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# Control of Male Gametophyte Development

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## INTRODUCTION

In a previous review of male gametophyte development (McCormick, 1993), it was noted that the two areas posed by Mascarenhas (1975) as fruitful areas for future research were the following. What are the differences in the two cytoplasms that determine the different cell fates of the generative and vegetative cells? And what are the functions of pollen-specific proteins? Now, 10 years later, the genome sequences of Arabidopsis and rice have been completed. There are extensive EST databases for many plants and several data sets from microarray hybridizations. There are extensive resources for disrupting the functions of genes. The pollen research community has made significant progress toward a deeper understanding of pollen development using community resources as well as novel techniques developed specifically for the analysis of pollen. This review will provide an overview of these advances and prospects for the future, focusing on male gametophyte development, strictly defined as postmeiosis development, after the formation of the haploid microspores. Meiosis will not be discussed in detail. Anther development and the role of the tapetum in pollen development are discussed by Dickinson and Scott in this issue.

The main features of pollen development are shown in Figure 1, which is based on an ultrastructural analysis of microsporogenesis in *Arabidopsis thaliana* (Owen and Makaroff, 1995). The male gametophyte, or pollen grain, is a three-celled organism that is derived by stereotypical cell divisions. Our story starts inside the anther, when sporogenous initial cells, also called pollen mother cells, undergo meiosis to form a tetrad of cells. Figure 2 shows Arabidopsis tetrads that have been extruded from one anther. Each tetrad is enclosed in a thick callose wall. The microspores in each tetrad are freed from their meiotic brothers by the action of callase, an enzyme produced by the tapetum. The tapetum is a nutritive cell layer that lines the locule containing the developing microsporocytes. The tapetum disintegrates in the later stages of pollen development. The microspores enlarge and then each undergoes an asymmetric mitosis. The mitosis is asymmetric because the dividing nucleus is adjacent to the wall, and the spindle orientation is such that after cytokinesis, one cell is much smaller than the other. The two cells of this bicellular pollen grain have strikingly different fates. The larger cell is called the vegetative cell, and the smaller cell is called the generative cell. The larger vegetative cell does not divide again but eventually will form the pollen tube. The generative cell is engulfed inside the cytoplasm of the vegetative cell. The generative cell undergoes mitosis, sometimes termed a second or pollen mitosis, to form the two sperm cells. The timing of this second pollen mitosis varies in different plant families, sometimes occurring within the anther (as in grasses and crucifers), although more commonly it occurs during pollen tube growth. In most plants, mature pollen grains are released from the anthers in a partially dehydrated state. Once on the stigma, the pollen grains hydrate and the vegetative cell extends a tube that grows by tip growth. As the tube extends, the vegetative cell nucleus and the two closely associated sperm cells move into the tube. Eventually, the entire vegetative cell exits the pollen grain and travels at the tip of the rapidly growing pollen tube. Pollen development is complete when the sperm cells are released into the embryo sac.

## MUTANT ANALYSES

A common way to dissect a developmental pathway is to isolate mutants that disrupt the pathway. This approach has been used extensively to dissect male meiosis and subsequent stages of pollen development. Comprehensive reviews of meiotic stages and cytology in Arabidopsis (Ross et al., 1996; Armstrong and Jones, 2003) as well as a time course for meiotic stages (Armstrong et al., 2003) now provide the necessary foundation for the interpretation of phenotypes in mutants that disrupt particular phases of meiotic development. Caryl et al. (2003) comprehensively reviewed the mutants known to affect meiosis in Arabidopsis. Some mutants that disrupt pollen development affect the function of the tapetum. Because the tapetum is sporophytic, such male-sterile mutants generally are recessive and all of the pollen grains within an anther are affected. Mutants are termed gametophytic if they disrupt genes that act after meiosis, in the haploid phase of pollen development, and thus only the pollen grains carrying the mutant allele are affected.

Male gametophytic mutants often exhibit segregation distortion (i.e., reduced transmission through the male) if the gene that is mutated is important for pollen development or function. Indeed, this feature was used successfully as a first-pass method in screens designed to identify gametophytic mutants (Feldmann et al., 1997; Bonhomme et al., 1998; Howden et al., 1998; Grini et al., 1999; Oh et al., 2003). The developmental stage affected in each mutant was determined later. In some cases, the phenotype was demonstrated to be attributable to a single gene disruption. For example, several of the male transmission–defective mutants described by Bonhomme et al. (1998) had

