transitory proteins of intermediate size and basicity, similar to the situation in animals (Reynolds & Wolfe, 1984, 1978). Variation in the coexistence of intermediate histone/protamine proteins and somatic histones indicates a remarkable adaptation of nucleoprotein composition exchange mechanism among early divergent species (Borg & Berger, 2015).

The spermatozooids of charophyte algae and basal land plants share the common feature of cytoplasmic reduction via macroautophagy, with animal spermatozoa. Macroautophagy is controlled by autophagy related proteins (ATGs) in mice as well as in the moss *P. patens*, suggesting recurrent adoption of an ancestral mechanism. ATG5 and ATG7 are essential for sperm fertility in *P. patens*, similar to the requirement for ATG7 in mice. Impaired autophagy is thought to impact microtubule and flagella development, as well as nuclear packaging, by reduced clearance of cytoplasmic repressors (Sanchez-Vera et al., 2017). In angiosperms, ATG genes evidently do not influence pollen morphology, but indirectly support pollen maturation and germination. Knock-out of rice *ATG7* resulted in sporophytic pollen defects, arising from reduced autophagic degradation in the anther tapetum (Kurusu et al., 2014). Furthermore, pollen germination defects observed in *A. thaliana* *ATG6* mutants are gametophytically determined, but attributed to functions other than autophagy (Fujiki et al., 2007). Thus, the role of autophagy in male reproduction has diversified with the evolution of non-motile sperm in seed plants. Cytoplasmic reduction is likely to be less important for the larger and non-motile sperm of seed plants than for spermatozooids of basal land plants. However, there is evidence that sperm cells of some angiosperms reduce their cellular volume by shedding cytoplasmic vesicles into the vegetative cell (Yu & Russell, 1992). This may

assist in sperm cell movement within the pollen tube and could limit the paternal cytoplasmic contribution to the zygote.

A signal perception apparatus used for chemotactic navigation by land plants may operate in mammalian sperm. The GLUTAMATE-RECEPTOR-LIKE (GLR) ion-channels are essential for moss spermatozoids to navigate to archegonia (Ortiz-Ramírez et al., 2017). In flowering plants, where immotile sperm no longer require chemoattraction, GLRs have been redeployed to regulate pollen tube growth and navigation in response to chemotactic factors released by the pistil (Michard et al., 2011). Although GLRs are expressed in mammalian sperm, their functions are currently unknown (Hu et al., 2004).

In monilophytes, spermatozoids can develop over 100 flagella (Figure 3B; Myles & Hepler, 1977), while those of the non-siphonogamous gymnosperms, possess much larger multiflagellate sperm, with around 1000 flagella for *G. biloba* (Figure 3C; Gifford & Lin, 1975). The cycads have the largest sperm with a diameters of 200-500 µm and an estimated 40,000 flagella for the genus *Zamia* (Norstog, 1975). The increase in flagella number is associated with increased sperm cell volume, the absence of coiled morphology and reduced chromatin compaction. With the loss of sperm cell motility in siphonogamous gymnosperms (e.g. in *G. gnemon* and the genus *Ephedra*) and angiosperms, only the angiosperms possess highly condensed chromatin (see Southworth & Cresti 1997), indicating that chromatin compaction may be atavistic in angiosperms. The sperm cells of flowering plants also possess a distinctive cytoskeleton when compared to somatic cells (reviewed in Palevitz & Tiezzi, 1992; Southworth & Cresti, 1997). This consists of microtubule bundles that form a basket or cage around the nucleus. This internal cytoskeleton is thought to be involved in the establishment or maintenance of cell shape, because isolated sperm and generative cells become spheroidal and lose microtubule bundles (Mogensen, 1992; Palevitz & Tiezzi, 1992). It remains unclear whether flowering plant sperm evolved from motile sperm by loss of flagella, but distinct microtubule organizing centers such as blepharoplasts, centrioles, or

basal bodies are absent and microtubules do not occur regularly or in a 9 plus 2 arrangement (Palevitz & Tiezzi, 1992).

