Pollen Development, a Genetic and Transcriptomic View

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Abstract The haploid gametophyte generation occupies a short but vital phase in the life cycle of flowering plants. The male gametophyte consists of just two or three cells when shed from the anthers as pollen grains. It is this functional specialization that is thought to be a key factor in the evolutionary success of flowering plants. Moreover, pollen development offers an excellent model system to study many fundamentally important biological processes such as polarity, cell fate determination, cell cycle regulation, cell signaling and mechanisms of gene regulation.

In the first part of this chapter we review the progress achieved through genetic analysis in identifying gametophytic mutants and genes required for key aspects of male gametogenesis. In the second part we discuss recent advances in genome-wide transcriptomic studies of haploid gene expression and a critical evaluation of data treatment methods. Finally we provide a perspective of the impact of these data on future strategies for understanding the gametophytic control of pollen development.

1
Introduction

During the past decade there have been significant advances in the genetic and genomic technologies exploiting *Arabidopsis thaliana* as a model. These include completion of the *Arabidopsis* genome sequence (Arabidopsis Genome Initiative 2000), establishment of public databases of large numbers of cDNAs (Seki et al. 2002) and sequenced insertion site mutants (Alonso et al. 2003), extensive transcriptomic data sets (Zimmermann et al. 2004) including those for the "pollen transcriptome" (Honys and Twell 2004; Pina et al. 2005), as well as new "pollen-specific" research tools (Johnson-Brousseau and McCormick 2004). Fuelled by these rapidly expanding shared resources, research in pollen development has made substantial progress. Here we focus on advances in our understanding of the gametophytic control of postmeiotic pollen development from free microspores up to the point of mature pollen in the model *Arabidopsis*. Several other recent reviews provide a wider discussion of other important features of male fertility and pollen development that involve diploid cells of the sporophyte, such as anther differentiation, meiotic

division, and the influence of the tapetum on pollen development and pollen wall patterning (Ma 2005; McCormick 2004; Scott et al. 2004).

2
Pollen Development

Pollen development begins in the young anther locules and consists of two major phases – microsporogenesis and microgametogenesis. The primary sporogenous layer gives rise to the microsporocytes or meiocytes. Meiotic division of the diploid microsporocytes produces tetrads of four haploid microspores enclosed within thick callosic walls, which are then separated into individual microspores by an enzyme complex (callase) secreted by the tapetum (Fig. 1). Microspore development is accompanied by progressive vacuole biogenesis, fusion and fission events (Owen and Makaroff 1995; Yamamoto et al. 2003). In association with vacuole morphogenesis and microspore expansion, the microspore nucleus becomes polarised to an eccentric position against the microspore wall. The polarised microspores then undergo asymmetric division at pollen mitosis I (PMI) giving rise to bicellular pollen grains.

Pollen mitosis I is an intrinsically asymmetric division that gives rise to two daughter cells with completely different structures and cell fates (Twell et al. 1998). The large vegetative cell has dispersed nuclear chromatin and constitutes the bulk of the pollen cytoplasm. In contrast, the smaller generative cell has condensed nuclear chromatin and contains relatively few organelles and stored metabolites. Whereas the vegetative cell exits the cell cycle at G_1 phase, the generative cell remains division-competent and completes pollen mitosis II (PMII) to form the two sperm cells. Asymmetric cytokinesis following PMI effectively seals the fate of the smaller generative cell and possesses two special features in that: (1) no preprophase band of microtubules marks the future division plane, and (2) a unique curved cell plate is formed to enclose the generative nucleus. Two general models have been proposed to account for differential cell fate arising from asymmetric division at PMI (Eady et al. 1995). Both assume that vegetative cell gene expression is the default pathway resulting from the accumulation of gametophytic factors, and provide alternative mechanisms to explain how vegetative cell-specific genes are repressed in the generative cell. In essence gametophytic factors may be simply excluded from the generative cell pole, or hypothetical generative cell repressors at the generative cell-pole may block vegetative cell-specific gene expression. However, it is possible that a combination of both mechanisms operate to specify and reinforce generative cell fate.

