Fertilisation and Cell Cycle in Angiosperms

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1 Introduction

Angiosperms, the large groups of seed plants better known as flowering plants, are the most successful land plants on our planet today, representing an estimate of 95% of all terrestrial vegetation (Simonin and Roddy, 2018). Their seeds germinate only during favourable growth conditions culminating in the development of a new generation of plants, which can show variable periods of vegetative growth depending on the species, habitat, and growth conditions. The transition of many of these angiosperm plants into the flowering phase is triggered by seasonal and environmental factors and involves a switch from vegetative growth into developing reproductive structures. Upon fertilisation, the embryo encapsulated in a seed ensures the survival of the species. The seeds of some flowering plant species can be stored for very long periods of time and retain their viability even deep in the soil. Disruption of the substrate by ploughing or digging changes the micro environment and disrupts seed dormancy (Hilhorst et al., 2010; Shen-Miller et al., 1995). A large variation in shape, nectar availability, volatile production, and colours exists among flowers. Hence most flowers are very attractive to the human eye. In Angiosperms successful reproduction depends on pollen grains, often carried by wind and insect pollinators, that land on the stigmatic surface and fertilise the ovules. During the relatively rapid development of flowers, male and female reproductive organs, anthers and pistils, respectively, are formed at the centre of the flower. Within anthers and pistils, sporophytic cells are recruited to generate the new germ line from which male and female gametophytes develop. The egg cell is specified inside the developing embryo sac, which is the female gametophyte of a plant. Two sperm cells (SCs) develop inside a single vegetative cell (VC) surrounded by several durable layers, forming and protecting the male gametophyte, referred to as the pollen grain. Once flowers are fully mature and ready for reproduction, they open and the pollen grains are released from the anthers.

In the 320 000 different flowering plant species, many different mechanisms have evolved that ensure outcrossing to maximise genetic variability in the offspring (Lughadha et al., 2016; Fujii et al., 2016; Hiscock, 2011).

After pollen lands on a stigma, a series of complex recognition events results in rejection of all foreign pollen by as yet unknown mechanisms. In addition, approximately half of all angiosperm species display a genetically determined mechanism, which prevents self-fertilisation called self-incompatibility (SI). Pollen rejection can vary between different SI mechanisms, either by preventing pollen germination immediately after pollen landing by blocking water uptake from the stigma, or by triggering calcium signalling mediated programmed cell death in incompatible pollen, or by the activity of pistil produced S-RNases after translocation into the growing pollen tubes (Allen et al., 2010; Fujii et al., 2016).

All 'own' and compatible pollen grains rapidly hydrate, followed by pollen grain germination and the outgrowth of a pollen tube, driven by the tip growth of the VC. The pollen tube accurately delivers two SCs into one embryo sac deep enclosed in the ovule inside the pistil. In flowering plants, upon pollen tube arrival and SC release, a unique double fertilisation takes place in the female embryo sac. One SC fuses with the egg cell, generating the diploid zygote that will form a new sporophyte (embryo), whereas the second sperm merges with the diploid large central cell (CC) and results in the formation of the triploid endosperm precursor, triggering seed development. The endosperm functions as a nutrient source for the developing plant embryo, either during seed development and maturation before release from the mother plant, or during the early stages of plant development after seed dispersal and germination (de Graaf et al., 2001; Rousseau et al., 2008; Mizuta and Higashiyama, 2018).

Here, we survey the critical processes for plant reproduction and explore this in the context of cell cycle regulation. The coordination of cell cycle is required to produce the gametes, synchronise the cell cycle phase for fertilisation as well as the creation of an embryo from a zygote.

2 Fertilisation

2.1 Gametophyte Development

The developmental pathways of both male and female gametophytes producing both sex cell types for plant reproduction are highly conserved throughout angiosperms. Nevertheless, there are considerable differences in the timing of developmental steps, this can lead to a different number of cells in the pollen grain. In some species, the last mitotic division has not been executed and we see bicellular pollen, instead of tricellular. The total numbers of cells in the female gametophyte depends on the species as well (Yadegari and Drews, 2004).

The final cell number in most male gametophytes (pollen) appears to be rigidly conserved in all higher plants, formed by a well-defined sequence of divisions. Meiotic rounds of division (I and II) of a single diploid microsporocyte (microspore mother cell) gives rise to a tetrad of four haploid microspores embedded in callose (β1,3 glucan). The tapetum cell layer in the anther, surrounding the developing microspores, is responsible for the degradation of callose, releasing free microspores. These microspores undergo a first round of mitotic and asymmetric division that results in the formation of a larger VC and a smaller generative cell (GC). At this point of development, the GC becomes invaginated by the VC giving rise to a cell within a cell generating a bicellular pollen grain. The anther tapetum and developing pollen are both responsible for the production and deposition of two extracellular pollen layers, the intine composed mainly of cellulose-pectic compounds and an exine layer – composed of sporopollenin, which besides its species-specific structural properties to optimise pollen transfer, is composed of a biochemically decay-resistant substance protecting the pollen once released from the anther upon flower opening (Borg and Twell, 2010; Twell, 2011; Gómez et al., 2015).

