Epigenetic Reprogramming in Plant Reproductive Lineages

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Monoecious flowering plants produce both microgametophytes (pollen) and megagametophytes (embryo sacs) containing the male and female gametes, respectively, which participate in double fertilization. Much is known about cellular and developmental processes giving rise to these reproductive structures and the formation of gametes. However, little is known about the role played by changes in the epigenome in dynamically shaping these defining events during plant sexual reproduction. This has in part been hampered by the inaccessibility of these structures—especially the female gametes, which are embedded within the female reproductive tissues of the plant sporophyte. However, with the recent development of new cellular isolation technologies that can be coupled to next-generation sequencing, a new wave of epigenomic studies indicate that an intricate epigenetic regulation takes place during the formation of male and female reproductive lineages. In this mini review, we assess the fast growing body of evidence for the epigenetic regulation of the developmental fate and function of plant gametes. We describe how small interfering RNAs and DNA methylation machinery play a part in setting up unique epigenetic landscapes in different gametes, which may be responsible for their different fates and functions during fertilization. Collectively these studies will shed light on the dynamic epigenomic landscape of plant gametes or ‘epigametes’ and help to answer important unresolved questions on the sexual reproduction of flowering plants, especially those underpinning the formation of two products of fertilization, the embryo and the endosperm.

Keywords: Epigenetics • Gametes • Reproduction.

Abbreviations: DDM1, DECREASE IN DNA METHYLATION 1; DME, DEMETER; DRM2, de novo DNA methyltransferase 2; MET1, DNA methyltransferase 1; miRNA, microRNA; MMC, megaspore mother cell; RdDM, RNA-dependent DNA methylation; siRNA, small interfering RNA; TE, transposable element.

Introduction

The life cycle of flowering plants alternates between a diploid sporophytic phase and a haploid gametophytic phase. As part of this cycle, most flowering plants establish their germline as sporophytic development approaches maturity. The meiotic products originate from separate male and female diploid sporophytes but, rather than differentiating directly into gametes, as is the case in animals, they instead give rise to multicellular structures known as haploid gametophytes. This developmental process is complex and its regulation is not yet fully understood (Harrison et al. 2010). Nonetheless, the final outcome is the formation of a male gametophyte or pollen, consisting of one large vegetative cell and two gametic sperm cells, and a megagametophyte or embryo sac with its two gametes—the egg cell and the central cell—and five accessory cells that provide support during double fertilization. Although the cellular events that give rise to these male and female gametophytes, respectively, are well understood, it is becoming increasingly clear that the production of male and female lineages is not only under genetic, but, more importantly, also under epigenetic control. Here we discuss recent evidence supporting the view that dynamic changes in small non-coding RNAs and DNA methylation are part of a complex epigenetic reprogramming that takes place during the development of plant reproductive lineages that in Angiosperms culminates with the formation of the two largely similar male and two very different female gametes.

Epigenetic Reprogramming in the Male Gametes

Sperm are formed within pollen grains, which are located in the anthers of flowering plants. Meiosis in the young anther generates four haploid meiotic products (microspores), which first undergo a cell cycle resulting in formation of bicellular pollen grains containing a vegetative and a generative cell. In a second cycle, the generative cell divides to form two haploid sperm (Berger and Twell 2011). In some taxa, this second mitotic division can occur either in the anther (e.g. Arabidopsis) or during pollen tube growth (e.g. Nicotiana). Thus the male germline sensu stricto arises very late in higher plants, and the gametes appear even later (Dickinson and Grant-Downton 2009). However, this truncation of microgametophyte development, when compared with lower plants, indicates that epigenetic
events taking place in the young gametophyte are likely to impact on the epigenomic landscape of the sperm themselves. To capture all the events that may contribute to epigenetic reprogramming in male gametes, this section of the review will discuss the epigenetic events taking place during microsporogenesis and microgametogenesis leading to the development of the tricellular pollen encapsulating two sperm cells.

A number of epigenetic pathway genes are required for male gametophyte development in toto. In Arabidopsis, studies of met1 mutants have shown that maintenance CG methylation is required for the inheritance of epigenetic marks throughout gametophyte generation (Saiz et al. 2003). Likewise, a number of changes in chromatin structure are reported to be required for successful development of the male gametophyte (Berr et al. 2010).

