Understanding megasporogenesis through model plants: contemporary evidence and future insights

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ABSTRACT The megasporangium serves as a model system for understanding the concept of individual cell identity, and cell-to-cell communication in angiosperms. As development of the ovule progresses, three distinct layers, the epidermal (L1), the subepidermal or the hypodermal (L2) and the innermost layers (L3) are formed along the MMC (megaspore mother cell). The MMC, which is the primary female germline cell, is initiated as a single subepidermal cell amongst several somatic cells. MMC development is governed by various regulatory pathways involving intercellular signaling, small RNAs and DNA methylation. The programming and reprogramming of a single nucellar cell to enter meiosis is governed by ‘permissive’ interacting processes and factors. Concomitantly, several nucellar sister cells are prevented from germline fate also by a set of ‘repressive’ factors. However, in certain angiosperms, anomalies in development of the female gametophyte have been observed. The sporophytic tissue surrounding the female gametophyte affects the gametophyte in multiple ways. The role of genes and transcription factors in the development of the MMC and in the regulation of various processes studied in selected model plants such as Arabidopsis is explained in detail in this paper. However, as angiosperms display enormous diversity, it is important to investigate early stages of megasporogenesis in other plant systems as well. Such studies provide valuable insights in understanding the regulation of megasporogenesis and the evolution of the female gametophyte from gymnosperms to flowering plants.

KEYWORDS: arabidopsis, cell-cell signaling, epigenetics, hormonal control, megasporogenesis, programmed cell death (PCD)

Introduction

Development of female gametophyte (FG) exhibits various patterns and processes in the flowering plants. The ovule/megasporangium is a composite of three distinct radial layers L1 (epidermis), L2 (first subepidermal layer), and L3 (innermost layer) enclosing a multicellular nucellus (Vijayan et al., 2021). Studies on model plant Arabidopsis suggest that the FG is a seven celled and eight nucleate structure depicting two-phase development, where megasporogenesis is followed by megagametogenesis. In the first phase, the distal cell in the subepidermal layer (L2) of nucellus develops into megaspore mother cell (MMC) which further undergoes a meiotic division resulting in four haploid megaspores. This established development observed is the manifestation of changes taking place at molecular level and is governed by genes. Studies carried out on Arabidopsis, tomato (Jiang and Zheng, 2022), rice (Ren et al., 2018) and maize (Wu et al., 2011) have revealed plethora of molecules and coordinated mechanisms that play a role in FG development, especially cell specification. Signals arise in the apical L1 cells, the companion cells (the hypodermal nucellus cells in immediate contact with MMC having potential to acquire MMC fate) and the lower L1 cells (Fig. 1). These signals are responsible for a single MMC formation and phase changes (Petrella et al., 2021). Mendes et al., (2020) elucidated the role of regulatory pathways involving small RNAs in differentiation of a single meiocyte during megasporogenesis from a pool of somatic nucellar cells. Post meiosis out of four megaspores formed in the tetrad, only one remains functional and this has been thoroughly investigated and documented. The other three nuclei undergo

Abbreviations used in this paper: MMC, megaspore mother cell; RdDM, RNA-dependent DNA methylation.
degeneration or programmed cell death (PCD) which is regulated by hormones (Papini et al., 2011; Schmidt et al., 2015a, 2015b, Pinto et al., 2019, Jiang and Zheng, 2022). Besides hormones, a variety of regulatory factors play a role in megaspore degeneration. Descriptive embryologists have observed variations in the process amongst the members of same family and have attributed it mostly to angiosperm diversity and occasionally to the environmental factors. Signaling molecules such as calcium plays a significant role in the megaspore degeneration as observed in *Lactuca sativa* (Qiu et al., 2005a, 2005b).

**Fresh insights into megasporogenesis**

During megasporogenesis, one cell from the meristicmatic tissue acquires a unique identity to initiate female germline development. The formation of MMC thus marks the first cell of the germline lineage. The commitment of this cell to switch to a reduction division from equational division is meticulously programmed (Kapil and Bhatnagar, 1980). The callose around the MMC acts as a barrier and a security guard and secludes the developing germline cell from the sporophyte, besides providing nutrients when required. There are many independent aspects which come into action during megasporogenesis, such as regulation of the cell cycle, hormonal interplay, ribosome biogenesis, protein degradation and epigenetic pathways (Simpson, 2019). All these pathways are interconnected and operate via cell-to-cell communication in the developing ovule and its components (Kaur and Koul, 2022).