2.2 Attraction to the Ovule

Among angiosperms, there are considerable structural differences in the size of reproductive organs, which also confers large differences in distance between stigma surface of the pistil, the landing platform for pollen, and the ovules inside the pistil. As such, the time for pollen tubes to reach an ovule depends on the species. For example, it takes approximately five hours for the fastest growing vegetative pollen tube cell of Arabidopsis to grow approximately 3 mm inside the pistil to deliver the SCs and it takes an additional four to five hours to fertilise every ovule (Jean-Emmanuel et al., 2002). In maize, pollen tubes need to grow up to 25 cm in length for approximately 24 h, growing at an astonishing rate of almost 1.0 cm h^{-1} (Dresselhaus et al., 2016; Zhou et al., 2017). In rice, it only takes 15–60 min after pollen tube germination to reach an ovule and two to five hours to

complete fertilisation (Lan et al., 2004; Dong and Yang, 1989). Moreover, pollen tube growth is affected by the structural, physical, and nutritious properties of the different pistil tissues. A switch in tube growth speed is clearly observed in bicellular pollen species, such as *Nicotiana tabacum* (see below), which was suggested to be correlated with the staging of the second mitotic division of the GC into two SCs while growing through the style transmitting tissue (TT) of the pistil. In Arabidopsis, however, a species with tricellular pollen upon anthesis, the tubes grow slower through the TT of the short style and the long septum in the centre of the ovary as compared to the initial stigma growth (Lennon et al., 1998; Cheung et al., 2010). Pollen tubes show directional growth with high precision due to structural and biochemical clues that are provided by the different pistil tissues, i.e. stigma, style, ovary, and ultimately, the embryo sacs inside the ovules.

During pollen–pistil interaction, specific members of a large family of membrane localised receptor-like kinases (RLKs) are involved in the guidance of the expanding pollen tube tip through the different pistil tissues. Both pollen tube (ANX1/2, BUPS1/2 and LIPS1/2) and ovule-specific RLKs (STRUBBELIG) are responsible for sensing directional clues for micropyle entrance into an ovule and for the communication between pollen tube tip and the different types of embryo sac cells such as the synergid cells with their filiform apparatus (FERONIA), the eggs and CC, but also the integument cell layers surrounding, protecting and communicating with the embryo sac during ovule development (Muschietti and Wengier 2018; Wang et al., 2016).

'Defensin-like' proteins, i.e. LUREs and EA1 produced by synergids and egg cell respectively (see below), are responsible for a species-specific close-range attraction and guidance of pollen tubes into the micropyle of the ovule (Márton et al., 2012; Okuda et al., 2013; Takeuchi and Higashiyama, 2012). Remarkably, the interaction between growing pollen tubes and female pistil tissues during the progamic phase of plant reproduction is essential to prime the pollen tubes to respond to the LURE protein attractants and to release the SCs inside the embryo sac (Okuda et al., 2013; Leydon and Johnson, 2013). Interestingly, an additional style TT produced component that triggers the competence of pollen tubes to respond to these LURE proteins is not a protein or peptide but is shown to be an arabinogalactan polysaccharide (AMOR) (Mizukami et al., 2016; Okuda et al., 2013). One of the two synergid cells at the entrance of the embryo sac collapses upon pollen tube arrival at this site, at least in Arabidopsis (Jean-Emmanuel et al., 2002) concomitantly triggering the arrest and rupture of the pollen tube tip, releasing its content including both SCs (Leydon and Johnson, 2013; Leydon et al., 2013).

Curiously, an important structural characteristic of all different types of cells within an embryo sac (Yadegari and Drews, 2004) but also of the pollen tube arriving at one of the synergid cells and as well as of the two released SCs is the development of a cell wall. This may create a potential structural barrier between cells and thus prevent direct membrane–membrane interaction and animal-like cell communication and fusion event(s) (Wang et al., 2010; Mori et al., 2014). Signalling between these cells and the communication by making use of membrane-localised receptors, such as the FERONIA receptor, GPI-anchored LORELEI protein or the SC-specific MDIS1-MIK receptor kinases, GCS1/HAP2, and GEX2 (Mori et al., 2005, 2014; Escobar-Restrepo et al., 2007; Igawa et al., 2013; Liu et al., 2016; Dresselhaus and Snell, 2014; Wang et al., 2016; Berger et al., 2008; von Besser et al., 2006), can only occur based on the release (exocytosis) of highly diffusible compounds, e.g. Ca^{2+} and signalling proteins such as the group of small secreted cysteine-rich globular proteins (CRPs), e.g. the LURE proteins (Sprunck et al., 2012; Dresselhaus et al., 2016; Mizuta and Higashiyama, 2018; Márton et al., 2012). However, it has been demonstrated that in *N. tabacum*, high molecular weight and glycosylated arabinogalactan-like proline-rich proteins from the pistil TT can traverse pollen tubes walls *in vivo* ending up against the pollen tube membrane (de Graaf et al., 2000). Cell wall modifying enzymes such as pectinases, glucanases, and expansins may play a role in changing the structural properties of these cell walls facilitating protein movement across plant cell walls, supporting cell–cell communication (de Graaf et al., 2000). The CRPs and other defensin-related small peptides, including the LURE proteins, and especially the short RALF (*r*apid *al*kalinization *f*actor) peptides (see above), may be able to traverse cell walls more easily, quickly, and specifically (Takeuchi and Higashiyama, 2012; Márton et al., 2012).