In summary, the shared features of motile plant and animal sperm, like flagella-based locomotion, protamine-mediated chromatin compaction or cytoplasmic reduction via autophagy, have evolved independently. Chromatin compaction mediated by protamines was apparently lost in vascular plants, while flagellar-based motility was lost in some gymnosperms and in the common ancestor of angiosperms. Furthermore, immotile plant sperm evolved a unique microtubule cytoskeleton with uncertain origins. Autophagosomal pathways and chemoattractant perception were also redeployed to support the complex reproductive systems of angiosperms.

3.2 Gene expression in male germ cells

Transcriptome data for reproductive gametophyte stages of basal land plant species is limited to the water fern *M. vestita* and the liverwort *M. polymorpha*. A remarkable feature of spermatogenesis in *M. vestita* is that the process is largely under post-transcriptional control. This facilitates rapid spermatogenesis, largely independent of the external environment and also shifts the control of gametophyte fitness towards the sporophyte. Messenger RNAs required for sperm cell development are stored in microspores as partially processed pre-mRNAs. After spores are rehydrated, pre-mRNAs are unmasked, processed, and translated according to a precise spatiotemporal pattern. Stored transcripts include those encoding tubulins and centrin (Tsai et al., 2004), as well as proteins important for mitosis and flagella formation, such as kinesins (Tomei & Wolniak, 2016). More detailed information is available for the transcriptome of developing antheridia of *M. polymorpha* (Higo et al., 2016). Among more than 700 genes specifically or preferentially expressed in antheridia, are genes associated with sperm motility (see section 3.1), cell cycle control, reproduction, regulation of gene expression and epigenetic processes, including homologs of angiosperm genes essential for male gametophyte and gamete formation (see section 3.3).

The male germ cells of angiosperms possess a distinct transcriptome compared to those of other tissues or organs. Data from cDNA-sequencing are available for isolated generative cells or sperm cells of several species (reviewed in Russell & Jones, 2015; Rutley & Twell, 2015). Larger scale microarray studies of sperm cells of *A. thaliana* or rice detected 5,829 or 10,732 different transcripts respectively (Borges et al., 2008; Russell et al., 2012). A similar proportion of these transcripts (18-22 %) were absent from seedlings of both species and were thus considered specific or enriched in sperm. In an independent RNA-sequencing study, 16,985 transcripts were reliably detected in rice sperm, while 18,611 transcripts were present in a vegetative cell-enriched fraction (Anderson et al., 2013). Similar to *A. thaliana*, male gamete-enriched transcripts in rice are associated with ubiquitin-mediated proteolysis, DNA modification, and chromatin remodelling (reviewed in Russell & Jones, 2015; Rutley & Twell, 2015). Ubiquitin mediated protein turnover is critical for male gametogenesis, and disruption of the ubiquitin proteasome system impairs male gametophyte development in *A. thaliana* (Doelling et al., 2007; Gallois et al., 2009). Further compelling evidence for the importance of the ubiquitin proteasome system in gametogenesis includes the role of the SCF^{FBL17} E3 ubiquitin ligase complex, which promotes generative cell cycle progression (see section 2.2, Figure 4).

Changes in the relationship between cell cycle progression and sperm development have led to different ontogenetic patterns in seed plants, and this is important for gamete synchrony at fertilization (Friedman, 1999). In essence, the timing of generative cell division and the stage of the cell cycle of sperm cells vary in relation to pollination and fertilization (Friedman, 1999). For example, *A. thaliana* and *P. zeylanica* sperm cells are in S-phase at anthesis and reach the G2-phase prior to fertilization, whereas grass species like rice and maize remain in G1-phase until fertilization (Friedman, 1999; Gou et al., 2009). In accord with predictions based on these ontogenetic patterns, cell cycle gene expression in sperm cells differs between species. Consequently, S-phase associated cell cycle genes are enriched in sperm cells of *A. thaliana* (Borges et al., 2008) and *P. zeylanica* (Gou et al., 2009), but not in rice (Anderson et

al., 2013) and transcripts of G1-type cyclins were not detected in isolated sperm of maize (Sauter et al., 1998).