**The epigenetics of microsporogenesis**

Little is known of the epigenetics of plant meiosis, but data from other organisms suggest that recombination and synapsis are guided by epigenetic marks (Borde et al. 2009, Takada et al. 2011). Furthermore, expression data from both rice and Arabidopsis show that normally silenced transposable elements (TEs) and neighboring sequences become transcribed during meiotic prophase (Chen et al. 2010, Yang et al. 2011), indicating a partial release of epigenetic repression. In addition, recent data indicate that DNA methylation and histone modifications play a pivotal role in the regulation of meiosis (Perrella et al. 2010, Melamed-Bessudo and Levy 2012, Mirozue et al. 2012). Further evidence for epigenetic reprogramming during meiosis is provided by increased transcription of 55 genes of putative mitochondrial origin inserted in a silenced centromeric chromosomal region (Chen et al. 2010).

Interestingly, TE transcripts are not over-represented in the microspore transcriptome (Honys and Twell 2004), indicating that this phase of activation is temporary and that epigenetic silencing pathways, presumably involving RNA-dependent DNA methylation (RdDM), are restored post-meiosis. Transcriptional analyses do not often help to identify key epigenetic regulators, instead mutant analyses have shown that in rice MEIOSIS ARRESTED IN LEPTOTENE 1 and 2 (MEL1 and 2) are required for meiosis (Nonomura et al. 2007, Nonomura et al. 2011). MEL1 is the ortholog of Arabidopsis AGO5, while MEL2 is an RNA recognition motif (RRF) protein, suggesting that interaction with intergenic and/or repeat-associated small interfering RNAs (siRNAs) in the case of MEL1, and specific RNA processing in the case of MEL2, are required for meiotic progression.

There is indirect evidence that young microspores remain developmentally labile, as both temperature and nutrient stress (Dickinson 1994) can divert them to a sporophytic pathway resulting in androgenic embryogenesis, a process greatly exploited by industry (Dunwell 2010). Sequence data are now available for rice microspores (Wei et al. 2011) and are showing that these cells contain a number of novel microRNAs (miRNAs) and stage-specific enrichment for certain siRNAs—but surprisingly not those likely to be involved in TE silencing. Likewise, small RNA sequence data are now available for four stages of male gametophyte development in Arabidopsis (Le Trionnaire et al. 2011), which has revealed new putative miRNAs, most of them specific to the microspore stage, suggesting that miRNA activity is essential for the establishment of male gametophytic ‘developmental commitment’.

**The epigenetics of microgametogenesis**

The first mitotic division of the pollen is unusual in that it is asymmetric. Recent studies have identified a number of genes including DUO1/3 (Brownfield et al. 2009), APC/C cell cycle regulators (Zheng et al. 2011) and also an miRNA (Palatnik et al. 2007) essential for this division. However, the processes controlling the formation of the cytoskeleton that asymmetrically partitions the cytoplasm into two daughter cells (i.e. the vegetative and generative cells), and why they pursue such different fates, remain unclear (Berger and Twell 2011). The answer may lie with a changing epigenetic landscape accompanying these formative cell divisions. Certainly, unraveling epigenetic events during pollen development is complicated by the fact that maturing male reproductive cell lines are invested by numbers of different and very active tissues, and that following pollen mitosis I, pollen daughter cells follow radically different developmental pathways. Immunohistochemical analysis of cytosine methylation has, however, shown differences between the two nuclei of tobacco pollen (Oakeley et al. 1997), but these chromatin differences are most probably established after pollen mitosis I. More recently, fractionation strategies have been developed to isolate both transcripts and small RNA populations from pollen cells at key developmental stages (Honys and Twell 2004, Le Trionnaire et al. 2011), as well as sperm cells from mature pollen grains (Borges et al. 2008). Combining these technologies with large-scale sequencing has indicated that epigenetics may play a unique and active role in plant male gametogenesis. From the earliest transcriptomic analyses it became clear that small RNA pathways are very active early in pollen development, including those mediating miRNA formation and action. The identification of RDR6 and DCL3 at these early stages has also pointed to the activity of siRNA processing pathways operating in pollen (Grant-Downton et al. 2009a). Intriguingly, many key components of small RNA pathways persist through pollen development into the mature pollen grain and are particularly active in the sperm (Borges et al. 2008, Borges et al. 2011), reinforcing the view that cells of the male gametophyte are under strict epigenetic control throughout development (summarized in Fig. 1).

