Establishing a framework for female germline initiation in the plant ovule

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Abstract

Female gametogenesis in flowering plants initiates in the ovule, where a single germline progenitor differentiates from a pool of somatic cells. Germline initiation is a fundamental prerequisite for seed development but is poorly understood at the molecular level due to the location of the cells deep within the flower. Studies in *Arabidopsis* have shown that regulators of germline development include transcription factors such as NOZZLE/SPOROCTELESS and WUSCHEL, components of the RNA-dependent DNA methylation pathway such as ARGONAUTE9 and RNADEPENDENT RNA POLYMERASE 6, and phytohormones such as auxin and cytokinin. These factors accumulate in a range of cell types from where they establish an environment to support germline differentiation. Recent studies provide fresh insight into the transition from somatic to germline identity, linking chromatin regulators, cell cycle genes, and novel mobile signals, capitalizing on cell type-specific methodologies in both dicot and monocot models. These findings are providing unique molecular and compositional insight into the mechanistic basis and evolutionary conservation of female germline development in plants.

Keywords: *Arabidopsis*, differentiation, epigenetics, expansion, germline, megasporogenesis, ovule.

Introduction

During plant growth, cells must undergo numerous transitions to facilitate the essential developmental processes of germination, vegetative growth, and flowering (Bäurle and Dean, 2006). Changes in cell identity that define these developmental events have been intensively studied in the model plant *Arabidopsis thaliana*, mainly because it is small, easy to manipulate genetically, and provides access to a diverse range of genomic resources (Pang and Meyerowitz, 1987; Arabidopsis Genome Initiative, 2000). Many *Arabidopsis* genes have been identified that contribute to cell identity in different contexts, but a few key examples illustrate the basic mechanisms that facilitate plant cell differentiation. For example, in the shoot apical meristem, the identity of pluripotent stem cells is regulated by a negative feedback loop involving the signal peptide CLAVATA3 (CLV3), the CLAVATA1 (CLV1) receptor kinase, and the homeodomain transcription factor (TF) WUSCHEL (WUS), which is expressed in a group of cells termed the 'organizing centre' (Schoof *et al.*, 2000). A similar TF receptor–ligand regulatory mechanism has been found in the root meristem involving the CLV3/ESR-like CLE40 peptide, the *Arabidopsis* CRINKLY4 (CR4) receptor-like kinase, and a homologue of WUS, WUSCHEL-RELATED-HOMEOBOX5 (WOX5) (Sarkar *et al.*, 2007). Similar to WUS in the shoot, WOX5 is expressed in a group of root organizer cells called the 'quiescent centre'. Other regulators of root cell differentiation include the mobile transcription factor SHORTROOT, and its transcription factor target SCARECROW, which activate miRNA165/166 repressors as part of an elegant pathway that involves plasmodesmata and intercellular signalling to control xylem and endodermis development (Di Laurenzio *et al.*, 1996; Koizumi *et al.*, 2012). Moreover, in the epidermis of leaves and roots, transcriptional regulators such as GLABRA1 (R2R3- MYB) and GLABRA2 (homeobox-leucine zipper), bHLH proteins, and the WD-40 protein TRANSPARENT TESTA GLABRA 1 act in multimeric complexes to control hair formation and patterning in specific cell types (Schiefelbein, 2003). In general, these pathways involve a combination of organizer and response cells that interact via signals to cue differentiation of distinct cell types and tissues.

Critical differentiation events also take place during reproductive development. The flower is one of the last structures to form during plant growth, but integrates an array of cell specification and differentiation events in a relatively short time frame. This is particularly evident in the ovules and anthers, where germline cells differentiate from pools of sporophytic (somatic) cells and quickly transition towards a gametophytic phase (Schmidt *et al.*, 2015). The germline cells, known as the microspore mother cell (MiMC) in the anther and megaspore mother cell (MeMC) in the ovule, are predisposed to initiate meiosis and reduce their chromosome number by half, a critical requirement for stable sexual reproduction. The capacity to differentiate a germline cell, promote its entry into meiosis, and support its development into a haploid phase highlights a specialized type of plant cell differentiation; one that shares some similarities with other meristem types and is highly conserved amongst plant species (Grossniklaus, 2011).

Unlike male tissues where multiple MiMCs will initiate in each anther, only a single MeMC will typically arise in each ovule. The plant ovule consists of sporophytic tissues including a proximal funiculus, central chalaza, and distal nucellus surrounded by integuments (Fig. 1). The transition from a somatic cell to a germline precursor occurs within the nucellus (Maheshwari, 1950), usually beginning with expansion of a somewhat ambiguously termed 'archesporial cell'. This cell or one of its products will give rise to a single MeMC that subsequently undergoes meiosis to produce four meiotic products in a process also referred to as

megasporogenesis (Pinto *et al.*, 2019). Only one of the meiotic products, the functional megaspore, will initiate the subsequent stages of germline development (megagametogenesis) and the other three megaspores degenerate. This contrasts with MiMC development in the anther where all four meiotic products become viable pollen grains.