Angiosperm male gametes express a full complement of DNA and chromatin modifying proteins to support gametogenesis and transgenerational epigenetic processes (Anderson et al., 2013; Borges et al., 2008). In common with *A. thaliana*, rice sperm cells preferentially express DNA *METHYLTRANSFERASE1* (*MET1*), consistent with the maintenance and epigenetic inheritance of CG-methylation through the male germline (Calarco et al., 2012; Saze, 2008; Saze et al., 2003). Moreover, silencing of the transcription factor ARID1 in *A. thaliana* sperm cells is dependent upon *MET1* and linked to gene body specific CG-methylation (Li et al., 2017). The epigenetic remodeling of sperm chromatin is also thought to involve the creation and removal of histone marks and the expression of histone lysine methyl transferase and demethylase enzymes (see Anderson et al., 2013). Further aspects of chromatin organization in the male germ cells of angiosperms are discussed in section 2.4. A key feature is that distinct histone variants and several core histones are specifically or preferentially expressed in the male germline and can be unique even among angiosperms. In contrast, the amino acid sequence of the *M. polymorpha* antheridium-specific H3 variant does not differ from that of somatic H3.3 (Higo et al., 2016). This indicates that the specialized roles proposed for unique histone variants of flowering plant sperm (section 2.4) may not operate in bryophytes and that the control of histone H3.3 abundance may be more important for gametogenesis.

Adaptation of the transcriptome of male gametes is evident from the recruitment of new genes to a sperm-cell network in *A. thaliana*. The *SHORT SUSPENSOR* (*SSP*) gene of *A. thaliana* encodes a membrane-associated IRAK/Pelle-like kinase, which regulates YODA mitogen-activated protein kinase signalling in early embryogenesis (Bayer et al., 2009). *SSP* is specifically transcribed in sperm, but transcripts are only translated after transmission to the zygote. Phylogenetic analysis has revealed the divergence and neofunctionalization of *SSP*

following a polyploidy event in Brassicaceae (Liu & Adams, 2010). Moreover, the recruitment of *SSP* to a sperm cell-specific network, leading to the paternal control of zygote development, is a recent evolutionary event (Liu & Adams, 2010).

3.3 The evolution of transcription factors regulating male gametogenesis

Comparative studies are starting to uncover the evolutionary origins of transcriptional networks in male gametophyte development. Functionally conserved modules, as well as the rewiring of gametophyte and germline gene networks, have been described.

Recent studies of MIKC-type MADS-domain transcription factors illustrate how closely related gene families can follow distinct evolutionary trajectories. Three of the six member MIKC^C-type MADS-domain family in *P. patens* are redundantly required for the formation of sperm with functional flagella (Koshimizu et al., 2018). Mutations in these genes also disturb cell division and growth in the stems of gametophores, which is important for the uptake of external water and for fertilization. In mutants deleted for all six *P. patens* MIKC^C-type genes, the expression of flagellum-related genes is disturbed, leading to defective sperm movement and less efficient entry of sperm into archegonia. In *Chara globularis*, MIKC^C-type MADS-box genes are expressed during sperm differentiation, suggesting an ancient and conserved function in the regulation of flagellum genes (Tanabe et al., 2005). In angiosperms, MIKC^C-type MADS-box genes seem to have lost their role in fertilization, but through amplification and neofunctionalization, these genes still control cell division and organ growth and identity (Koshimizu et al., 2018). In contrast, the closely related MIKC* MADS-genes are thought to have retained conserved roles in the gametophyte (Kwantes et al., 2012). MIKC* MADS-genes evolved from an MIKC^C-like ancestor (Henschel et al., 2002) and function in heteromeric complexes regulating pollen development (see 2.4). Unlike the MIKC^C class, the MIKC* MADS-genes did not acquire sporophytic expression in angiosperms (Kwantes et al., 2012). Although the function of MIKC* MADS-genes in bryophytes is yet to be determined, the successful complementation of *A. thaliana* mutants lacking MIKC* complexes with the

single *M. polymorpha* ortholog, supports a conserved molecular mechanism in land plants (Zobell et al., 2010).