Expression data from both large-scale sequencing (Grant-Downton et al. 2009b, Le Trionnaire et al. 2011) and microarrays (Chambers and Shuai 2009) have confirmed the presence of pollen non-coding small RNA populations and that the majority of pollen miRNAs are also present in the
sporophyte. However, detailed expression analysis has revealed that not all male gametophytic miRNAs are able to cleave their target sequences, which suggests that they may act to regulate gene expression by translational repression (Grant-Downton et al. 2009b). This complex mode of regulation may be significant in pollen as it is maintained in a dormant state prior to release from the anther. Further, sequence data from sperm cells have also identified abundant small RNAs and, more importantly, miRNAs, specifically enriched in the sperm (Slotkin et al. 2009, Borges et al. 2011). While molecular analyses have shown the presence of both miRNAs and their ‘products’, evidence of their function is essentially circumstantial. However, it has been shown recently that artificial miRNAs are able to down-regulate endogenous target transcripts not only in mature pollen (Coimbra et al. 2009) but also in the germline (Slotkin et al. 2009). The presence of abundant trans-acting siRNAs in developing pollen (Grant-Downton et al. 2009b, Le Trionnaire et al. 2011) coupled with the impaired pollen tube growth observed in mutants defective in the siRNA pathway suggests that trans-acting siRNAs may also play an important part in development pre- and post-germination. In addition, natural antisense siRNAs (nat-siRNA) would also appear to be important in male gametophyte as shown by the misregulation of a nat-siRNA in Arabidopsis which results in impaired fertilization (Ron et al. 2010).

The very late formation of a germline, sensu stricto, in plants suggests that the complex mechanisms evolved by animals to suppress TE activity in the germline (Klattenhoff and Theurkauf 2008) may be absent in the male gametophyte. Analysis of epigenetic pathways and small RNA populations in the sperm and vegetative cells of Arabidopsis supports this hypothesis (Slotkin et al. 2009). Late in pollen development the chromatin remodeling factor DECREASE IN DNA METHYLATION 1 (DDM1) becomes down-regulated in the vegetative nucleus with a concomitant increase in transcripts of retrotransposons. Strikingly, analysis of sperm small RNAs revealed the presence of high levels of TE-derived siRNAs, leading to the proposal that siRNAs generated in the vegetative nucleus are translocated to the sperm where they increase the silencing of TEs in the gametic genome by reinforcing RdDM (Slotkin et al. 2009).
In support of this intercellular movement of small RNAs, it has been shown that expression of a transgenic sperm-specific green fluorescent protein (GFP) was down-regulated by an artificial miRNA expressed in the vegetative cell under the LAT52 promoter (Slotkin et al. 2009). Importantly, the LAT52 promoter is expressed in both the microspore and binucleate stages of pollen development (Eady et al. 1995), so it remains possible that this silencing mechanism is established well before pollen mitosis II, with vegetative cell siRNAs first silencing germ-line TEs in the generative cell. The possibility of siRNA translocation between the vegetative cell and the sperm is supported by the evidence for sperm cell cytoplasmic projections that link the vegetative nucleus with the sperm within the male germ unit (McCue et al. 2011).

Intriguingly, the DNA glycosylase DEMETER (DME) has recently been found to be restricted to the pollen vegetative cell (Schoff et al. 2011). In the female central cell, DME acts to hypomethylate specific DNA sequences, and is responsible for activating the maternal alleles of certain imprinted genes (Hsieh et al. 2011). Transmission of the mutant dme allele through pollen is reported to be reduced for some Arabidopsis ecotypes, indicating that it is required for gametophyte and/or progamic development—presumably by establishing the DNA methylation landscape of the vegetative cell. While DME is expressed in the principal accessory cells of both male and female gametophytes, its absence from the sperm indicates that it has no influence on the methyleome of male gametes, which may play a key part in establishing the parent-of-origin methylation asymmetry observed at some imprinted loci in plant gametes (Gutierrez-Marcos et al. 2006; see also Ikeda 2012). It is possible that DME-induced hypomethylation of TEs in the vegetative nucleus results in their transcriptional activation and may contribute to an increase in TE-derived siRNAs. Therefore, the abundant siRNAs reported in pollen vegetative cells may be a product of both chromatin remodeling and DNA hypomethylation. However, it is not yet clear whether DME specifically targets TEs for demethylation in the vegetative cell.