Considerable knowledge has been generated regarding male germline development in Arabidopsis and rice, and this has recently been reviewed (Lockhart, 2014; Russell and Jones, 2015; Vogler *et al.*, 2017). Although the same amount of knowledge is lacking in regards to female germline development, recent studies provide key insight into the pathways involved. In this review, we explore these findings in relation to key regulatory mechanisms involved in female germline formation in Arabidopsis and angiosperms in general. We discuss (i) cues that influence the number of germline cells forming in the ovule; (ii) morphological features that define germline formation; and (iii) factors that may influence the privileged location of the germline cell.

Megasporogenesis: there can be only one... most of the time

Many studies in diverse angiosperms have provided a descriptive summary of megasporogenesis, highlighting the remarkably conserved nature of the process (Tilton and Lersten, 1981; Kennell and Horner, 1985; Bajon *et al.*, 1999; Russell, 2001). Indeed, only one MeMC is typically reported in most angiosperms and gymnosperms. This is illustrated by the examples of the gymnosperm *Larix decidua* (larch; Fig. 2A), the monocot *Hordeum vulgare* (barley; Fig. 2C), the early divergent angiosperm *Annona cherimola* (cherimoya; Fig. 2D), the model Brassicaceae Arabidopsis (Fig. 2E), and the Asteraceae member *Hieracium pilosella* (mouse-ear hawkweed; Fig. 2G). However, multiple MeMCs have been reported in species such as *Trimenia moorei*, which belongs to the ancient angiosperm clade Austrobaileyales (Bachelier and Friedman, 2011). This feature, can also be observed in atypical gymnosperms such as *Gnetum* (Fig. 2B) where up to 12 MeMCs form. Some of these cells degenerate, yet around five female MeMCs enter meiosis (Takaso and Bouman, 1986). In other specialized cases, such as *Hieracium aurantiacum* (orange hawkweed; Fig. 2F), multiple ‘germline-like’ cells can be observed in close proximity to a typical MeMC. These cells are called aposporous initials (AIs) and contribute to the process of asexual seed development (apomixis). It is unlikely, however, that these AI cells are extra MeMCs, since compositional and molecular studies suggest they share greater similarity with the functional megaspore than the MeMC (Tucker *et al.*, 2003; Juranic *et al.*, 2018).

Based on cytological studies, one of the key differences during megasporogenesis in angiosperms is the nucellus layer surrounding the female germline. This consists of several epidermal layers in crassinucellate ovules (e.g. *Annona* species; Fig. 2D) or a single epidermal nucellus layer in tenuinucellate ovules (e.g. Arabidopsis and *Hieracium*; Fig. 2E–G). The crassinucellate ovule has been considered the ancestral type of ovule in angiosperms (Sporne, 1969). It is generally found in living gymnosperms such as *Pinus*, *Larix*, and *Gnetum* (Fig. 2A, B; Takaso and Bouman, 1986; Fiordi, 1987), basal/earlydivergent angiosperms (e.g. *Annona*; Fig. 2D; Endress, 2011; Lora *et al.*, 2017), and in cereal monocots (Fig. 2C; Rudall, 1997), although this is debated in species such as barley depending on the developmental stage. In contrast, tenuinucellate ovules are found in evolutionarily derived angiosperms such as the eudicot Arabidopsis (Fig. 2E; Bajon *et al.*, 1999; Endress, 2011). The nucellus is an intriguing tissue,

fulfilling roles in germline development, nutrient flow, and mechanical support (see Wilkinson *et al.*, 2018 and Lu and Magnani, 2018 for recent reviews).

Diverse factors influence germline formation

Apart from genes identified via genetic screens for reduced fertility, details of the molecular and genetic mechanisms underlying megasporogenesis have been difficult to dissect. Indeed, most of the classic megasporogenesis-related genes were reported in *Arabidopsis* some time ago (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999; Gross-Hardt *et al.*, 2002). More recently, progress has been facilitated through improved microscopic and genomic technologies, and details of these pathways and their role during megasporogenesis are considered below (Fig. 3). Among them, the *KNUCKLES* (*KNU*) gene has been used extensively as a marker of MeMC identity (Payne *et al.*, 2004; Tucker *et al.*, 2012a). *KNU* encodes a C2H2 zinc-finger protein involved in the basal pattern elements along the proximal–distal axis of the *Arabidopsis* gynoecium prior to the initiation of female germline development (Payne *et al.*, 2004). Although *KNU* has been shown to accumulate in the MeMC and possibly even the archesporial cell, its role in MeMC development remains unknown.