Comparative analysis is also beginning to uncover the evolutionary origins of germline specification factors. In *M. polymorpha*, the bHLH-family transcription factor *BNB* is required for the initiation of specialised male and female reproductive shoots (gametangiophores), which bear male and female gametangia, while two *A. thaliana* orthologues of *BNB* are redundantly required for male gametogenesis (Yamaoka et al., 2018). *MpBNB* is expressed in gametangiophore and gametangia initial cells of both sexes, and in the germ cell lineage of antheridia and archegonia. Although *MpBNB* is expressed in immature eggs cells and in spermatogenous cells prior to proliferative cell divisions, it is not maintained in maturing gametes. In *A. thaliana*, *AtBNB1* expression is restricted to the generative cell of developing pollen and is absent from sperm cells. This shift in expression pattern of *BNB* from *M. polymorpha* to *A. thaliana* reflects the absence of gametangiophores and antheridal initial cells from angiosperms, and the maintenance of expression in the immature sperm cell lineage (i.e. generative cells) (Figure 2B and F). Mutation of both *AtBNB* paralogs abolishes the expression of the sperm cell differentiation marker H3.10, which is under the control of the *DUO1-DAZI* module (Yamaoka et al., 2018). Moreover, *DUO1* and *DAZI* are specifically expressed in antheridia of *M. polymorpha* (Higo et al., 2016). The overlapping expression of *BNB* and *DUO1* in generative cells of *A. thaliana* highlights the potential role of *BNB* as a conserved upstream regulator of the *DUO1-DAZI* module (Figure 4).

DUO1 and *DAZI* orthologs are also co-expressed in sperm cells of rice, together with homologues of several *DUO1* target genes, indicating conservation of a key germline regulatory network in angiosperms (Li et al., 2010; Russell et al., 2012; Rutley & Twell, 2015). The antheridia-specific expression of the single copy *DUO1* and *DAZI* orthologs in *M. polymorpha* (Higo et al., 2016) and the presence of two *DUO1* orthologues in *P. patens*

(Brownfield et al., 2009a), suggest a conserved role for the *DUO1-DAZI* module in bryophytes. Moreover, homologs of several genes essential for angiosperm gametogenesis are specifically or preferentially expressed in antheridia of *M. polymorpha* (Higo et al., 2016). These include homologs of the DUO1-target genes, *GEX2* and *GCSI/HAP2*, which highlights the question of when these fertilization genes were recruited to the *DUO1-DAZI* network.

There is growing evidence for the adaptation and rewiring of germline gene networks in the 450-500 million years of evolution separating liverworts and angiosperms. The transcription factors *RKD* and *BNB* evolved sex-specific roles in angiosperms from broader ancestral roles in bryophytes. In *M. polymorpha*, *MpRKD* is required for egg and sperm differentiation, while in *A. thaliana*, *RKD* family genes are associated with embryogenesis and egg cell formation, but not with sperm differentiation (Koi et al., 2016; Rövekamp et al., 2016). Similarly, the *M. polymorpha* bHLH protein *BNB* has an essential role in male and female gametangia development, while the function of the *A. thaliana* orthologues (*BNB1/2*) is restricted to the male germline (Yamaoka et al., 2018). Evolutionary rewiring has also changed the regulatory network of GAMYB transcription factors. GAMYB proteins have retained an ancient role in the control of sporopollenin biosynthesis, which is required for spore and pollen grain development in land plants (Aya et al., 2011, 2009). In vascular plants, sporopollenin biosynthesis controlled by the GAMYB transcriptional network was adopted by the newly evolved gibberellin (GA) signal transduction module (Aya et al., 2011). Duplication and neofunctionalisation diversified the roles of GAMYBs in angiosperms to include inflorescence and anther development (Gocal et al., 2001; Kaneko et al., 2004), pollen tube differentiation (see section 2.3, Figure 4; Leydon et al., 2013; Liang et al., 2013), and non-reproductive pathways, including GA-signalling during seed germination (Tsuji et al., 2006)