In both bicellular and tricellular pollen species, the GC and the two SCs are spatially, structurally, and temporally connected with the vegetative tube cell nucleus (VN), defined as a male germ unit (MGU). The GC and SCs are surrounded by a continuous vegetative membrane which has originated from the microspore membrane during pollen mitosis I – during pollen development in the anther locule – when the GC becomes 'endocytosed' from the vegetative pollen cell after asymmetric division (see above). An intercellular matrix or 'periplasm-like' region, rich in pectins and arabinogalactan proteins (AGPs), separates both SCs from the vegetative tube cell. One end of the SCs forms a characteristic hook structure and creates an intimate contact between the VN of the tube cell and either the GC or SCs and is suggested to play a role in communication (Jiang et al., 2015; Grant-Downton et al., 2013; Slotkin et al., 2009).

However, plasmodesmata-like cytoplasmic connections between both cell types appear to be absent (van Aelst and van Went, 1992; Cresti et al., 1987; Southworth and Kwiatkowski, 1996). After pollination and pollen tube germination, the MGU becomes translocated into the growing tube where it migrates with and at a specific distance from the growing pollen tube apex. Importantly, once pollen tubes grow inside the female pistil, the interaction between the male gametophyte and maternal tissues triggers different gene expression in the pollen tube, compared to *in vitro* growing pollen tube cultures. This indicated that pistil pollen tube communication is essential for directed pollen tube growth, and indeed pollen tubes respond to the LURE protein attractants produced by the female gametophyte (Lin et al., 2014; Qin et al., 2009). In addition, maturation of the SCs in the tricellular pollen of Arabidopsis seems to be completed only by the time pollen tubes reach the embryo sacs (Friedman, 2006). An important question that needs to be resolved is whether SCs, when transported through a growing pollen tube – are indeed actively involved in pollen tube guidance to an ovule and fertilisation (Mori et al., 2005). When the SC expressed HAP2/GCS1 protein is absent, these pollen tubes are disrupted in their ability to find the micropyle of the ovules, and those that do arrive fail to initiate fertilisation (von Besser et al., 2006). Recently, however, Glöckle et al. (2018) demonstrated that mutant pollen – in which the second mitotic division of the GC into two SCs is blocked – are still capable of producing a pollen tube, and find their way to an ovule in vivo. These results suggest that SCs are not important for the function of the pollen VC, which is the formation, polarised and directional growth of the pollen tube (Glöckle et al., 2018; Zhao et al., 2012). How communication is established between all the different cell types needs to be resolved.

2.3 Double Fertilisation

Recently, it has been demonstrated that besides reactive oxygen species (ROS), RALF peptides specifically expressed in the pollen tubes and in the ovule are involved in the actual disruption of the pollen tube tip membrane, at least in tissue culture (Ge et al., 2017; Mecchia et al., 2017; Duan et al., 2014). Remarkably, reducing the level of these RALF peptides does not result in a 'nonbursting' pollen tube phenotype in plants which suggests that other pollen and or ovule components are involved in the sperm release from pollen tubes. Following the release of the SC pair in the embryo sac, one of the synergids degenerates. Initially, both SCs move in close proximity showing a Brownian-like movement inside the embryo sac, up to the point that they separate, and move in different directions: one travels to the egg cell, whereas the second one find its way to the CC to

fuse and form the new zygote (sporophyte) and endosperm, respectively (Zhang et al., 1999; Huang and Russell, 1994; Kawashima et al., 2014), such events have now been captured by live imaging (Hamamura et al., 2011). The SCs have a protoplast-like morphology (Jean-Emmanuel et al., 2002), decorated with myosin, and are proposed to be guided precisely to the egg and CC by two actin coronas for double fertilisation to take place (Huang and Russell, 1994; Zhang et al., 1999; Kawashima et al., 2014; Ohnishi et al., 2014). At this stage, the vegetative membrane surrounding the two SCs and the pectic/AGP matrix must be removed to be able to make the contact between sperm membrane and the actin coronas and to achieve fusion with the egg and CC ultimately, with a lag time of eight minutes between sperm release and actual fusion (Hamamura et al., 2011; Denninger et al., 2014). Interestingly, in rice, the chalazal end of the egg cell does not present a proper cell wall which is suggested to be the site of SC fusion whereas formation of a cell wall early after sperm-egg fusion in Arabidopsis and maize is suggested to be important to prevent polyspermy (Kranz et al., 1995; Okamoto, 2010; Tekleyohans et al., 2017).

