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REVIEW PAPER

Male gametophyte development: a molecular perspective

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Abstract

Pollen grains represent the highly reduced haploid male gametophyte generation in flowering plants, consisting of just two or three cells when released from the anthers. Their role is to deliver twin sperm cells to the embryo sac to undergo fusion with the egg and central cell. This double fertilization event along with the functional specialization of the male gametophyte, are considered to be key innovations in the evolutionary success of flowering plants. This review encompasses important recent advances in our understanding of the molecular mechanisms controlling male gametophyte development. A brief overview of pollen development is presented, followed by a discussion of genome-wide transcriptomic studies of haploid gene expression. The progress achieved through genetic analysis of landmark events of male gametogenesis is discussed, with a focus on sperm cell production, and an emerging model of the regulatory network governing male germline development is presented. The review concludes with a perspective of the impact these data will have on future research strategies to further develop our understanding of the gametophytic control of pollen development.

Key words: Arbidopsis, cell cycle, cell fate, development, double fertilization, germline, male gametophyte, pollen, sperm cell, transcriptomics.

Introduction

Higher plants have a complex life cycle that alternates between the growth of a diploid sporophytic organism and a highly reduced haploid gametophytic form. In flowering plants, the male gametophyte (or pollen grain) plays a vital role in plant fertility and crop production through the generation and delivery of the male gametes to the embryo sac for double fertilization. Apart from its intrinsic importance in sexual reproduction, the simple cell lineage and highly orchestrated development of the male gametophyte also represents a microcosm of cellular development. This makes it an attractive system in which to dissect the fundamental processes of cell polarity, control of the cell cycle, the regulation of gene expression and cell specification, and how these processes are integrated.

During the past decade, major advances in genetic and genomic technologies have fuelled significant progress in understanding male gametophyte development at the molecular level (Twell *et al.*, 2006). Resources include the highly annotated *Arabidopsis thaliana* genome (The *Arabidopsis* Genome Initiative, 2000), its associated public databases and readily available sequenced insertion site mutants (Alonso *et al.*, 2003). There are also comprehensive transcriptomic data sets from *Arabidopsis* (Zimmermann *et al.*, 2004) including those from the male gametophyte (Honys and Twell, 2004; Pina *et al.*, 2005) and recently from isolated sperm cells (Borges *et al.*, 2008). The use of several new male gametophyte-specific techniques (Johnson-Brousseau and McCormick, 2004) and the identification of various gametophytic mutants through genetic screening (discussed in this review) are also valuable additions. The establishment of sequenced EST libraries from sperm cells of *Zea mays* (Engel *et al.*, 2003) and *Plumbago zeylanica* (SD Russell, unpublished results), and from generative cells of *Lilium longiflorum* (Okada *et al.*, 2006), as well as the arrival of newly sequenced plant genomes, will enable a molecular phylogenetic perspective of male germline development.

Some of the key advances made in understanding male gametophyte development at the molecular level, from unicellular microspores to the point of shed pollen, are reviewed here. We focus on analyses of gametophytic gene

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expression and traditional genetic studies of the developing haploid male gametophyte. Discussion of aspects of male fertility and pollen development that involve diploid sporophytic tissues, including anther differentiation, male meiosis, and the influence of the tapetum on pollen wall patterning, can be found in other recent reviews (Ma, 2005; Scott *et al.*, 2004; Wilson and Yang, 2004). Although an overview of development and a brief discussion of transcriptomic advances is provided, several recent reviews discuss this in greater depth (Honys *et al.*, 2006; Twell *et al.*, 2006; Becker and Feijo, 2007; Singh and Bhalla, 2007). The discussion is then focused on the genes involved in 'landmark' cell division and cell specification events during male gametophyte development. The opportunity is taken to propose a theoretical model of the emerging regulatory network governing male germline specification and maintenance during male gametophyte development.

Overview of male gametophyte development

In animals, cells destined to become germline cells are determined early in embryogenesis and remain as a distinct population of stem cells throughout life (Hayashi *et al.*, 2007; Strome and Lehmann, 2007). By contrast, flowering plants maintain various populations of undifferentiated stem cells mostly in meristems. Meristematic tissue is capable of growth and differentiation to form vegetative tissues and organs, eventually giving rise to reproductive organs containing diploid sporogenous cells. A strict male germline is only established after meiosis when haploid microspores divide asymmetrically to form a small germ cell and a large vegetative cell. Unlike the animal germline, the plant male germline does not regenerate itself through mitotic stem cell divisions. Rather it undergoes a single round of mitosis to produce the functional twin sperm cells required for double fertilization.

Formation of the male gametophyte in flowering plants takes place within specialized male reproductive organs called the stamens and consists of two distinct sequential phases, microsporogenesis and microgametogenesis (Fig. 1). During microsporogenesis, diploid pollen mother cells undergo meiotic division to produce tetrads of haploid microspores. This stage is completed when distinct unicellular microspores are released from the tetrad by the activity of a mixture of enzymes secreted by the tapetum, the inner nutritive layer of the stamen (Scott *et al.*, 2004). During microgametogenesis, the released microspores enlarge and a single large vacuole is produced (Owen and Makaroff, 1995; Yamamoto *et al.*, 2003). This is accompanied by migration of the microspore nucleus to a peripheral position against the cell wall. The microspore then undergoes an asymmetric cell division known as Pollen Mitosis I (PMI). The small germ cell, representing the male germline, is subsequently engulfed within the cytoplasm of the larger vegetative cell to create a novel cell-within-a-cell structure. This engulfing process involves degradation of the hemispherical callose wall that separates the newly formed vegetative and germ cells. The fully engulfed germ cell forms a spindle-like shape that is maintained by a cortical cage of bundled microtubules (Palevitz and Cresti, 1989; Cai and Cresti, 2006). The asymmetric division at PMI is essential for the correct cellular patterning of the male gametophyte, since the resulting two daughter cells each

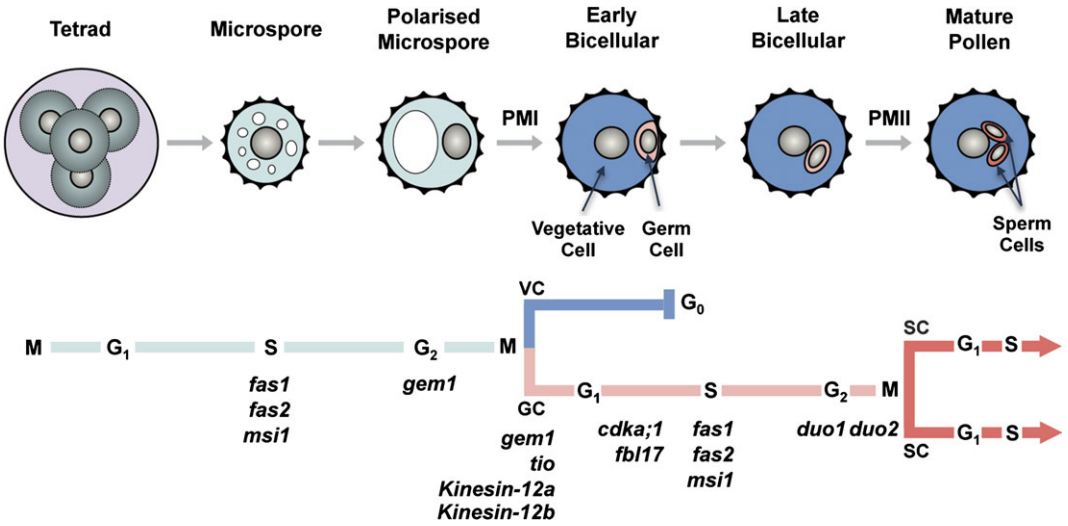

Fig. 1. Male gametophyte development in *Arabidopsis*. Schematic diagram representing the distinct morphological stages of male gametophyte development in *Arabidopsis* along with a colour-coded timeline of the cell cycle progression of each cell type. Known mutations critical during male gametophyte development are listed below the time-line at the point that they are known to act. During microsporogenesis, microsporocytes undergo a meiotic division to produce a tetrad of four haploid microspores. During microgametogenesis, the released microspores undergo a highly asymmetric division, called Pollen Mitosis I (PMI), to produce a bicellular pollen grain with a small germ cell engulfed within the cytoplasm of a large vegetative cell. Whilst the vegetative cell exits the cell cycle, the germ cell undergoes a further mitotic division at Pollen Mitosis II (PMII) to produce twin sperm cells. The sperm cells then continue through the cell cycle to reach G_2 prior to karyogamy and double fertilization. VC, vegetative cell; GC, germ cell; SC, sperm cell.

harbour a distinct cytoplasm and possess unique gene expression profiles that confer their distinct structures and cell fates (Twell *et al.*, 1998). Induction of symmetrical division at PMI has demonstrated that vegetative cell gene expression is the default developmental pathway and that division asymmetry is critical for correct germ cell differentiation (Eady *et al.*, 1995).

After PMI, the large vegetative cell has dispersed nuclear chromatin and exits the cell cycle in G_1. The vegetative cell nurtures the developing germ cell and gives rise to the pollen tube following successful pollination. This pollen tube grows through the stylar tissues of the gynoecium to deliver twin sperm cells to the embryo sac. During pollen maturation, the vegetative cell accumulates carbohydrate and/or lipid reserves along with transcripts and proteins that are required for rapid pollen tube growth (Pacini, 1996). Osmoprotectants, including disaccharides, proline and glycine-betaine, which are thought to protect vital membranes and proteins from damage during dehydration, are also accumulated (Schwacke *et al.*, 1999).