sRNA pathways: a possible trigger for germline development

At early developmental stages the morphology and size of most ovule cells (particularly those in the sub-epidermal L2 layer) are similar, leading to the suggestion that multiple cells have the potential to become an MeMC (Nonomura, 2003). However, differences in cell volume appear relatively quickly once germline development initiates; in *Arabidopsis*, L2 ovule cells increase in volume before stabilizing at the flanks of a centrally positioned germline precursor, the archesporial cell, which continues to increase in volume and becomes the MeMC (Fig. 1; Lora *et al.*, 2017). A concomitant increase of the nuclear volume of the MeMC during these early developmental steps may reflect a change in the epigenetic landscape. Indeed, specific chromatin decondensation and reprogramming were observed during MeMC differentiation in *Arabidopsis* (She *et al.*, 2013). Several mutants also suggest that epigenetic pathways may restrict expansion of L2 cells in general (Armenta-Medina *et al.*, 2011). For example, mutations in *ARGONAUTE 9* (*AGO9*), *RNA-DEPENDENT POLYMERASE 6* (*RDR6*), and *SUPPRESSOR OF GENE SILENCING 3* (*SGS3*), among other RNA-directed DNA methylation (RdDM) silencing components, result in the simultaneous expansion of multiple L2 cells adjoining the MeMC in *Arabidopsis* (Fig. 3A; Olmedo-Monfil *et al.*, 2010). While *RDR6* is involved in the biogenesis of siRNAs (Peragine *et al.*, 2004), *AGO* family proteins bind diverse small RNA (sRNA) molecules and induce silencing of complementary sequences by post-transcriptional gene silencing or DNA methylation (Fang and Qi, 2016). Recently, multiple MeMC-like cells were also reported in mutants of the THO/TREX complex genes (*TEX1*, *HPR1*, and *THO6*) in *Arabidopsis*. The THO complex is involved in the biogenesis of TAS3-derived *trans*-acting siRNA (ta-siRNA) molecules that spatially restrict the expression of the *AUXIN RESPONSE FACTOR 3* (*ARF3*) gene to the chalazal region of the ovule, while *TEX1* is localized in the nuclei of the epidermal cell layer (Fig. 3A; Su *et al.*, 2017). These results suggest that sRNA-guided repression of specific target genes in the young ovule limits the capacity for L2 cells to expand and prevents them from assuming germline fate.

AGO9 preferentially binds 24 nucleotide siRNAs derived from transposable elements (Olmedo-Monfil *et al.*, 2010) and has been observed in cytoplasmic foci of most somatic cells in the ovule primordia and in the nucleus of the MeMC (Rodríguez-Leal *et al.*, 2015; Su *et al.*, 2017). AGO9 belongs to the AGO4 clade, and mutations in the remaining AGO4 clade members (AGO4, AGO6, and AGO8) also show multiple enlarged germline-like ovule cells (Fig. 3A). This is not the case for other ovule-expressed AGOs such as AGO1, AGO5, and AGO10 (Tucker *et al.*, 2012a; Hernandez-Lagana *et al.*, 2016). Notably, the combination of *ago4* and *ago9* mutants leads to an increased frequency of somatic cells showing aberrant expansion in the ovule primordia, suggesting that members of the AGO4 clade may act on similar targets (Hernandez-Lagana *et al.*, 2016). Interestingly, variation is observed in the number of L2 cells that 'naturally' enlarge during ovule development, suggesting that the contribution of epigenetic pathways may vary between ecotypes. This is often accompanied by variations in the expression pattern and localization of AGO9, indicating that differences in upstream regulators or interacting partners might contribute to natural variability in germline initiation potential (Rodríguez-Leal *et al.*, 2015).

In addition to their role in Arabidopsis, AGO proteins have been shown to function during megasporogenesis in diverse species, although the timing and role of homologous genes sometimes differs. For example, the AGO104 gene from maize is related to AGO9 and is similarly expressed in somatic cells surrounding the female germline (Singh *et al.*, 2011). However, AGO104 acts to repress somatic cell fate in the germline, and mutants show alterations in chromatin condensation, spindle formation, and chromosome segregation. DNA methylation analysis suggests that AGO104 is involved in non-CG methylation in heterochromatin (Singh *et al.*, 2011), which may establish an epigenetic landscape that is essential for subsequent female germline development. Another conserved AGO protein, AGO5, accumulates in the cytoplasm of Arabidopsis nucellus cells that also express AGO9, but loss-of-function mutants show no obvious reproductive phenotype (Tucker *et al.*, 2012a). Curiously, a truncated semi-dominant AGO5 protein that lacks the catalytic PIWI-domain appears to affect the transition to megagametogenesis in the functional megaspore (Fig. 1), independent of AGO9 (Olmedo-Monfil *et al.*, 2010; Tucker *et al.*, 2012a). A similar phenotype was observed using P1/Hc-Pro, a viral suppressor protein that binds siRNA and inhibits AGO-dependent pathways (Kasschau *et al.*, 2003; Tucker *et al.*, 2012a). In rice, alterations of chromosome condensation were observed through mutation of the AGO gene, *MEIOSIS ARRESTED AT LEPTOTENE* (*MEL1*), which is phylogenetically closest to the AGO group of AGO5. Chromosome condensation is arrested during meiosis in *mel1* mutants (Nonomura *et al.*, 2007), while MEL1 protein is located in the cytoplasm of the germ cells and preferentially binds 21 nucleotide phase siRNA (Komiya *et al.*, 2014).