When exactly SCs are fully mature and capable to fuse with the egg and CC s inside the embryo sac is currently not known. *In vitro* egg-sperm fusion experiments, using tissue cultured tobacco pollen tubes for the isolation of the SCs and egg cell protoplasts, suggests that the sperm is 'more or less' fully mature and capable of fusing with the egg cells immediately after division (Jean-Emmanuel et al., 2002; Khalequzzaman and Haq, 2005; Kranz et al., 2008). However, in tissue culture fertilisation assays, it has become evident that pollen tube attraction by ovules, the release of SCs and the fusion between sperm and egg cell is much more efficient when ovules, sperm, and egg cells are isolated from pre-pollinated pistils. Recently, it has been suggested that the small cysteine-rich EC1 protein, secreted by the egg cell, is important for rapid SC 'activation' to fuse with the egg cell more efficiently *in vivo* (Dresselhaus et al., 2016). Although it has been clearly demonstrated that pollen tubes need the interaction and communication with the different pistil tissues and the embryo sac containing the egg cell, respectively, it is still debated whether within a single male gametophyte (pollen), the VC or VN communicates directly with one or both SCs (Zhang et al., 2017a; McCue et al., 2011; Slotkin et al., 2009; Grant-Downton et al., 2013; Jiang et al., 2015).

The diploid CC inside the embryo sac fuses with one of the two SCs and this fusion product, the endosperm initial, develops a triploid endosperm, which functions as a temporary nutrient source for the actual developing zygote into the new embryo and seedling ultimately. The nutrient storage capacity and composition of endosperm tissue varies greatly between different species (Esau, 1962). Remarkably, in

gymnosperms, producing naked seeds, although two SCs are being delivered by a single pollen tube, only a single fertilisation event takes place due to the absence of a CC. The second sperm degenerates during further seed development.

2.4 Preventing Polytuby

In Arabidopsis, upon pollen tube arrival and fertilisation, ovules also seem to become reprogrammed and either stop producing the short and – most probably – long distance guidance clues, or perhaps start producing repellent-like products preventing additional pollen tubes to grow towards and into an 'unavailable' ovule (Maruyama et al., 2015; Völz et al., 2013). For example, the production of LURE, a pollen tube attractant is turned off upon fertilisation (Palanivelu and Preuss, 2006; Maruyama et al., 2013). This process prevents the attraction of excess pollen tubes and the degeneration and fusion of the remaining synergid with the CC is key in this process. In Arabidopsis, this remaining synergid, the second synergid nuclei, degenerates after fertilisation, and this cell fuses with the CC in response to ethylene produced by the newly formed zygote (Maruyama et al., 2015; Völz et al., 2013). FIS-PRC2 is a chromatin-modifying complex involved in gene silencing via trimethylation of lysine 27 on histone H3 (H3K27me) (Kohler et al., 2012), and the activity of this complex is required for mitosis associated degradation of the synergid's nucleus (Maruyama et al., 2015). The fusion of the persistent synergid with the CC results in the dilution of the synergid produced attractants. JAGGER, an AGP is required for the degeneration of the remaining synergid (Pereira et al., 2016a, b). Interestingly, in *Oryza sativa* (rice, a monocotyledon), the degeneration of one of the synergids starts just before anthesis, thus even before pollination and this seems to be developmentally regulated rather than by an approaching pollen tube (Dong and Yang, 1989). However, in Arabidopsis, mutants that do allow more than one pollen tube to enter an ovule, the second set of SCs released were not able to fertilise the already fertilised egg or CC (Scott et al., 2008).

3 Cell Division and Fertilisation

The formation of a gametophyte involves three basic steps, the recruitment of diploid germ line cells in floral tissues, meiosis to generate haploid cells and one or more rounds of mitosis to generate the cells that will populate the gametophyte, including the gametes. During the mitotic

cell cycle, the duplication of genetic material during the S phase and segregation of the two copies in two different cells during M phase is temporally separated by two gap phases (G), G_1 and G_2 during which nutrient status and developmental signals can be integrated and DNA integrity control can be performed. The meiotic cell cycle is a modified cell cycle with a single S-phase but with two rounds of chromosome segregation generating haploid daughter cells.

In all eukaryotes, progression through both the meiotic and mitotic cell cycle is driven by the action of cyclin (CYC)/cyclin-dependent kinases (CDKs) complexes (De Veylder et al., 2007). These kinases phosphorylate key actors in cell cycle progression and directly or indirectly provide transcriptional and post-translational control over the core cell cycle machinery. The CDK/CYC kinases act in feedforward networks, wherein controlled destruction of cell cycle factors results in irreversible switches that provide direction to cell cycle progression (Zhao et al., 2012). To this end, cell cycle regulators are degraded by the proteasomes upon tagging by ubiquitin, implicating the action of ubiquitin ligases in cell cycle regulation. SCF (SkP, Cullin, F-box containing) complexes ubiquitinate factors involved in both G_1/S and G_2/M transitions, and the APC/C (anaphase-promoting complex/cyclosome) controls the metaphase–anaphase transition during mitosis (Genschik et al., 2014).