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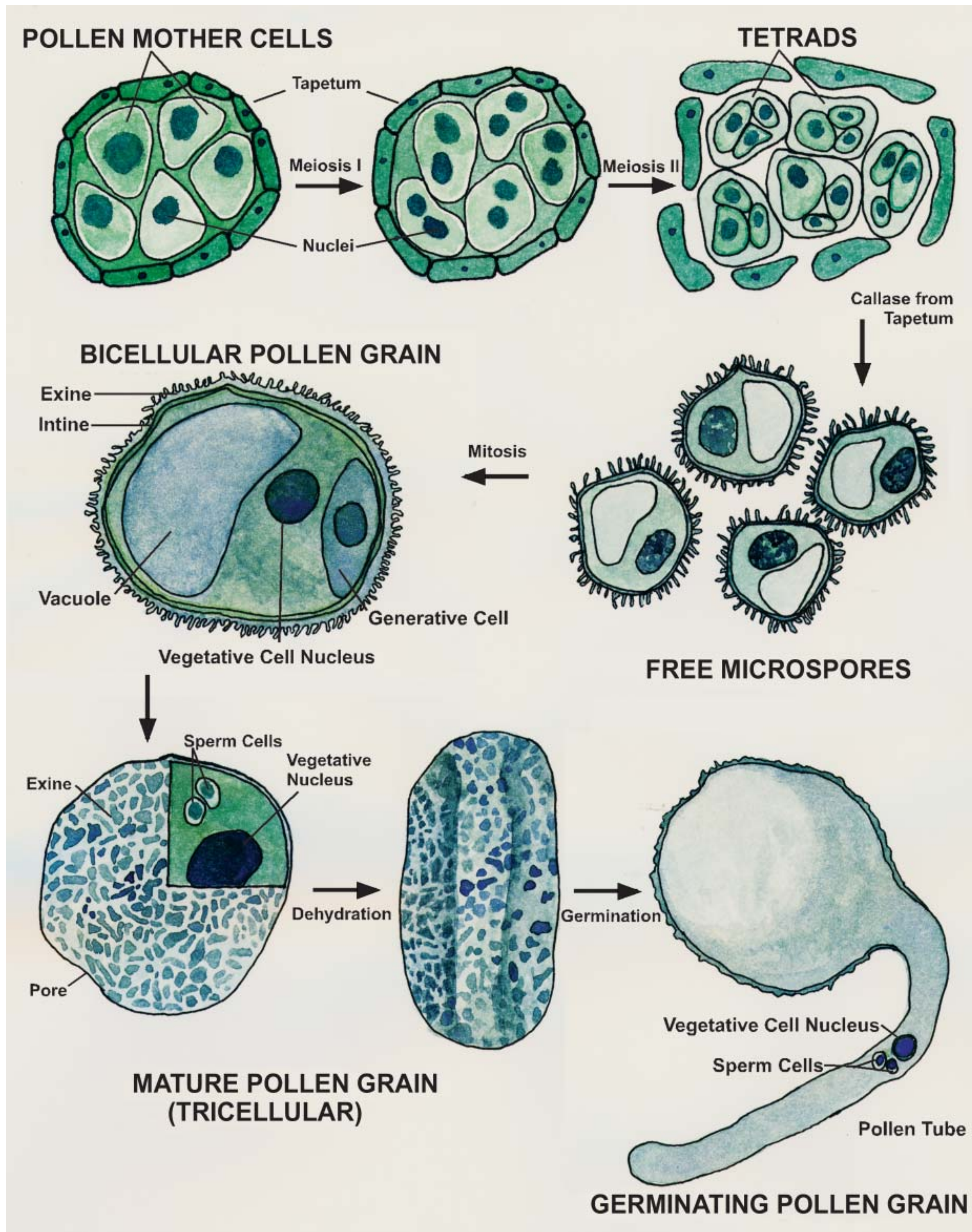


**Figure 1.** Scheme of Microsporogenesis.

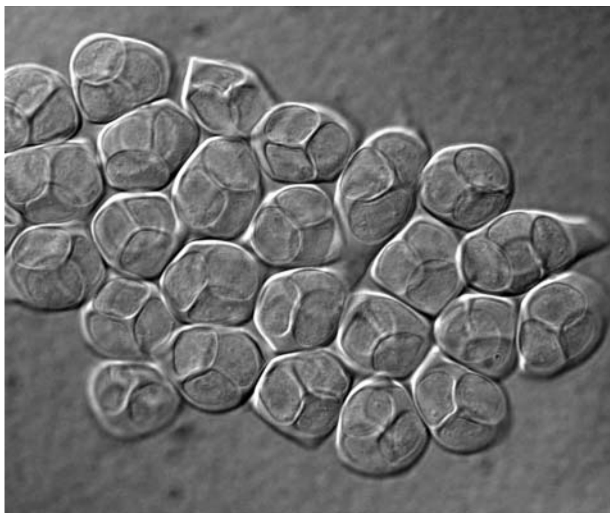

**Figure 2.** Tetrads of Arabidopsis.

Male meiosis is essentially synchronous within an anther.

similar phenotypes, and upon further mapping and cloning they were found to be alleles. The disrupted gene was renamed *kinky pollen* because pollen grains harboring a mutant allele exhibited aberrant tube growth (Procissi et al., 2003). However, frequently, mutations identified from T-DNA insertion screens were unlinked to the T-DNA insertion or exhibited complex molecular lesions. In such cases, it was difficult to identify the molecular basis for the transmission defect. For example, the T-DNA insertion mutant named *halfman* (Oh et al., 2003) was demonstrated to have a gametophytic defect with low penetrance. Molecular analysis showed that the T-DNA insertion in *halfman* had induced an adjacent deletion of 150 kb that encompassed 38 genes; the loss of 1 or more of these genes might have been responsible for the observed phenotype, in which ∼30% of the pollen aborted at approximately the bicellular stage of development.

Assiduous screening for mutants based on morphology or staining characteristics of pollen from individual M1 or M2 plants also has yielded very interesting phenotypes (Chen and McCormick, 1996; Johnson and McCormick, 2001; Lalanne and Twell, 2002). As illustrated in Figure 1, pollen development undergoes stereotypical cell divisions to yield the three-celled male gametophyte. To test whether the asymmetric division that resulted in the formation of the vegetative cell and the generative cell could be disrupted mutationally, or if the number of cell divisions typical of pollen development could be disrupted mutationally, pollen of mutagenized populations was examined after 4′,6-diamidino-2-phenylindole staining. Such screens yielded, for example, the mutants *sidecar pollen* (Chen and McCormick, 1996) and *gemini pollen* (Park et al., 1998). At the mature pollen stage in *sidecar pollen* heterozygotes, ∼50% of the pollen was normal, ∼43% was aborted, and ∼7% showed the sidecar phenotype, namely an extra cell within the pollen exine. Analyses of earlier stages of pollen development revealed that microspores carrying the *sidecar pollen* mutant allele frequently underwent a premature and symmetric cell division. For unknown reasons, one of the resulting two cells then was able to undergo the normal mitotic divisions to form the generative cell and the two sperm cells. Despite the apparent equal sizes of the two cells, this finding suggests that an inherent asymmetry must persist. The molecular lesion in *sidecar pollen* is still unknown.