In summary, these studies suggest that several sRNA-related pathways have repressive functions during distinct stages of germline development. Although there is evidence of diversified functions in different species, the role of AGO proteins and sRNAs appears to be a fundamental requirement for correct germline specification. One key mechanistic question that remains unanswered is how these pathways inhibit growth/expansion of sub-epidermal cell types while selectively allowing expansion of a single germline cell (MeMC).

Cell competency factors are required for germline formation

Additional regulatory pathways contributing to megasporogenesis have been revealed via mutants that fail to differentiate an MeMC or, similar to the epigenetic pathways described above, produce additional MeMC-like cells. One of the earliest mutants described that shows

defective MeMC cell differentiation is *Arabidopsis nozzle/sporocyteless (nzz/spl)* (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). Normal female and male sporogenesis are blocked in *nzz/spl* mutants, meaning that plants are unable to differentiate any MiMC or MeMC. Mutations also affect surrounding somatic cells, since development of the anther wall is blocked and the ovule nucellus forms an elongated finger-like structure. The *NZZ/SPL* gene, which encodes a nuclear protein related to MADS-box transcription factors, appears to be expressed in several ovule tissues including the germline during sporogenesis (Fig. 3B; Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). While the *SPL* promoter monitored by β -glucuronidase (GUS) activity is observed at the micropylar end of the ovule throughout germline development (Yang *et al.*, 1999), *in situ* hybridization shows *NZZ/SPL* expression in the ovule primordia and later in the integuments and weakly in the MeMC (Schiefthaler *et al.*, 1999; Balasubramanian and Schneitz, 2000). The GUS expression pattern from an *NZZ*-GUS reporter gene is broader and is also observed in the funiculus and chalaza (Ito *et al.*, 2004). Despite reports suggesting otherwise, the cell type-specific requirements for *NZZ/SPL* function remain unclear.

Recently, orthologues of the *Arabidopsis NZZ/SPL* gene have been reported in important food crops such as the monocot, rice (Ren *et al.*, 2018), and the eudicots, tomato (Rojas-Gracia *et al.*, 2017) and cucumber (Liu *et al.*, 2018). Although overall sequence similarity is limited, all three genes show conservation in regions encoding the SPL, NLS, and EAR motifs. Expression of the *NZZ/SPL* gene in rice and tomato is observed in male and female tissues, and mutations produce defective stamens and ovules. Interestingly, while the formation of the MiMC and MeMC is affected in the tomato *hydra (nzz/spl)* mutant (Rojas-Gracia *et al.*, 2017), female sporogenesis is not obviously affected in the rice *Osspl* mutant, which successfully produces an MeMC in the ovule and only arrests during the later developmental process of meiosis (Ren *et al.*, 2018). A molecular function for *NZZ/SPL* has only recently been reported in *Arabidopsis*, suggesting that it may act as a transcriptional co-repressor (Chen *et al.*, 2014; Wei *et al.*, 2015). In both male and female sporogenesis, *NZZ/SPL* uses the EAR motif to recruit a TOPLESS/TOPLESSRELATED (TPL/TPR) and histone deacetylase (HDAC) complex to inhibit CINCINNATA (CIN)-like TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) transcription factors, thereby promoting meiotic fate in the anther and ovule (Chen *et al.*, 2014; Wei *et al.*, 2015). In rice, *OsSPL* also interacts with TPL and TCP in the anther (Ren *et al.*, 2018). These studies provide support for a generally conserved role of the *NZZ/SPL* gene.

Another study related to disruption of germline development in *Arabidopsis* investigated ovule phenotypes in *wuschel (wus)*, *windhose1-windhose2 (wih1wih2)*, and *tornado (trn)* mutants (Fig. 3B; Gross-Hardt *et al.*, 2002; Lieber *et al.*, 2011). At the molecular level, *NZZ/SPL* appears to promote expression of *WUS* in the nucellus, which in turn up-regulates expression of the small peptides *WIH1* and *WIH2* (Lieber *et al.*, 2011). *WIH1/2* genes act downstream of *WUS*, and double mutants of both genes show defective megasporogenesis in which ~30% of ovules fail to produce an MeMC (Fig. 3B). Similar phenotypes were also observed in *trn1* and *trn2* mutants (Lieber *et al.*, 2011). In contrast to *NZZ/SPL*, *WUS* mRNA and protein have only been observed in the nucellar cells surrounding the MeMC during ovule development (Zhao *et al.*, 2017).

Multiple inputs contribute to MeMC identity

Genes such as *NZZ/SPL* and *WUS* presumably act upstream of factors involved in the establishment and/or maintenance of MeMC fate. Some of these factors may have been revealed through transcriptomic analysis of different ovule cell types. For example, the first

transcriptomic analysis of the Arabidopsis MeMC and surrounding nucellar cells using laser capture microdissection (LCM) revealed an enrichment of transcriptional regulators and RNA helicases in the MeMC transcriptome. This led to the discovery of the MNEME(*mem*) gene, encoding an RNA helicase that is expressed in the germline from where it restricts germline identity in surrounding cells (Schmidt *et al.*, 2011). Mutations in *mem* lead to the formation of multiple germline-like cells in the L2 layer (somewhat reminiscent of *ago9*) and show epigenetic perturbations in gametophytic nuclei (Schmidt *et al.*, 2011). Although MEM has not been directly linked to the RdDM pathway, it is tempting to speculate that they may regulate similar targets.