In higher plants, the G_1/S transition of the mitotic cell cycle is driven by the action of CDKA/CYC kinase complexes, whereas progression through G_2/M involves the action of both CYC/CDKA and CYC/CDKB kinases (Polyn et al., 2015). The activity of these CDK kinases is post-translationally controlled by phosphorylation. Activating phosphorylations that stabilise the T loop of the CDK are mediated by CDK-activating kinases (CAKs), while WEE1 kinases reduce activity by phosphorylating residues in the ATP-binding pocket. Interaction with inhibitors, of which the Kip-related proteins (KRPs)/interactor-inhibitor of cyclin-dependant kinase (ICK) and SIAMESE related (SIMS) are the best documented, and the control of CYCLIN levels by proteolysis are important negative feedback mechanisms. A key target of G_1/S CYCD/CDK and CYCA/CDK kinases is the retinoblastoma-related protein. Hyperphosphorylation of retinoblastoma related protein (RBR) by these kinases reduces its potential to interact with transcription factors and factors involved in chromatin structure and cell cycle progression. The action of phosphatases at the end of M-phase strips RBR of phosphate groups resulting in its reactivation. Proteolysis of the KRP inhibitors stimulated by the action of CYC/CDK via inactivation of RBR provides a positive feedback resulting in irreversible switch-like behaviour of the G_1/S cell cycle transition (Zhao et al., 2012). However, G_1/S kinases also regulate levels of RBR (Dewitte et al., 2003),

and E2Fa-RBR complexes suppresses E2F targets genes involved in cell differentiation (Magyar et al., 2012). This indicates that both RBR bound and free E2F fractions can have a role in suppressing differentiation and activating cell division. Further research into the role and stoichiometry of these fractions is required to elucidate this mechanism.

3.1 Gamete Formation and Cell Division

In preparation for sexual reproduction, male and female haploid gametes are produced in hermaphrodite flowers containing both male and female reproductive organs, anthers and pistils, or in male or female flowers that are formed on the same plant or on separate individuals, termed monoecious and dioecious plants respectively (Barrett and Hough, 2013). During flower development after the recruitment of a germ cell in the floral organs of the sporophyte, a series of meiotic and mitotic divisions, in combination with cell differentiation and programmed cell death, results in the formation of mature dry pollen grains and embryo sacs starting from diploid microsporocytes and megasporocytes, respectively.

Cyclins have been shown to be involved in meiosis, the correct execution of meiosis in the male microspore mother cell requires the action of six CYCAs, one CYCB type cyclin and the SOLODANCERS (SDS), related cyclins (Bulankova et al., 2013), which are activated in different stages of the process. CYCA1;2 is required for timely entry and prevents premature exit from meiosis after the first division. Its mutant alleles are known as *TARDY ASYNCHRONOUS MEIOSIS* (d'Erfurth et al., 2010). CDKA;1-CYCA1;2 phosphorylates and destabilises THREE DIVISION MUTANT1/MALE STERILE5 (TDM1) until meiosis I is successfully performed. TDM1 is required for meiotic exit by stimulating the APC/C activity at the end of meiosis II (Cifuentes et al., 2016). OSD1 seems to be also involved in controlling levels of APC activity, and *osd1 tam1* mutants even fail to enter into meiosis I (d'Erfurth et al., 2010). Several cyclins play a role in male meiosis. CYCA2s play an important role for the segregation of meiotic chromosomes. CYCB3;1 is localised to the meiotic spindles, and together with SDS represses the premature onset of cell wall formation (Bulankova et al., 2013). SDS is required for chromosome pairing. Meiotic exit requires the activation of TDM1 and the nonsense-mediated RNA decay factor encoding SMG7 genes (Bulankova et al., 2010). Male meiosis is sensitive to elevated temperatures, and the CDKG1-CYCLINL complex is involved in RNA splicing and is critical for chromosome pairing and recombination and the robustness of the meiotic process at ambient temperatures (Huang et al., 2013; Zheng et al., 2014b).

Secondly, after meiosis, several rounds of mitosis provide the appropriate cell number in the gametophyte. The male gametophyte contains three cells at the time of fertilisation. The pollen grain contains the SCs enveloped in the VC. This acquisition of new daughter cells fates involves RBR action as *rbr* mutant VCs retain aspects of the microspore whilst reiterating these unequal divisions (Chen et al., 2009). A second mitotic division of the GC generates the two SCs. Whilst this process is strictly conserved among all flowering plants, the timing of these divisions with respect to pollination and fertilisation seems to be variable in different species (Friedman, 1999). Degradation of KRP inhibitors by the SKP1-Cullin1-F-box protein (SCF) mediated by the FBL17 F-box protein is required to trigger the symmetrical division of the GC in order to produce two SCs (Kim et al., 2008), and CDKA;1 activity is required for their timely division (Nowack et al., 2006; Iwakawa et al., 2006; Aw et al., 2010). Cell cycle arrest of the VC requires the action of RBR, as well as suppression of CDKA;1 activity by the KRP inhibitors (KRP6 and KRP7 in Arabidopsis). SAMBA-mediated APC/C activity destabilisation of CYCA2;3 is required for progression through mitosis I (Eloy et al., 2012). The activation of CDKs by the combined action of the CDKD CAK kinases has proved to be essential for pollen mitosis I and II (Takatsuka et al., 2015).