In contrast to *sidecar pollen*, in *gemini pollen*, arrest occurs after the first division, so that at the mature pollen stage the affected pollen grains have two cells, each with decondensed chromatin and each able to express a vegetative cell–specific marker (Park et al., 1998). *Gemini pollen* encodes a microtubule-associated protein (MAP215 family), and the *gemini pollen* phenotype is now believed to be attributable to a defect in the correct positioning of the cytokinetic phragmoplast at pollen mitosis I (Twell et al., 2002). It was hypothesized that the aberrant partitioning of cytoplasmic factors led to the alterations in cell fate. In another mutant, *duo*, the first mitotic division occurs asymmetrically, yielding one larger cell with decondensed chromatin and a vegetative cell fate and a smaller generative cell with condensed chromatin. However, the second mitotic division of the generative cell, to form two sperm cells, fails. The molecular lesion in *duo* is not yet known. A gametophytic male-sterile mutant (gaMS-2) that affects this stage of development was described in maize (Sari-Gorla et al., 1997); in microspores carrying the mutant allele, the vegetative nucleus and the generative nucleus exhibit altered identities and sometimes undergo extra divisions.

Mutants that affect these division patterns also were isolated from the T-DNA screens. For example, in *limpet pollen* (Howden et al., 1998), now named *ingressus* (http://www.le.ac.uk/biology/research/pollen/ing.html), the generative cell divides to form two sperm cells, but the sperm cells cannot migrate inward and instead remain near the pollen tube wall. More recently, Lalanne and Twell (2002) found that the association between the sperm cells and the vegetative nucleus could be disrupted mutationally. In *gum* (*germ unit malformed*), the vegetative nucleus stays near the pollen wall, although the sperm cell pair associates and migrates inward, as in the wild type. In *mud* (*male germ unit displaced*), the sperm cells associate with the vegetative nucleus, as in the wild type, but this group then remains near the pollen wall. The phenotypes in double mutants allowed these authors to infer that GUM acts earlier than MUD.

It is usually the case that gametophytic mutants that affect pollen development or function must be maintained as heterozygotes. If the female gametophyte also is affected (Howden et al., 1998), the chance of obtaining homozygous lines is slight. However, it was possible (Chen and McCormick, 1996) to obtain a homozygous *sidecar pollen* line, albeit at a much lower than expected frequency, indicating that pollen grains carrying the *sidecar pollen* mutant allele could transmit the gene to the next generation. The *gum* and *mud* mutants (Lalanne and Twell, 2002) show only minor transmission defects through the male, and homozygous lines of these mutants were obtained readily. Consequently, visual screening facilitated the discovery of these mutants, because they would not have been detected readily in a segregation distortion screen. If homozygous lines can be generated, it will be possible to test for phenotypes in the sporophyte. The *gum* and *mud* homozygotes show no obvious vegetative phenotypes.

Identifying additional alleles is a standard approach that facilitates the positional cloning of genes. For a pollen mutant whose phenotype is visible only after microscopic examination, this task is not easy. Additional visual screens of mutagenized populations will undoubtedly yield additional phenotypes of some sort, but there is no guarantee that the desired phenotype will be seen in the next 5000 plants screened. However, screening for new alleles can be simplified for certain mutants with striking phenotypes. For example, in anthers of *raring-to-go* (*rtg*) (Johnson and McCormick, 2001), some of the pollen stains with decolorized aniline blue, a stain specific for callose, a component of the pollen tube wall. Because pollen does not normally hydrate and begin germination until it contacts the stigma, finding the precocious pollen germination phenotype in *rtg* was serendipitous. To obtain additional *rtg* alleles, mutagenized populations were screened by collecting pollen from 100 plants at a time and screening the pollen in bulk after staining with decolorized aniline blue. This directed screen yielded several probable alleles of *rtg* as well as other mutants (*gift-wrapped pollen*, *polka dot pollen*, and *emotionally fragile pollen*) whose pollen grains exhibited unusual staining patterns; the existence of these staining patterns could not have been predicted (Johnson and McCormick, 2001).

There are undoubtedly more mutants to find from such visual screens. Libraries that generate cDNA–green fluorescent protein (GFP) fusions can provide hints about the normal locations of proteins of unknown function (Cutler et al., 2000). A high-throughput screen for GFP localization in leaves that had been infected transiently with a cDNA-GFP library was remarkably successful at identifying proteins that are associated with plasmodesmata (Escobar et al., 2003). Unfortunately, these existing resources do not provide tools that can be used to study subcellular locations for pollen proteins; Cutler et al. (2000) used the 35S promoter of *Cauliflower mosaic virus* (CaMV), which is expressed poorly during later stages of male gametophyte development, whereas Escobar et al. (2003) used viral infection to deliver the constructs to leaves. Nonetheless, it should be straightforward to generate new transformed lines using a pollen-expressed promoter driving cDNA-GFP libraries. In also might prove fruitful to generate transgenic lines harboring GFP fusions to selected pollen proteins and then to mutagenize these lines and screen for mislocalization of the GFP fusion protein. Such a screen was performed for mutants that mislocalized a GFP fusion protein of the double-stranded RNA binding protein Staufen during *Drosophila* embryo development (Martin et al., 2003).

## *QUARTET* AND *TETRASPORE*, USEFUL TOOLS FOR MUTANT ANALYSIS

Two mutants in Arabidopsis, *quartet1* and *tetraspore*, have greatly facilitated the analysis of male gametophytic mutations. *quartet1* is a sporophytic recessive mutation. In *quartet1/quartet1* plants, all of the products of a single meiosis are held together in a tetrad throughout pollen development (Preuss et al., 1994), but each pollen grain is otherwise normal and can germinate. Tetrad analysis is used to test whether pollen phenotypes result from a gametophytic mutation or from a dominant sporophytic mutation. This test is important because many mutants that affect pollen phenotypes exhibit variable expressivity and penetrance: an observation that ~50% of the pollen has an altered phenotype is not, in and of itself, proof of gametophytic action. To perform tetrad analysis, *mutant*/+ plants are crossed as females to homozygous *quartet1* plants. Those F1 progeny whose pollen exhibit the mutant phenotype are self-pollinated, and the F2 progeny are scored to identify the *mutant*/+ *quartet1*/*quartet1* double mutants. If a mutation is expressed sporophytically but has low expressivity, it might be expected that the numbers of normal and affected pollen resulting from meiosis would vary in each tetrad. However, if a mutation is gametophytic, the ratio of normal to affected pollen resulting from meiosis should be 2:2. Figures 3A to 3D show an example of such a test: after staining with aniline blue, two of the pollen grains in the tetrad are wild type and the other two show tubules within the grain that are diagnostic of the *gift-wrapped pollen1* phenotype (Johnson and McCormick, 2001).