Collectively, these studies reveal that all components of the epigenetic machinery play a pivotal role in the development and reprogramming of the male germline.

**Epigenetic Reprogramming in Female Gametes**

The female gametes are formed within the female gametophyte, which is generated after a series of divisions from a single surviving haploid megaspore cell (Berger and Twell 2011). The megaspore undergoes three syncytial divisions to form an eight-nucleate sac/female gametophyte that then cellularizes into seven cells that differentiate into four morphologically distinct cell types. Two of these adjacent cells differentiate into the haploid egg and the homodiploid central cell gametes. Although it is not clearly understood how these two genetically identical female gametes differentiate during cellularization, it is possible to speculate that this process probably involves epigenetic processes initiated during the syncytial developmental phase of the female gametophyte and that culminates in cellularization. Although this hypothesis is not yet supported by experimental data, the analysis of mutants which affect nuclear and cellular proliferation in the embryo sac leading to the production of extra gametes (Evans 2007, Purugganan and Fuller 2009) suggests that the fate of female gametes is not only determined by positional information but could also involve global epigenetic changes such as differences in chromatin structure (Garcia-Aguilar et al. 2010, Pilott et al. 2010). This section of the review will focus on the epigenetic events, mainly non-coding RNAs and DNA methylation, that take place during the formation of the female gametophyte and megagametogenesis (summarized in Fig. 1).

**The epigenetics of megagametogenesis**

The megaspore mother cells (MMCs) are specified in a subepidermal position of the sporangia. Intriguingly, the identification of an argonaute mutant in Arabidopsis that develops multiple MMCS indicates that the fate of the female spore mother cell is epigenetically regulated, most probably by the action of small non-coding RNAs (Olmedo-Monfil et al. 2010). However, AGO9 is not expressed in the subepidermal layer where the MMC is specified, but instead is present in the adjacent epidermal cell layer, which could indicate the non-cell-autonomous action of AGO9. More recently, analysis of a mutant allele for AGOS has revealed that the small RNA pathway also acts in somatic nucellar cells to promote megagametogenesis in megaspores (Tucker et al. 2012). This analysis also revealed that AGOS acts independently of AGO9, thus pointing to the complex role that small RNAs play in megagametophyte development (Fig. 1). These findings are perhaps not surprising as non-coding small RNAs have recently been implicated in the non-cell-autonomous regulation of gene expression in other plant organs by the RdDM pathway (Dunoyer et al. 2010, Molnar et al. 2010). The hypothesis that similar non-cell-autonomous mechanisms may take place in the specification of sporogenic cell fate is further supported by the recent analysis of maize transgenic lines with down-regulated homologs of Arabidopsis de novo DNA methyltransferase 2 (DRM2), which also exhibit ectopic embryo sac formation from supernumerary MMCS (Garcia-Aguilar et al. 2010).

Intriguingly, AGO9 and de novo methyltransferases interact with TE-derived siRNAs, whose activity is required to silence TEs in the female gametophyte (Duran-Figueroa and Vienne-Calzada 2010, Olmedo-Monfil et al. 2010, Singh et al. 2011). However, it remains to be shown if discrete protein-coding loci are affected by this epigenetic regulation and if they directly participate in the development of the female gametophyte.

Regulation of the transition from mitosis to meiosis in the female gametophyte is also epigenetically regulated. This view is
supported by the discovery of a rice meiotic mutant, *meiosis arrested at leptotene 1* (mel-1), whose affected gene encodes a sporogenous cell-specific Argonaute protein. However, the role of non-coding RNAs in the mitotic to meiotic switch in other species remains unclear. It is envisaged that further investigation to reveal the identity of MEL1 targets could lead to the discovery of other key epigenetic factor(s) regulating the meiotic switch in plants.