After pollen mitosis I, the generative cell migrates inward resulting in a "cell within a cell" structure, enabling gamete transport within the pollen tube. Generative cell migration follows degradation of the hemispherical cal-
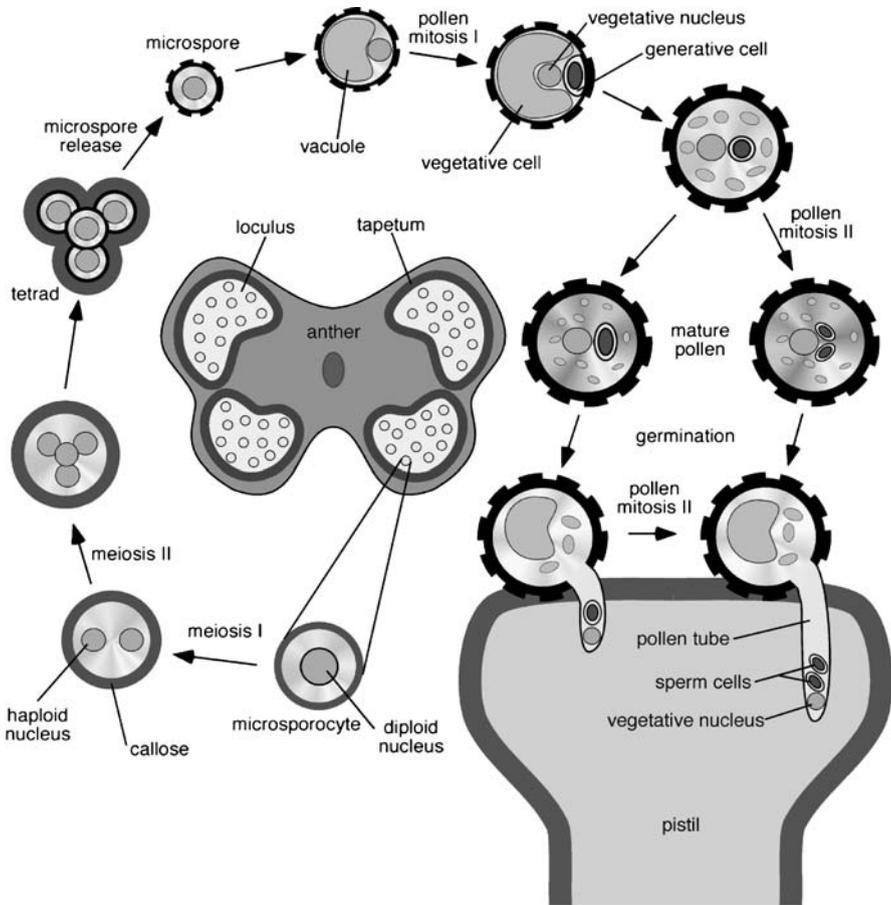
Fig. 1 Morphological stages of microsporogenesis and microgametogenesis. During microsporogenesis, microsporocytes undergo two nuclear divisions at meiosis followed by cytokinesis to produce a tetrad of four haploid microspores. During microgametogenesis, microspores undergo two stereotypical mitotic divisions, pollen mitosis I and pollen mitosis II to produce bicellular (70% of species) or tricellular pollen grains (e.g., *Arabidopsis*). In species with bicellular pollen grains, pollen mitosis II occurs in the growing pollen tube within the pistil

lose wall that separates the vegetative and generative cells. This presumably involves targeted secretion of β(1-3)-glucanases. Subsequently, the generative cell forms an elongated, or spindle-like shape that is maintained by a cortical cage of bundled microtubules (Cai and Cresti, this volume). Pollen mitosis II takes place within a membrane bound compartment of the vegetative cell cytoplasm and a physical association is established between the gametic cells and the vegetative nucleus known as the male germ unit (MGU). The MGU exists in both bicellular and tricellular pollen system and is thought

to be important for the coordinated delivery of the gametes and sperm cell fusion events (Dumas et al. 1998). In *Arabidopsis* the MGU is first assembled in tricellular pollen (Lalanne and Twell 2002). During pollen maturation the vegetative cell accumulates carbohydrate and/or lipid reserves required for the demands of plasma membrane and pollen tube wall synthesis (Pacini 1996). Pollen grains are usually strongly dehydrated when finally released from the anthers. The accumulation of sugars and amino acids as osmoprotectants, including disaccharides and proline or glycine-betaine, is thought to protect vital membranes and proteins from damage during dehydration (Schwacke et al. 1999).

2.1
Gametophytic Mutants Affecting Pollen Development

Mutants affecting pollen development provide excellent material to analyze processes of cell fate specification and cellular function (Sects. 2.1.1–2.1.4). There have been three major screening strategies to isolate gametophytic mutants and genes affecting pollen development in *Arabidopsis*; gametophyte-targeted forward genetic screens, gametophyte-directed reverse genetic screens and non-gametophyte-directed reverse genetic screens.