Because pollen is haploid, it is not straightforward to determine if a mutation is dominant or recessive. To combine a mutant allele with a wild-type allele in one cell for a dominance/recessiveness test, the mutant can be crossed, as female, with diploid pollen from a tetraploid plant. However, the resulting F1 progeny are triploid. Pollen from triploid plants typically have a high degree of pollen abortion that might interfere with scoring for the pollen phenotype of interest. Another alternative is to introgress the mutant allele into a tetraploid background and then examine the phenotype in the diploid pollen produced by the tetraploid plant. This method has been used infrequently (Kamps et al., 1996) in maize. A third option, in Arabidopsis, is to cross the pollen mutant of interest with *tetraspore*. Because plants that are homozygous for a *tetraspore* mutant allele fail to undergo cytokinesis after meiosis, large multinucleate pollen grains are formed (Hulskamp et al., 1997; Spielman et al., 1997; Yang et al., 2003). This feature provides a way to determine the dominance relationships of male gametophytic genes. In a *tetraspore* (*tes*/*tes*) homozygote that also is heterozygous for the gametophytic gene being tested, pollen grains carrying both a mutant allele and a wild-type allele will exist.

Figure 3E shows anthers of *polka dot pollen* heterozygous plants after staining with decolorized aniline blue, a stain specific for callose (Johnson and McCormick, 2001); ~50% of the pollen grains show blobs of callose. A *polka dot pollen* heterozygote (*pdp*/+) was crossed into a *tes*/*tes* line. The F1 plants had normal-sized pollen, because *tes* is a sporophytic recessive mutant. Pollen from each F1 plant were examined, and plants with the *pdp* phenotype were self-pollinated. Then, *tes*/*tes* plants were identified among the F2 progeny. If the mutant phenotype is not observed in the large pollen grains, then the gametophytic mutation is an effective recessive, because the wild-type allele can compensate. However, some of the large pollen grains in the *tes*/*tes* +/*pdp* plants showed blobs of callose (Figures 3F and 3G). Because the mutant phenotype for *pdp* is observed in the large pollen grains, we infer that the lesion in *pdp* is unlikely to be a loss-of-function mutation. Instead, the mutant allele might encode a nonfunctional protein that acts as a dominant negative, or it might be a gain-of-function mutation. That a lesion in a candidate gene is responsible for the observed mutant

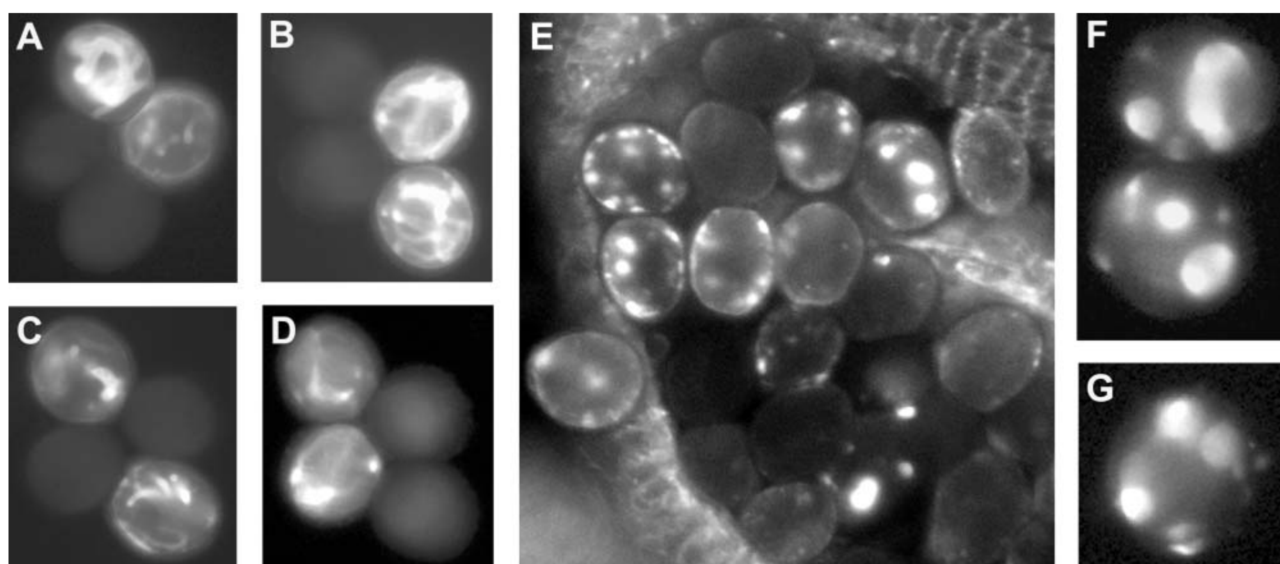


**Figure 3.** Crosses with *quartet1* or *tetraspore*: Useful Tools for the Analysis of Gametophytic Mutants.

**(A)** to **(D)** Tetrads from a *giftwrapped pollen1*/+ *quartet1*/*quartet1* plant.
**(E)** Pollen grains within an anther of a *polka dot pollen*/+ plant.
**(F)** and **(G)** Large pollen grains from a *pdp*/+ *tes1*/*tes1* plant.

phenotype usually is confirmed by transformation. For gametophytic mutants, crosses with *tes* are useful because for transformation it is important to know whether to introduce a wild-type copy of the gene into the mutant background or to introduce the mutant version of the gene into the wild-type background.