The smaller germ cell has condensed nuclear chromatin and continues through a further round of mitosis, called Pollen Mitosis II (PMII), to produce twin sperm cells. In species that shed tricellular pollen, such as *Arabidopsis thaliana*, PMII takes place within the pollen grain prior to anthesis. This is in contrast to the majority of species that shed their pollen in a bicellular state, such as *Lilium longiflorum*, with PMII taking place in the growing pollen tube. Following PMII, a physical association between the sperm cells and the vegetative nucleus is established that is referred to as the male germ unit (MGU). Mutations affecting either the assembly (germ unit malformed or *gum* mutants) or positioning (MGU displaced or *mud* mutants) of the MGU in *Arabidopsis* pollen lead to reduced male transmission (Lalanne and Twell, 2002). The MGU is common to both bicellular and tricellular pollen systems and is thought to be important for the co-ordinated delivery of the gametes and sperm cell fusion events (Dumas *et al.*, 1998).

Studies of gene expression in the male gametophyte

In recent years, new high-throughput technologies have enabled the analysis of male gametophyte gene expression on a global scale. Pioneering studies exploiting serial analysis of gene expression (SAGE) technology (Lee and Lee, 2003) and 8K Affymetrix AG microarrays (Becker *et al.*, 2003; Honys and Twell, 2003) provided analysis of the male gametophyte based on approximately one-third of the *Arabidopsis* genome. The development of the Affymetrix 23K *Arabidopsis* ATH1 array, which covers approximately 80% of *Arabidopsis* genes, enabled transcriptomic analysis of the male gametophyte on a much larger scale in three major studies, each with publically available datasets.

The first of these microarray datasets covers four stages of male gametophyte development (uninucleate microspores, bicellular pollen, tricellular pollen, and mature pollen) from ecotype Landsberg *erecta* (Honys and Twell, 2004). The two remaining datasets were derived from mature pollen grains from ecotype Columbia (Zimmermann *et al.*, 2004; Pina *et al.*, 2005). Consideration of these datasets together estimated the total number of genes expressed in the mature male gametophyte to lie between 5000 and 7000. When the analysis is extended to cover the pollen developmental series, the expression of approximately 14 000 genes was detected (Honys and Twell, 2004; Twell *et al.*, 2006).

An increasing number of publicly available sporophytic datasets has allowed comparative analyses to be performed with the male gametophyte transcriptome. Initial estimates indicated that around 10% of genes expressed in mature pollen were specific to the male gametophyte (Honys and Twell, 2004; Pina *et al.*, 2005). Recently, tools that enable comparison between large numbers of datasets, including those of pollen, have been assembled in a normalized database incorporating visualization tools: *Arabidopsis* Gene Family Profiler (aGFP) (Dupl'akova *et al.*, 2007). Using these tools with a greater number of sporophytic datasets to analyse gametophytic–sporophytic overlap, the estimated number of pollen-specific genes drops to approximately 5% of expressed genes (Twell *et al.*, 2006). With the increasing analysis of specialized sporophytic tissues this number may drop further. Other pollen-expressed genes have been described as showing enhanced expression in pollen with estimates varying between 26% (Pina *et al.*, 2005) and 10% (Twell *et al.*, 2006) of pollen-expressed genes. Although the estimates of the number of specific or enhanced pollen-expressed genes varies, it is still generally higher than estimates for most other plant tissues, which have up to approximately 3% of genes showing specific or enhanced expression (Ma *et al.*, 2005). This relatively high level of specific or enhanced expression reflects the functional specificity of the male gametophyte. Consistent with this, male gametophyte-specific genes are often characterized by very high expression levels. Many of these genes encode proteins with predicted functions related to pollen germination or tube growth (Honys and Twell, 2004; Pina *et al.*, 2005, Twell *et al.*, 2006)

Male gametophyte development is often divided into two major phases, an early phase that comprises microspore and bicellular pollen, and a late phase including tricellular and mature pollen. Differences between these stages are reflected in their transcriptomic profiles. More genes are expressed in the early phase with nearly 12 000 active genes in microspores and bicellular pollen. This number progressively declines to just over 7 000 in mature pollen (Fig. 2A**)** (Honys and Twell, 2004). Although there is significant overlap with the majority of genes expressed in both phases, the percentage of specific genes increases from unicellular microspores to mature pollen (Fig. 2A**)** (Honys and Twell, 2004). The reduction in complexity and switch to a late programme is also accompanied by an increase in genes involved in such processes as cell wall metabolism, cytoskeleton functions, and cell signalling, which are important for pollen maturation, germination, and rapid pollen tube growth (Honys and Twell, 2004; Pina *et al.*, 2005; Twell *et al.*, 2006).

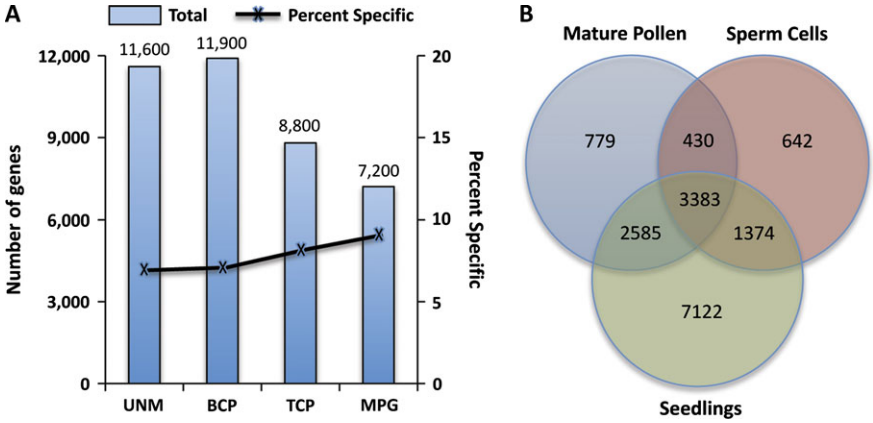

Fig. 2. Gene expression in the male gametophyte. (A) Transcriptome profile of the total number of expressed genes during male gametophyte development overlaid with a line graph representing the proportion of male gametophyte-specific genes. This diagram highlights the two distinct early and late transcriptional programmes of male gametophyte development. Whilst the number of genes expressed is reduced during the late phase of development, there is a clear overrepresentation of highly expressed genes within the male gametophyte-specific subset. (B) Venn diagram showing the quantitative overlap between the genes expressed in *Arabidopsis* seedlings (14 464), mature pollen (7177), and sperm cells (5829) (Borges *et al.*, 2008). This diagram highlights the large overlap between the genes expressed in sperm cells and seedlings (4757 or 82% of genes expressed in sperm). Approximately 11% of sperm cell-expressed genes (642) appear to be unique to sperm.

The large number of genes expressed and the switch between the early and late phases of male gametophyte development demands co-ordinated regulation of numerous genes at the transcriptional level. Accordingly, 612 out of approximately 1350 predicted transcription factors present on the *Arabidopsis* ATH1 Genome Array (Affymetrix) are expressed in developing male gametophytes (Twell *et al.*, 2006). Of these, 27 are pollen-specific and represent strong candidate factors that are likely to play important roles in pollen regulatory networks. Accordingly, a pioneering study has combined transcriptomic and mutational analysis on a group of transcription factors to establish a late pollen regulatory network. Analysis of transcriptomic data identified a subset of five pollen-specific MIKC* MADS box proteins (AGL30/65/66/94/104) that are expressed during pollen maturation (Honys and Twell, 2004; Pina *et al.*, 2005). Double mutant combinations affect *in vitro* pollen germination and pollen fitness *in planta* (Verelst *et al.*, 2007*a*) and transcriptomic analysis of pollen from single, double, and triple mutant combinations of interacting MIKC* proteins allowed the identification of putative target genes of the five pollen-specific MIKC* MADS box complexes (Verelst *et al.*, 2007*b*). These include structural and regulatory genes including two non-MIKC* MADS proteins (AGL18 and AGL29) that were identified as downstream regulators of a subset of MIKC* regulated genes. Further analysis of the architecture of this late network together with ongoing mutational analysis of different classes of pollen transcription factors, will establish more complex regulatory networks and determine their significance in pollen development and fitness.

Gene expression in the male germline

There is considerable interest in identifying and characterizing germline-expressed genes since they may play vital roles in germline development and fertilization. The discovery of germline-expressed genes had until recently remained elusive due to the small size and inaccessible nature of the germ cells within the vegetative cell. An insight into this size difference is highlighted in a study of *Plumbago zeylanica* pollen, in which the size ratio between the sperm cell lineage and the vegetative cell was found to be in the order of 1:1000 at anthesis (Russell and Strout, 2005). Given that RNA from the vegetative cell comprises the large majority of RNA extracted from whole pollen, important germline-expressed genes with a low level of expression may be missed in whole pollen transcriptomic analyses.

Pioneering studies of male germline expression focused on species other than *Arabidopsis* that have more accessible germline cells. This has enabled the development of techniques for the isolation and purification of male germ cells (Russell, 1991; Tanaka, 1988; Zhang *et al.*, 1998; Uchiumi *et al.*, 2006) that involve mechanical or enzymatic disruption of either mature pollen grains or *in vitro*-grown pollen tubes. Germline cells are then separated from the vegetative cell debris by density gradient centrifugation (Tanaka, 1988; Xu *et al.*, 2002), micromanipulation (Zhang *et al.*, 1998; Chen *et al.*, 2006) or fluorescence-activated cell sorting (FACS) based on DNA staining (Engel *et al.*, 2003). These techniques provide purified germline cells that are suitable for gene expression studies and, as such, cDNA libraries have been constructed from germ cells of *Lilium longiflorum* (lily) (Okada *et al.*, 2006) and from sperm cells of *Zea mays* (maize) (Engel *et al.*, 2003), *Plumbago zeylanica* (white leadwort) (SD Russell, unpublished data; Singh *et al.*, 2008) and *Nicotiana tabacum* (tobacco) (Xu *et al.*, 2002).