An enrichment of transcriptional regulators, including *WUS* and *NZZ/SPL*, was also reported in a second LCM-based transcriptional study of the Arabidopsis nucellus (Tucker *et al.*, 2012a). In total, ~440 genes were identified that are significantly up-regulated in the nucellus relative to the rest of the ovule, reflecting a diversity of cell type-specific expression patterns in the epidermis, functional megaspore, and proximal nucellus. Another study analysed the transcriptome of the *nzz/spl* mutant, and identified four key genes involved in meiosis; *Atg04880*, *At1g13710*, *At1g13870*, and *At2g33640* (Zhao *et al.*, 2014). Further characterisation of *At1g13710*, which encodes a cytochrome P450 monooxygenase named KLUH (KLU) (Anastasiou *et al.*, 2007; Adamski *et al.*, 2009), revealed a role in chromosome pairing during female meiosis (Zhao *et al.*, 2014). Similar anomalies were also reported in mutants of *ACTIN RELATED PROTEIN 6 (ARP6)*, which is a component of the chromatin remodelling SWR1 complex (Qin *et al.*, 2014). Interestingly, the *arp6klu* double mutant revealed a genetic interaction between *KLU* and *ARP6* that suggests that they are normally involved in preventing multiple MeMC-like cells from forming in the L2 layer (Fig. 3: Zhao *et al.*, 2018). Further studies, including a transcriptional analysis using wildtype, *klu*, *arp6*, and *arp6klu* plants revealed how the transcription factor, WRKY28, which is exclusively expressed in the L2 cells adjacent to the MeMC, acts to repress germline fate. The expression of WRKY28 is controlled by the deposition of the histone variant H2A.Z mediated by SWR1 (Fig. 3C). H2A.Z deposition is dependent on the non-cell-autonomous function of *KLU*, which is expressed in the chalaza and inner integument of the ovule primordia (Fig. 3C; Zhao *et al.*, 2018).

The capacity to maintain multiple germline cells varies between species and mutants

Other components of germline formation may have been revealed in monocot systems. In rice, one pathway that prevents cells from entering into male and female sporogenesis involves the leucine-rich repeat receptor-like kinase MULTIPLE SPOROCTE (MSP1), since *msh1* mutants produce multiple MeMCs and MiMCs (Nonomura *et al.*, 2003). The TPD1-like protein of rice, TAPETUM DETERMINANT1/ MICROSPORELESS 2 (OsTLD1A/MIL2), binds to MSP1 in yeast and is co-expressed in somatic cells surrounding the MiMC and MeMC. *OsTLD1A*-RNAi lines only phenocopy *msh1* in the ovule (Zhao *et al.*, 2008; Hong *et al.*, 2012), suggesting the existence of alternative interacting partners of MSP1 in the anther. Similar protein pairs (EXS/EMS1– TPD1) have been reported in Arabidopsis (Zhao *et al.*, 2002; Canales *et al.*, 2002; Yang *et al.*, 2003, 2005; Jia *et al.*, 2008) and maize (e.g. *MAC1*, an orthologue of rice *TDL1A*; Sheridan *et al.*, 1996, 1999; Wang *et al.*, 2012), while an *MSP1* homologue has been revealed through transcriptome profiling of the maize anther (Ma *et al.*, 2007). These studies suggest that in addition to *NZZ/SPL* and its co-repressors, conserved receptor– ligand pathways contribute to megasporogenesis and microsporogenesis in multiple species. How these transcription factor and receptor pathways interact remains unclear, since the identification of targets, particularly in the ovule, is yet to be elucidated.

One curious finding is that mutants showing extra ‘germlinelike’ cells exhibit key differences in their ability to enter meiosis and/or produce a female gametophyte. For example, the extra germline-like cells in Arabidopsis RdDM mutants typically fail to enter meiosis and infrequently initiate gametophyte development (see Pinto *et al.*, 2019 for a review). In Arabidopsis *tex1* and *tas3* mutants, the extra cells occasionally start dividing as a gametophyte (Su *et al.*, 2017). Contrasting this, the rice *msp1* mutant (Nonomura *et al.*, 2003) and maize *mac1* mutant (Sheridan *et al.*, 1996) produce extra germline cells in the ovule that initiate meiosis and contribute to the formation of multiple disorganized gametophytes. It is unclear why this capacity to initiate meiosis varies between species and mutants. It may simply relate to different roles of the receptor–ligand EXS/EMS1 and RdDM pathways in female germline development. For example, RdDM pathways may fine-tune the ‘archesporial state’ to limit the pool of archesporocytes, while EXS/EMS1 pathways may influence both cell identity and competency, providing a capacity to produce multiple archesporocytes and subsequently MeMC. An alternative possibility indirectly raised by Nonomura *et al.* (2003) is that the enlarged nucellus in rice and maize provides a more suitable environment to support development of multiple MeMCs, somewhat reminiscent of the multi-MeMC phenotypes observed in *Trimenia* and *Gnetum* (Fig. 2). Based on the large size of the cereal nucellus (Wilkinson and Tucker, 2017), one could envisage it provides more nutritional support or more ‘organizer’ cells that are capable of producing signals that stimulate entry into meiosis. If such a stimulatory factor does exist, it must still be present to some degree in RdDM mutants of Arabidopsis, which still produce one fully functional MeMC that is capable of entering meiosis.