## DETERMINING PROTEIN FUNCTION

In addition to mutant screens, there are several alternative ways to determine the roles of genes that are expressed in the male gametophyte. These include the analysis of T-DNA insertion lines (Alonso et al., 2003) in particular genes and analysis of RNA interference or antisense lines for particular transcripts (for example, Muschietti et al., 1994; Gupta et al., 2002). For example, because calcium levels and calmodulin were known to be important for pollen function, it was reasonable that Golovkin and Reddy (2003) chose to study the Arabidopsis homologs of a pollen-specific calmodulin binding protein (no pollen germination1 [NPG1]) from maize. There are three closely related members in the Arabidopsis genome; *NPG1* was expressed only in pollen, whereas the two related genes were expressed in pollen as well as in other tissues. Homozygotes for the T-DNA insertion in *NPG1* could not be obtained. Reciprocal crosses indicated that there was a problem with transmission through the male. Further examination revealed that although NPG1 was not required during pollen development, it was essential for pollen germination. Another example involves GTPases. An important role for Rop (Rho-like GTPases of plants) proteins in pollen tube growth was discovered by analyzing lines in which mutant versions of Rop were expressed in pollen (reviewed by Yang, 2002). A surprise came from the analysis of a maize mutant that is disrupted in Rop2, one of two very closely related Rops that are expressed in maize pollen (Arthur et al., 2003). The *rop2* mutant allele is poorly transmitted through the male in heterozygotes, implying that the mutant pollen is at a competitive disadvantage. Surprisingly, pollen tube growth in the mutant appeared normal. Further analysis might reveal a previously unsuspected role for Rops, perhaps during targeting of the pollen tube to the ovule.

Such approaches have serendipitously allowed the identification of gene products that must play a crucial, but previously unpredicted, role in pollen development. For example, Kang et al. (2003) found that they could not identify homozygous T-DNA insertions in a dynamin-like protein, ADL1C, and subsequently noticed that in heterozygotes, 50% of the pollen grains were collapsed and failed to germinate. Further examination revealed that this defect was first evident during microspore maturation and that the affected microspores appeared to have plasma membrane defects. Further work will be required to determine precisely why the absence of ADL1C leads to pollen abortion. Another recent example involves apyrases (Steinebrunner et al., 2003). Apyrases are known to hydrolyze nucleoside phosphates; in animals, they are important in several signaling pathways, but their roles in plants are not well understood. In an effort to study the roles of apyrases in plants, gene disruptions were generated. There are two apyrase genes in Arabidopsis, and disruption of either gene alone had no discernible phenotype. However, the double knockout could not be obtained, because pollen grains carrying both disrupted alleles could not germinate. Again, further work will be required to determine what processes during pollen germination are affected by the absence of apyrases.

In many multigene families, only a few isoforms are expressed in pollen, and mutant phenotypes can sometimes be obtained

even when the expression of one isoform might in principle compensate for the lack of another (Arthur et al., 2003; Golovkin and Reddy, 2003). Sometimes, however, redundancy does thwart efforts to obtain a phenotype and thus infer a function for a protein. For example, there are several monosaccharide transporters that are expressed late during pollen maturation; a homozygous T-DNA gene disruption in one of them, AtSTP6, showed no apparent defects in pollen germination or transmission through the male (Scholz-Starke et al., 2003). Does this mean that monosaccharide transporters (or any other genes whose mutants lack a discernible phenotype) are not important? Not necessarily. Edelman and Gally (2001) suggest that gene degeneracy should be considered in addition to redundancy, that important developmental processes may be "covered" by several proteins, any of which can accomplish the task in the absence of another.

## GENE REGULATION

Two groups (Becker et al., 2003; Honys and Twell, 2003) have recently used microarrays to analyze gene expression patterns in mature pollen of Arabidopsis. The results are not yet global (only ~8000 of the predicted ~27,000 genes of Arabidopsis were analyzed), but they can be extrapolated to the whole genome. The two studies differ quantitatively and qualitatively, probably because of differences in sample preparation and methods of data analysis. Nonetheless, it is noteworthy that the estimates for the numbers of genes expressed in pollen and for the numbers of genes that might be pollen specific are similar to those estimated from cDNA–poly(A) RNA hybridization kinetics (Mascarenhas, 1975, 1990, 1993). Microarray experiments are extremely useful in identifying targets for further analyses, especially for genes currently annotated as hypothetical or of unknown function. But such experiments are only a start and must be confirmed by other independent tests. Many transcripts have low abundance and their expression will not be assayable by global expression methods such as microarray analysis. Indeed, for several genes called "Absent" in the microarray experiments (Becker et al., 2003; Honys and Twell, 2003), their probable homologs are indeed present in tomato pollen cDNA libraries (Tang et al., 2002; Van der Hoeven et al., 2002) or in a maize sperm cell cDNA library (Engel et al., 2003). A different approach, serial analysis of gene expression (SAGE), was used by Lee and Lee (2003) to obtain gene expression data from pollen from Arabidopsis plants grown under normal or chilling conditions in an effort to understand why plants raised under cold conditions exhibit impaired male fertility. Although most transcript levels were not affected by cold treatment, the SAGE analysis provided a possible explanation for why pollen of some species is not cold tolerant: transcripts involved in cold tolerance in sporophytic cells were poorly represented in pollen mRNA. In plants for which there are limited sequence data, techniques such as cDNA–amplified fragment length polymorphism transcript profiling have proved useful for identifying genes for further study. For example, Cnudde et al. (2003) used this technique to characterize gene expression patterns in *Petunia* anthers at different developmental stages.

