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Pattern formation in miniature: the female gametophyte of flowering plants

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Summary

Plant reproduction involves gamete production by a haploid generation, the gametophyte. For flowering plants, a defining characteristic in the evolution from the 'naked-seed' plants, or gymnosperms, is a reduced female gametophyte, comprising just seven cells of four different types – a microcosm of pattern formation and gamete specification about which only little is known. However, several genes involved in the differentiation, fertilization and post-fertilization functions of the female gametophyte have been identified and, recently, the morphogenic activity of the plant hormone auxin has been found to mediate patterning and egg cell specification. This article reviews recent progress in understanding the pattern formation, maternal effects and evolution of this essential unit of plant reproduction.

Key words: Embryo sac, Gametophyte, Flowering plant, Reproduction, Gametes

Introduction

The life cycle of land plants involves an alternation of generations between a haploid gametophyte (see Glossary, Box 1) and a diploid sporophyte (see Glossary, Box 1). Whereas animal gametes are formed directly after meiosis, plant gametes are produced only after growth of the multicellular haploid gametophyte. The morphological complexity of the haploid generation ranges from the macroscopic moss gametophytes, which dwarf the sporophyte, to the three-celled male gametophyte (pollen) and seven-celled female gametophyte (embryo sac) that are characteristic of most flowering plants (Maheshwari, 1950). The latter evolved through an extreme reduction from the female gametophytes of the gymnosperms (see Glossary, Box 1), which frequently contain over a thousand cells, and is considered a key innovation in the evolution of flowering plants (reviewed by Friedman and Williams, 2003). These 'stripped down to essentials' female gametophytes confer two major defining characteristics of the flowering plants. First, they are small enough to be packaged within an ovary. Second, they generate two gametes that undergo double-fertilization to produce the nutritive tissue called endosperm concordantly with the embryo, which allows more efficient resource allocation to fertilized seeds (reviewed by Raghavan, 2003). The reduced female gametophyte of flowering plants enabled much more rapid seed setting (i.e. the production of seeds during reproductive growth) than is possible in gymnosperms, allowing for habitat adaptations that require short reproductive cycles and facilitating the expansion of flowering plants into diverse ecological niches.

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Box 1. Glossary

Angiosperms. Flowering plants, in which seeds are produced within the female reproductive organs.

Anthers. The anther, which carries the pollen grain (the male gametophyte), sits on top of a filament to form the stamen or male organ of a flower.

Embryo sac. The female gametophyte of flowering plants, which produces the two female gametes – the egg cell and central cell – for double-fertilization by the two sperm cells of the male gametophyte (pollen grain).

Eudicots. The term means, literally, 'true dicotyledons', as this group contains the majority of plants that have been considered dicotyledons and have typical dicotyledonous characters.

Filiform apparatus. Structure formed at the micropylar (distal) pole of the synergid cell wall that is thickened, forming finger-like projections into the synergid cell cytoplasm.

Gametophyte. The haploid generation of a plant produced by meiosis from the sporophyte. The mature gametophyte produces male or female gametes (or both in lower plants). The fusion of male and female gametes produces a diploid zygote that develops into a new sporophyte.

Gymnosperms. Group of seed-bearing plants from which the angiosperms are presumed to have descended. The name comes from the Greek word gymnospermos, meaning 'naked seeds', as seeds are found unenclosed on the scales of a cone or similar structure.

Ovule. The ovule, which carries the female gametophyte, is located in the part of the carpel (the female organ of a flower) known as the ovary, which ultimately becomes the fruit. Depending on the plant, flowers can have one or multiple ovules per ovary.

Sporophyte. The diploid generation of a plant, which is initiated with the zygote.

Stigma. In a flower, the stigma is the terminal portion of the carpel, which has no epidermis and is meant to receive pollen grains.

Despite the crucial importance of the female gametophyte of flowering plants, much remains to be learnt about its development and overall biology, partly because it is a highly inaccessible structure. The past few years, however, have seen some exciting progress in the field. Here, we focus primarily on the female gametophyte of the model plant *Arabidopsis*, which has been studied more extensively than that of other plants, and review recent advances in the understanding of the patterning, the maternal effects and the evolution of the female gametophyte.

Female gametophyte development

In angiosperms (see Glossary, Box 1), the male gametophytes (the pollen grains) develop within the anthers (see Glossary, Box 1) and the female gametophytes (megagametophytes or embryo sacs) develop within the ovule (see Glossary, Box 1). There has been over a century of developmental studies on gametophyte development in a large number of diverse plant species (Friedman et al., 2008). In

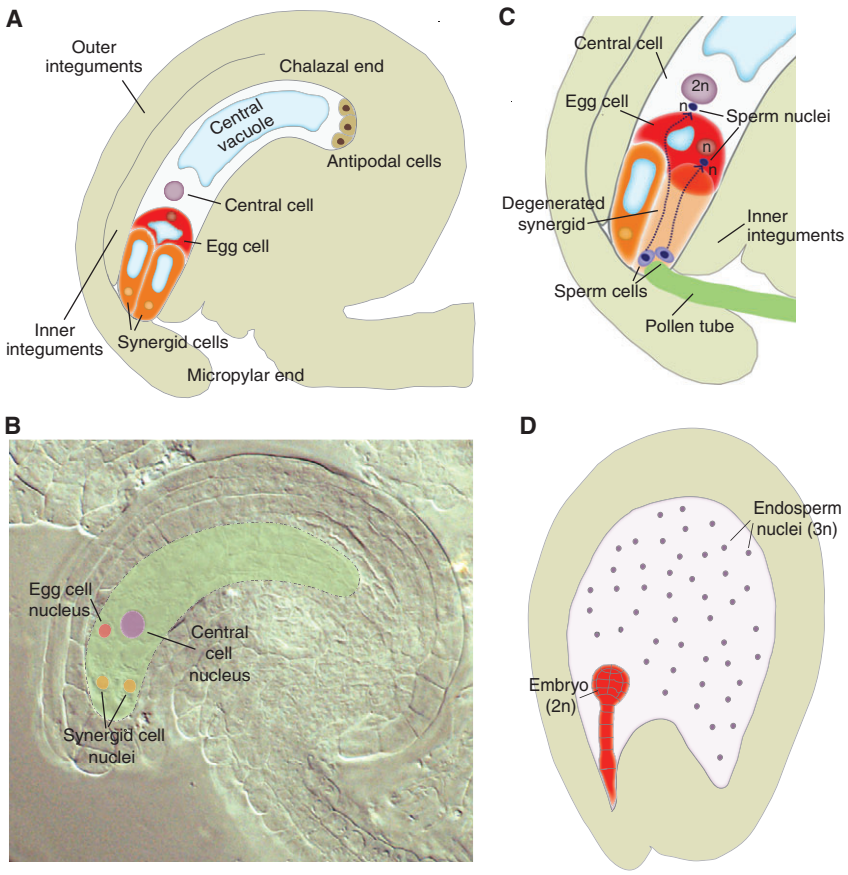

Fig. 1. Cell types and reproductive functions of the female gametophyte (embryo sac). (**A**) Sketch of a seven-celled embryo sac (*Polygonum*-type), based on the model plant *Arabidopsis*, that shows the four cell types within the maternal tissues of the ovule: the synergid cells, the egg cell, the central cell and the antipodal cells. (**B**) Wild-type *Arabidopsis* ovule showing a mature embryo sac (FG7 stage, green dashed) with the two synergid cell nuclei (yellow), the egg cell nucleus (red) and the central cell nucleus (purple). (**C**) Sketch of pollen tube reception within a degenerated synergid cell in the micropylar end of the embryo sac. Two sperm cells (violet) are released upon pollen tube reception. One sperm nucleus (dark blue) fuses to the egg cell nucleus (n), and a second sperm nucleus fuses to the central cell nucleus (2n) during double fertilization. (**D**) Sketch of a fertilized seed, with an embryo (2n) at the globular stage and with endosperm nuclei (3n) before cellularization occurs.