Cell fate transition (somatic cell to germline cell transition) is accompanied by a large-scale chromatin reprogramming which is partially synchronous with meiotic S phase. It is marked by several changes such as chromatin decondensation, reduction in heterochromatin, depletion of linker histones (H1.1, H1.2, LHP1 and H2A.Z), changes in core histone variants and histone modification organization (She et al., 2013). In a normal embryo sac, only one MMC differentiates in the ovule. However, sometimes two or three MMCs are formed. In such a situation, the reproduction is either apomictic or may lead to abnormal female gametophyte development (Schmidt et al., 2014). Remodeling helps MMCs to establish an epigenetic and transcriptional status that is distinct from the pool of the surrounding nucellar tissue. Reprogramming facilitates subsequent meiotic events and post-meiotic events that determine the cell fate. Selection of functional megaspore (FMS) of the tetrad and PCD of the other three (Papini et al., 2011; Singh et al., 2017) lead to development of monosporic embryo sac/FG (Evans and Grossniklaus, 2009). The molecular and genetic basis of this differentiation has been investigated extensively (Schmidt et al., 2015a; Cao et al., 2018).

Single-cell RNA sequencing (scRNA-seq) and other throughput technology has been used to elucidate the role of potential nucellar cells that adapt to a particular germline fate. One MMC develops per ovule and many genes establish or/and maintain germline fate during early ovule development e.g., *MULTIPLE ARCHESPORIAL CELLS 1* (MAC1) gene in maize (Sheridan et al., 1996; Wang et al., 2012), and *MULTIPLE SPOROCYES* (MSP1) (Nonomura et al., 2003), *OsERECTA2* (OsER2) receptor-like kinase gene, *OsTDL1A* (Zhao et al., 2008) have been identified in rice. However, these genes work in coordination with several other genes, transcription factors, proteins, and hormones during female germline differentiation. The interaction results in maintenance of MMC which is programmed to switch from mitotic divisions to meiotic divisions (Zhao et al., 2020).

Since MMC lies deeply embedded in nucellar (ovular) tissue, it is less accessible and thus understudied compared to pollen mother cells. High throughput techniques that have elucidated the molecular mechanisms underlying developmental program in its counterpart are now being used. Techniques such as gene profiling through bulk RNA sequencing, single- cell type RNA sequencing, laser-assisted micro-dissection, or fluorescence-assisted cell-sorting techniques have helped in isolating deep-seated MMC. Such studies have assisted in revealing the signaling network that regulates specification and differentiation in the female germ line cell (Zhao et al., 2014, Zhao et al., 2020). In high-through put single- cell transcriptomic study, Hou et al., (2021) have analyzed the developmental trajectory of female germ line – associated cell in *Arabidopsis thaliana*. The studies from pseudo time trajectory of MMC reveal bifurcation of ovule cells at a branch point into two – 1) a germ line branch (GB) and 2) a non-germ line branch (NGB).

Thus, a heterogeneity exists among the cells contributing to ovule primordial and those involved in germline specification (Hou et al., 2021). Such isolation has led to identification of the germ line lineage and the surrounding somatic sub populations. The studies indicate an increased expression of cell cycle genes along the germ line growth. Three germ line-associated cell clusters, AC.1, MMC1.10 and MMC2.6, have been identified with sub clusters (AC-MMC2) nested in them. These sub clusters are enriched for gene expression and macromolecule biosynthesis besides metabolic processes. Many genes and proteins in these are activated during the developmental processes. The germ line sub cluster also revealed enriched GO (Gene Ontology) related to peptide biosynthesis and transport. Such an abundance facilitates peptide-mediated cell communication, an important phenomenon during germ line specification (Zhao et al., 2008, Lieber et al., 2011). At MMC2 stage, the sub cluster shows enrichment of genes involved in ‘chromosome organization’ and microtubule-based processes (Hou et al., 2021). This observation is supported by events such as chromatin remodeling, cell-cell regulation and cell wall composition which are associated with differentiation of MMC (Lora et al., 2017, Nelms and Walbot, 2019, Jiang and Zheng, 2022).