Several components of this ‘meiotic competency’ may have been revealed through study of cell cycle mutants. The triple mutant of *KRP* [interactor/inhibitor of cyclin-dependent kinase (CDK)/kip-related proteins] genes, *krp4krp6krp7*, produces multiple MeMCs as a result of mitotic division of the initial MeMC, which fails to directly enter meiosis (Fig. 3C; Zhao *et al.*, 2017). A similar effect was found in the mutant of *RETINOBLASTOMA-RELATED1* (*RBR1*), a key cell cycle regulator that is a target of CDKA:1. *RBR1*, a transcriptional repressor, binds the promoter of *WUS* to induce direct downregulation (Zhao *et al.*, 2017). While *WUS* is observed in the L1 layer of the nucellus in the wild type, *WUS* was observed in the MeMCs of *krp4krp6krp7* and *rbr1* mutants, and depletion of *WUS* limits the formation of multiple MeMCs in the *rbr1* background. Intriguingly, ectopic expression of *WUS* in the MeMC (of wild-type ovules) appears to have no effect, suggesting that the normal function of *WUS* is to organize developmental signals from somatic cells to the germline. These signals may be conserved between male and female tissues, but regulated by different factors, since multiple MiMCs were also observed in the anther of *krp4krp6krp7* where *WUS* is not normally expressed (Zhao *et al.*, 2017).

ICK/KRPs also appear to function redundantly to restrict MeMC formation to one cell; the inactivation of seven ICK/KRPs led to the formation of multiple MeMCs in random positions, suggesting the loss of a positional signal in addition to de-regulated *WUS* (Cao *et al.*, 2018). The canonical E2Fs, core cell cycle regulators, are also involved in this pathway. The *e2fa e2fc e2fc* triple mutant was reported to produce multiple MeMCs, but these may actually reflect multiple megaspores that failed to degenerate during functional megaspore selection (Yao *et al.*, 2018). Taken together, these findings detail a range of pathways involved in the molecular control of germline formation and progression. Importantly, links are emerging between developmental regulators (e.g. *WUS*) and essential cellular pathways (e.g. core cell cycle machinery such as *RBR1* and the ICK/KRPs), providing novel insight into the molecular requirements for germline initiation. Despite this, the nature of the signals that facilitate

differentiation into a fully functional MeMC remains unknown. One possibility, based on studies from a number of species, is that phytohormones are involved.

Megasporogenesis is highly polarized by auxin and cytokinin maxima

Phytohormones act as a key player in physiological processes during plant development, in particular by providing positional information that supports localized differentiation events (van den Berg *et al.*, 1995). During gynoecium development, the pattern of auxin and cytokinin distribution is complementary and, while auxin is observed distally, cytokinin distribution is proximal (Marsch-Martínez *et al.*, 2012; Schaller *et al.*, 2015). Similarly, distal auxin and proximal cytokinin distribution polarizes ovule primordia development (see Shirley *et al.*, 2019 for a recent review). In brief, the number of ovule primordia is reduced by inhibition of a regulator of polar auxin transport *Pin-formed 1 (PIN1)* (Okada *et al.*, 1991) or by mutation of a cytokinin receptor (Riefler *et al.*, 2006; Kinoshita- Tsujimura and Kakimoto, 2011). In contrast, the number of ovule primordia is increased in mutants of a cytokinin oxidase/ dehydrogenase-deactivating enzyme (Bartrina *et al.*, 2011). Importantly, Bencivenga *et al.* (2012) linked the two hormones in the ovule and showed that *PIN1* expression is controlled by cytokinin.

The polarized accumulation of both phytohormones continues during megasporogenesis, and appears to be dependent upon a combination of local biosynthesis and transport. The *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1)* gene from Arabidopsis encodes a protein involved in the biosynthesis of auxin (Zhao, 2012). During early ovule development, *TAA1* is expressed in the inner integument and funiculus (Ceccato *et al.*, 2013). Contrasting this, auxin accumulates in the tip of the nucellar epidermis (based on the synthetic auxin sensor, *DR5*; Fig. 4), most likely via *PIN1* (Benková *et al.*, 2003; Pagnussat *et al.*, 2009) and *PIN3* (Ceccato *et al.*, 2013) expression in the epidermis and recycling downwards towards the funiculus. On the other hand, cytokinin has been observed in the basal part of the ovule (Fig. 4; Zurcher *et al.*, 2013). Thus, the female germline in Arabidopsis develops while polarized by somatic auxin and cytokinin. Interestingly, the distal auxin maximum in the nucellus overlaps closely with a number of key regulators of female germline development including *WUS*, *AGO5*, and *WIH1/2* (Lieber *et al.*, 2011; Tucker *et al.*, 2012a; Zhao *et al.*, 2017). This auxin maximum was not observed in *nzz/spl* ovules, consistent with *NZZ/SPL* being required for *PIN1* expression (Fig. 3B; Bencivenga *et al.*, 2012).