Male gametophyte-targeted forward genetic screens have identified altered pollen phenotypes caused by chemical or physical mutagens (Chen and McCormick 1996; Park et al. 1998; Grini et al. 1999), or by insertional mutagens of T-DNA (Bonhomme et al. 1998; Howden et al. 1998; Johnson et al. 2004) or transposon origin (Lalanne et al. 2004b). Disrupted genes have been identified by map-based cloning (Twell et al. 2002; Rotman et al. 2005; Oh et al. 2005) or by generating flanking sequence tags (Lalanne et al. 2004a,b; Johnson et al. 2004). Non-gametophyte-directed reverse genetic approaches have in several cases uncovered unexpected gametophyte-defective phenotypes caused by mutations in genes of interest (Robertson et al. 2004; Niewiadomski et al. 2005; Dettmer et al. 2005). While this approach is generally not gametophyte-targeted, prior analysis of the expression patterns of genes of interest can direct research toward testing gametophytic function. For example gametophyte functions for the tyrosine phosphatase *AtPTEN1* (Gupta et al. 2002; Yokota and Shimmen, this volume) were discovered by RNAi-directed down-regulation in pollen using the vegetative-cell-specific *LAT52* promoter (Twell 1992).

At the time of writing, we were able to compile data on 45 gametophytic mutants that show defects during post-meiotic male gametophyte development before pollen release (Table 1). The most dominant class of 28 mutants displays pollen cell death phenotypes at various developmental stages. A second major class of nine mutants show specific division-related phenotypes either affecting microspore division (*scp*, *gem1,2*, *tio*) or generative cell division (*duo1,2,3,4,5*). Different approaches have led to the identification of 14 genes

Table 1 Arabidopsis gametophytic mutants and genes that affect pollen development

Mutant [a,b]	Gene [b]	Status [c]	Protein identity	Map position	Mutant Phenotype	Function	Refs.
abnormal gametophytes	AGM	V	Putative transmembrane protein	At5g44860	Pollen degenerates at late microspore stage	Microspore development and/or division	Sorensen et al. 2004
arabidopsis dynamin-like 1C	ADL1C	V	Dynamin-like protein	At1g14830	Pollen abortion during maturation	Pollen plasmamembrane maintenance, cell wall synthesis	Kang et al. 2003
arabidopsis phosphatase and tensin homologue	AtPTEN	V	Phosphatase and tensin homologue [Tyrosine/PIP3 phosphatase]	At5g39400	Pollen death after pollen mitosis II	Pollen maturation	Gupta et al. 2002
arabidopsis H+-ATPase 3	AHA3	V	Plasma membrane H+-ATPase	At5g57350	Aborted mature pollen	Microspore/pollen nutrient transport	Robertson et al. 2004
arabinogalactan protein1 [AtBCP1]	AGP1	V	Arabinogalactan protein	At1g24520	Pollen aborts at bicellular stage	Tapetum, microspore and bicellular pollen viability	Xu et al. 1995
both male and female defective (1,2,3)	BOD	—	—	Chr 1 ~ 108/18/88 cM	Pollen arrested at bicellular stage with pleiotropic effects	Pollen maturation after pollen mitosis I	Grini et al. 1999
duo pollen1	DUO1	V	R2R3 MYB transcription factor (MYB125)	At3g60460	Bicellular pollen: generative cell fails to enter pollen mitosis II	Regulator of gene expression required for mitotic entry	Durbarry et al. 2005 Rotman et al. 2005
duo pollen2	DUO2	—	—	Chr 5 ~ 54 cM	Bicellular pollen: generative cell arrested at prometaphase	Required for mitotic progression	Durbarry et al. 2005

Table 1 (continued)

Mutant[a,b]	Gene[b]	Mutant Phenotype	Map position	Status[c]	Protein identity	Function	Refs.
duo pollen3	DUO3	Bicellular pollen: generative cell fails to enter pollen mitosis II	Chr 1 ~95 cM	—	—	Required for mitotic entry	Durbarry & Twell, unpublished
duo pollen4	DUO4	Pollen fails to enter or complete generative cell division	Chr 4 ~75 cM	—	—	Generative cell morphogenesis and cell cycle progression	Twell 2002; Durbarry & Twell, unpublished
duo pollen5	DUO5	Pollen fails to enter or complete generative cell division	Chr 4 ~93 cM	—	—	Generative cell morphogenesis and cell cycle progression	Twell 2002; Durbarry & Twell, unpublished
emotionally fragile pollen1	EFP1	Pollen shows diffuse callosic staining	—	—	—	Repression of callose synthesis during pollen maturation	Johnson et al. 2001
female gametophyte3	FEM3	Aborted mature pollen	Chr 1 ~84 cM	—	—	Pollen viability	Christensen et al. 1998
gemini pollen1	GEM1	Twin-celled and binucleate pollen: abnormal divisions at pollen mitosis I	At2g35630	V	MAP215 family of microtubule associated proteins	Microspore polarity and cytokinesis through microtubule organisation	Park et al. 1998; Park and Twell 2001; Twell et al. 2002