The coordination of the final mitotic cell division and fate adoption during SC formation is intriguing. In gymnosperm male gametophytes, we also see a mitotic cycle that produces two SCs, even if this is unnecessary as only a single sperm is required for fertilisation, suggesting that cell cycle progression is not only necessary to generate the correct cell number but also a requirement for cell fate acquisition. However, cell division and sperm fate acquisition can be uncoupled in mutants impaired in the chromatin assembly factor 1 pathways in which single fertile SCs are formed (Chen et al., 2008), suggesting that defects in histone deposition might overcome the requirement of normal cell cycle progression to adopt a new cell fate. This in combination with the observation that in mutants of ARID1, which physically interacts with histone deacetylase, histone acetylation expands which is normally a feature of the SC, also expands into the VCs chromatin (Zheng et al., 2014a), suggest that regulation of histone deposition and activity is key in the process of SC specification. The coordination of fate acquisition and cell division during sperm formation requires the action of DUO1, a germline-specific Myb protein and a positive regulator of CYCB1;1 amongst other targets. DUO1 is itself a target of the ARID1 transcription factor. Both *duo1* and *arid1* mutant alleles display a high proportion of blocked divisions in the GCs (Durbarry et al., 2005; Brownfield et al., 2009). The expression of a subset of DUO1

targets requires the action of DUO3, a homeobox-related factor (Brownfield et al., 2009), which acts independently of CYCB1;1, but has a wider role in sporophytic cell division and embryo patterning.

In order to ensure an ovule produces a single seed, it is essential that only a single cell inside the female ovule primordium enters the meiotic programme. Several key factors in this process have been discovered. Entry into meiosis requires the action of SWITCH/AMEIOTIC 1 (Mercier et al., 2001; Pawlowski et al., 2009). Furthermore, RBR plays a key dual role in this and suppresses WUSCHEL, a factor controlling stem cell identity involved in meiocytes specification, and limits the number of mitotic divisions to trigger meiosis in a single cell (Zhao et al., 2017). During evolution, the development of the female gametophyte has evolved from a four nucleate, four-celled gametophyte created by two mitotic divisions of the megaspore into seven-celled, eight nuclei-containing structures by nuclear migration followed by the duplication of modules (Friedman and Ryerson, 2009). Additional variations in the pattern of division led to a wide flexibility of the cell (and nuclei) number in the female gametophyte (Schmid et al., 2015). The plant retinoblastoma-related protein is a key factor to limit the divisions in the female gametophyte (Ebel et al., 2004), and local elevated expression of a D-type cyclin can prevent the arrest of the CC (Sornay et al., 2015). The current working hypothesis for cell fate acquisition in the female gametophyte is that this is mediated through an auxin gradient in combination with accurate nuclear migration and positioning (Sprunck and Gross-Hardt, 2011).

Maintenance of DNA integrity is essential in the context of gamete production and integral to cell cycle progression. Hence, it is worth noting that cell cycle machinery and DNA repair are closely linked. E2F activity is suppressed by the conserved DNA damage protein SNI, and they act in a negative feedback loop, linking the G_1/S checkpoint with DNA repair (Magyar et al., 2012). E2Fa-RBR complexes are recruited to sites of DNA damage and interact with AtBRCA1, a conserved DNA repair-associated protein. Furthermore, RBR is required to recruit RAD51 to sites of DNA damage, suggesting a central role for RBR in complexes involved in DNA repair (Horvath et al., 2017; Biedermann et al., 2017). Specific CDK activity is essential for DNA repair to overcome mitotic arrest, *CYCB1;1* is rapidly induced upon DNA damage, the attraction of RBR and RAD51 to damaged loci is dependent on cycb1;1/CDKB kinase action and RAD51 is a substrate of CYCB1/CDKB1 kinases (Weimer et al., 2016; Schnittger and de Veylder, 2018). The WEE1 kinase arrests the cell cycle by phosphorylation of CDK interfering with ATP binding, and its action is essential to block the cell cycle in case of replication defects (De Schutter et al., 2007).

3.2 Coordination of Cell Cycle for Fertilisation

Fertilisation is the result of plasmogamy followed by karyogamy, the fusion of cells followed by the fusion of nuclei. In angiosperms, double fertilisation requires two mature SCs to physically interact and fuse with the egg cell and CC to form the zygote and endosperm precursors (see above).

Angiosperms can be divided into two groups based on pollen morphology: one group, comprising approximately 70% of the total number of species, releases pollen at anthesis at the bicellular stage after pollen dehydration and anther locule degeneration. In the second group of angiosperms ($\pm$30%), pollen will continue their development following a second mitotic step of the GC into two SC before pollen dehydration and anthesis, which is called tricellular pollen (McCue et al., 2011).