most flowering plants, including *Arabidopsis* and maize, the embryo sac has a characteristic organization called the *Polygonum*-type (Maheshwari, 1950), containing seven cells of four distinct cell types (Fig. 1A,B). These four cell types are, first, the egg cell, which gives rise to the embryo, second, two accessory cells called synergid cells, which are important for pollen tube attraction, third, the central cell, which gives rise to the endosperm, and fourth, three cells of unknown function called antipodal cells. The entire embryo sac is enclosed within diploid sporophytic tissues called integuments, which will constitute the seed coat in the mature seed. When pollen lands on the stigma (see Glossary, Box 1), it forms a structure called the pollen tube that penetrates the embryo sac through an opening in the integuments called the micropyle and delivers two sperm cells by entering a synergid cell, which subsequently degenerates (Fig. 1C). Eventually, the other synergid cell will also degenerate. The two gametic cells of the embryo sac, the egg cell and the central cell, will undergo double-fertilization by the two sperm cells of the pollen (Fig. 1C). The fertilization of the egg cell within the embryo sac by one of the sperm cells delivered by the pollen tube results in the diploid zygote, which will initiate the next sporophyte generation (Fig. 1C,D). The fertilization of the central cell by the second sperm cell results in the triploid endosperm, which functions as a nutritive source for the embryo or the germinating seedling (Fig. 1C,D).

A schematic outline of the development of a seven-celled embryo sac, based on the model plant *Arabidopsis*, is depicted in Fig. 2. In the developing ovule, a single cell from the internal tissue (called the nucellus) is specified as an ‘archesporial’ cell, which enlarges and differentiates into the female meiocyte or megaspore mother cell (MMC; Fig. 2A). The MMC undergoes meiosis to generate four spores (Fig. 2B), three of which undergo programmed cell death, leaving only the proximal (chalazal) megaspore as the functional megaspore [FM stage or female gametophyte stage 1 (FG1); Fig. 2C] in each ovule. The megaspore then undergoes three sequential mitotic nuclear divisions, designated as stages FG2 to FG5 (Christensen et al., 1997), to generate the eight nuclei that will contribute to the differentiated embryo sac, four at each pole (Fig. 2D,E). Subsequent cellularization results in the formation of seven cells owing to the nuclear migration of two of these nuclei called the polar nuclei (Fig. 2E), which will constitute the central cell nucleus. In the seven-celled embryo sac, the micropylar (distal) end of the ovule is adjacent to the egg cell and the two synergid cells, whereas the chalazal (proximal) end of the ovule is adjacent to the three antipodal cells. The homo-diploid (2n) central cell occupies much of the embryo sac and contains a large central vacuole. All of the cells within the embryo sac are highly polarized and possess distinctive morphologies (Huang and Russell, 1992).

The precise migration and positioning of the nuclei at the eight-nucleate FG5 stage marks the distinction between these nuclei in their cell-fate specification (Huang and Sheridan, 1994; Webb and Gunning, 1994). At the micropylar pole, the two most distal nuclei will form the future synergids. Of the remaining two nuclei, the more distal nucleus of the pair will form the future egg cell, whereas the more central nucleus will become one of the two polar nuclei of the central cell. The most centrally located nucleus of the four nuclei from the chalazal pole will constitute the other polar nucleus of the central cell. This nucleus migrates towards the micropylar pole and becomes positioned close to the first polar nucleus as the embryo sac undergoes cellularization to form the seven-celled embryo sac (Webb and Gunning, 1994). Although the mechanism of cellularization is not well understood, the microtubular cytoskeleton

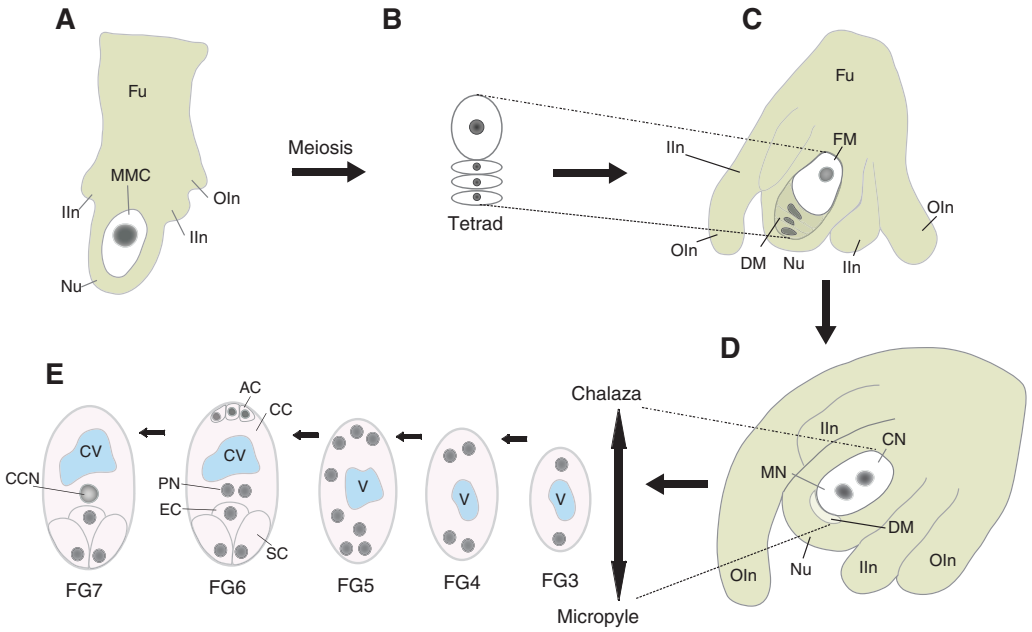

Fig. 2. Schematic of female gametophyte development, based on the model plant *Arabidopsis*. (**A**) The megaspore mother cell (MMC) is surrounded by epidermal cells of the nucellus (Nu) prior to undergoing meiosis to generate four spores. At this stage, the formation of the outer (OIn) and inner (IIn) integuments has just initiated. (**B**) Diagram of MMC asymmetric meiosis that generates four spores (tetrad). Three of these undergo programmed cell death. The proximal (chalazal) megaspore becomes the functional megaspore (FM). (**C**) FG1 stage. The FM is teardrop-shaped and undergoes the first mitotic division. (**D**) FG2 stage. The female gametophyte comprises two nuclei. The nucellus (Nu) is enclosed by the OIn, but not the IIn integuments. (**E**) Stages FG3 to FG7. The female gametophyte comprises two nuclei, separated by a large vacuole (V), that undergo second and third mitotic divisions to generate the eight-nucleate mature embryo sac at the FG5 stage. Subsequent cellularization (FG6 stage) results in the formation of seven cells: two synergid cells (SC); one egg cell (EC); one central cell (CC) carrying two polar nuclei (PN); and three antipodal cells (AC). By FG7, the two polar nuclei have fused to form the central cell nucleus (CCN), and the antipodal cells degenerate. CN, chalazal nucleus; CV, central vacuole; DM, degenerating megaspores; Fu, funiculus; IIn, inner integuments; MMC, megaspore mother cell; MN, micropylar nucleus; Nu, nucellus; OIn, outer integuments.