Table 1 (continued)

Mutant[a,b]	Gene[b]	Mutant Phenotype	Map position	Status[c]	Protein identity	Function	Refs.
gemini pollen2	GEM2	Similar to *gem1*, but less severe	Chr 5 ~ 95 cM	—	—	Control of microspore polarity and cytokinesis	Park et al. 2004
gametophytic factor3	GFA3	Vacuolated mature pollen	Chr 2 ~ 73 cM	—	—	Vacuole morphogenesis	Christensen et al. 1998; Feldmann et al. 1997
gametophytic factor4	GFA4	Aborted mature pollen	Chr 3 ~ 98 cM	—	—	Pollen viability	Christensen et al. 1998; Feldmann et al. 1997
gametophytic factor5	GFA5	Aborted mature pollen	Chr 4 ~ 25 cM	—	—	Pollen viability	Christensen et al. 1998; Feldmann et al. 1997
glucose-6-phosphate translocator1	GPT1	Aborted pollen. Reduced lipid bodies, vesicles and vacuoles. Defects at tri-cellular stage	At5g54800	V	glucose-6-phosphate translocator	Glc6P import for starch, fatty acid biosynthesis and carbon for OPPP	Niewiadomski et al. 2005
gift wrapped pollen 1	GWP1	Pollen contains internal callose tube-like structures	Chr 2 ~ 50 cM	—	—	Regulation of callose synthesis	Johnson et al. 2001

Table 1 (continued)

Mutant[a,b]	Gene[b]	Mutant Phenotype	Protein identity	Status[c]	Map position	Function	Refs.
gift wrapped pollen 2	GWP2	Pollen contains internal callose tube-like structures	—	—	—	Regulation of callose synthesis	Johnson et al. 2001
male germ unit malformed(1,2)	GUM(1,2)	Male germ unit does not form: vegetative nucleus separated from two sperm cells	—	—	Chr 4 ~ 24.2 cM	Male germ & stability unit assembly	Lalanne & Twell 2002
halfman	HAM	Pollen degenerates during bicellular stage	~ 150 kb deletion including 38 predicted genes	V	At4g28490 -28830	Pollen maturation after pollen mitosis I	Oh et al. 2003
hapless5	HAP5	Aborted mature pollen	Cation-chloride cotransporter	T	At1g30450	Ion homeostasis during development	Johnson et al. 2004
hapless12	HAP12	Aborted mature pollen	Contains AP2 domain (RAP2.10)	T	At4g36900	Transcriptional regulator of pollen gene expression	Johnson et al. 2004
hapless16	HAP16	Slightly collapsed mature pollen	—	—	—	Pollen maturation	Johnson et al. 2004
limpet pollen	LIP	Generative cell remains attached to pollen wall	—	—	—	GC internalisation	Johnson et al. 2001
male defective1	MAD1	Pollen arrested during bicellular development, some internal dividing walls	—	—	Chr 1 ~ 118 cM	Vegetative cell maturation and GC division	Grini et al. 1999

Table 1 (continued)

Mutant[a,b]	Gene[b]	Mutant Phenotype	Map position	Status[c]	Protein identity	Function	Refs.
male defective2	MAD2	Pollen arrested during bicellular development, diffuse GC nuclei	Chr 1 ~84 cM	—	—	Pollen maturation and GC differentiation	Grini et al. 1999
male defective3	MAD3	Smaller mature pollen, GC arrested late during bicellular development, intine defects	Chr 1 ~104	—	—	Involved in intine synthesis required for pollen maturation and GC division	Grini et al. 1999
male germ unit displaced1	MUD1	Male germ unit displaced to the cortical cytoplasm in mature pollen	Chr 3 ~41.6 cM	—	—	Regulation of nuclear-cytoplasmic organisation	Lalanne & Twell 2002
male germ unit displaced2	MUD2	Male germ unit displaced to the cortical cytoplasm in mature pollen	Chr 2 ~71.7 cM	—	—	Regulation of nuclear-cytoplasmic organisation	Lalanne & Twell 2002
polka dot pollen	PDP	Pollen shows abnormal localised callose staining	—	—	—	Regulation of callose synthesis	Johnson et al. 2001
ungud(1,2)	UNG(1,2)	Aberrant microspore divisions and bicellular pollen abortion	—	—	—	Microspore and pollen development, division at pollen mitosis I	Lalanne et al. 2004b
ungud3	UNG3	Progressive cell death from microspore through bicellular pollen stages	At2g34550	T	Gibberellin-2-oxidase	Control of gibberellin levels required for cell development	Lalanne et al. 2004b
ungud4	UNG4	Smaller pollen showing bicellular and tricellular arrest	—	—	—	Bicellular pollen development	Lalanne et al. 2004b