Initial studies of isolated germ cells focused on the identification of germline-specific transcripts. This led to the identification of several such genes, which include *Lily Generative Cell1* (*LGC1*) (Xu *et al.*, 1999*b*), *gcH2A* and

gcH3 encoding histone isoforms (Xu *et al.*, 1999*a*), a poly-ubiquitin gene *LG52* (Singh *et al.*, 2002) and *Generative Cell Specific1* (*GCS1*) from lily germ cells (Mori *et al.*, 2006), along with two unique germ cell-specific genes, *NtS1* and *NtS2,* from tobacco (Xu *et al.*, 2002). A wider perspective of germline expression has been gained through various EST sequencing projects, which have identified 5176 sequences from maize (recent number from NCBI) (Engel *et al.*, 2003), 1522 sequences from *Plumbago* sperm cells (Singh *et al.*, 2008) and 886 sequences from lily germ cells (Okada *et al.*, 2006). The germline-specificity of the transcripts identified in lily was investigated by hybridization to a spotted cDNA microarray prepared from germ cells and sporophytic tissues (Okada *et al.*, 2007). This approach highlighted the high ratio of cell-specific or enhanced transcripts in the lily male germline, with 356 out of the 430 genes investigated (83%) showing up-regulation.

Analysis of the conservation between male germline transcriptomic datasets has revealed that the expression of male germline genes overlaps in germ cells across different plant taxa (Okada *et al.*, 2006). Of 637 non-redundant lily germ cell ESTs, 168 showed significant similarity to maize sperm cell ESTs while 129 showed significant similarity to *Arabidopsis* male gametophyte-specific genes identified by microarray analysis (Honys and Twell, 2004). Moreover, 55 of these shared significant similarities to sequences from both the maize sperm cell and *Arabidopsis* male gametophyte datasets.

Functional characterization of these ESTs shows that not only are some genes conserved, but similar classes of genes are expressed in the germ cells of different species. Commonly represented classes include genes associated with general metabolism, cellular organization, DNA synthesis, chromatin structure, and protein degradation. The maize genome is characterized by being very abundant in retroelements, and this is also reflected in sperm cells, where the most abundant class of EST sequences represent components of retroelements (19% of total) (Engel *et al.*, 2003). In the maize ESTs, there was also a high number of genes encoding proteins known to or predicted to be plasma membrane associated or secreted (Engel *et al.*, 2003), while in the lily ESTs, communication and signal transduction categories were highly represented (Okada *et al.*, 2006). Such genes could be involved in signalling processes required during fertilization. Interestingly, proteins involved in the ubiquitin-mediated proteolysis pathway are highly represented in plant germline cells. This pathway plays an essential role in controlling spermatogenesis in metazoans, as well as being important for gametogenesis and fertilization (reviewed by Baarends *et al.*, 1999; Sakai *et al.*, 2004). The recent discovery and functional characterization of the F-box protein FBL17, which controls cell cycle machinery to promote male germ cell division in *Arabidopsis* (Kim *et al.*, 2008), is a novel example supporting the importance of the ubiquitination pathway in male gametophyte development (discussed in further detail below).

Transcriptomic datasets generated from lily, maize, and *Plumbago* have provided invaluable insight into the transcriptional programme of the plant male germline. However, 40–60% of the ESTs have no matches or encode unknown proteins. Incomplete genome annotation in these species, coupled with the limited prospect of reverse genetics, is a drawback of these systems. *Arabidopsis* currently provides the most tractable experimental and genetic model that is supported by recent determination of the transcriptome of isolated sperm cells (Borges *et al.*, 2008).

Initial studies on germline-expressed genes in *Arabidopsis* focused on the identification of genes homologous to those expressed in the germline of other species. A homologue of the lily gene *GCS1* (*HAP2*), and three genes homologous to maize sperm cell expressed genes (*GEX1*, *GEX2*, and *GEX3*), are examples of *Arabidopsis* germline-expressed genes identified using this comparative approach (Engel *et al.*, 2005; Mori *et al.*, 2006; von Besser *et al.*, 2006; Alandete-Saez *et al.*, 2008). Although no direct homologues of the lily H3 histones *gcH2A*, and *gcH3* were identified in *Arabidopsis*, analysis of the histone family did reveal a germline-specific histone H3 in *Arabidopsis* called *MGH3* (also referred to as *HTR10* on ChromDB*)* (Okada *et al.*, 2006). The identification of these germline-specific genes has allowed the isolation of *Arabidopsis* sperm cells for transcriptomic analysis (Borges *et al.*, 2008). Mechanically disrupted pollen from transgenic *Arabidopsis* plants expressing a sperm-specific eGFP marker (driven by the germline-specific *GEX2* promoter) was subjected to FACS in order to purify GFP-marked sperm cells based on their GFP signal, size, intracellular complexity, and presence of DNA. The resulting mRNA populations were then analysed with Affymetrix ATH1 arrays to provide the most comprehensive male germline transcriptomic dataset to date.

A total of 5829 genes (27% of the total genes on the array) gave a reliable expression signal in sperm cells in comparison to 7177 (33%) and 14 464 (64%) genes in pollen and seedlings, respectively (Borges *et al.*, 2008). Comparative analyses of the sperm cell transcriptome with that of pollen and seedlings again highlighted the distinct and diverse repertoire of germline-expressed genes (Fig. 2B). Interestingly, the majority of genes expressed in sperm cells (82% of the sperm cell transcriptome) were also expressed in seedlings. Despite this large overlap, approximately half of all sperm cell-expressed genes showed enriched expression in sperm cells (Borges *et al.*, 2008). Unsurprisingly, the vast majority of these sperm cell-expressed genes (3813) were also detected in mature pollen (Borges *et al.*, 2008). It is evident that the sperm cell transcriptome is complex, with the majority of expressed genes shared amongst other tissues, but there are still a significant number of genes (642, 11% of sperm cell-expressed genes) that appear to be sperm cell-specific (Fig. 2B). These putative sperm cell-specific genes are attractive candidates for further analysis in order to broaden our knowledge of unique sperm cell functions at the molecular level. This is exemplified by essential sperm cell-specific genes such as GCS1, which encodes a membrane-associated protein required for pollen tube guidance and fertilization (Mori *et al.*, 2006; von Besser *et al.*, 2006). Functional classification of sperm cell-enriched transcripts has revealed a high representation of gene ontology

categories associated with DNA repair, ubiquitin-mediated proteolysis, and cell cycle progression, which are similar to germline-expressed gene categories in other species. Small RNA and DNA methylation pathways also seem to be up-regulated in *Arabidopsis* sperm cells compared with vegetative cells, indicating active maintenance of the epigenetic state in the male germline (Borges *et al.*, 2008).

Genetic studies of male gametophyte development

Asymmetric division at PMI is a vital process during male gametogenesis as it is the first point at which the germline is set-aside during pollen development. The resulting germ cell will then undergo a further cell division (PMII) along with cell specification to produce twin sperm cells. A number of genetic studies have provided insights into the genes important during these processes and these are discussed below. The critical genes are then combined into an emerging model of germline specification and maintenance during male gametophyte development

Genes regulating asymmetric division and male germline formation

The asymmetry of division at PMI is critical for the formation of the male germline as induced symmetric division results in two daughter cells that both exhibit vegetative cell fate (Eady *et al.*, 1995). Several mutants have been isolated that demonstrate the importance of genes and cellular processes in patterning male gametophyte development (Table 1). *sidecar pollen (scp)* is a male-specific

Table 1. Summary of *Arabidopsis* genes and loci known to affect microspore development, asymmetric division and male germline development