A distal auxin maximum was also reported in the eudicot *Hieracium* (Tucker *et al.*, 2012b) and in the monocot maize (Forestan *et al.*, 2012; Lituiev *et al.*, 2013), and proposed in the early divergent angiosperms *Annona cherimola* (cherimoya) and *Persea americana* (avocado) (Lora *et al.*, 2017). These studies suggest a conserved hormonal microenvironment (in terms of auxin transport at least) around the female germline in flowering plants and a role for the nucellar epidermis as a signalling conduit. Remarkably, this does not seem to be adversely influenced by the number of layers that separate the MeMC from the tip of the ovule. Maize, cherimoya, and avocado produce a crassinucellate ovule compared with the tenuinucellate ovule of Arabidopsis and *Hieracium* (Fig. 2), meaning they show differences in the number of cell layers surrounding the female germline. Irrespective, both types of ovules show similar auxin accumulation. Interestingly, while only one *PIN1* gene has been identified in Arabidopsis and cherimoya, two *PIN1* (*PIN1.1* and *PIN1.2*) orthologues have been identified in avocado. The *PIN1* orthologue of cherimoya is observed in all 'epidermal' nucellus layers surrounding the female germline, while expression of avocado *PIN1.1* is only observed in cell layers surrounding the MeMC. However, the expression of a *PIN1.1* orthologue (also called *SISTER*

OF *PIN1* in Arabidopsis; O'Connor *et al.*, 2014) in avocado is mainly observed in the distal cell layers of the nucellus and complements the *PIN1.1* expression in the neighbouring cell layers around the MeMC (Lora *et al.*, 2017). How auxin transport and response might be achieved during female germline development in gymnosperms such as *Larix*, *Gnetum*, *Pinus*, or *Ginkgo* that produce even more layers between the germline and tip of the ovule (Fiordi, 1987) is currently unknown. While the central location of the MeMC is consistent, the 'depth' at which it is specified and/or develops within the nucellus must require specialized inputs depending on the species.

Cell wall components may allow germline cells to enter distinct developmental pathways

The defined position of the MeMC, the polarized nature of its meiotic products, and the involvement of distinct epidermal and subepidermal cells in specification suggest a need to precisely control the flow of information during megasporogenesis. The cell wall and its constituent polysaccharides, glycoproteins, and phenolic compounds provide an ideal matrix to regulate signal flow and facilitate changes in cell identity (reviewed in Tucker and Koltunow, 2014; Tucker *et al.*, 2018). For example, callose (1→3)-β-d-glucan has been identified as a molecular filter that can regulate the movement of molecules through plasmodesmata (Maule *et al.*, 2012). In angiosperms, callose generally appears around the MiMC just before meiosis (Blackmore *et al.*, 2007), and is typically observed around the MeMC and the megaspores of the ovule (Tucker *et al.*, 2001; Musiał *et al.*, 2015). Deposition in the MeMC wall precedes the initiation of meiosis, and may provide a transient molecular filter to restrict crosstalk with surrounding somatic cells. Plasmodesmata are observed in the MeMC and the lateral and chalazal side of the functional megaspore, but are absent in the functional megaspore wall that is adjacent to the degenerating megaspores of Arabidopsis (Bajon *et al.*, 1999). A recent study in apomictic *Hieracium* revealed localized callose deposits between the functional megaspore and dormant aposporous initials (ectopic functional megaspore-like cells, somewhat similar to those observed in RdDM mutants in Arabidopsis), potentially limiting access to signals that facilitate progression through the S/G2 phase of the cell cycle and the continuation of germline development (Juranic *et al.*, 2018). In gymnosperms, while callose has scarcely been observed around the MeMC and/or during meiosis (Rodkiewicz, 1970; Rafińska and Bednarska, 2011), the MeMC shows a thickening of the cell wall such that plasmodesmata are occluded during meiosis (Fiordi and Maugini, 1977; Fiordi *et al.*, 1988, 1991). In contrast, the gymnosperm *Taxus baccata* shows unchanged wall thickenings near plasmodesmata, and this coincides with the appearance of up to four functional megaspores (Fiordi *et al.*, 1991).