Friedman and colleagues established that there are three options for karyogamy in seed plants (Carmichael and Friedman, 1995). G_1 karyogamy, whereby the zygote undergoes DNA replication, or S-phase where karyogamy is initiated in G_1 but nuclei pass through S-phase before completing fusion, or G_2 karyogamy where nuclei fuse after S-phase and once completed enter M-phase. The sperm nuclei are guided towards the egg cell by interaction with the actin cytoskeleton, and once in close proximity, the egg cell chromatin moves into the sperm nucleus (Ohnishi et al., 2014). The synchrony of gametes required for karyogamy is a challenge from the point of view of cell cycle regulation. Male SCs have to reach the correct cell cycle phase, and the egg cell has to remain quiescent until karyogamy. It implies that core cell cycle regulators are involved that blocks gamete cells either in G_1 or in G_2, especially in the waiting or quiescent egg cell, and that in both the male and female gametophyte key regulatory pathways ensure this coordination. Progress in gamete isolation (Englhart et al., 2017; Schoft et al., 2015; Santos et al., 2017) and developments in transcriptomics and proteomics (Zhang et al., 2017b) are anticipated to put us in a position to identify the key core cell cycle regulators in this process. Although our knowledge is still fragmented due to our limits of detection, the emerging data on sperm (Table 1) and egg cells are in line with our knowledge about the cell cycle regulators involved in the key cell cycle transitions. Rice gametes fuse in G_1, and transcripts for key factors such as KRPs, CDKB1;1, WEE1, and KRP2 were detected by RT PCR (Sukawa and Okamoto, 2018). An array-based analysis revealed significant levels of transcripts encoding for CDKB1;1, CYCB1-2, and KRP1 whereas proteomics detected only CDKA;1. This suggests that rice

SCs, while still in G_1 express a number of cell cycle genes, which have been implicated in DNA repair and G_2/M transition. In the egg cell, on the other hand, mainly transcripts encoding for KRP1 and CDKA;1 were detected (Sukawa and Okamoto, 2018). One can speculate that DNA repair mechanism are activated in SCs given that pollen are more exposed to environmental factors compared to the female gametophyte deep inside the ovary, alternatively, the paternal genome could be the main source of G2/M factor encoding transcripts.

Table 1 Transcriptomics and Proteomics in Male Gametes

	G_1	S	G_2
Rice, single-cell proteomics (Abiko et al., 2013a) Rice sperm, transcriptomics (Abiko et al., 2013b)	PCNA, CDKA;1 *KRP1* *CDKB1;1* *CYCB1-2*		
Arabidopsis pollen (Grobei et al., 2009) (Weimer et al., 2018) (Zhao et al., 2012) Arabidopsis sperm RNA (Borges et al., 2008)		CDKA;1, CDKB2;1, CDKB2;2, CYCA2;1, CYCA3;1, CYCB1;3, CYCB2;4, CYCD2;1, CYCD4;1, RBR CYCD7;1-GFP CDKA;1-YFP *CDKA;1 (−−), CDKB1;2(++) CDKB2;2 (++), CDKB2;1 (−−), RBR (++), CYCB3;1 (++), CYCA1s (+), CYCA3 (−−), CYCD2;1 (−−), RBR (++), CYCD3;1 &D3;3 s (−−), CYCD3;2 (+), CYCD4;1 (+)* *CYCD1;1 (+), CYCD5;1 (−), CYCD2; 1(−), KRP1 (−), KRP2 (−), KRP3 (−−)), CDC6, CDC25/ACR2, CDC48*	
Lilly (Zhao et al., 2013) Tobacco pollen transcriptomics (Hafidh et al., 2012)			SC enriched in SCF complex-mediated proteolysis components and CDKB1;1 *CDKA;1* *CYCB1;1* *CDKB2;2* *KRP3*

Identified proteins or transcripts in species with pollen described to be in G_1, S or G_2.
Blue. transcripts: purple. native proteins: green. translational fluorescent protein fusions.

[ntbz001.eps]

In Arabidopsis pollen CYCLIND, CDKA and CDKB transcripts and the proteins they encode were detected. Whilst some KRP transcripts were detected their levels were relatively low, suggesting that if they are involved in the S-phase arrest of the SCs, the SCs are preloaded with KRP proteins after the last division. The localisation of CYCD7;1 in the SCs is intriguing given that CYCD7;1-GFP accumulates in the precursor of stomatal guard cells, an unrelated cell type (Weimer et al., 2018).

3.3 Fertilisation and Activation of Cell Division in the Zygote

Fertilisation of both the egg and the CC forms the zygote (2n) and endosperm precursor, which is the starting point for development of the new sporophyte and the endosperm inside the developing seed. The origin of the double fertilisation mechanism and the gain of fitness associated with this development is an intriguing problem in developmental biology. The requirement for the contribution of paternal gene expression to sustain proliferation of the endosperm is ensured by genomic imprinting. The need for the paternal contribution can be bypassed in mutants in the FIS-class genes (Nowack et al., 2007).

Sperm and CC plasmogamy is sufficient to trigger the first divisions of the endosperm precursor, evidenced by the pollination with *cdka;1* mutant sperm which prevents karyogamy of the homodiploid CC and the sperm nucleus but does trigger the first rounds of mitosis (Aw et al., 2010). This suggests that the contribution of the second sperm for the fertilisation of the CC is more involved in the timing of the division, rather than a strict requirement for the division mechanism. In combination with the autonomous endosperm development in some mutant backgrounds, this observation supports the hypothesis of a gametophytic origin of the endosperm (Strassburger, 1900; Nowack et al., 2007), and that the fertilisation by the second sperm, in combination with imprinting provides a mechanism to synchronise the proliferation of the endosperm with the formation of the zygote.