Gene ID	Gene	Mutant	Mutant phenotype	Protein identity	Protein function	References
At1g65470	*FAS1*	*fasciata1*	Microspore and male germ cell cycle arrest at G_2/M	Chromatin Assembly Factor-1 (CAF-1) p150 subunit	Nucleosome/chromatin assembly during replication	Chen *et al.*, 2007
At5g64630	*FAS2*	*fasciata2*	Microspore and male germ cell cycle arrest at G_2/M	Chromatin Assembly Factor-1 (CAF-1) p60 subunit	Nucleosome/chromatin assembly during replication	Chen *et al.*, 2007
At3g48750	*CDKA;1*	*cyclin-dependent kinase A;1*	Bicellular pollen: S-phase progression inhibited in germ cell	Cyclin-dependent kinase	Germ cell S-phase progression	Nowak *et al.*, 2005 Iwakawa *et al.*, 2005
At3g60460	*DUO1*	*duo pollen1*	Bicellular pollen: germ cell fails to enter PMII	R2R3 MYB transcription factor (MYB125)	Regulator of germ cell specification and required for G_2/M transition.	Durbarry *et al.*, 2005 Rotman *et al.*, 2005
Unknown	*DUO2*	*duo pollen2*	Bicellular pollen: germ cell arrested at prometaphase	Unknown	Unknown	Durbarry *et al.*, 2005
At3g54650	*FBL17*	*F-box-like 17*	Bicellular pollen: S-phase progression inhibited in germ cell	F-Box protein	Targeted proteolysis of CDKA inhibitor KRP6 in male germ line	Kim *et al.*, 2008
At2g35630	*GEM1*	*gemini pollen1*	Twin-celled and binucleate pollen: abnormal divisions at PMI	MOR1/GEM1: Homologous to chTOGp/XMAP215 family of microtubule associated proteins	Microspore polarity and cytokinesis through microtubule organization.	Park *et al.*, 1998 Park and Twell, 2001 Twell *et al.*, 2002
At4g14150	*Kinesin-12A*	*kinesin-12a*	Microspores fail to complete PMI with phragmoplast defects	Kinesin-12 family	Phragmoplast microtubule organization	Lee *et al.*, 2007
At3g23670	*Kinesin-12B*	*kinesin-12a*	Microspores fail to complete PMI with spindle defects	Kinesin-12 family	Phragmoplast microtubule organization	Lee *et al.*, 2007
At5g58230	*MSI1*	*multicopy supressor of IRA1*	Microspore and male germ cell cycle arrest at G_2/M	Chromatin Assembly Factor-1 (CAF-1) p48 subunit/pRbAp48 homologue	Nucleosome/ chromatin assembly during replication	Chen *et al.*, 2007
Unknown	*SCP*	*sidecar pollen1*	Extra-celled pollen: abnormal divisions at PMI	Unknown	Unknown	Chen and McCormick, 1996
At1g50230	*TIO*	*two-in-one*	Microspores fail to complete cytokinesis at PMI	Homologous to FUSED-kinases	Signalling role in cell plate/phragmoplast expansion	Oh *et al.*, 2005
At3g45150	*TCP16*	*tcp16*	Microspore nuclear DNA loss and abortion	bHLH protein, TCP PCF-subfamily	Regulator of microspore gene expression	Takeda *et al.*, 2006

mutant affecting microspore division and cellular pattern (Chen and McCormick, 1996). *scp* microspores undergo a symmetrical division, followed by asymmetric division of only one of the daughter cells to produce mature pollen with an additional vegetative cell. *gemini pollen1 (gem1)* affects both male and female transmission and displays a range of microspore division phenotypes including equal, unequal, and partial divisions (Fig. 1) (Park *et al.*, 1998). GEM1 is identical to MOR1 (Whittington *et al.*, 2001). MOR1/GEM1 belongs to the MAP215 family of microtubule-associated proteins and plays a vital role in microspore polarity and cytokinesis by stimulating growth of the interphase spindle and phragmoplast microtubule arrays (Twell *et al.*, 2002).

In the *two-in-one* (*tio*) mutant, microspores complete nuclear division but fail to complete cytokinesis resulting in binucleate pollen grains (Fig. 1). TIO is the plant homologue of the Ser/Thr protein kinase FUSED (Oh *et al.*, 2005), which is a key component of the hedgehog-signalling pathway in fruitflies and humans (Lum and Beachy, 2004). TIO localizes to the phragmoplast mid-line where it plays an essential role in centrifugal cell plate expansion. The recently reported PAKRP1/Kinesin-12A and PAKRP1L/Kinesin-12B are two functionally redundant microtubule motor kinesins that also localize to the middle region of the phragmoplast (Lee *et al.*, 2007). Dividing microspores of double *kinesin-12A/kinesin-12B* mutants show disrupted microtubule organization and fail to form an antiparallel microtubule array between reforming nuclei. Although nuclear division is not affected in *gem1, tio*, and *kinesin-12A/kinesin-12B* microspores, symmetrical cell divisions and cytokinetic defects disrupt patterning of the male gametophyte and lead to failure of germline formation (Fig. 1). These observations strengthen the hypothesis that correct differentiation of the germ cell lineage depends on persistent cell fate determinants being correctly segregated between the unique daughter cells at PMI.

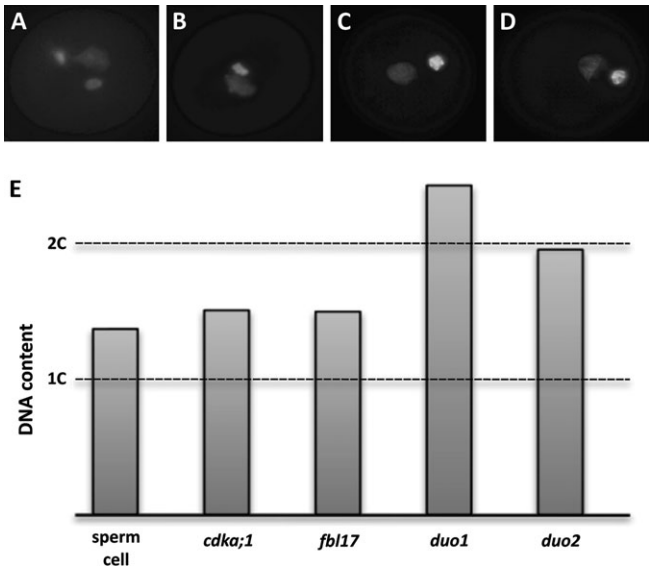

Fig. 3. Phenotype of mutants defective in germ cell division. DAPI-stained images of pollen grains depicting the wild-type tricellular phenotype in (A) compared with the bicellular phenotype of *fbl17* (B) (also representative of *cdka;1*), *duo1* (C) and *duo2* (D). In (E), the average DNA content of a wild-type sperm cell nucleus is compared with that of germ cell nuclei of *cdka;1*, *fbl17*, *duo1*, and *duo2* mutant pollen. *cdka;1* and *fbl17* germ cell nuclei show delayed S-phase and arrest before pollen mitosis II. This is reflected in the slightly increased DNA content of *cdka;1* and *fbl17* germ cell nuclei in comparison to sperm cell nuclei (Nowack *et al.*, 2006; Kim *et al.*, 2008). By contrast, *duo1* germ cell nuclei skip mitosis altogether and continue through another round of S-phase, increasing their DNA content to approximately twice that of sperm cell nuclei (Durbarry *et al.*, 2005). Similarly, *duo2* germ cell nuclei complete germ cell S-phase but enter pollen mitosis II and thus have an increased DNA content when compared to sperm cell nuclei (Durbarry *et al.*, 2005). However, the DNA content of *duo2* germ cell nuclei is less than that in *duo1* since they arrest at prometaphase and do not enter a further round of S-phase.

Genes required for germ cell division

Following asymmetric division at PMI, the vegetative cell exits the cell cycle in G_1 while the germ cell continues through a further round of mitosis at PMII. This differential control of cell cycle progression is paramount in ensuring that the germ cell produces the twin sperm cells required for double fertilization. A number of mutants have been described in *Arabidopsis* in which bicellular pollen (a single germ cell within the vegetative cell) is produced due to failure of germ cell division (Table 1).

Components of the canonical cell cycle machinery are expected to play essential roles in germ cell division, but only recently has functional evidence emerged. Analysis of T-DNA insertion mutants in the single A-type cyclin-dependent kinase (*CDKA;1*) in *Arabidopsis* revealed an essential role in germ cell division (Fig. 1) (Iwakawa *et al.*, 2006; Nowack *et al.*, 2006). In *cdka;1* mutants, germ cell division fails and DNA synthesis (S) phase of the cell cycle is delayed (Fig. 3). This single germ cell, however, is able to fertilize exclusively the egg cell. This preferential fertilization may arise from positional constraints, signalling within the embryo sac, or involve incomplete gamete differentiation (Nowack *et al.*, 2006). Moreover, the fact that the single germ cell is capable of fertilization demonstrates that key features of germ cell differentiation can be uncoupled from cell division.

A very similar phenotype to the *cdka;1* mutant is observed when the *F-box-Like 17* (*FBL17*) gene is disrupted (Fig. 3) (Kim *et al.*, 2008). F-box proteins associate with Skp1 and CUL1 to form SKP1-CUL1-F-box protein (SCF) E3 ubiquitin protein ligase complexes. These SCF complexes are involved in the ubiquitination of proteins targeted for proteasome-dependent degradation (Petroski and Deshaies, 2005; Smalle and Vierstra, 2004). Substrate specific F-box proteins play a critical role in controlling the cell cycle and diverse developmental processes through targeted degradation of various proteins (Cardozo and Pagano, 2004; Lechner *et al.*, 2006). FBL17 is transiently

expressed in the male germline after PMI and targets the CDK inhibitors KRP6 and KRP7 for proteasome-dependent degradation, enabling the germ cell to progress through S-phase (Fig. 1) (Kim *et al.*, 2008). Conversely, vegetative cell cycle progression is inhibited since FBL17 is not expressed in the vegetative cell and persistent levels of KRP6/7 continue to inhibit CDKA;1. The *fbl17* mutant pollen phenotype and its similarity to *cdka;1* mutant pollen is explained by stabilization of KRP6/7 in the germ cell in the absence of FBL17, resulting in continued inhibition of CDKA;1. Germline-specific expression of FBL17 thus enables differential control of the cell cycle in the germ and vegetative cells, and licenses the progression of germ cells through S-phase (Fig. 3).