What are the roles of the mRNAs that are present in pollen? Based on results from early experiments with transcriptional and translational inhibitors, it was widely accepted that pollen would contain mRNAs that are synthesized during pollen development and stored for translation during pollen germination (for review, see Mascarenhas, 1993). However, many proteins corresponding to such mRNAs already are present in mature, ungerminated pollen, as was discovered once antibodies were generated (Muschietti et al., 1994, 1998; Kim et al., 2002). The results from the microarray experiments (Becker et al., 2003; Honys and Twell, 2003) indicate that mRNAs that encode signal transduction and cell wall biosynthesis proteins were highly represented, whereas transcription and translation proteins were underrepresented. SAGE analysis (Lee and Lee, 2003) confirmed that mRNAs that encode cell wall biogenesis were highly expressed in pollen. The potential roles for one category of genes expressed prevalently in pollen were tested collectively by Lalanne et al. (2004) by examining the pollen phenotypes in T-DNA insertion lines that disrupted the *SETH1* and *SETH2* genes. *SETH1* and *SETH2* encode proteins required for the addition of glycosylphosphatidylinositol (GPI) anchors to proteins. Lalanne et al. (2004) found that preventing the addition of GPI anchors to proteins resulted in defects in pollen tube growth; further studies now can be focused on determining precisely which GPI-anchored proteins are important and why. Similar experiments (Alfieri et al., 2003) were used to show that GPI-anchored proteins were important for sperm–egg adhesion in mammals.

A few groups have tried to identify mRNAs that are specific to the germination phase of pollen development. In *Petunia*, flavonoids are required for germination. Guyon et al. (2000) took advantage of this requirement to precisely control the initiation of germination in flavonoid-deficient pollen. Thus, they performed a differential screen for cDNAs that were transcribed or upregulated soon after the addition of flavonoids to the germination medium. They identified ~20 such cDNAs; almost all were pollen specific, and many corresponded to low-abundance mRNAs. Despite such intricate cDNA selection schemes (Guyon et al., 2000; see also Mu et al., 1994), it has been difficult to identify genes that are transcribed only upon pollen germination or to identify mRNAs that are essentially not translated until after pollen germination (Wittink et al., 2000).

Given that most mRNAs are presynthesized before pollen maturation, it is curious that only a few studies have examined the potential for post-transcriptional control of gene expression during pollen development. Ylstra and McCormick (1999) tested the long-held assumption that pollen mRNAs were long-lived. For 10 messages, they confirmed that the mRNA half-life was very long. However, two mRNAs were relatively short-lived in pollen, and an mRNA that was very unstable in somatic cells was relatively stable in pollen. Bate et al. (1996) used transient expression assays and stable transgenic plants to demonstrate that the 5′ untranslated region (UTR) of the late anther tomato52 (LAT52) gene acted as a translational enhancer in pollen. No significant increase in translational efficiency was seen when constructs with the LAT52 5′ UTR were introduced into sporophytic cells. In pollen, the translational enhancement was maximal during the last stages of pollen development. Although LAT52 transcripts can be detected at approximately the time of first microspore mitosis (Eady et al., 1994), there might be

mechanisms in place to prevent significant accumulation of late gene products such as LAT52 until near pollen maturity. Bate et al. (1996) suggested that pollen-specific translation initiation factors, such as eiF-4a (Brander and Kuhlemeier, 1995), might play a role in this regulation. Curie and McCormick (1997) studied the 5′ UTR of the LAT59 gene, which, when removed from constructs, enhanced transgene expression in transient assays. In this case, the effect was not pollen specific: including the LAT59 5′ UTR in gene constructs reduced gene expression of the reporter gene in both pollen and in somatic cells.

The most detailed analyses of pollen-specific promoters were performed with the LAT52 (Eyal et al., 1995; Bate and Twell, 1998) and the LAT59 (Eyal et al., 1995) genes. Gain-of-function and loss-of-function gene constructs were tested by transient assays in pollen and in somatic cells as well as in stably transformed plants. In this way, 30-bp elements that could confer pollen specificity to a heterologous promoter (CaMV35S) were defined. Competition experiments using constructs with concatamers of these elements suggested that the promoter elements might compete for binding to a shared *trans*-acting factor. We still do not know what transcription factors bind to these identified promoter elements, so our understanding of the determinants for pollen-specific gene expression is incomplete. It may be informative to use microarrays to identify all of the genes with similar expression profiles in pollen and then use bioinformatics tools (Rombauts et al., 2003) to examine their promoter regions for common elements. However, the critical sequence elements identified in the tomato LAT59 promoter (Eyal et al., 1995) are not well conserved in the promoter of Nt59, its tobacco homolog (Kulikauskas and McCormick, 1997). This result suggests that sequence identities between promoter regions might not be sufficient to explain the regulation of pollen-expressed genes.

The lack of defined transcription factors for well-characterized pollen-specific promoters does not mean that pollen-specific transcription factors are not known. For example, Zachgo et al. (1997) characterized a pollen-specific MADS box transcription factor in *Antirrhinum*, as did Heuer et al. (2000) in maize; because expression of the maize MADS box gene continued during pollen tube growth, it was surmised that this factor might be needed during the later phases of pollen tube growth. Lohrmann et al. (2001) demonstrated the pollen-specific expression of a transcription factor that regulates nuclear genes required for mitochondrial function. Kobayashi et al. (1998) reported that seven different zinc-finger transcription factors in *Petunia* were expressed transiently and sequentially at different stages of pollen development. They suggested that such transcription factors might constitute a regulatory cascade and that each might have specific target genes, including the next-acting transcription factor in the cascade.

The extensively characterized LAT52 promoter (Twell et al., 1991; Eyal et al., 1995) is the workhouse for gene expression analyses in pollen (Muschietti et al., 1994; Lee et al., 1996; Cheung, 2001). However, there is a need for other promoters of differing strengths, and certain experimental designs require multiple promoters. For example, coexpression of a reporter gene probably should not use the same promoter as the gene whose function is being assayed, in case the transcription factors required for promoter activation are limiting. Gene expression driven by the LAT52 promoter can be detected in uninuclear microspores in Arabidopsis (Eady et al., 1994), and this promoter drives strong gene expression at all developmental stages occurring after the bicellular pollen stage, even after delivery into mature pollen via particle bombardment (Twell et al., 1991; Eyal et al., 1995). If a narrower window of gene expression of a transgene is desired, the LAT52 promoter may not be the best choice. For example, Lee et al. (1996) were unable to assess whether the pollen receptor kinase PRK1 played a role during pollen germination, because antisense expression driven by the LAT52 promoter resulted in pollen abortion at the unicellular microspore stage.