Upon the division of the egg cell, the two daughters not only differ in size but also in developmental fate, hence the first division of the zygote is asymmetric and differential, generating a smaller terminal cell and a larger basal cell. This division establishes the apical–basal axis of the future embryo. Proliferation of the apical cell will develop all the embryonic structures apart from the quiescent centre and root cap. Proliferation of the basal cell will form the quiescent centre and root cap and the suspensor (ten Hove et al., 2015). *De novo* transcription of cell-type specific homeobox genes in both cell types guarantees the polarised development of the embryo proper and suspensor cells (Ueda et al., 2011; Armenta-Medina et al., 2017). This first asymmetric division of the zygote requires extensive reorganisation of the cytoskeleton (Kimata et al., 2016). Upon fertilisation, microtubules form a transverse ring to drive apical cell growth. Actin filaments reorganise to position the nucleus in the apical end of the zygote before the asymmetric division takes place. Furthermore, the anaphase-promoting complex ubiquitin ligase has to be active to target CYCB1;1 for destruction for proper formation of the new cell wall (Guo et al., 2016).

From a cell cycle perspective, this poses several questions. How does successful karyogamy triggers cell cycle progression, and how is an asymmetric division established?

One of the key players during the whole process of sexual reproduction in plants, as in animals and humans, is calcium (Fan et al., 2008; Edel et al., 2017). This universal signalling component is important in many – if not all – intracellular plant cell signalling events. Calcium as second messenger functions either at the ion level or by highly cell type-specific changes in free cytoplasmic concentrations or changes in stored calcium in various intracellular compartments. These so-called 'calcium signatures' can be triggered by intracellular and extracellular stimuli (Edel et al., 2017). Pollen development in the anther requires Ca influx from the surrounding sporophytic tissue, and upon landing on the stigma, pollen tube growth through the pistil tissues towards and into the ovule is highly dependent on calcium. Upon entering the micropyle, the entrance site of the ovule near the egg and synergid cells, the growing pollen tube first encounters one of the two synergid cells (SY). The physical interaction between growing pollen tube tip and this SY cell initiates a calcium response of continuous oscillations in the SY, leading to programmed cell death (PCD) and collapse of only this particular SY cell and the release of stored calcium into the cavity where the pollen tube resides (Denninger et al., 2014). This localised increase of free calcium may coincide with slowing down pollen tube growth (Holdaway-Clarke et al., 2003; Hill et al., 2012).

A distinctive short calcium spike response is observed in the egg cell upon SC release whereas upon the arrival of the sperm at the egg cell, which shows a distinctive deformation, a second transient rise in intracellular calcium is detected in the egg cell upon fertilisation, but only after successful karyogamy (Denninger et al., 2014). This second, longer lasting rise in calcium trigger *de novo* cell wall synthesis of the fertilised egg and prevents the unlikely event of polyspermy. Once fertilisation has occurred, the production of the LURE protein, which is the pollen tube attractant, is turned off (Palanivelu and Preuss, 2006; Maruyama et al., 2013).

The second calcium signature, a transient broader spike, detected upon successful karyogamy (Denninger et al., 2014; Palanivelu and Preuss, 2006), besides preventing polyspermy, may also be the intrinsic autonomous and decisive signal in the fertilised egg cell to re-start the cell cycle, and thus early embryogenesis. Importantly, immediately after karyogamy, the first division of the single-celled zygote is asymmetric, producing a small apical cell that will develop further into the embryo and new sporophyte ultimately, whereas a much larger basal cell is responsible for the formation of the suspensor (Yadegari and Drews, 2004). It is tempting to propose a role for various $Ca2^+$/calmodulin-dependent protein kinases in this process. Also, of particular interest in this process is the

ZAR1 receptor kinase. *Zygotic arrest 1* was identified as a mutant in which the asymmetric division and the daughter cell fate acquisition was perturbed *ZAR1* encodes a receptor-like kinase/Pelle kinase (Gish and Clark, 2011) that interacts with calmodulin and the heterotrimeric G protein Gβ. In *zar1* the basal cell is short and the apical cell has basal cell fate (Yu et al., 2016).

De novo transcription of cell-type specific homeobox genes and miRNA biosynthesis in both cell types guarantees the polarised development of the embryo proper and suspensor cells (Ueda et al., 2011; Armenta-Medina et al., 2017).

4 Concluding Remarks

The complexity of the interactions required for the coordination of fertilisation in seed plants is emerging and advanced live imaging is revealing new features. In this process, the control of the cell cycle is vital to generate the gametes, instigate cell cycle synchrony for gamete fusion and trigger cell division upon successful germination. The identification of cell cycle factors involved has started, and links with coordinating pathways discovered. This research has the potential to provide us with new breeding strategies to enable fertilisation and thus seed setting under challenging conditions.

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