appears to establish and maintain organelle polarity and function in the migration and arrangement of the nuclei during embryo sac development (Russell, 1993; Webb and Gunning, 1994), and radiating perinuclear microtubules have been observed during cellularization (Huang and Sheridan, 1994; Webb and Gunning, 1994). Following cellularization in *Arabidopsis*, the polar nuclei fuse to form the homo-diploid central cell nucleus (Fig. 1A,B; Fig. 2E), and the antipodal cells will have degenerated at the time of fertilization (Fig. 2E).

In cereals, the overall development of the embryo sac is very similar to that in *Arabidopsis* and most other eudicots (see Glossary, Box 1) but with some notable differences at the late stages. The two polar nuclei are generally unfused until fertilization, when they will fuse with a sperm nucleus to form the triploid endosperm (Mol et al., 1994). Another difference is that the three antipodal cells do not degenerate, but instead begin to proliferate by nuclear division and cytokinesis. In maize, at the time of fertilization, proliferation results in up to 40 antipodal cells (Huang and Sheridan, 1994). The antipodal cells in cereals have cell walls that are covered with small outgrowths known as papillae at the boundaries between the maternal tissue and the antipodal cells and between the antipodal cells and the developing endosperm. As these cell walls resemble those of plant cells involved in nutrient transfer (known as transfer cells), it has been hypothesized that the antipodal cells might perform an essential function in the transfer of nutrients from maternal tissues to the cereal endosperm (Diboll and Larson, 1996; Maeda and Miyake, 1997). However, in the majority of flowering plants, including *Arabidopsis*, the antipodals degenerate before fertilization, and their function, if they have any, remains a mystery.

Female gametophyte genes and mutants

The small size of the female gametophyte and its inaccessibility, which is owing to it being embedded within the sporophytic tissues of the ovule, have made it a particularly challenging and intractable system for developmental studies. Large-scale forward genetic screens have been employed, using insertional mutagens such as T-DNA or transposons, to identify genes with important functions in the embryo (Bonhomme et al., 1998; Christensen et al., 1998; Feldmann et al., 1997; Howden et al., 1998; Pagnussat et al., 2005). Most of the above screens employed a selectable marker carried by a transposon or T-DNA, and relied on the principle that female gametophyte mutants exhibit a markedly reduced transmission through the female gametes of a marker linked to the mutation (Brink, 1925). These screens have revealed genes that affect embryo sac development, its capacity for fertilization and the post-fertilization development of the seed. In the majority of cases, the mutations are in genes that control essential functions, such as cellular metabolism or the cell cycle, although mutations in putative regulatory or signaling genes can also be found (Pagnussat et al., 2005). However, such screens are generally limited to the discovery of single genes that result in phenotypes when mutated, and are less effective in the identification of multiple redundant genes that might be required for a developmental process.

Expression profiling studies can overcome the above limitation of forward genetic screens but the difficulty of isolating the embryo sac without contamination with sporophytic tissues has been a technical hurdle. This problem has been partially overcome for the egg cell, which can be isolated from the embryo sac in wheat and maize and used for expression analysis by cDNA library construction and sequencing, but this approach is not suitable for the much smaller embryo sac of *Arabidopsis* (Le et al., 2005; Sprunck et al., 2005; Yang et al., 2006). An alternative approach is comparative expression profiling, in which the expression profiles of whole wild-type ovules are compared with those of mutants that do not form embryo sacs to deduce the set of embryo sac-specific genes. This approach has been employed by several labs and has led to the identification of hundreds of genes that are specifically expressed or enriched in the embryo sac, many of which display cell-specific patterns of expression (Johnston et al., 2007; Jones-Rhoades et al., 2007; Steffen et al., 2007; Yu et al., 2005). For most of these genes, their functions in embryo sac development still await systematic reverse genetic studies using loss-of-function mutants; even the outcome of these, however, is not straightforward to interpret owing to redundancy, as noted above.

From both forward and reverse genetics approaches, functions for several transcription factors involved in cell-type differentiation have been uncovered. For example, the ATMYB98 transcription factor was identified by its synergid-specific expression and appears to be required for the formation of the filiform apparatus (see Glossary, Box 1) by the synergid cells (Punwani et al., 2007; Punwani et al., 2008). *AGL80* and *AGL61*, two *Arabidopsis* genes that are expressed specifically in the central cell and that belong to the type I MADS-box family of transcription factors, appear to be important for the normal differentiation and function of the central cell. Plants that carry mutations in these genes fail to express central cell markers, to achieve fertilization of the central cell or to initiate endosperm development (Bemer et al., 2008; Portereiko et al., 2006). Because these genes are expressed relatively late after cellularization, it is probable that they establish central cell identity and function after cell specification. Mutations in another type I MADS-box transcription factor gene, *AGL23*, arrest embryo sac development at the FM stage; however, the phenotype is incompletely penetrant, and the exact role of this gene in gametophyte development remains to be determined (Colombo et al., 2008).

Mutants that alter cell-fate specification are very infrequent, and only four such mutants have been described. In the *eostre* mutant, the mis-expression of the Knox-TALE homeodomain gene *BEL-like homeodomain 1* (*BLH1*) in the embryo sac produces nuclear migration abnormalities from stage FG3 onwards that correlate with the production of an extra functional egg cell in place of a synergid (Pagnussat et al., 2007). These observations suggested that the determination of egg cell fate might depend on a location-specific mechanism within the syncytial female gametophyte (see below). However, *eostre* is a gain-of-function mutation, and as the loss-of-function of *BLH1* has no phenotype, and as *BLH1* is not expressed in wild-type embryo sacs, it is probable that another *BLH* gene from the same family of ~13 genes in *Arabidopsis* functions in nuclear positioning in the wild-type embryo sac. The Knox-TALE family of transcription factors is found in both animals and plants (Burglin, 1997), and members of this family typically function as heterodimers. In plants, the BLH proteins interact with Knotted-like homeobox (Knox) proteins to activate transcription (Bellaoui et al., 2001). Interestingly, the gain-of-function *eostre* mutant is suppressed by loss-of-function of *KNAT3*, which encodes a type II Knox protein; these form a sub-class of Knox proteins conserved even in lower plants, but with as yet no defined functions. It is possible that nuclear positioning during embryo sac development is mediated by *KNAT3* in association with an unidentified *BLH* gene product.