Recent analysis of Chromatin Assembly Factor-1 (CAF-1) pathway mutants (*fas1, fas2, msi1*), indicates that chromatin integrity is also important for germ cell division (Chen *et al.*, 2008). CAF-1 pathway mutants display a range of phenotypes with some failing to divide at PMI, some failing to divide at PMII, and some successfully dividing to produce tricellular pollen. This indicates that the CAF-1 pathway has a wide role in male gametophyte cell division that could involve direct or epigenetic deregulation involving nucleosome and chromatin reassembly following replication (Chen *et al.*, 2008). CAF-1 deficient pollen are able to fertilize and the bicellular pollen correctly expresses germ cell-fate markers (Chen *et al.*, 2008). Interestingly, while *cdka;1* and *fbl17* mutants preferentially fertilize the egg cell, CAF-1 deficient pollen can fertilize either the egg or central cell. The reason for this difference is unclear, but it could relate to incomplete specification of the germ cell in *cdka;1* and *fbl17* mutants, or to some tricellular CAF-1 deficient pollen containing only one functional sperm cell that is able to fertilize either the egg or central cell.

A single germ cell phenotype is also present in *duo pollen* (*duo*) mutants. In these mutants, asymmetric microspore division at PMI is completed, however, the resulting germ cell fails to undergo cell division at PMII (Fig. 1) (Durbarry *et al.*, 2005). Heterozygous *duo1* and *duo2* mutants produce approximately 50% bicellular pollen containing a single germ cell showing complete penetrance of the mutations (Fig. 3). *duo2* mutant germ cells enter mitosis but arrest at prometaphase suggesting a specific role for DUO2 in mitotic progression (Durbarry *et al.*, 2005). By contrast, mutant germ cells in *duo1* complete S-phase but fail to enter mitosis (Fig. 3) (Durbarry *et al.*, 2005). DUO1 encodes a novel R2R3 MYB protein specifically expressed in germline cells (Rotman *et al.*, 2005). Unlike *fbl17*, *cdka;1* and CAF-1 pathway-deficient mutant pollen, *duo1* pollen cannot fertilize. This suggests that, in addition to cell cycle defects, key features of gamete differentiation and function are incomplete in *duo1*. DUO1 may therefore act as a germ cell fate determinant linking cell division and gamete specification. *DUO1* orthologues are present throughout the angiosperms (Rotman *et al.*, 2005) and recent identification of *DUO1* orthologues in basal angiosperms indicates the evolutionary conservation of this critical male germline specific regulator (M Borg and D Twell, unpublished results).

Genes associated with germline specification

As discussed earlier, there is compelling evidence for a unique germline transcriptome profile (Engel *et al.*, 2003; Okada *et al.*, 2006; Borges *et al.*, 2008) that is presumably important in the specification of functional sperm cells. Several male germline genes have been characterized in *Arabidopsis* and some have been shown to be useful cell fate markers (Table 2). *DUO1*, which appears to be important for specification (see above), is one such gene. DUO1 is

Table 2. Characterized male germline-expressed genes in *Arabidopsis* useful as molecular markers

Gene ID	Gene	Expression[a]	Protein annotation	Localization	Reference
At3g60460	*DUO1*	GC–SC	R2R3 MYB transcription factor (MYB125)	Nucleus	Durbarry *et al.*, 2005 Rotman *et al.*, 2005
At1g19890	*MGH3/HTR10*	GC–SC	H3.3 histone	Nucleus	Okada *et al.*, 2005 Ingouff *et al.*, 2007
At5g55490	*GEX1*	OV–RTS–GDC–SC	Three transmembrane domain protein	Plasma membrane	Engel *et al.*, 2005
At5g49150	*GEX2*	EC–GC–SC	Six transmembrane domain protein	Plasma membrane	Engel *et al.*, 2005
At5g16020	*GEX3*	EC–VC–SC–SIL	Single transmembrane domain protein with four extracellular β-propellor (PQQ) domains	Plasma membrane	Alandete-Saez *et al.*, 2008
At4g11720	*GCS1/HAP2*	GC –SC	Single transmembrane domain with a histidine rich C-terminal tail	Plasma membrane	Mori *et al.*, 2005 von Besser *et al.*, 2006
At4g05440	*AKV*	MS–VC and GC in BCP–SC only in MPG	D123-like protein	Unknown	Rotman *et al.*, 2005

[a] VC, vegetative cell; GC, germ cell; SC, sperm cell; OV, ovules; RTS, roots; GDC, guard cells; SIL, siliques; MS, microspore; BCP, bicellular pollen; MPG, mature pollen.

expressed exclusively in the male germline, with expression first detected in the germ cell soon after asymmetric division at PMI (Fig. 4) (Rotman *et al.*, 2005). The *Arabidopsis* male germline-specific histone H3 gene *MGH3* is another useful marker (Okada *et al.*, 2005; Ingouff *et al.*, 2007). *In situ* hybridization and monitoring of promoter activity with the β-glucuronidase (GUS) reporter demonstrates that MGH3 is specifically expressed in both the germ cell and sperm cells (Okada *et al.*, 2005). The expression of *DUO1* and *MGH3* early in the germ cell before PMII indicates that the regulatory network controlling sperm cell specification is initiated soon after asymmetric division (Fig. 4) (Okada *et al.*, 2005; Rotman *et al.*, 2005). The abundant and specific expression of *MGH3* in sperm cells also suggests that MGH3 may have an important role in chromatin structure in the germline. However, an *MGH3* insertion mutant did not show aberrant phenotypes and may arise from functional redundancy among histone H3 genes (Okada *et al.*, 2005). Recent studies of the behaviour of the MGH3-mRFP1 marker and the centromeric histone H3 marker HTR12-GFP in zygote and endosperm nuclei indicate active replication-independent replacement of paternal histone H3.3 in the zygote and replication-coupled removal in the endosperm. This study also revealed a spatial segregation of paternal chromatin (marked by HTR12-GFP) from maternal chromatin (known as gonomery) in the endosperm, but not in the zygote. Thus, the differential paternal chromatin remodelling involving histone H3 variants, which may also be coupled to parental imprinting of the endosperm, distinguishes the two products of fertilization (Ingouff *et al.*, 2007).

Sperm-surface proteins are likely to play important roles in the guidance, recognition, and/or fusion of gametes during double fertilization. *Generative Cell-Specific 1* (*GCS1*) was isolated from lily male germ cells by differential display (Mori *et al.*, 2006) and a homologue, *HAP2*, has been identified in *Arabidopsis* (von Besser *et al.*, 2006). *GCS1* encodes a gamete surface protein required for pollen tube guidance and fertilization (Mori *et al.*, 2006; von Besser *et al.*, 2006). Recently, *GCS1* homologues have been identified in the green alga *Chlamydomonas reinhardtii* and the rodent malaria parasite *Plasmodium berghei*, and their characterization revealed that they are required for fertilization (Hirai *et al.*, 2008; Liu *et al.*, 2008). In the diverse organisms *C. reinhardtii* and *P. berghei*, GCS1 is required for membrane fusion (Liu *et al.*, 2008) and it is likely that GCS1 has a similar role during fertilization in flowering plants.

Several other membrane-associated proteins expressed in male germ cells in *Arabidopsis* (*Gamete Expressed 1* to *3, GEX1-3*) were identified by comparative analysis of the maize sperm cell EST library with the *Arabidopsis* genome (Alandete-Saez *et al.*, 2008; Engel *et al.*, 2005). GEX1 and GEX2 are predicted to have three and six transmembrane domains, respectively, while GEX3 is predicted to have only one. Fluorescent protein fusions indicate that all three proteins are plasma membrane-associated. Within the male gametophyte, *GEX1* is expressed specifically in sperm cells,

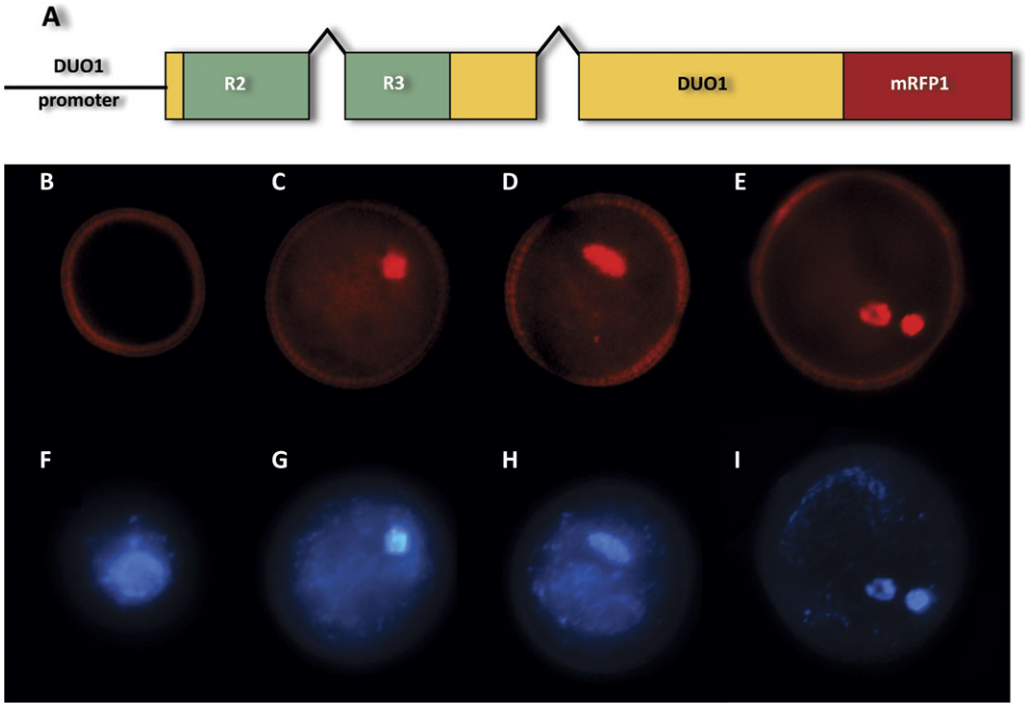

Fig. 4. Germline-specific expression of DUO1 during male gametogenesis. Subcellular localization of a DUO1 genomic fragment fused to mRFP1 and driven by the native DUO1 promoter (A) during male gametophyte development (Rotman *et al.*, 2005). mRFP1 fluroesence is absent from the microspore (B) and is first detectable in the germ cell nucleus at the early biceullar stage (C). mRFP1 fluorescent intensity increases at the late bicellular stage (D) and persists in sperm cell nuclei following pollen mitosis II (E). (F), (G), (H), and (I) show corresponding DAPI-stained nuclei of pollen.