**Functional megaspore selection**

Recent investigations have revealed that switching over of MMC from mitotic to meiotic division is due to cyclin-dependent kinase (CDK) inhibitor called KIP-RELATED PROTEIN (KRP). The KRP inhibits CDKA:1 and ensures entry of MMC into meiosis. Likewise, CDKA:1 targets RETINOBLASTOMA-RELATED1 (RB1) in *Arabidopsis* and inhibits designated MMC from entering mitosis (Zhao et al., 2017). In *Arabidopsis*, it has been found that ANTIKEVORKIAN (AKV) gene is involved in regulating megaspore survival. The evidence is provided by the akv mutant in which all four megaspores survive in 10% of the ovules. The number and position of surviving megaspores is variable in akv mutants (Yang and Sundaresan, 2000). Calcium is known to play an important role in megaspore degeneration (Qiu et al., 2008a). It is further corroborated that PCD of megaspores in *Lettuce* is closely related with change in calcium content (Qiu et al., 2008b). Of the four megaspores, a cell that shows differential calcium degeneration and further synthesis develops into a functional megaspore. The
MMC differentiation and the SPL pathway

SPL/NZZ (SPOROCYTELESS) in Arabidopsis, Os-SPOROCYTELESS (OsSPL) in rice and AG-NZZ/SPL in pineapple are the genes that promote germline specification (Zhao et al., 2021). SPL/NZZ (SPOROCYTELESS), a key regulator of megasporogenesis is expressed at the tip of the ovule in apical cell of L1 layer and establishes proximal-distal pattern formation within the ovule (Balasubramanian and Schneitz, 2000; Zhao et al., 2020). The SPL signaling pathway includes SPL/NZZ, several genes and factors such as WUSCHELL (WUS), WINDHOSE 1 (WH1), WH2, TORNADO 2 (TRN2), and CIN-CINNATA (CIN).

AGAMOUS (AG) activates SPL/NZZ transcription factor in the ovules of pineapple. The homeodomain transcription factor WUSCHELL (WUS) is activated by SPL/NZZ expression in the nucellus, and both promote MMC differentiation. However, the proximity, the breadth and the magnitude of the interaction cascades are least understood. According to Groß-Hardt et al., (2002, 2003), WUS has a role in MMC differentiation, and acts via SPL/NZZ. SPL/NZZ and WUS are promoted by the cytokinin receptors ARABIDOPSIS HISTIDINE KINASE4/CYTOKININ RESPONSE1 (AHK4/CRE1), AHK2 and AHK3. In this pathway, WUS activates expression of the small WINDHOSE 1 and 2 (WH1/2) peptides. WH1/2 genes encoding small peptides work together with tetraspanin-type protein (TRN2) and the leucine rich-repeat protein TRN1 to promote megasporogenesis. These two proteins are partners of WH1 and WH2 and their coordination is required for MMC to function efficiently (Leiber et al., 2011). Auxins also play a key role in MMC specification and YUCCA family of enzymes have been found to control the biosynthesis of auxins. SPL represses the expression of YUCCA genes that are required for auxin biosynthesis (Li et al., 2008). Auxins accumulate at the tip of the nucellus (Ceccato et al., 2013). SPL transcription is repressed in all the nucellar cells of L1 except in the apical cells. In this cell, SPL/NZZ can promote cell enlargement by activation/repression of proteins that will generate a signal for the cell below to enlarge (Fig. 1).
MMC differentiation and role of ARGONAUTE