Another prominent yet confounding component of the female germline cell wall are the arabinogalactan proteins (AGPs). These glycoproteins are ubiquitous in plants and contain an extremely variable sugar chain that can be detached from the cell wall and secreted to the apoplast (Majewska-Sawka and Nothnagel, 2000; Ellis *et al.*, 2010), possibly acting as a signal for cell–cell communication. During Arabidopsis ovule development, AGP18 is required for selection of the functional megaspore (Demesa-Arévalo and Vielle-Calzada, 2013). Although AGP18 expression is only observed in the basal part of the integument during meiosis, overexpression results in survival of multiple megaspores after meiosis, suggesting that the transcript may be a non-cell-autonomous component of megaspore selection (Demesa-Arévalo and Vielle-Calzada, 2013). AGP localization, revealed using monoclonal antibodies such as JIM8 and JIM13, is dynamic during ovule development and varies between species. For example, although AGP epitopes are not detected in the Arabidopsis MeMC, AGPs are observed in the

MeMC of strawberry (Szczuka and Leszczuk, 2017; Leszczuk and Szczuka, 2018) and *Hieracium* species (Juranic et al., 2018). Moreover, AGPs have been observed in the cell wall of the MeMCs in the crassinucelate ovule of *A. cherimola* and *P. americana* (Lora et al., 2017).

These studies of the cell wall provide some insight into compositional changes that occur during the acquisition of germline identity. However, it is unclear how components such as callose and AGPs are integrated into the network of typical primary cell wall polysaccharides such as cellulose, pectin, and xyloglucan, and how they contribute to the process of MeMC growth. Although evidence supports the presence of these polysaccharides in ovule cells of several species (Lora et al., 2017; Juranic et al., 2018), their precise role has yet to be elucidated. Converging signals and mechanical changes may promote germline expansion. As discussed above, rapid expansion of the primary germline cell (compared with surrounding somatic cells) is a feature of MeMC maturation in many divergent plant species (Fig. 2; Lora et al., 2017). The signal source for this growth could be adjacent nucellar cells, which provide a positional cue that prompts the change in cell size. As described above, AGO9 represses ectopic 'MeMC' growth and division through an sRNA pathway, possibly acting from non-germline cells to inactivate genes involved in mitosis (Fig. 3; Olmedo-Monfil et al., 2010). At the same time, auxin forms a local maximum in nucellar cells, just above the distal end of the MeMC (Fig. 4; Benková et al., 2003; Pagnussat et al., 2009). In maize, a similar auxin maximum in the ovule primordium is observed, and the distal end of the maize MeMC expands more significantly than its proximal end (Lituiev et al., 2013). Auxin can be a trigger for either cell division or cell expansion (Perrot-Rechenmann, 2010), and in the context of MeMC development it seems feasible that auxin might result in cell expansion via inhibition of a cell division pathway. The arrested development of the germline precursor in the *Arabidopsis nzz/spl* mutant might simply be a result of disordered auxin distribution, since *NZZ/SPL* acts upstream of *PIN1* (Bencivenga et al., 2012). In general terms, auxin induces the acidification of the extracellular space, and activation of cell wall relaxants such as expansins and xyloglucan endotransglycosylase/hydrolases (XTHs) (Perrot-Rechenmann, 2010). The auxin maximum in the ovule primordium could provide similar stimuli to the MeMC. Accordingly, the accumulation of methylesterified homogalacturonan in the cell wall of the MeMC and adjacent cells also indicates the decrease of tissue rigidity in this area (Fig. 4; Pérez-Pastrana et al., 2018).

Although the surrounding somatic cells must create a favourable environment for the MeMC to expand, this expansion event is not just passively driven. The rice *msp1* mutant allows multiple germline precursors to develop, and each MeMC-like cell expands to a larger volume than somatic cells, giving rise to a wider nucellus in the mutant (Nonomura et al., 2003). Could positive regulators function within the MeMC to promote its expansion? As MeMC cell expansion progresses, the cell produces a large number of organelles and establishes a cellular polarity, with the nucleus localized at a more proximal position, most of the plastids in the area beneath the nucleus, and cisternae of endoplasmic reticulum (ER) arranged in a parallel manner in the distal part of the cell (Stewart and Gifford, 1967; Russell, 1979; Bajon et al., 1999; Siddiqi et al., 2000). Only a few small vacuoles are detected in the MeMC, which might not provide sufficient oncotic pressure to drive cell expansion. This indicates that the expansion requires coordination with surrounding cells, as well as the complex activities of pre-meiotic preparation and epigenetic reprogramming. The polarized distribution of the ER is intriguing, suggesting a potential protein and/or mRNA gradient in the MeMC and local regulators of cell behaviour. It is still unclear if there is any connection between cell expansion

and organelle polarization, but disturbing specific microtubule or F-actin-binding proteins involved in organelles movement may give clues to this puzzle.

Conclusions

Female germline development in plants is a fascinating form of cell differentiation. The specification of the MeMC is a remarkably conserved process across diverse plant species, one that appears to be independent of considerable natural variation in nucellus size and epidermal layer number. Important recent findings have revealed a new set of genes supporting MeMC formation that are expressed outside of the germline, reaffirming the role of signals in positioning the germline and influencing its development. These signals involve RdDM, receptor–ligand complexes, and cell cycle- and chromatin-related machinery, which, along with several key unknown factors, appear to provide the capacity to differentiate and enter meiosis. Importantly, the point at which these pathways merge remains elusive, as does the mechanistic basis for selective growth of the MeMC. This provides scope for future studies into MeMC development at an even more refined level than those described to date.

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