Table 1 (continued)

Mutant[a,b]	Gene[b]	Mutant Phenotype	Map position	Status[c]	Protein identity	Function	Refs.
raring-to-go	RTG	Precocious germination of pollen within the anther locule	Chr 3 ~ 30 cM	—	—	Regulation of pollen hydration status	Johnson et al. 2001
sidecar pollen	SCP	Pollen with an extra vegetative cell: early symmetric microspore division	Chr 3 ~ 80 cM	—	—	Regulation of microspore cell cycle or polarity establishment	Chen & McCormick 1996
T-DNA transmission defective (6,17,38,40)	TDT	Pollen aborted at pollen mitosis I or in mature anthers	—	—	—	Microspore or pollen viability	Bohomme et al. 1998; Procissi et al. 2001
two-in-one	TIO	Microspores initiate, but fail to complete cytokinesis at pollen mitosis I	At1g50230	V	TIO: Homologous to FUSED-kinase family	Signalling role in phragmoplast expansion	Oh et al. 2005
Vacuolar-ATPase V1 subunit A	VHA-A	Pollen aborted at bicellular stage and later: Swollen ER cisternae	At1g78900	V	Vacuolar-ATPase V1 subunit A	pH homeostasis, secondary active transport, Golgi organization	Dettmer et al. 2005

a Only gametophytic mutants that show abnormal phenotypes detectable before pollen shed are included.

b Numbers in parenthesis refer to individual mutants or genes with the same mutant symbol prefix.

c Gene function status: V = verified by independent methods [alleles, KO, antisense, RNAi or complementation]; T = tagged gene(s) showing 100% linkage, but not yet independently verified.

— = not determined

responsible for male-gametophyte developmental mutations (Table 1). Half of these were identified through forward genetics, by positional cloning (*GEM1, DUO1, TIO*), or by isolating flanking sequence tags (*UNG3, AGM, HAP5, HAP12*). The other half were functionally identified using reverse genetics approaches (*AGP1, AtPTEN, ADL1C, AGM, AHA3, GPT1, VHA-A*). Proteins with a variety of cellular roles have been defined including those with roles in cell division (GEM1, DUO1, TIO) nutrition and metabolism (AHA3, GPT1), pH regulation and ion transport (VHA-A, HAP5), and other groups with roles in transcriptional regulation (DUO1, HAP12) or post-translational (AtPTEN, TIO) intracellular signaling events (Table 1).

Two forward genetic strategies have been adopted to identify gametophytic mutants affecting pollen development. First, by morphological screening of pollen from mutagenized plants using histochemical staining for DNA (Chen and McCormick 1996; Park et al. 1998) or callose (Johnson and McCormick 2001). Second, by analysis of pollen phenotypes in plants showing marker segregation ratio distortion (Bonhomme et al. 1998; Grini et al. 1999; Howden et al. 1998; Johnson et al. 2004; Lalanne et al. 2004a,b; Procissi et al. 2001). Morphological screens are labor intensive, but have proven effective in the identification of several important mutants with novel phenotypes (Table 1). These include mutants that disturb asymmetric cell division at pollen mitosis I (Chen and McCormick 1996; Twell et al. 1998, 2002; Oh et al. 2005), generative cell division (Grini et al. 1999; Durbarry and Twell 2005; Rotman et al. 2005), generative cell migration (Howden et al. 1998), the positioning and structure of the male germ unit (Lalanne and Twell 2002), callose wall deposition and the repression of pollen germination within the anther (Johnson and McCormick 2001).