GEX2 is expressed in both the germ and sperm cells, and *GEX3* is expressed in the vegetative cell and sperm cells. *GEX1* is also expressed in some sporophytic tissues (Engel *et al.*, 2005), while *GEX2* and *GEX3* also have a low level of expression in the egg cell of the female gametophyte (Alandete-Saez *et al.*, 2008). The function of GEX1 and GEX2 has not yet been elucidated, while analysis of transgenic GEX3 knockdown and overexpression lines revealed reduced seed set caused by a female defect (Alandete-Saez *et al.*, 2008). The discovery of these different gamete surface proteins with potential roles in signalling during pollen tube guidance and fertilization hint at complex communications between the different cells of the male and female gametophyte.

Although positive regulators such as DUO1 may be required for the activation of genes required for sperm cell specification, a repressor protein isolated from lily suggests that a transcriptional derepression mechanism is also likely to be involved. The male germline-specificity of the lily gene *LGC1* was associated with a 43 bp silencer in its regulatory region (Singh *et al.*, 2003). The protein Germline Restrictive Silencing Factor (GRSF) was shown to specifically bind to an 8–9 bp motif in the *LGC1* silencer region (Haerizadeh *et al.*, 2006). It has been proposed that ubiquitous expression of GRSF in somatic and non-germ cell lineages leads to the repression of target genes like *LGC1*, while its absence from the male germline in lily alleviates this repression specifically in germ cells (Haerizadeh *et al.*, 2006).

Putative GRSF binding sites found in the regulatory regions of three germline expressed genes, *DUO1*, *MGH3*, and *GEX2*, have been proposed to mediate similar control in *Arabidopsis* (Haerizadeh *et al.*, 2006), although *GEX2* has also been shown to be expressed in the female gametophyte (Alandete-Saez *et al.*, 2008). The *LGC1* silencer is active in *Arabidopsis* leaves, however, a role for a GRSF-like protein regulating the expression of genes by derepression in the germline is yet to be shown. In contrast to previous analyses of the *LGC1* promoter (Singh *et al.*, 2003; Haerizadeh *et al.*, 2006), our analysis of the *DUO1* promoter has revealed only positive regulatory elements in controlling germline expression and no apparent role for the putative GRSF binding site (M Borg and D Twell, unpublished results). This indicates that GRSF-independent regulatory pathways also operate to control germline-specific expression in *Arabidopsis*. An emerging model of male germline development incorporating this data from lily and *Arabidopsis* is presented in Fig. 5.

Perspectives

The availability of comprehensive transcriptomic data from different plant species, coupled with both forward and reverse genetic approaches, has had a significant impact on our understanding of male gametophyte development (Honys *et al.*, 2006; Twell *et al.*, 2006; Singh and Bhalla, 2007). The particular value of transcriptomic studies lies in the massively increased knowledge base of the complexity and dynamics of haploid gene expression in the developing gametophyte and germline cells (Honys and Twell, 2004). However, progress in studying male germline expression in a range of plant systems has been hindered by inadequate genomic annotation and the inherent limitations for testing the function of interesting genes. The recent completion of the *Arabidopsis* sperm cell transcriptome represents a further milestone in male gametophyte biology. Along with high quality genome annotation, the advantage of genetic and transgenic manipulation of *Arabidopsis* provides the ideal platform to elucidate germline gene functions. This will allow for more targeted application of functional genomic approaches that have been successful with previously available resources. Moreover, the synergy of comprehensive data from different plant species will also enable valuable comparative analyses and provide a phylogenetic perspective of male gametophyte development.

The developmental male gametophyte transcriptome has also enabled the discovery of network level responses, exemplified by the discovery of the late MIKC* MADS box network involved in pollen maturation (Verelst *et al.*, 2007*a*, *b*). Efforts merging this developmental data with the newly available sperm cell resource, along with a sustained effort in understanding the role of critical cell fate determinants like DUO1, will aid in establishing the significance and interaction of the key regulatory networks influencing male germline development and fertilization.

Despite the wealth of molecular evidence already available, transcriptomic data of the male gametophyte is still limited to those genes represented on the Affymetrix *Arabidopsis* ATH1 Genome Array (80% of *Arabidopsis* genes). The availability of the Affymetrix *Arabidopsis* tiling array, which comprises over 3.2 million probe pairs tiled through the complete non-repetitive *Arabidopsis* genome, offers the opportunity to gain a truly comprehensive view of haploid gene expression. Moreover, the introduction and refinement of novel high-throughput sequencing technologies, such as 454 sequencing (Margulies *et al.*, 2005), polony sequencing (Shendure *et al.*, 2005) and SOLiD technology (Applied Biosystems), also offers the opportunity to perform in-depth transcriptomic analysis, including the discovery of small RNAs (Mardis, 2008). Small RNA pathways are implicated in a variety of processes including development, genome stability, regulation of gene expression, and adaptive responses to stress (Bonnet *et al.*, 2006; Mallory and Vaucheret, 2006). It was previously suggested that such regulatory pathways are inactivated in the late male gametophyte (Pina *et al.*, 2005), but re-analysis of all available mature pollen datasets provided evidence to the contrary (Twell *et al.*, 2006). Moreover, the effective use of hairpin-based RNAi constructs (Gupta *et al.*, 2002; Takeda *et al.*, 2006) and the recent identification of small RNA pathway components in the sperm cell transcriptome further support the function of small RNAs in pollen.

The application of proteomic technologies in the field will further define the developmental synthesis and functional roles of proteins involved in germline development, pollen

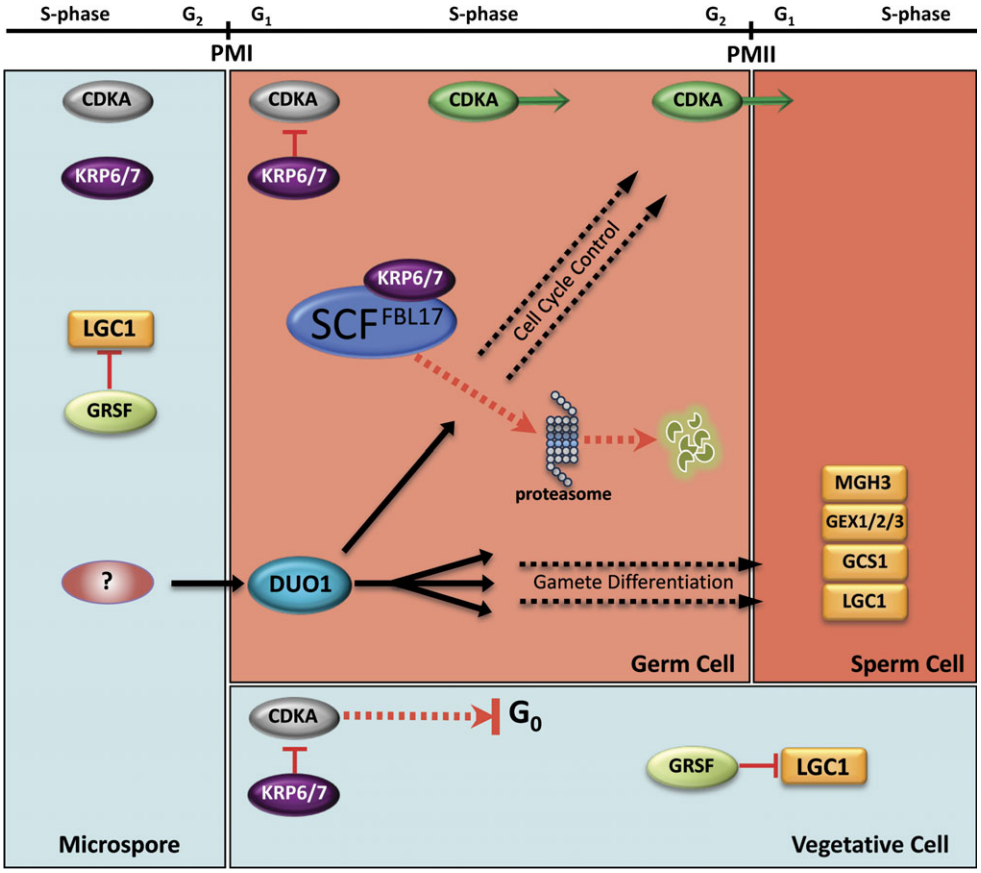

Fig. 5. An emerging model of male germline specification and maintenance. A schematic model of male germline development with data incorporated from lily and *Arabidopsis*. A highly asymmetric division at PMI results in two daughter cells with vastly different morphologies and cell fates. The cytokinesis machinery ensures that cell fate determinants are differentially segregated between the resulting daughter cells. After PMI, the cell cycle inhibitors KRP6 and KRP7 are present in both the germ and vegetative cells. Transient expression of FBL17 in the germ cells leads to the degradation of these KRPs, enabling CDKA activation and entry into S-phase. FBL17 is not expressed in the vegetative cell and so CDKA activity continues to be inhibited by high levels of KRP6 and KRP7, preventing entry into the cell cycle. Once S-phase has been completed, the DUO1-dependent activation of G_2/M phase regulators coupled to CDKA activation, promotes the germ cell to progress through the G_2/M checkpoint and enter mitosis. DUO2 appears to be required for the timely progression through prophase and completion of germ cell mitosis (Durbarry *et al.*, 2005). Germline specification begins shortly after PMI, when the germ cell possesses factors that promote the germline differentiation pathway. Such factors may include suppressors of GRSF expression, which, in turn, would alleviate the repression of germline-specific genes like *LGC1*. Positive factors are proposed to activate DUO1, which, in turn, activates genes required for sperm cell specification, therefore integrating differentiation with cell cycle progression. Ultimately, the co-ordinated association of these parallel pathways results in a pair of fully differentiated sperm cells equipped with a complement of essential germline factors such as GCS1.