Three other genes, *LACHESIS* (*LIS*), *GAMETOPHYTIC FACTOR1* [*GFA1*; *CLOTHO* (*CLO*)] and *ATROPUS* (*ATO*) have been found to be required to restrict gametic cell fate (Coury et al., 2007; Gross-Hardt et al., 2007; Moll et al., 2008). Mutants in these genes show changes in cell identities inside the embryo sac that only become apparent after cellularization, after an initially normal development, including normal nuclear positioning within the syncytium (Gross-Hardt et al., 2007; Moll et al., 2008). In the embryo sacs of *lis*, *gfa1* (*clo*) and *ato* mutants, cells that are normally specified as synergid cells can express egg cell-specific markers, and cells that are normally specified as antipodal cells can express central cell-specific markers. In addition, in *clo* and *ato* mutants, cells that are normally specified as central cells can express egg cell-specific markers. Also, pollen tube attraction is reduced and no zygotes are produced, which suggests pleiotropic effects on other embryo sac functions, including fertilization. It has been proposed that a mechanism of lateral inhibition by the gametic cells, i.e. the egg cell and the central cell, operates to prevent the formation of excess gametic cells inside the female and to maintain the cell fate of the accessory synergids and antipodals (Gross-Hardt et al., 2007). All three genes, *LIS*, *GFA1/CLO* and *ATO*, encode pre-mRNA splicing factors that are conserved among all eukaryotes (Bartels et al., 2003; Horowitz et al., 1997) and are expressed throughout the *Arabidopsis* plant. In the mature embryo sac, *CLO* and *ATO* are expressed in all cells, whereas *LIS* is restricted to gametic cells. It is not clear why such altered cell-fate phenotypes arise from mutations in genes that encode components of the core splicing machinery. It is possible that the splicing defects might disrupt signaling mechanisms required for the maintenance of cell fates specified at cellularization. Alternatively, the splicing of auxin response factors involved in cell fate specification (discussed in the next section) might be affected in these mutants, leading to abnormal auxin responses and to the mis-specification of cell fates.

In summary, the extensive forward genetic screens carried out over the past decade, together with expression profiling studies, have led to the identification of many important genes that affect specific functions of the embryo sac. However, the mutant screens have been disappointing in terms of revealing the overall principles and mechanisms that govern embryo sac cell specification, or, more broadly, how the embryo sac is patterned. Indeed, a recent review has commented that "The signaling events underlying cell specification in the female gametophyte are, however, completely unknown" (Evans and Grossniklaus, 2009). This situation has now changed owing to some recent progress, which is discussed in the next section.

Patterning of the embryo sac by auxin

As described earlier, the embryo sac first develops as a syncytium, and the establishment of cell identity coincides with cellularization, which suggests that cell fates are programmed prior to this stage. Moreover, in the *eostre* mutant, the aberrant positioning of the nuclei during embryo sac development is correlated with the production of an extra functional egg cell in place of a synergid (Pagnussat et al., 2007). These observations suggest that the determination of egg cell fate might depend on a location-specific mechanism within the syncytial female gametophyte. In addition, the mutation of the maize *indeterminate gametophyte* gene, which encodes a transcription factor related to *Arabidopsis ASYMMETRIC LEAVES 2* (Evans, 2007),

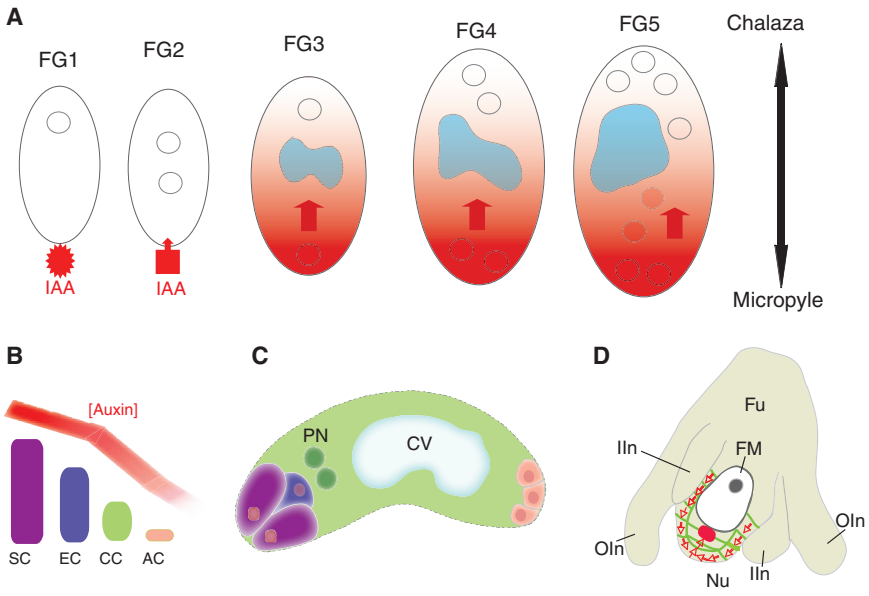

Fig. 3. Cell specification within the *Arabidopsis* female gametophyte by auxin. (**A**) Schematic of the different stages of female gametophyte development from the one-nucleate FG1 stage (left) to the eight-nucleate FG5 stage (right), showing the position of nuclei relative to the auxin gradient (red) during embryo sac development. The auxin source (IAA) is located in the sporophytic tissue at FG1 and FG2. The micropylar end of the female gametophyte becomes the auxin source from FG3 until FG5. (**B**) Model for cell-fate specification upon cellularization by an auxin gradient (red line) in the syncytial embryo sac. Synergid cells (SC) are formed from nuclei exposed to the highest auxin, the egg cell (EC) from a nucleus in position of high auxin, the central cell (CC) from a nucleus in intermediate to low auxin and antipodal cells (AC) are derived from nuclei exposed to no or very low auxin. (**C**) Sketch of the female gametophyte after cellularization (FG6 stage) showing the two synergid cells (purple), the egg cell (blue), the central cell (green) and the antipodal cells (pink). CV, central vacuole; PN, polar nuclei. (**D**) FG1 stage. Diagram showing the initial specification of the micropylar auxin source through PIN-FORMED 1 (PIN1)-dependent auxin efflux in the sporophytic nucellus. PIN1 (green) molecules transport auxin (red arrows) towards the nucellus, leading to the formation of an auxin maximum (red circle) that is transported inside the female gametophyte at FG2. FM, functional megaspore; Fu, funiculus; IIn, inner integuments; Nu, nucellus; OIn, outer integuments.

results in the formation of supernumerary nuclei in the embryo sac that, upon cellularization, appear to assume specific cell fates correlated with their position (Guo et al., 2004). On the basis of these findings, an attractive hypothesis is that positional information within the syncytium might rely on the asymmetric distribution of a morphogenetic determinant, similar to the positional information provided during the development of the *Drosophila* embryo by the proteins Bicoid and Nanos (van Eeden and St Johnston, 1999).