The Arabidopsis genome also contains 10 genes in three clades encoding ARGONAUTE (AGO) proteins that are effectors in all small RNA (sRNA)-related pathways. AGO proteins - AGO9, AGO4, AGO6, AGO8 out of 10 such proteins are known to play crucial role in gametophyte development. They belong to one clade and are involved in MMC formation (Hernández-Lagana et al., 2016). AGO9 is in the basal L1 cells, where it produces a mobile signal that moves to subepidermal cells and restricts megaspore fate. The MMC is known to exhibit a 60% reduction in heterochromatin that moves to subepidermal cells and restricts megaspore fate. In maize, AGO104, a homolog of A. thaliana AGO9, encodes for AGO protein that can silence heterochromatin within young ovule primordia. Though both AGOs are homologs, yet they act in different ways. While AGO104 represses somatic fate in germ cells, AGO9 represses germine fate in somatic cells. A mutation in AGO104 results in the failure of meiosis in MMC and an unreduced embryo sac is formed (Singh et al., 2011). Both the AGO9 and AGO104 proteins are present in the subepidermal companion cells (L2) surrounding the developing MMC but are not reported in the MMC (Fig. 1). It indicates that the surrounding cells produce an AGO104-dependent mobile signal influencing the meiocyte differentiation (Carman, 1997). Asynchronous expression of duplicate genes in angiosperms triggers apomixis, bisporic, tetrasporic, and polyploidy which can occur on account of certain signals promoting meiosis or repressing further cell divisions in MMC (Singh et al., 2011).

MMC differentiation and epigenetic regulation

The first visible change in the presumptive MMC is marked by cell enlargement and elongation (Schneitz, 1995) and further increase in the size of the nucleus and nucleolus (Schulz and Jensen, 1981; Armstrong and Jones, 2003; Sniezeko, 2006). These changes are correlated to the chromatin condensation and heterochromatin formation (Wang et al., 2013). According to She et al. (2013) there is 60% reduction in heterochromatin content and fewer chromocenters of the MMC nucleus in Arabidopsis. This chromatin reprogramming in the MMC promotes post-meiotic competence leading to the development of the pluripotent gametophyte with different types of cells (egg, synergids, central cell and antipods). Furthermore, the patterns of DNA methyltransferase (enzymes that bring methylation) expression, DNA methylation, and the distribution of histone variants and histone modifications have been reported to enhance the epigenetic differentiation between the somatic cells and the MMC (Ingouff et al., 2007; Schöft et al., 2009; Ingouff et al., 2010; Pillot et al., 2010; Houben et al., 2011; Ibarra et al., 2012; Jullien et al., 2012). Histones H2A, H2B, H3 and H4 along with H1, a linker DNA bring condensation in the nucleosome (Hergeth and Schneider, 2015). In Arabidopsis H1 is encoded by three genes – H1.1, H1.2 and H1.3 (Ascensi and Gantt, 1997). While H1.1 and H1.2 are down regulated in MMC, H1.3 is mildly detected at the ovular primordia stage, suggesting a shift from the ordinary somatic cell to a cell with germline fate (She et al., 2013). Accompanied with chromatin condensation, histone variants are also markers of germline cells. The variants are proteins substituting for the core canonical histones (H3, H4, H2A, H2B) in nucleosomes of eukaryotes. They often confer specific structural and functional features to nucleosomes and are involved in chromatin remodeling and nucleosome stability (Talbert and Henikoff 2021). Among multiple histone variants, H3.1 is related to inactive transcription (Jacob et al., 2014) and this variant is gradually evicted in MMC, which is indicative of distinguished cell identity. Another variant H2A.Z is also evicted from the early MMC stage but is reincorporated later.

MMC specification is also due to small-RNA-dependent DNA methylation pathways acting in the nucellus (Olmedo-Monfil et al., 2010; Singh et al., 2011). The small RNAs play an important role in MMC formation. The small RNA population usually is divided into three groups – micro-RNA (miRNA), small interfering RNA (sRNA) and trans-acting small interfering RNA (tasi-RNA) (Borges and Martienssen 2015). The tasi-RNA and siRNA are widely produced during initial ovule development. While the former impedes the surrounding somatic cells to obtain germline identity, the latter strengthens the repressive chromatin status in these cells (Figs. 1 and 2). The tasi-RNA acts through inhibiting ARF3 (AUXIN RESPONSE FACTOR3) expression in nucellar region (Su et al., 2017, 2020), and the siRNA acts by guiding DNA methylation and /or histone modification. The MMC instead shows a higher level of a
is highly expressed in MMC (Berr et al., 2010, Guo et al., 2010), while a key regulator LHP1 for H3K27me3 is barely expressed (She et al., 2013).