tube growth and fertilization (recently reviewed by Becker and Feijo, 2007; Chen *et al.*, 2007). Currently, there have been five proteomic analyses of mature pollen, two in the *Arabidopsis* ecotype Columbia (Holmes-Davis *et al.*, 2005; Noir *et al.*, 2005), one in ecotype Landsberg *erecta* (Sheoran *et al.*, 2006), one in mature and germinated pollen in *Oryza sativa* (Dai *et al.*, 2006) and one in tomato (Sheoran *et al.*, 2007). Although relatively low numbers of proteins were identified in these studies, reflecting the limitations of proteomic technologies, high throughput methods currently being applied to *Arabidopsis* pollen promise significant advances (Becker and Feijo, 2007).

The future therefore promises to deliver some exciting and novel discoveries in male gametophyte biology. Much knowledge has already been gained through the genetic and transcriptomic approaches discussed in this review. The future challenge is to apply systematic approaches and to exploit new knowledge of specifically expressed genes to formulate new hypotheses. Concerted efforts are thus expected to be able to deliver a corroborated and system level perspective of the mechanisms controlling male gametogenesis and gamete functions in plant sexual reproduction.

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References

Alandete-Saez M, Ron M, McCormick S. 2008. GEX3, expressed in the male gametophyte and in the egg cell of *Arabidopsis thaliana*, is essential for micropylar pollen tube guidance and plays a role during early embryogenesis. *Molecular Plant* **1,** 586–598.

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R. 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301,** 653–657.

Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408,** 796–815.

Baarends WM, Roest HP, Grootegoed JA. 1999. The ubiquitin system in gametogenesis. *Molecular and Cellular Endocrinology* **151,** 5–16.

Becker JD, Boavida LC, Carneiro J, Haury M, Feijo JA. 2003. Transcriptional profiling of *Arabidopsis* tissues reveals the unique characteristics of the pollen transcriptome. *Plant Physiology* **133,** 713–725.

Becker JD, Feijo JA. 2007. How many genes are needed to make a pollen tube? Lessons from transcriptomics. *Annals of Botany* **100,** 1117–1123.

Bonnet E, Van de Peer Y, Rouzé P. 2006. The small RNA world of plants. *New Phytologist* **171,** 451–468.

Borges F, Gomes G, Gardner R, Moreno N, McCormick S, Feijo JA, Becker JD. 2008. Comparative transcriptomics of *Arabidopsis thaliana* sperm cells. *Plant Physiology* **148,** 1168–1181.

Cai G, Cresti M. 2006. The microtubular cytoskeleton in pollen tubes: structure and role in organelle trafficking. *Plant Cell Monographs* **3,** 157.

Cardozo T, Pagano M. 2004. The SCF ubiquitin ligase: insights into a molecular machine. *Nature Reviews Molecular Cell Biology* **5,** 739–751.

Chen SH, Liao JP, Kuang AX, Tian HQ. 2006. Isolation of two populations of sperm cells from the pollen tube of *Torenia fournieri*. *Plant Cell Reports* **25,** 1138–1142.

Chen T, Wu X, Chen Y, Böhm N, Lin J, Šamaj J. 2007. Pollen and pollen tube proteomics. In: Šamaj J, Thelen JJ, eds. *Plant proteomics*. New York, USA: Springer, 270–282.

Chen Y, McCormick S. 1996. Sidecar pollen, an *Arabidopsis thaliana* male gametophytic mutant with aberrant cell divisions during pollen development. *Development* **122,** 3243–3253.

Chen Z, Tan JLH, Ingouff M, Sundaresan V, Berger F. 2008. Chromatin assembly Factor 1 regulates the cell cycle but not cell fate during male gametogenesis in *Arabidopsis thaliana*. *Development* **135,** 65–73.

Dai S, Li L, Chen T, Chong K, Xue Y, Wang T. 2006. Proteomic analyses of *Oryza sativa* mature pollen reveal novel proteins associated with pollen germination and tube growth. *Proteomics* **6,** 2504–2529.

Dumas C, Berger F, Faure JE, Matthys-Rochon E. 1998. Gametes, fertilization and early embryogenesis in flowering plants. *Advances in Botanical Research* **28,** 232–261.

Dupl'akova N, Renak D, Hovanec P, Honysova B, Twell D, Honys D. 2007. *Arabidopsis* gene family profiler (aGFP) user-oriented transcriptomic database with easy-to-use graphic interface. *BMC Plant Biology* **7,** 39.

Durbarry A, Vizir I, Twell D. 2005. Male germ line development in *Arabidopsis*: duo pollen mutants reveal gametophytic regulators of generative cell cycle progression. *Plant Physiology* **137,** 297–307.

Eady C, Lindsey K, Twell D. 1995. The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. *The Plant Cell* **7,** 65–74.

Engel ML, Chaboud A, Dumas C, McCormick S. 2003. Sperm cells of *Zea mays* have a complex complement of mRNAs. *The Plant Journal* **34,** 697–707.

Engel ML, Holmes-Davis R, McCormick S. 2005. Green sperm. Identification of male gamete promoters in *Arabidopsis*. *Plant Physiology* **138,** 2124–2133.

Gupta R, Ting JTL, Sokolov LN, Johnson SA, Luan S. 2002. A tumor suppressor homolog, AtPTEN1, is essential for pollen development in *Arabidopsis*. *The Plant Cell* **14,** 2495–2507.

Haerizadeh F, Singh MB, Bhalla PL. 2006. Transcriptional repression distinguishes somatic from germ cell lineages in a plant. *Science* **313,** 496–499.

Hayashi K, de Sousa Lopes SMC, Surani MA. 2007. Germ cell specification in mice. *Science* **316,** 394–396.

Hirai M, Arai M, Mori T, Miyagishima S, Kawai S, Kita K, Kuroiwa T, Terenius O, Matsuoka H. 2008. Male fertility of malaria parasites is determined by GCS1, a plant-type reproduction factor. *Current Biology* **18,** 607–613.

Holmes-Davis R, Tanaka CK, Vensel WH, Hurkman WJ, McCormick S. 2005. Proteome mapping of mature pollen of *Arabidopsis thaliana*. *Proteomics* **5,** 4864–4884.

Honys D, Renák D, Twell D. 2006. Male gametophyte development and function. *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* **1,** 76–87.

Honys D, Twell D. 2003. Comparative analysis of the *Arabidopsis* pollen transcriptome. *Plant Physiology* **132,** 640–652.

Honys D, Twell D. 2004. Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*. *Genome Biology* **5,** R85.

Ingouff M, Hamamura Y, Gourgues M, Higashiyama T, Berger F. 2007. Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. *Current Biology* **17,** 1032–1037.

Iwakawa H, Shinmyo A, Sekine M. 2006. *Arabidopsis* CDKA;1, a cdc2 homologue, controls proliferation of generative cells in male gametogenesis. *The Plant Journal* **45,** 819–831.

Johnson-Brousseau SA, McCormick S. 2004. A compendium of methods useful for characterizing *Arabidopsis* pollen mutants and gametophytically-expressed genes. *The Plant Journal* **39,** 761.

Kim HJ, Oh SA, Brownfield L, Hong SH, Ryu H, Hwang I, Twell D, Nam HG. 2008. Control of plant germline proliferation by SCF^{FBL17} degradation of cell cycle inhibitors. *Nature* **455,** 1134–1137.

Lalanne E, Twell D. 2002. Genetic control of male germ unit organization in *Arabidopsis*. *Plant Physiology* **129,** 865–875.

Lechner E, Achard P, Vansiri A, Potuschak T, Genschik P. 2006. F-box proteins everywhere. *Current Opinion in Plant Biology* **9,** 631–638.

Lee JY, Lee DH. 2003. Use of serial analysis of gene expression technology to reveal changes in gene expression in *Arabidopsis* pollen undergoing cold stress. *Plant Physiology* **132,** 517–529.

Lee YRJ, Li Y, Liu B. 2007. Two *Arabidopsis* phragmoplast-associated kinesins play a critical role in cytokinesis during male gametogenesis. *The Plant Cell* **19,** 2595–2605.

Liu Y, Tewari R, Ning J, *et al*. 2008. The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in *Chlamydomonas* and *Plasmodium* gametes. *Genes and Development* **22,** 1051–1068.

Lum L, Beachy PA. 2004. The Hedgehog response network: sensors, switches, and routers. *Science* **304,** 1755–1759.