A recent study provides an overall mechanism for the patterning of the female gametophyte within the concept of morphogenetic determinants (Pagnussat et al., 2009). The phytohormone auxin has been shown to act as a positional determinant that regulates cell specification in the female gametophyte of *Arabidopsis*. Auxin has previously been shown to provide positional signals in sporophytic developmental processes, but the action of auxin in the embryo sac has several unusual aspects. In this study, the distribution of auxin was followed by the synthetic auxin-responsive reporter *DR5::GFP* (Ulmasov et al., 1997), and a highly asymmetric distribution of auxin in the developing embryo sac was observed (Fig. 3A). At the FG1 stage of female gametophyte development, the auxin signal is strong in the nucellus, outside the functional megaspore (FM). As the female gametophyte develops, a strong signal is localized within the embryo sac at the micropylar end up to the FG5 stage, when the third mitotic division has been completed and eight nuclei are positioned prior to cellularization. At later stages, post-cellularization, the auxin signal is weak in all cells of the mature embryo sac.

The asymmetric distribution of auxin was disrupted by driving the expression of the auxin biosynthetic gene *YUC1* (Zhao et al., 2001) in the embryo sac. The resulting female gametophytes showed high auxin levels throughout the female gametophyte from stages FG1 to FG5. Cell specification and morphologies were abnormal, and the expression of synergid-specific markers was found to occur in multiple cells, including in those normally specified as egg cells, central cells and antipodal cells (Pagnussat et al., 2009). In some cases, egg cell markers were also expressed at the antipodal location, whereas the expression of antipodal-specific attributes was significantly reduced. Also consistent with the ectopic acquisition of synergid cell fates, additional pollen tubes entering the female gametophyte were detected in these embryo sacs. These observations show that high auxin levels throughout the embryo sac alter cell identities, resulting in the conversion of chalazal (proximal) cell identities to micropylar (distal) cell identities. Importantly, auxin over-production did not result in any abnormalities in nuclear positioning during syncytial embryo sac development, indicating that the nuclear positioning pathway is auxin-independent.

When auxin signaling is attenuated, which mimics the effect of lower auxin levels, cell-fate specification shifts away from synergid towards egg cell identity. The response to auxin is mediated by a family of transcription factors called Auxin Response Factors (*ARF*s; Guilfoyle and Hagen, 2007). Using an artificial microRNA (*amiR-ARFa*) that targets a subset of the *ARF*s (*ARF1-8* and *ARF19*), defective embryo sacs were formed in which all three micropylar cells expressed egg cell markers, which suggests that synergid identity had been replaced with egg cell identity. These embryo sacs did not attract any pollen tubes, consistent with the loss of synergid fate. A partial conversion of synergid cell to egg cell identity was also observed in mutants that are defective in certain auxin receptors of a family of F-box family proteins encoded by the genes *TIR1* and *AFB1-3* (Dharmasiri et al., 2005). Thus, the attenuation of auxin signaling results in micropylar cell fates (synergids) shifting towards chalazal cell fates (egg cells).

These observations indicate that differences in auxin concentration specify cell fates. An auxin gradient is formed in the syncytial embryo sac with a maximum at the micropylar pole and a minimum at the chalazal pole (Fig. 3A). An independent pathway regulates nuclear positioning; this might possibly involve the BLH and KNAT homeodomain proteins, as revealed by the *eostre* mutant (Pagnussat et al., 2007). The superposition of the auxin gradient on the nuclear positioning pathway would lead to each of the nuclei being exposed to different local concentrations of auxin. Each nucleus would then

select a different cell fate as a read-out of the auxin signal (Fig. 3B,C). Thus, synergid fate would correspond to the highest auxin concentration and antipodal fate to the lowest, with egg cell and central cell fates representing different intermediate auxin levels. This model has predictable consequences when auxin biosynthesis or signaling is altered, which are consistent with the experimental outcomes.

Auxin sources in the female gametophyte

How might this asymmetric auxin distribution arise? In sporophytic development, auxin maxima arise from the polar transport of auxin, which occurs through the action of auxin efflux carriers encoded by the *PIN-FORMED* (*PIN*) gene family (Galweiler et al., 1998). However, none of the seven *PIN* genes examined is expressed in wild-type female gametophytes. Rather, auxin appears to arise from localized synthesis by the auxin-biosynthetic products of the *YUCCA* (*YUC*) genes (Cheng et al., 2006) because the expression of *YUC* genes overlaps with the auxin signal in the ovules. *YUC* expression appears first at the micropylar region outside of the embryo sac at FG1, and then localizes to the micropylar pole of the gametophyte from FG3 through to the later developmental stages, while being undetectable in the sporophyte (Fig. 3A). Interestingly, the *PIN1* gene is expressed in the diploid tissues of the ovule until the FG1 stage, but disappears soon thereafter (Pagnussat et al., 2009).

These observations suggest a sequential source mechanism for the formation of the gametophytic auxin gradient. The early auxin flux in the nucellus, as indicated by PIN1 localization, might be involved in establishing an initial auxin maximum, which could then provide the trigger for events that establish gametophyte patterning by specifying a sporophytic auxin source (Fig. 3D). Auxin from the sporophytic source leads to the specification of secondary auxin sources within the gametophyte at the micropylar pole, which, in turn, result in a gradient of auxin along the micropylar-chalazal axis (Fig. 3A). The outcome is the graded specification of different cell types according to nuclear position (Fig. 3B,C). This model for auxin in the embryo sac implies that it acts as a morphogen, i.e. a source-derived gradient that directs the concentration-dependent specification of different cell types, comparable to the morphogen systems in animal development. However, it cannot be excluded that the original sporophytic signal is not auxin, but another unknown signaling molecule that induces auxin synthesis at the micropylar pole.

The maintenance of an auxin gradient within the syncytial embryo sac raises some interesting questions. The highly asymmetric expression of a GFP reporter can be accounted for by the partitioning of the syncytial embryo sac into cytoplasmic domains (Brown and Lemmon, 1991; Brown and Lemmon, 1992; Webb and Gunning, 1994), which might restrict the translation of mRNAs to the domain that contains the nucleus and limit the subsequent movement of GFP. However, a small molecule like auxin would probably diffuse rapidly even with cytoplasmic partitioning, which suggests that other mechanisms might be required to maintain asymmetric auxin distribution. Potential candidates include unidentified non-PIN auxin carriers that export auxin from the embryo sac at the chalazal pole or, alternatively, the inactivation of auxin by conjugation or degradation at the chalazal pole. Further studies are required to answer these questions.