**MMC differentiation and the RdDM pathway**

The siRNA can trigger DNA methylation through RNA-dependent DNA methylation (RdDM) pathway (Xie and Yu 2015) which confines female germline to a single cell in the nucellar tissue (Aslam et al., 2022). The siRNAs required for MMC formation are produced from double stranded RNAs generated by RDR6. The RdDM pathway proteins, DOMAINS REARRANGED METHYLASES (DRM1 and DRM2) are involved in DNA methylation (Groth et al., 2014). DRM2 is specifically associated with the transcribed locus inducing DNA cytosine methylation (Zhong et al., 2014). In maize, DMT102 and DMT103, the homologs of DRM1 and DRM2 are expressed in a restricted portion of the nucellus during meiosporogenesis. This pathway silences transposable elements SPL/NZZ or the repetitive sequences. SPL/NZZ can also be silenced directly by mRNA degradation which is brought about by ARGONAUTE9 (AGO9) and/or by methylation of SPL/NZZ genomic region. While AGO9 is preferentially expressed in the somatic cells of the L1 or companion of the pre-meiotic ovule, it is transiently and sporadically expressed in the nucleus of the MMC (Rodriguez-Leal et al. 2015). The AGO9 also engages with transposable elements (TEs) that are silenced during the process. Another gene, SEEDSTICK (STK), directly regulates AGO9 and RDR6 expression in the ovule and indirectly regulates SPL/NZZ expression and control meiosporocyte specification and female gamete formation. MIR822, the AGO9-interactor is involved in regulation of meiosporocyte formation (Tovar-Aguilar et al., 2023).

THO/TREX (Transcription export protein complex) is another important component of the meiosporogenesis machinery. It plays an important role in small interfering RNA-dependent processes in plants and its impairment leads to defects in siRNA biogenesis, export, processing and transporting ta-siRNA precursors from the nucleus to the cytoplasm (Jauvion et al., 2010; Yelina et al., 2010) (Francisco-Mangilet et al., 2015). The ta-siRNA restricts the cell specification as MMC by restricting the expression of ARF3-AUXIN RESPONSIVE FACTOR-3 to the nucellar region (Su et al., 2020). A non-autonomous activity for ARF3 in female germline specification serves as a positive regulator for MMC identity. A protein TEX 1 (TRANSCRIPTION EXPORT) is known to prevent somatic cells from adopting MMC fate in a non-cell autonomous manner through Trans Acting Small Interfering RNAs (TAS3)-mediated restriction of ARFs expression. Hence, any mutation in TEX1 leads to the formation of multiple MMCs (Su et al., 2017). Zhao et al., (2017) found that a balance between WUS activation and inactivation is crucial. When activated, WUS specifies MMC in all mutants of the WUS/WIH pathway, in place of the germ cell, while parenchyma-like cells resembling somatic cells are formed (Lieber et al., 2011). However, when RETINOBLASTOMA RELATED1 (RBR1) directly represses WUSCHEL (WUS), germline entry into meiotic division is facilitated. Thus, RBR1 plays a pivotal role in meiosporocyte differentiation, and its repressive potential is currently unknown.

**Fig. 2.** The ovular primordium showing chromatin landscape of the Megaspore Mother Cell (MMC). Although SPL/NZZ presents the core transcription factor for germline differentiation, but the differentiation is also associated with epigenetic regulation at multiple layers. The entire battery of histone variants, histone modifications, small RNAs and DNA methylation is involved.

permisive chromatin marked by active histones. If the activities of siRNA or DNA methylation are disrupted, more MMCs start to differentiate. Thus, epigenetic dimorphism is seen between MMC and the surrounding somatic cells of the nucellus (Fig. 2). The dimorphism is based on differential patterns of DNA methylation, small RNA activities, distribution of histone variants and histone modifications (Jiang and Zheng, 2022). All these regulatory pathways ensure specification and differentiation of single MMC per primordia.