Several groups have made progress in filling in the gaps for gene expression at other critical stages during pollen development. For example, Klimyuk and Jones (1997) characterized a meiosis-specific promoter, AtDMC1, and showed that it directs reporter gene expression exclusively in meiocytes. Custers et al. (1997) characterized the promoter of the tobacco NTM19 gene and showed that it will direct gene expression only in microspores. They also characterized the Bp4 promoter and showed that it was active a bit later, when bicellular pollen is being formed. The transcripts for the tomato receptor kinases LePRK1, LePRK2, and LePRK3 are detected only in mature pollen (Muschietti et al., 1998; Kim et al., 2002). Although not yet tested, it is likely that these promoters, or the promoters of their Arabidopsis homologs (Kim et al., 2002), might provide a way to express transgenes only at the latest stages of pollen development. Numerous other candidates for precisely timed promoters may become obvious as microarray analyses (Becker et al., 2003; Honys and Twell, 2003) are extended to other stages of pollen development. Despite the small size of the anthers in which meiosis occurs, pollen mother cells can be extruded from anthers at the appropriate stage. It is reasonable to anticipate that molecular analyses of isolated meiocytes will be achievable and that analyses with such material might eventually provide promoters that will be suitable for directing gene expression at precise stages of meiotic development.

Molecular analyses of the generative cell were first accomplished with lily. Lily has a large generative cell that is easily isolated free of the vegetative cell, and generative cell–specific histones were characterized (Ueda and Tanaka, 1995; Xu et al., 1999a; Ueda et al., 2000). Xu et al. (1999b) used a cDNA library prepared from lily generative cells to isolate and characterize a generative cell–specific gene, *lily generative cell1* (*LGC1*). The LGC1 protein was localized to the surface of the generative cell and sperm cells, suggesting that it might play a role in gamete interactions. Subsequently, Singh et al. (2003) characterized the *LGC1* promoter in transgenic tobacco plants. From their preliminary deletion analyses, they suggest that generative cell–specific expression driven by the LGC1 promoter is attributable to a repressor element, because when the promoter fragment was truncated, the reporter gene was expressed more widely. Xu et al. (2002) constructed a cDNA library from partially purified tobacco sperm and, after differential screening of this library and analysis of $\sim$400 cDNAs, reported that two cDNAs were sperm specific: one was similar to a polygalacturonase and one was similar to a protein of unknown function (At3g23860).

More recently, Engel et al. (2003) sequenced ~5000 ESTs from a cDNA library prepared from sperm of maize that had been purified away from vegetative cytoplasm by fluorescence-activated cell sorting and used reverse transcriptase–mediated PCR and in situ hybridizations to characterize the expression patterns for some of these mRNAs.

For ~4% of the maize sperm ESTs, the best database matches were to proteins that were annotated as hypothetical (i.e., not in other available EST databases). We reasoned that those might be good candidates for sperm-specific messages. Accordingly, the promoters of the Arabidopsis homologs were tested (M. Engel and S. McCormick, unpublished data) to determine if any would confer sperm-specific expression to a reporter gene, eGFP. In transgenic lines harboring one such construct, the two sperm cells are brightly fluorescent. Figure 4 shows a pollen grain from one line: note that one sperm has a long cytoplasmic extension that connects it to the vegetative nucleus (M. Engel and S. McCormick, unpublished data). A cytoplasmic extension between one of the two sperm cells and the vegetative nucleus was already documented from transmission electron microscopy analyses in *Plumbago* (Russell, 1984), and extensions of the vegetative nucleus toward one of the two sperm cells are visible in 4′,6-diamidino-2-phenylindole–stained pollen (Lalanne and Twell, 2002). It will be interesting to determine if the sperm cell that is "in communication" with the vegetative nucleus is destined for a particular fusion partner (egg or central cell), a situation that has been documented in *Plumbago* (Russell, 1985). Genetic evidence for fusion partner selectivity comes from studies of maize lines that carry supernumerary (so-called B) chromosomes. In maize lines carrying B chromosomes, the B centromere frequently undergoes nondisjunction at the second mitosis, so that one sperm cell of a pair acquires two B centromeres and the other acquires none (Rusche et al., 1997). Genetic markers linked to the B centromere revealed preferential transmission to the embryo. But Faure et al. (2003) were not able to discern any preference of the B chromosome–containing sperm when sperm pairs from one pollen grain were used for in vitro fertilization. However, when sperm are isolated from pollen grains, connections to the vegetative nucleus are necessarily disrupted. Sperm cytoplasm that is marked with eGFP, in conjunction with improvements for live-cell imaging (Feijo and Cox, 2001), should allow a more detailed analysis of sperm dynamics at all stages of their transit through the pollen tube for delivery to the embryo sac.

**Figure 4.** Arabidopsis Pollen Grain from a Line Expressing a Sperm Promoter:eGFP Construct.

Image is a maximum projection of the central Z-sections acquired with a two-photon microscope. The GFP image is inverted to more clearly show that one of the two sperm cells has a long cytoplasmic extension that appears to partially encircle the vegetative cell nucleus.

Given that generative cells and sperm cells are thought to be relatively quiescent transcriptionally, it is unclear why sperm have diverse mRNAs (Engel et al., 2003). Perhaps some of these mRNAs are not translated until they are delivered to the central cell or the egg cell. In Arabidopsis, numerous genes have been tested for expression from the paternal genome, and most (Vielle-Calzada et al., 2000, 2001) but not all (Baroux et al., 2001; Weijers et al., 2001) were not expressed from the paternal genome until a few days after fertilization. However, Scholten et al. (2002) used a transgenic line of maize harboring a CaMV35S promoter driving GFP expression to test whether there was expression from the paternal allele soon after fertilization. There were no detectable GFP transcripts in sperm of this line, but when sperm carrying the transgene were fused in vitro with wild-type eggs, paternally derived *GFP* transcripts were detectable within the first few hours after fertilization and GFP was detectable soon thereafter. It will be important to test how widespread such paternal expression might be, preferably using endogenous, rather than virally derived, promoters to drive transgene expression.