Evolution of the flowering plant female gametophyte

The highly reduced seven-celled female gametophyte is considered to be a key innovation during the evolution of flowering plants. Because of the small size of the embryo sac compared with gymnosperm gametophytes consisting of more than 1000 cells, the ovules of flowering plants are tiny. This enables multiple ovules to be packaged into an ovary, which is a crucial feature of flowers and fruits. Moreover, the small size of the embryo sac permits more rapid cycling of the gametophytic generation, which is in large part accomplished by shifting nutritive tissue development from the gametophyte to the seed through the processes of double fertilization and endosperm formation. Studies of extant basal angiosperms suggest that the earliest angiosperms might have possessed a four-celled embryo sac that comprised two synergids, an egg cell and a haploid central cell (Williams and Friedman, 2002) (Fig. 4A). A modular hypothesis for embryo sac evolution has been proposed, according to which the seven-celled/eight-nucleate *Polygonum*-type embryo sac that characterizes the majority of the angiosperms is the result of a duplication of an ancestral four-celled/four-nucleate developmental module (Friedman and Williams, 2003). This duplication would result in two modules that form two sets of four-nucleate embryo sacs within a single syncytium, of which only one, the one closest to the micropyle where the pollen tube will enter, forms a functional egg cell and synergids (Fig. 4B). The other module, comprising the four nuclei at the chalazal pole, contribute a single nucleus to the central cell, generating a central cell that is diploid and endosperm that is triploid, upon fertilization. The remaining three nuclei from this module form the antipodal cells, which have been proposed to be evolutionary relics or 'spandrels' that have no essential functions (Friedman et al., 2008). Remarkably, the results of delocalization of auxin biosynthesis suggest that antipodals have retained the potential to differentiate into egg cells or synergids, despite the estimate of ~120 million years ago for the modular duplication, which is when the basal angiosperm lineage split from the other flowering plant lineages. Why might the proposed modular duplication not result in embryo sacs with two sets of egg apparatus (egg cell and synergids), one at each pole? According to the auxin patterning model, after the initial duplication, the potential egg apparatus located at the chalazal pole would be within a field of low auxin owing to its distal position relative to the auxin source, which could lead to the acquisition of antipodal cell identity as a default fate (Friedman, 2009).

The modular hypothesis helps explains some of the variation in flowering plant embryo sacs. Although the eight-nucleate *Polygonum*-type embryo sac is by far the most frequent (found in >70% of all analyzed angiosperm species), a wide variety of other embryo sac configurations, ranging from four- to sixteen-nucleate, have been documented (Maheshwari, 1950). The most interesting cases are those in which one or both meiotic divisions are not accompanied by cytokinesis, which results in bisporic or tetrasporic embryo sacs containing haploid nuclei that are genetically distinct products of meiosis. In the *Penaea*-type embryo sac, which is tetrasporic, each of the four genetically distinct nuclei undergoes patterns of division that recapitulate that of the modular four-nucleate embryo sac, terminating with four modules at the four sides of the mature embryo sac (Friedman et al., 2008) (Fig. 4C). Only one of these modules generates a functional egg apparatus (i.e. an egg cell and synergids), but all four modules contribute nuclei to the central cell, resulting in a pentaploid endosperm that contains five distinct haploid genomes after fertilization. In other variations within flowering plants, the endosperm ploidy can be as high as decapentaploid (15*n*, with *n* being the number of haploid chromosomes present). Yet in all cases of multisporic embryo sacs, a unique egg cell is specified, resulting in monozygotic seed for all angiosperm species. The auxin patterning model provides a mechanistic basis for this occurrence because it predicts that the egg

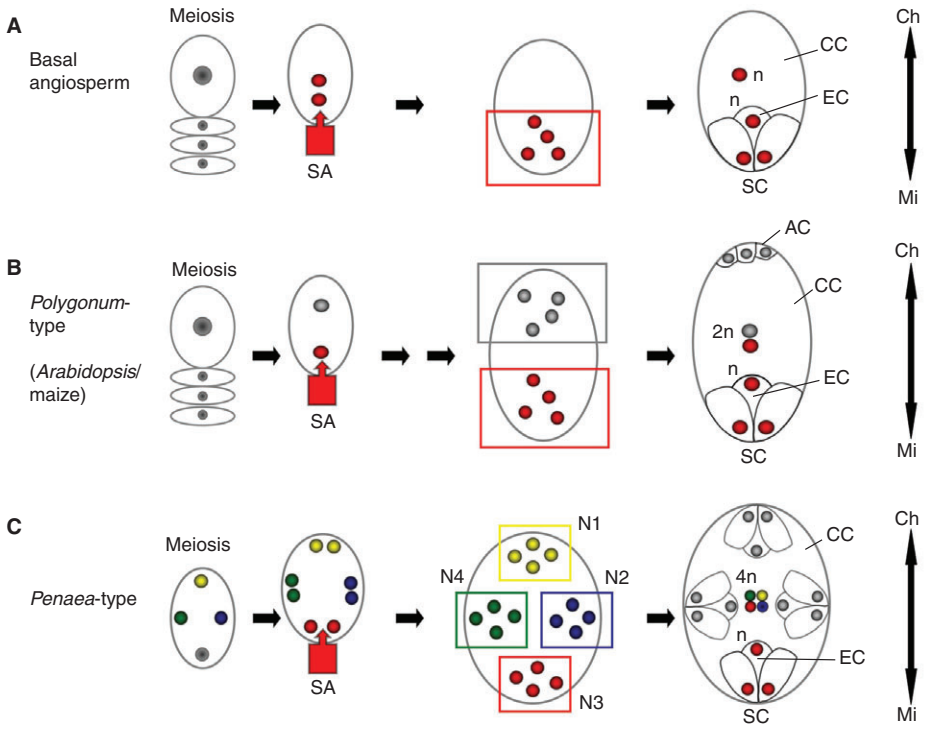

Fig. 4. The modular hypothesis for embryo sac evolution can be combined with the auxin gradient patterning model. (**A**) After asymmetric meiosis in a basal angiosperm (*Nuphar polysepalum*), a functional megaspore undergoes two sequential mitotic divisions that generate the modular four-nucleate (red box) female gametophyte. The auxin source (SA) at the micropylar end determines which nuclei will become the synergid cells (SC), the egg cell (EC) or the central cell (CC). Both the EC and the CC are haploid (n). (**B**) In the *Polygonum*-type embryo sac (*Arabidopsis*, maize), the functional megaspore undergoes three sequential mitotic divisions that generate the seven-celled/ eight-nucleate female gametophyte. The first mitotic division is hypothesized to result in a duplication of the four-nucleate module observed in basal angiosperms (red and gray boxes). The proximity to the sporophytic auxin source induces a functional module (red box) at the micropylar end with cell fates similar to the basal angiosperm module (i.e. SCs, EC and CC), whereas the other module (gray box) contributes a nucleus to the CC, which becomes diploid (2n), and the other three nuclei will specify antipodal cells (ACs). (**C**) In the *Penaea*-type embryo sac (*Euphorbia procera*), after meiosis, all four spores undergo two sequential mitotic divisions, which terminate with four modules (N1-N4; yellow, blue, red and green boxes) within one embryo sac (nuclei depicted in the same color are identical). The auxin source at the micropylar end determines which module will form the egg cell (n) and the synergid cells. All modules contribute one nucleus to the tetraploid CC. Ch, chalaza; Mi, micropyle.

apparatus will be always specified proximal to the micropylar auxin source, which is initially derived from the sporophyte (Fig. 4). Variations in endosperm ploidy can impact the paternal versus maternal genome dosage that controls seed sizes (Scott et al., 1998), but in the embryo, the occurrence of unique egg cells imposes an obligate equal ratio of parental genomes. Consequently, multisporic embryo sacs result in very unusual ratios for the heterozygosity of the endosperm and the relatedness between embryo and endosperm, on which evolutionary selection can operate (Friedman et al., 2008).