2.1.1
Mutants Affecting Gametophytic Cell Divisions

sidecar pollen (*scp*) is a male-specific mutant that affects microspore division and cell patterning (Chen and McCormick 1996). Mutant *scp* microspores first undergo a premature symmetrical division, followed by asymmetric division of only one of the daughter cells to produce mature pollen with a supernumerary vegetative cell. Although polarity is not visibly expressed when *scp* microspores divide, polarity is not defective, since it is re-established in one daughter cell. *scp* provides evidence for the importance of coordinating the mitotic cell cycle with the expression of polarity. Although *SCP* remains to be identified, the mutant phenotype supports the hypothesis of an asymmetric distribution of polarity determinants before division in *scp* (Chen and McCormick 1996).

gemini pollen1 (gem1) affects male and female transmission and displays a range of microspore division phenotypes including equal, unequal and partial divisions (Park et al. 1998). In contrast to *scp*, symmetrical divi-

sions in *gem1* do not occur precociously and neither daughter cell completes a further division. Cell fate analysis in *gem1* supports the role of cell size or nuclear/cytoplasmic ratio as a factor in determination of cell fate (Park et al. 1998; Park and Twell 2001). *GEM1* was the first male gametophytic gene to be positionally cloned, revealing its identity to the MOR1 (Whittington et al. 2001). MOR1/GEM1 is homologous to the MAP215 family of microtubule-associated proteins that stimulate plus-end microtubule growth. MOR1/GEM1 is associated with interphase, spindle and phragmoplast microtubule arrays and plays a vital role in microspore polarity as well as cytokinesis (Twell et al. 2002). To understand the role of MOR1/GEM1 in more detail we have recently expressed a GFP alpha-tubulin fusion in developing pollen that will allow the analysis of microtubule dynamics in wild type and mutant genetic backgrounds (S-A. Oh, J.A. Johnson, D. Twell unpublished).

In the *two-in-one* (*tio*) mutant, microspores complete nuclear division but fail to complete cytokinesis resulting in binucleate pollen grains. In contrast to *gem1, tio* shows normal nuclear polarity before pollen mitosis I, but displays cytokinesis-specific defects including the formation of incomplete callosic cell plates that fail to expand (Fig. 2). TIO was recently identified as
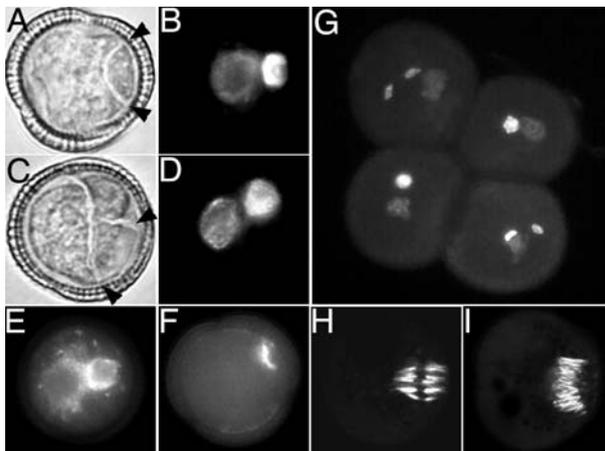

Fig. 2 Pollen phenotypes of *Arabidopsis* gametophytic mutants and GFP::tubulin expression (**A**–**D**) Bright field (**A,C**) and corresponding DAPI-stained (**B,D**) images of wild type (**A,B**) and *gem1* (**C,D**) pollen at early bicellular stage. Wild type pollen shows a typical hemispherical cell wall but *gem1* pollen grains often produce misplaced and branched internal cell walls. **E** DAPI-stained *tio* pollen with typical binucleate phenotype resulting from failed cytokinesis at pollen mitosis I. **F** *tio* pollen immediately after nuclear division at PMI stained with aniline blue showing an incomplete and transient callose cell plate. **G** Tetrad from heterozygous *duo1* mutant in the *quartet1* background showing 2 : 2 segregation for wild type and *duo1* mutant pollen. (**H, I**) Spindle (**H**) and phragmoplast microtubules (**I**) at pollen mitosis I visualised in transgenic tobacco microspores expressing GFP::TUA6

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 is localised to the phragmoplast midline where it has an essential role in centrifugal cell plate expansion. Although nuclear division is not affected in *gem1* and *tio* microspores, daughter nuclei do not divide further, strengthening the hypothesis that PMII depends on persistent cell fate determinants that may be sealed in the generative cell cytoplasm at PMI.