Ma H. 2005. Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. *Annual Review of Plant Biology* **56,** 393–434.

Ma L, Sun N, Liu X, Jiao Y, Zhao H, Deng XW. 2005. Organ-specific expression of *Arabidopsis* genome during development. *Plant Physiology* **138,** 80–91.

Mallory AC, Vaucheret H. 2006. Functions of microRNAs and related small RNAs in plants. *Nature Genetics* **38,** S31–S36.

Mardis ER. 2008. The impact of next-generation sequencing technology on genetics. *Trends in Genetics* **24,** 133–141.

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437,** 376–380.

Mori T, Kuroiwa H, Higashiyama T, Kuroiwa T. 2006. Generative cell specific 1 is essential for angiosperm fertilization. *Nature Cell Biology* **8,** 64–71.

Noir S, Bräutigam A, Colby T, Schmidt J, Panstruga R. 2005. A reference map of the *Arabidopsis thaliana* mature pollen proteome. *Biochemical and Biophysical Research Communications* **337,** 1257–1266.

Nowack MK, Grini PE, Jakoby MJ, Lafos M, Koncz C, Schnittger A. 2006. A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. *Nature Genetics* **38,** 63–67.

Oh SA, Johnson A, Smertenko A, Rahman D, Park SK, Hussey PJ, Twell D. 2005. A divergent cellular role for the FUSED kinase family in the plant-specific cytokinetic phragmoplast. *Current Biology* **15,** 2107–2111.

Okada T, Bhalla PL, Singh MB. 2006. Expressed sequence tag analysis of *Lilium longiflorum* generative cells. *Plant and Cell Physiology* **47,** 698–705.

Okada T, Endo M, Singh MB, Bhalla PL. 2005. Analysis of the histone H3 gene family in *Arabidopsis* and identification of the male-gamete-specific variant AtMGH3. *The Plant Journal* **44,** 557–568.

Okada T, Singh MB, Bhalla PL. 2007. Transcriptome profiling of *Lilium longiflorum* generative cells by cDNA microarray. *Plant Cell Reports* **26,** 1045–1052.

Owen HA, Makaroff CA. 1995. Ultrastructure of microsporogenesis and microgametogenesis in *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (Brassicaceae). *Protoplasma* **185,** 7–21.

Pacini E. 1996. Types and meaning of pollen carbohydrate reserves. *Sexual Plant Reproduction* **9,** 362–366.

Palevitz BA, Cresti M. 1989. Cytoskeletal changes during generative cell division and sperm formation in *Tradescantia virginiana*. *Protoplasma* **150,** 54–71.

Park SK, Howden R, Twell D. 1998. The *Arabidopsis thaliana* gametophytic mutation gemini pollen1 disrupts microspore polarity, division asymmetry and pollen cell fate. *Development* **125,** 3789–3799.

Petroski MD, Deshaies RJ. 2005. Function and regulation of cullin-RING ubiquitin ligases. *Nature Reviews Molecular Cell Biology* **6,** 9–20.

Pina C, Pinto F, Feijo JA, Becker JD. 2005. Gene family analysis of the *Arabidopsis* pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. *Plant Physiology* **138,** 744–756.

Rotman N, Durbarry A, Wardle A, Yang WC, Chaboud A, Faure JE, Berger F, Twell D. 2005. A novel class of MYB factors controls sperm-cell formation in plants. *Current Biology* **15,** 244–248.

Russell SD. 1991. Isolation and characterization of sperm cells in flowering plants. *Annual Reviews in Plant Physiology and Plant Molecular Biology* **42,** 189–204.

Russel SD, Strout GW. 2005. Microgametogenesis in *Plumbago zeylanica*. 2. Quantitative cell and organelle dynamics of the male reproductive cell lineage. *Sexual Plant Reproduction* **18,** 113–130.

Sakai N, Sawada MT, Sawada H. 2004. Non-traditional roles of ubiquitin–proteasome system in fertilization and gametogenesis. *International Journal of Biochemistry and Cell Biology* **36,** 776–784.

Schwacke R, Grallath S, Breitkreuz KE, Stransky E, Stransky H, Frommer WB, Rentsch D. 1999. LeProT1, a transporter for proline, glycine betaine, and {Gamma}-amino butyric acid in tomato pollen. *The Plant Cell* **11,** 377.

Scott RJ, Spielman M, Dickinson HG. 2004. Stamen structure and function. *The Plant Cell* **16,** S46.

Shendure J, Porreca GJ, Reppas NB, Lin X, McCutcheon JP, Rosenbaum AM, Wang MD, Zhang K, Mitra RD, Church GM. 2005. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science* **309,** 1728–1732.

Sheoran IS, Ross ARS, Olson DJH, Sawhney VK. 2007. Proteomic analysis of tomato, (*Lycopersicon esculentum*) pollen. *Journal of Experimental Botany* **58,** 3525–3535.

Sheoran IS, Sproule KA, Olson DJH, Ross ARS, Sawhney VK. 2006. Proteome profile and functional classification of proteins in *Arabidopsis thaliana* (Landsberg *erecta*) mature pollen. *Sexual Plant Reproduction* **19,** 185–196.

Singh M, Bhalla PL, Xu H, Singh MB. 2003. Isolation and characterization of a flowering plant male gametic cell-specific promoter. *FEBS Letters* **542,** 47–52.

Singh MB, Bhalla PL. 2007. Control of male germ-cell development in flowering plants. *BioEssays* **29,** 1124–1132.

Singh MB, Bhalla PL, Russell SD. 2008. Molecular repertoire of flowering plant male germ cells. *Sexual Plant Reproduction* **21,** 27–36.

Singh MB, Xu H, Bhalla PL, Zhang Z, Swoboda I, Russell SD. 2002. Developmental expression of polyubiquitin genes and distribution

of ubiquitinated proteins in generative and sperm cells. *Sexual Plant Reproduction* **14,** 325–329.

Smalle J, Vierstra RD. 2004. The ubiquitin 26S proteasome proteolytic pathway. *Annual Review of Plant Biology* **55,** 555–590.

Strome S, Lehmann R. 2007. Germ versus soma decisions: lessons from flies and worms. *Science* **316,** 392–393.

Takeda T, Amano K, Masa-aki O, Nakamura K, Sato S, Kato T, Tabata S, Ueguchi C. 2006. RNA interference of the *Arabidopsis* putative transcription factor *TCP16* gene results in abortion of early pollen development. *Plant Molecular Biology* **61,** 165–177.

Tanaka I. 1988. Isolation of generative cells and their protoplasts from pollen of *Lilium longiflorum*. *Protoplasma* **142,** 68–73.

Twell D, Oh S, Honys D. 2006. Pollen development, a genetic and transcriptomic view. *Plant Cell Monographs* **3,** 15.

Twell D, Park SK, Hawkins TJ, Schubert D, Schmidt R, Smertenko A, Hussey PJ. 2002. MOR1/GEM1 plays an essential role in the plant-specific cytokinetic phragmoplast. *Nature Cell Biology* **4,** 711.

Twell D, Park SK, Lalanne E. 1998. Asymmetric division and cell-fate determination in developing pollen. *Trends in Plant Science* **3,** 305–310.

Uchiumi T, Komatsu S, Koshiba T, Okamoto T. 2006. Isolation of gametes and central cells from *Oryza sativa* L. *Sexual Plant Reproduction* **19,** 37–45.

Verelst W, Saedler H, Münster T. 2007*b*. MIKC* MADS-protein complexes bind motifs enriched in the proximal region of late pollen-specific *Arabidopsis* promoters. *Plant Physiology* **143,** 447.

Verelst W, Twell D, de Folter S, Immink R, Saedler H, Munster T. 2007*a*. MADS-complexes regulate transcriptome dynamics during pollen maturation. *Genome Biology* **8,** R249.

von Besser K, Frank AC, Johnson MA, Preuss D. 2006. *Arabidopsis* HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. *Development* **133,** 4761–4769.

Whittington AT, Vugrek O, Wei KJ, Hasenbein NG, Sugimoto K, Rashbrooke MC, Wasteneys GO. 2001. MOR1 is essential for organizing cortical microtubules in plants. *Nature* **411,** 610–613.

Wilson ZA, Yang C. 2004. Plant gametogenesis: conservation and contrasts in development. *Reproduction* **128,** 483–492.

Xu H, Swoboda I, Bhalla PL, Singh MB. 1999*a*. Male gametic cell-specific expression of H2A and H3 histone genes. *Plant Molecular Biology* **39,** 607–614.

Xu H, Swoboda I, Bhalla PL, Singh MB. 1999*b*. Male gametic cell-specific gene expression in flowering plants. *Proceedings of the National Academy of Sciences, USA* **96,** 2554–2558.

Xu H, Weterings K, Vriezen W, Feron R, Xue Y, Derksen J, Mariani C. 2002. Isolation and characterization of male-germ-cell transcripts in *Nicotiana tabacum*. *Sexual Plant Reproduction* **14,** 339–346.

Yamamoto Y, Nishimura M, Hara-Nishimura I, Noguchi T. 2003. Behavior of vacuoles during microspore and pollen development in *Arabidopsis thaliana*. *Plant and Cell Physiology* **44,** 1192.

Zhang Z, Xu H, Singh MB, Russell SD. 1998. Isolation and collection of two populations of viable sperm cells from the pollen of *Plumbago zeylanica*. *Zygote* **6,** 295–298.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. 2004. Genevestigator. *Arabidopsis* microarray database and analysis toolbox. *Plant Physiology* **136,** 2621–2632.