The female gametophyte in fertilization

The mature female gametophyte provides essential functions required for fertilization, including pollen tube attraction and the release of the male gametes (sperm cells) from the pollen tube. As this topic has been covered in a recent review (Crawford and Yanofsky, 2008), only a brief synopsis is presented here. Synergid cells play an essential role in pollen tube guidance and pollen tube reception (Higashiyama et al., 2001; Rotman et al., 2003; Sandaklie-Nikolova et al., 2007). The synergids have characteristic cell wall ingrowths at the micropylar end termed the filiform apparatus (see Glossary, Box 1), which are located at the site of pollen tube entry (Huang and Russell, 1992). In maize, a small protein, EA1, which is synthesized in the egg apparatus (i.e. the synergids and the egg cell), is required for micropylar pollen tube guidance (Marton et al., 2005). In *Arabidopsis*, the synergid-specific transcription factor ATMYB98 seems to regulate a battery of genes that encode small peptides, although these have not yet been demonstrated to be pollen tube attractants (Kasahara et al., 2005; Punwani et al., 2007; Punwani et al., 2008). Recently, a significant advance was reported in *Torenia fournieri*, showing that small cysteine-rich polypeptides called LUREs, which are synthesized in the synergids, function as pollen tube attractants in that plant species (Okuda et al., 2009). Besides synergid-expressed genes, it appears that the membrane protein GEX3 and the nuclear protein CCG, which are expressed in the egg cell and central cell, respectively, also cause micropylar pollen tube guidance defects in *Arabidopsis* (Alandete-Saez et al., 2008; Chen et al., 2007), which suggests that signaling mechanisms between the egg and/or the central cell and the synergids might be important for this process.

After entry into the synergid cell, the process of pollen tube reception and male gamete release requires the plasma membrane receptor kinase FERONIA, which might play a role in species recognition (Escobar-Restrepo et al., 2007), as well as LORELEI, a membrane GPI-anchored protein (Capron et al., 2008). Also required for successful pollen tube reception is the expression of the peroxisomal protein encoded by *abstinence by mutual consent* in both the male and the female gametophytes, which serves to illustrate the active participation of the male gametophyte in gamete release (Boisson-Dernier et al., 2008), in addition to the fusion of the sperm and the egg cell by the male-specified GCS1 protein (Mori et al., 2006).

Female gametophytic control of seed development: maternal effect genes and epigenetic mechanisms

In animals, the diploid mother establishes the local environment for the zygote, including the positional signaling that enables the patterning of the embryo. Maternal effect mutations that affect the development of the embryo, but not that of the mother, have provided important insights into animal embryogenesis. By contrast, embryogenesis in plants is only indirectly affected by the diploid sporophyte, and the environment and signals for the development of

the products of fertilization depend more directly on the haploid gametophytic generation. Gametophytic maternal effect mutations in plants can be broadly defined as mutations that permit the formation of functional embryo sacs, but affect embryo and/or endosperm growth and development. Gametophytic mutants cannot be rescued by wild-type pollen, which distinguishes them from zygotic mutants. There are two general mechanisms for such maternal effects. The first arises from a maternal gene product that is made and sequestered in the egg and/or the central cell and is required for the initiation or progression of embryo and/or endosperm development. This might include genes in essential cellular processes, such as metabolism, protein translation, cell cycle and so forth. In a large-scale genetic screen for female gametophyte mutants, nearly half of the mutants identified appeared to have maternal effect mutations, i.e. functional embryo sacs could be formed, but not viable embryos, even when pollinated with normal pollen. The majority of the disrupted genes appeared to be involved in basic cellular processes consistent with this first interpretation (Pagnussat et al., 2005).

A second, but less frequent, type of maternal effect mutation involves epigenetic mechanisms, such as chromatin modifications or altered methylation of genetic loci in the female gametophyte, which then affect gene expression after fertilization. The first such mutation to be characterized was in the gene *MEDEA* (*MEA*) (Grossniklaus et al., 1998). A mutant allele of *MEA* results in the abortion of the seeds when transmitted through the female, but not the male. *MEA* is a homolog of *Drosophila Enhancer of zeste* [*E(z)*] and a member of a plant Polycomb group (PcG) complex called the fertilization independent seed (FIS) complex, which also includes *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*), which is the homolog of *Drosophila extra sexcombs* (*esc*), and *FERTILIZATION INDEPENDENT SEED2* (*FIS2*), which is the homolog of *Drosophila Su(z)12*. All three genes are expressed in the central cell of the female gametophyte, where they function to repress genes involved in endosperm growth (Chaudhury et al., 2001; Hsieh et al., 2003). Loss-of-function mutations in any of these genes result in divisions of the central cell nucleus without a need for fertilization, which give rise to a diploid endosperm referred to as ‘autonomous endosperm’. This endosperm growth eventually arrests and is followed by seed abortion (Chaudhury et al., 1997). The FIS PcG complex acts in the nucleus of the central cell of the embryo sac to repress genes that promote endosperm growth; it does so by chromatin modifications that involve the methylation of Histone H3 at the lysine residue 27 (K27) and eventual heterochromatinization (Makarevich et al., 2006). These loci are therefore maintained in a transcriptionally silent state in the central cell nucleus until fertilization. The *MEA* gene itself is under positive regulation by another gene called *DEMETER* (*DME*), which encodes a DNA demethylase that only is active in the central cell (Choi et al., 2002). In *dme* mutants, *MEA* is not expressed and the result is a non-functional FIS complex and autonomous endosperm. The formation of autonomous endosperm in *fis* mutants is blocked by the *glauce* mutant; however, the identity of the *GLAUCE* gene is currently unknown (Ngo et al., 2007). In addition to the above genes, the product of a fourth gene, *MSI1* (the *Arabidopsis* homolog of the yeast gene *MSI*), a WD-40 protein, is also required for FIS complex activity (Guitton et al., 2004; Kohler et al., 2003). Embryo sacs that inherit a mutant allele of *MSI1* exhibit autonomous endosperm growth, as would be expected for an activator of the FIS complex. Interestingly, *msi1* mutant embryo sacs also show infrequent parthenogenic embryos that arise from the growth and division of the egg cell; these embryos do not, however, progress beyond a very immature stage and eventually abort (Guitton and Berger, 2005). These observations suggest that *MSI1* might function in the egg cell in addition to the central cell.