The *duo pollen* (*duo*) mutants complete normal asymmetric division at PMI, but fail to enter or complete generative cell division (Durbarry et al. 2005). Heterozygous *duo* mutants produce ∼ 50% bicellular pollen containing a vegetative cell nucleus and a single "generative-like" cell with condensed nuclear chromatin (Fig. 2). DUO1 was recently identified as a novel R2R3 MYB protein specifically expressed in generative and sperm cells (Rotman et al. 2005). DUO1 represents the first germ line specific regulator to be identified and DUO1 homologues in maize, rice and tobacco all possess a supernumerary lysine signature in the R3 MYB domain that defines the DUO1 family (Rotman et al. 2005). Mutant generative cells in *duo1* pollen fail to enter PMII and enter a partial endocycle, suggesting that DUO1 may be a specific regulator of genes required for G2 to M-phase transition (Durbarry et al. 2005). In contrast, mutant generative cells in the *duo2* mutant do enter mitosis, but arrest at prometaphase suggesting the role of DUO2 in mitotic progression (Durbarry et al. 2005).

2.1.2
Mutants Affecting Pollen Cell Morphogenesis

Several interesting mutants have been isolated that affect pollen cell morphogenesis after PMI using different screens. In the *limpet pollen* (*lip*) mutant, identified in a T-DNA segregation distortion screen, the generative cell fails to migrate inward after PMI suggesting defects in mechanisms of generative cell engulfment. Moreover, the generative cell is able to divide to form two marginalized sperm cells outside the vegetative cell cytoplasm suggesting that positional information is not essential for generative cell division (Howden et al. 1998). The *mud* (*male germ unit displaced*) and *gum* (*germ unit malformed*) mutants were identified from morphogenesis screens as male-specific gametophytic mutants affecting the MGU (Lalanne and Twell 2002). *mud* pollen displays normal MGU assembly, but the MGU is displaced to an eccentric position in mature pollen. In *gum* pollen however, the MGU fails to assemble and the vegetative nucleus is separated from the two sperm cells. Double mutant analysis suggests that *GUM* acts upstream of *MUD* in a pathway required for MGU assembly and positioning. From independent morphological screens a range of mutants were isolated that show unusual callose staining (Johnson and McCormick 2001). These

include *raring-to-go* (*rtg*) that shows premature pollen hydration and germination within the anther locules. Other mutants producing excess callose in pollen grains, *emotionally fragile pollen* (*efp*), *gift-wrapped pollen* (*gwp*) and *polka dot pollen* (*pdp*), may be regulators of callose synthase and/or components pathways that normally repress callose production until pollen germination.

2.1.3
Segregation Ratio Distortion Screens for Male Gametophytic Mutants

Forward genetic screens using marker segregation ratio distortion have also been successful in identifying genes involved in microgametogenesis. These screens have mostly employed T-DNA or transposon insertion populations that harbor dominant antibiotic or herbicide resistance markers. For example, if an insertion inactivates an essential male gametophytic gene, then the ratio of resistant to sensitive progeny will deviate significantly below the expected 3 : 1 ratio toward 1 : 1. Such screens are inclusive in that mutants affecting both pollen developmental and progamic phases are recovered as well as those affecting female gametogenesis.

Insertional segregation ratio distortion screens have the advantage of straightforward identification of the mutated sequences. A number of these cause aborted pollen phenotypes that could arise from various mechanisms (Table 1). For example *abnormal gametophytes* (*agm*) encodes putative transmembrane protein; *vha-A*, a subunit of the vacuolar H$^+$ ATPase, tagged sequences in *hap5*, encode a putative AP2 domain transcription factor and in *hap12*, a cation-chloride transporter. The *ung3* mutant results in early bicellular pollen arrest (Lalanne et al. 2004b). Tagged sequences in *ung3* encode a GA-2-oxidase suggesting a role for GA in pollen development, as well as its recently discovered role in pollen tube growth (Singh et al. 2002).

A different segregation distortion screen for EMS-induced gametophytic mutants using multiple visible markers on chromosome 1 was devised by Grini et al. (1999). Three male-specific lines (*mad1*, *mad2*, *mad3*) showed defects during pollen development, and three other lines showed variable defects in both male and female gametophytes (*bod1*, *bod2*, *bod3*). Although most mutants showed pleiotropic effects, the most common phenotypes included pollen arrested at the bicellular stage. Interestingly, *mad1* also showed dividing walls at mid-bicellular stage reminiscent of phenotypes in *gem1* (Park et al. 1998).