4. Perspective

The evolutionary history of male gametogenesis has left a trail of adaptive changes, which enabled plants to safeguard their reproduction on land. Our understanding of these changes has progressed with advances in microscopy, genetics, and omics-approaches, but knowledge at the molecular level has been largely limited to angiosperms. Important evolutionary questions remain unresolved. How and when did anisogamy evolve in streptophyte algae? Which regulators and gene networks are responsible for the distinct development of male and female gametangia? A central question in angiosperms is how the germline *sensu stricto* is specified by asymmetric division of the microspore. The role and diversity of the changing chromatin landscape of germline versus non-germ cells is another fundamental issue for which there is limited knowledge. Given the reduced costs of DNA sequencing, progress can be made to clarify the origins and the evolution of genes and networks responsible for sexual reproduction. Sexual reproduction for most basal charophytes is unknown, and nuclear genome sequences are only available for one charophyte species, *Klebsomidium nitens* (Hori et al., 2014). Whole genome sequencing and transcriptome studies of gametangia of charophytes, as well as basal land plants, like hornworts, will help to uncover pathways crucial for reproductive adaptation to terrestrial habitats. The availability of a full range of tools for gene manipulation in bryophytes, like *M. polymorpha* and *P. patens*, are already beginning to advance understanding of reproductive development and the adaptations of basal land plants (Koi et al., 2016; Koshimizu et al., 2018; Rövekamp et al., 2016; Yamaoka et al., 2018). These comparative studies span the major reproductive transitions, from exo- to endosporic gametophytes, from motile to immotile sperm, and the innovation of double fertilisation. These key transitions are expected to involve the rewiring of gene networks such as those controlled by deeply conserved regulators such as *BNB* (Yamaoka et al., 2018), *DUO1* and *DAZI* (Brownfield et al., 2009a; Higo et al., 2016).

Collaborative efforts, such as the multinational EVOREPRO consortium (<http://www.evorepro.org/>), are providing new tools for comparative transcriptomics. Transcriptome data from various stages of gametogenesis from model and from crop plants,

representing major phylogenetic clades of land plants, will accelerate discovery through an open source database (Proost & Mutwil, 2018). The inclusion of data from *M. polymorpha* and *P. patens*, alongside *Amborella trichopda*, maize, rice, *A. thaliana*, and tomato, will provide new opportunities to identify novel and missing components involved in gamete development and fertilization. Moreover, rapidly developing methods, like chromatin immunoprecipitation sequencing (ChIPseq) (Kaufmann et al., 2010) and Transposase-Accessible Chromatin using sequencing (ATACseq) (Bajic et al., 2018; Buenrostro et al., 2013; Lu et al., 2017), offer new horizons in the decoding of pollen and gamete regulatory networks. These approaches would allow the binding between transcription factors and their target loci to be resolved, and to decipher how these interactions are integrated with the changing chromatin landscape. The precision of such methods will be further enhanced by the use of fluorescence-activated cell sorting (FACS) and single cell transcriptome analysis (Borges et al., 2012; Santos et al., 2017; Schoft et al., 2015). Future studies using established and newly emerging models promise major advances in our understanding of the development and evolution of male gametogenesis in plants.

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Figure 1

Figure 2

Figure 3

Figure 4