Although transcriptome analyses are useful (Becker et al., 2003; Honys and Twell, 2003) for selecting candidates for functional analyses, it is equally important to determine the protein complement of the cells of the male gametophyte. To begin to develop information about the proteins present at different stages of pollen development, Kerim et al. (2003) used two-dimensional gel electrophoresis and mass spectrophotometry to analyze the protein complement of rice anthers. This study was not comprehensive, but it was able to identify ~150 protein spots that varied between different stages of development. Because multiple isoforms for several of these proteins were found, the authors inferred that post-translational modifications might play a significant role during pollen development. Proteomics efforts also are warranted in plants whose genomes are not yet sequenced, because there are extensive EST databases available from male gametophytes of several crops (for example, Van der Hoeven et al., 2002). Nonetheless, for many plants, it will be difficult to collect large quantities of male gametophytes or of their component cells, so future proteomics efforts probably will

use more sensitive methods (Koller et al., 2002) that do not rely on the need for two-dimensional gels.

## EXPECTATIONS FOR THE NEXT 10 YEARS

Because comparative studies had indicated that many of the structural and molecular aspects of microsporogenesis are conserved throughout evolution (McCormick, 1993), a concerted molecular, morphological, and cell biological description of microsporogenesis in a few plant species was suggested (McCormick, 1993) to provide a framework for future studies. It also was suggested that we take advantage of the increasing knowledge and methodology available from nonplant organisms to make more rapid progress in understanding meiosis, cell fate determinants, and cell–cell interactions. Such efforts have paid off. Researchers studying pollen can select a plant species most suitable for the question they are asking and then rather easily move to other species to apply different methodologies. And new methods continue to be developed or adapted. For example, Touraev et al. (1997) pioneered the biolistic introduction of gene constructs into unicellular microspores of tobacco, followed by in vitro maturation of these microspores to mature pollen. When the resulting pollen grains were used for pollination, some transgenic plants were obtained, although the efficiency was quite low ($<10^{-3}$). Aziz and Machray (2003) recently optimized this protocol for tobacco and reported up to 15% transgenic progeny. If this efficiency can be achieved routinely, it will be an attractive way to generate transgenic plants, especially if in vitro maturation of microspores can be developed for other plant species.

The ambitious goal of the NSF2010 project is to elucidate the functions of all of the proteins of Arabidopsis by the year 2010. Can we meet this goal for the proteins in the male gametophyte? From gene disruptions and the other methods discussed in this review, we have learned about particular proteins that are important for pollen development. But much of our knowledge is still discrete; how various proteins interact is largely unknown. In some fully sequenced organisms, protein–protein interaction networks are beginning to be elucidated (reviewed by Zhu and Snyder, 2002). Yeast two-hybrid screens have not yet been used extensively in pollen biology. But where such screens have been performed (Skirpan et al., 2001; Tang et al., 2002), it is already clear that some proteins identified from such screens also are pollen specific. Thus, signaling pathways already thought to be elucidated in sporophytic cells might have different players in the male gametophyte, and proteins that are expressed widely might have unique roles in the male gametophyte.

Two recent studies (Levsky et al., 2002; Jongeneel et al., 2003) offer inspirations for the future: truly global expression analyses for all of the developmental stages of the male gametophyte, and true single-cell analysis of the male gametophyte transcriptome. Jongeneel et al. (2003) used the sensitive technique called massively parallel signature sequencing to analyze gene expression in two different mammalian cell lines. They found that almost 60% of the genes were expressed at such a low level that they would not have been detected by standard techniques such as microarray analysis. Furthermore, the complexity of their expression profiles was limited; one cell line expressed $\sim$10,000 genes, and the other expressed $\sim$15,000 genes. And there were $\sim$2000 mRNAs identified that corresponded to no known (i.e., annotated) genes. Most methods used to analyze gene expression necessarily rely on RNA samples prepared from many cells. Levsky et al. (2002) pioneered a method to examine gene expression of multiple genes at the true single-cell level. In this method, sites of de novo transcription are visualized by fluorescence in situ hybridization; up to 11 genes can be assayed at once. By using spectrally distinct fluorophore labels for the gene-specific probes, the authors were able to have a unique bar code identify each transcript. In addition to transcriptional changes, a gene expression difference deduced from a microarray experiment encompasses the abundance of the message before the start of the experiment and the message stability over the course of the experiment. Thus, our lesson is that microarray analyses (Becker et al., 2003; Honys and Twell, 2003) are only a start to a global understanding of male gametophyte development. Single-cell transcriptome analyses might even allow the detection of subtle differences in gene expression in different pollen grains in a tetrad. The recent availability of GFP-tagged sperm (Figure 4) offers the promise of global transcriptome (and proteome) analyses of the gametes, assuming that large enough quantities can be purified for such analyses.

In the last 10 years, more and more emphasis has been put on model systems such as Arabidopsis. But there is still a place for nonmodel systems. Some very interesting phenomena are not known or their analysis is not tractable in the well-studied model plants. As just one example, a type of segregation distortion mediated by so-called gametocidal genes is known in several species (wheat, maize, rice, and tomato). In wheat lines that are hemizygous for a gametocidal gene (Gc2/−), gametophytes that lack the Gc2 allele undergo DNA fragmentation, presumably induced during meiosis by allelic interaction with the Gc2 allele. Friebe et al. (2003) recently obtained an ethyl methanesulfonate–induced mutant at Gc2; the mutant plants are fully fertile because the (−) gametophytes do not die. Map-based cloning of Gc2, and molecular analyses of this interesting phenomenon, should soon be possible.

That vertebrates have >1000 genes for olfactory receptor proteins was no surprise, but that some olfactory receptors were expressed in human spermatogenic cells was considered bizarre. Now, Spehr et al. (2003) have shown that one of these odorant receptors mediates human sperm chemotaxis in vitro, detecting an odorant similar to a compound found in rose petals. Kim et al. (2003) recently demonstrated that a small protein that is secreted from the style of lily flowers acts as a chemoattractant for pollen tube growth. There is every reason to believe that plant reproductive biology will tempt us with such surprises for years to come.

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