At least one of these additional functions of *MSI1* appears to require the activity of its binding partner RBR, the gene product of *RBR* (*RETINOBLASTOMA-RELATED PROTEIN*), which is the plant homolog of the animal retinoblastoma protein Rb. In both animals and plants, RBR/Rb binds and inhibits transcription factors of the E2F family that promote the progression through the G1/S transition of the cell cycle. In *Arabidopsis*, the *rbr* mutant has a female gametophytic phenotype (Ebel et al., 2004). Specifically, there is an over-proliferation of nuclei in the female gametophyte, which results in embryo sacs with more than eight nuclei. In the *rbr* mutant, gametophytic development is initially normal until stages FG5/FG6, when the normal number of nuclei is formed. From this stage onwards, over-proliferation occurs. The supernumerary nuclei are more pronounced at the micropylar end and can cellularize to generate additional synergids and egg cells, as well as multinucleate central cells. Interestingly, the cell fates adopted by these supernumerary nuclei appear to be specified by their positions within the mutant embryo sacs, consistent with the auxin gradient model.

Recent studies have revealed a novel function for *RBR*, together with *MSI1*, in the female gametophyte in regulating the methylation state of the gametes (Johnston et al., 2008; Jullien et al., 2008). It was shown that RBR represses the transcription of the DNA

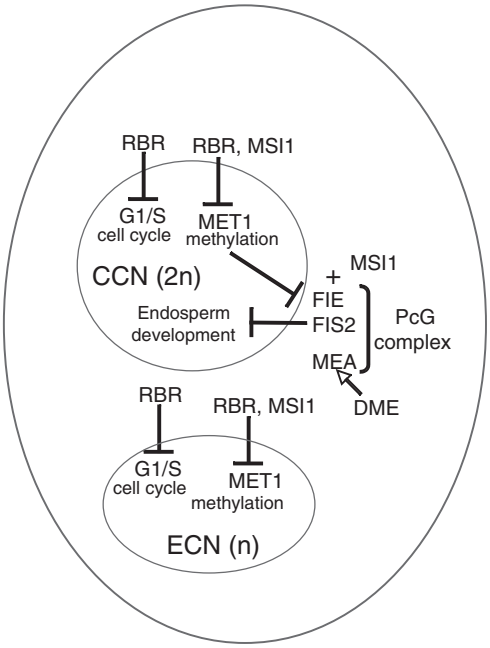

Fig. 5. Maternal gene networks and epigenetic modifications in the *Arabidopsis* embryo sac. Different sets of genes act on the central cell nucleus (CCN; top) and the egg cell nucleus (ECN; bottom). RBR acts on both the CCN and ECN to keep the cells in G1, and, together with MSI1, suppresses the maintenance methylase MET1. In the CCN, the Polycomb group (PcG) complex formed by FIE, FIS2 and MEA acts together with MSI1 to suppress endosperm development. MEA is activated in the CCN by a specific demethylation activity of DME. See text for details. DME, DEMETER; FIE, FERTILIZATION-INDEPENDENT ENDOSPERM; FIS2, FERTILIZATION-INDEPENDENT SEED 2; MEA, MEDEA; MET1, METHYLASE 1; MSI1, MULTICOPY SUPPRESSOR OF IRA1; RBR, RETINOBLASTOMA-RELATED protein.

methylase MET1 (which maintains ^{5m}C methylation at CpG sites and is the homolog of the animal maintenance methylase *DNMT1*) during *Arabidopsis* embryo sac development (Fig. 5). The mechanism appears to require association with MSI1 to bind the promoter of *MET1* at a presumptive S-phase-promoting E2F transcription factor binding site. The result is the decreased methylation of the egg cell and central cell genomes, presumably due to the loss of maintenance of methylation during the mitotic divisions of the gametophyte (Jullien et al., 2008). This general demethylation appears to be required to de-repress the PcG gene *FIS2* in the central cell. In *rbr* mutants, concomitant with *FIS2* repression, an increase in heterochromatin was observed in the central cell nucleus, although curiously not in the egg cell nucleus (Johnston et al., 2008). The increase in heterochromatin was attributed to the presumptive increased methylation of the central cell genome. Because *MEA* expression in *rbr* mutants is unaffected, the suppression of *MET1*-dependent maintenance-methylation might not be sufficient to relieve the repression of the *MEA* promoter, which shows low overall methylation. It might be that *MEA* expression in the central cell is exclusively dependent upon the demethylation activity of *DME*. Therefore, there are two types of demethylation processes that occur during gametogenesis (Jullien et al., 2008). The first is a passive, genome-wide decrease in DNA methylation due to the repression of *MET1* by *RBR* and *MSI1*, which is expected to be common to both the egg cell and the central cell. The second might be an active demethylation at specific loci, as in the case of *DME* acting on *MEA*, and perhaps on other PcG genes as well. Recent studies indicate a more extensive role for *DME* in demethylation of the maternal genome (Gehring et al., 2009; Hsieh et al., 2009). This type of demethylation is specific to the central cell, which gives rise to the triploid endosperm after fertilization and is a tissue in which parental imprinting has been extensively demonstrated. The above processes of demethylation probably play a key role in the parent-of-origin-dependent expression observed for imprinted genes during the growth of fertilized seeds. A recent study analyzed short interfering RNA (siRNA) expression in the fertilized endosperm of *Arabidopsis* and found that these siRNAs are primarily of maternal origin, which implicates the female gametophyte as their source and suggests that imprinting might be more widespread in the *Arabidopsis* genome than at the few loci described to date (Mosher et al., 2009).

Conclusions

The importance of the embryo sac as the site of female gamete formation was recognized over a century ago. This was followed by the discovery of double-fertilization, and the realization that the alternation of generations is integral to the plant life cycle. However, nearly all the subsequent advances in plant biology have focused on the diploid sporophytic generation, which led to the description of haploid gametophytes as the 'forgotten generation' (Brukhin et al., 2005). Over the past few years, this situation has rapidly begun to be remedied. Several large-scale forward mutant screens, in combination with expression profiling, are uncovering genes that contribute to fundamental processes in the embryo sac. Major findings include the identification of genes that play key roles in the differentiation of different cell types, pollen tube attraction and fertilization, and the recognition of maternal effects on seed development, which include the epigenetic modifications of the maternal chromosome set. New studies have provided insights into the mechanisms of pattern formation in the female gametophyte and indicate that morphogenetic gradients of the hormone auxin act in combination with precisely orchestrated nuclear positioning to specify the various gametophytic cell fates. However, much remains to be understood, particularly with respect to the central questions of patterning. How is the auxin gradient maintained in a syncytium? What is the mechanism by which auxin concentrations specify different cell types? For example, are the different fates attributable to different auxin response factors that activate different target genes depending on the auxin concentration? What are the mechanisms that restrict gametic cell fate to the egg cell and the central cell once initial patterning is established? Exactly how is nuclear positioning controlled, given that it must remain independent of auxin for patterning to work? The answer to this last question must involve the regulation of the embryo sac cytoskeletal machinery, but other than a poorly defined role for Knox-TALE proteins, nothing at all is known about such mechanisms. These examples demonstrate that major unanswered questions remain regarding pattern formation in this miniature multicellular structure so crucial to plant reproduction, but we are at last able to formulate conceptual frameworks that allow us to begin addressing these questions systematically.

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Competing interests statement

The authors declare no competing financial interests.

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