In addition, several recent studies indicate that large-scale epigenetic modifications take place during female gametophyte development. In particular, chromatin organization of the egg and central cell gametes differs from that of accessory cells (Ingouff et al. 2010, Pilott et al. 2010), thus suggesting that distinct epigenetic features are established prior to or during the cellularization of the female gametophyte. Intriguingly, the epigenetic landscape of the two female gametes differs not just at the chromatin level (Garcia-Aguilar et al. 2010, Ingouff et al. 2010, Pilott et al. 2010) but also in terms of the DNA methylation patterns (Gutierrez-Marcos et al. 2006, Jahnke and Scholten 2009). This asymmetry in DNA methylation between female gametes is largely due to the activity of DME in the central cell (Choi et al. 2002, Gehring et al. 2006) and by the transcriptional repression of DNA methyltransferases (namely MET1) by the retinoblastoma pathway during maturation of the female gametophyte (Julien et al. 2006, Costa and Gutierrez-Marcos 2008). This global epigenetic reprogramming taking place in central cells may be associated with the high transcriptional activity of TEs (Le et al. 2005, Yang et al. 2011), which probably may contribute to an abundant siRNA population in the central cell. It has been proposed that the global demethylation found in the central cell may be directed by siRNAs, which in turn could direct DME to discrete portions of the genome (Bourc’his and Voinnet 2010). However, DME and DME-like proteins appear preferentially to target gene-neighboring chromosomal regions and not particularly TEs (Gehring et al. 2009, Schoft et al. 2011). An alternative hypothesis is that DME is directed to transcriptionally active regions of the genome featuring a specific chromatin conformation. This view is supported by the recent identification of an Arabidopsis mutant (*ssrp1*), which codes for a chromatin protein, FACT histone chaperone, that is required for DNA demethylation in the central cell (Ikeda et al. 2011). Thus, an attractive possibility raised by this study is that remodeling of chromatin may precede genome-wide DNA demethylation in the central cell.

Exactly why the central cell undergoes such a massive epigenetic reprogramming remains a mystery. One intriguing explanation is that mobile small RNAs from the central cell are able to act non-cell-autonomously to silence TEs in the neighboring egg cell and later in the zygote (Mosher and Melnyk 2010), but this theory awaits experimental confirmation. Epigenetic changes in the central cells could also be pivotal for marking genes in the female central cell prior to fertilization, which could later establish the asymmetric gene expression observed at a significant number of imprinted gene loci in plants (reviewed by Ikeda 2012). Molecular experimental approaches such as a genome-wide epigenetic profiling of female gametes could be easily performed to test this hypothesis.

### Future Challenges

While a combination of refined cell fractionation techniques and large-scale sequencing has resulted in a clearer understanding of the epigenetic control of male and female gametophyte development in higher plants, there are a number of areas where lack of data is hampering significant advances. For example, microspore-based systems for making double haploid plants would dramatically improve the rate of improvement of some crop species such as wheat, and a better understanding of the role played by epigenetics in establishing embryogenic developmental commitment would transform our ability to achieve this goal. Also, the mechanism by which the pollen exists in a transcriptionally dormant state in the anther until it is activated on the stigma surface remains unknown. However, the observation that pollen tube growth is compromised in *dicer* mutants indicates that the transcriptional activity of mature pollen may be regulated by siRNAs. Further, the putative silencing of sperm TEs by siRNAs generated in the vegetative cell seems to be reflected by a similar gamete or accessory cell interaction in the female gametophyte (Mosher and Melnyk 2010). It is important that robust data are now obtained to ascertain the precise timing of these events, and the process by which small RNAs regulate gametogenesis. Another outstanding question is: do small non-coding RNAs play a role in female gamete function? This could be revealed by the systematic functional analysis of AGO members that are specifically expressed in female gametes (Wuest et al. 2010). Equally, mobile signals produced in the female gametophytic accessory cells and/or surrounding maternal tissues may also influence gamete function and thus warrant further investigation. Certainly, these are exciting times for studying the dynamic nature of plant epigenomes (see Kinoshita and Jacobsen 2012), and in particular the part played by epigenetic factors in shaping the unique genomes of developing male and female reproductive lineages and gametes.

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