It is equally important for MMC to maintain the acquired permissive chromatin state and for neighboring cells to continue with the repressive state. This heterochromatin state is achieved through decreased active histone modifications such as H1, H3K9me2 and H3K27me3 in somatic cells, while H3K4me3 histone modification is seen in MMC (Jiang and Zeng, 2022). In contrast, a 2.7-fold increase is seen in H3K4me3, while H3K27me1, H3K9me2 and H3K27me3 are reduced in MMC (Jiang and Zheng, 2022). Correspondingly a large protein, SET DOMAIN GROUP 2 (SDG2), a writer for H3K4me3...
activity affects cell proliferation. Finally, the number of MMCs and subsequent embryo sacs in the Arabidopsis ovule is also regulated by a subset of redundantly acting KIP-RELATED PROTEIN (KRP/ICK) and cyclin-dependent kinase (CDK) inhibitors which restrict one MMC and one FM per ovule. The KRPs act by restricting CDKA1-dependent inactivation of RBR1 (Cao et al., 2018). Once the MMC specification is over, the KRPP-CDKA1-RBR1 pathway ensures that MMC enters meiotic pathway by inhibiting the expansion of WUS into the MMC (Jiang and Zheng, 2022).

Based on the studied mechanisms, Mendes et al., (2020) developed a new model for the genetic and epigenetic control for specification of the single MMC. The model proposes that STK activates RdDM gene expression in the lower L1 layer cells. The siRNAs present in these cells act either via mRNA cleavage/repression or methylation to suppress SPL/NZZ expression and synthesis. One of the pioneer transcription factors, SPL/NZZ and WUS, play an important role in promoting MMC formation. These are synthesized and expressed only at the tip of the ovule primordium/L1 layer. The spatial distribution of SPL, WUS and WRKY28 away from the centred position of the nucellus region is the prerequisite of MMC specification and MMC differentiation, respectively. The specification of a female germline precursor can be achieved via several possible mechanisms. The movement of an effector molecule, possibly through the plasmodesmata, to the subtending L2 cell presumably initiates MMC expansion and eventual MMC specification (Figs. 1 and 2). The nature of this effector, its mode of operation and its target(s) in the L2 cell are still being explored. Hou et al., (2021) provided insights into the molecular mechanisms underlying germline specification in Arabidopsis ovule primordia. They further highlighted the complexity and importance of gene expression, regulation, and cellular processes during the developmental stages. It was observed through serial thin sections at MMC2 stages that cellular differentiation in the germline involves genes linked to chromosome organization and microtubule-based process. Such cell-specific changes in chromatin organization, cell-cycle regulation, and cell wall composition have been related to evolution among angiosperms (Lora et al., 2017).

Conclusions

The first committed cell of female germline lineage is the megaspore mother cell (MMC) which originates from a mass of somatic cells of the nucellar origin. A single MMC is differentiated per ovule and acquires a germline fate. It switches to meiotic divisions from a mitotic cell division plan leading to formation of a tetrad of megaspores; three of these spores (monosporic FG) undergo PCD while the FMS (Functional megaspore) embarks on the second phase of FG formation. The entire development occurs with precision and involves pathways where several genes, factors, hormones, epigenetic mechanisms, and chromosome modelling form the basis.

The MMC first enlarges, and chromosomes and histones undergo various changes. This is followed by MMC specification. For the formation of MMC, inactivation of WUS by RBR1 repressor is mandatory. Disruption of RBR1 and KRP/ICK gene functions leads to supernumerary MMC formation. The expression of another gene, WRKY28 prevents neighboring somatic cells to acquire germline fate. Auxin signaling which acts through ARFs and SPL/NZZ is an important component of MMC specification pathway.

The RdDM pathway with a battery of genes namely, AG09, DRM1, DRM2, also help to limit the number of MMC to one per ovule. RdDM gene expression in lower L1 cells is activated by STK gene which binds to AG09 and RDR6, and the complex then silences TE, repetitive sequences and SPL/NZZ. Further, AG09, DMR1 and DMR2 also silence SPL/NZZ in the subtending cell. The SPL/NZZ essential for MMC differentiation, is synthesized and expressed only in apical L1 but is required for MMC specification in L2 subtending cell. Therefore, SPL/NZZ pathway works in a non-cell autonomous manner. Thus, spatial distribution of SPL (along with WUS and WRKY28) away from L2 subtending presumptive MMC is crucial for germline specification.

Research using modern techniques has largely elucidated the regressive pathways. However, there is paucity of information on mechanisms that promote MMC specification. Considerable amount of variation is displayed in the pattern and pathways operating during megasporogenesis in angiosperms. Such interacting pathways can result in overexpression or underexpression of the genes involved in germline specification and its regulation. Further studies are warranted in this field and encouraging results from single-cell studies on model plant germline trajectory have started to emerge.

References