Despite cloning successes complications can arise in insertion-based screens, and chromosomal rearrangements involving inversion, translocation and deletion have been reported (e.g., Tax and Vernon 2001). Many mutants with lethal phenotypes remain to be identified including several members of the, *both male and female defective*, *T-DNA transmission defective* and *ungud* mutant classes (Table 1). Failure to clone flanking sequences

could arise from such rearrangement of insertion sequences (Lalanne et al. 2004b; Johnson et al. 2004). For example in the *ham* mutant, insertion of a *Ds* element caused an approximately 150 kb deletion, hampering identification of the responsible gene(s) (Oh et al. 2003). Therefore it is important that the function of even apparently tagged mutants are independently verified by isolation of new alleles, complementation and/or by targeted downregulation.

2.1.4
Non-gametophyte Targeted Identification of Male Gametophytic Genes

In an increasing number of cases, gametophytic roles have been discovered by characterising loss of function phenotypes caused by knockout or knockdown mutants in genes of interest. This approach has led to the identification of four genes, *AtPTEN*, *ADL1C*, *AHA3* and *GPT1*, with important roles during microgametogenesis (Table 1). In some cases progress was directed by knowledge of pollen-enhanced gene expression. For the dynamin-like *ADL1C*, gene expression profiles and promoter-GUS analysis hinted at a role for *ADL1C* in pollen. T-DNA insertions in *ADL1C* caused plasma membrane and cell wall defects that could suggest a role in for ADL1C in plasma membrane maintenance (Kang et al. 2003).

The requirement of two ATPases for pollen development was also demonstrated by isolating T-DNA insertion alleles. Knockout of a single member of the 11 plasma membrane proton H^+-ATPases, AHA3, leads to pollen abortion that suggests a role in secondary ion transport and microspore nutrition (Robertson et al. 2004). A T-DNA insertion in the A subunit of the vacuolar H^+ ATPase, VHA-A, leads to mutant pollen with curved and swollen Golgi cisternae suggesting a role in Golgi organization (Dettmer et al. 2005). The essential role of a plastidic glucose 6-phosphate/phosphate translocator (GPT1) in pollen was recently demonstrated by isolating knockout mutants in one of the two homologous GPT genes (Niewiadomski et al. 2005). *gpt1* mutant pollen shows reduced formation of lipid bodies and vacuoles essential for cell viability. The suggested role for GPT1 in pollen is to support Glc6P import into plastids as a source of carbon for starch and fatty acid biosynthesis, or as a starter for the oxidative pentose phosphate pathway.

2.2
Perspectives from Male Gametophytic Mutant Studies

Currently we know about 45 gametophytic mutants for which 14 corresponding genes have been identified (Table 1). Progress has been slowed because of the limitations of positional cloning and the effort required for large-scale segregation distortion screening. Saturation screening by segregation distortion remains a daunting task, since it is estimated that 180 000 T-DNA

insertions are required to achieve one insertion in every gene in *Arabidopsis* with 95% probability (Krysan et al. 1999). With the establishment of large FST (Flanking Sequence Tag) databases and corresponding germplasm stocks more than 80% of protein coding genes now have intron or coding regions FST matches likely to produce knockout mutations (Schoof et al. 2004). With such resources lines in hand, the total number of insertions that need to be screened to achieve saturation is more realistic and similar to the total number of annotated genes in *Arabidopsis*. However, it will remain difficult to identify essential genes like *TIO* with critical functions in both gametophytes (Oh et al. 2005) due to their extremely reduced transmission. Moreover, many genes with redundant roles will not be revealed until double and multiple mutant combinations can be assembled.

3
Pollen Transcriptomics

Characterisation of the complexity of male gametophyte gene expression has reflected the available methodologies. First, isozyme studies suggested significant overlap of gametophytic and sporophytic gene expression ranging from 60 to 72% (Tanksley et al. 1981; Pedersen et al. 1987). RNA hybridisation studies confirmed this overlap and suggested that *Tradescantia paludosa* and *Zea mays* pollen express $\sim 20\,000$ to $24\,000$ individual mRNA sequences which was significantly lower than in roots (Willing et al. 1988). Subsequently, hybridisation studies of pollen cDNA libraries corrected the extent of gametophytic-sporophytic overlap suggesting that only 10% of pollen-expressed mRNAs may be pollen-specific (Stinson et al. 1987; Mascarenhas 1990).

Until recently, gene-by-gene characterization led to the identification of approximately 150 pollen-expressed genes from different species, with strong evidence for pollen-specific expression in about 30 (reviewed in Twell 2002). Moreover only 23 pollen-expressed genes were identified in *Arabidopsis*. The availability of new high-throughput technologies has enabled analysis of the haploid transcriptome on a global scale. Three initial studies, exploiting serial analysis of gene expression (SAGE) technology (Lee and Lee 2003) and 8K Affymetrix AG microarrays (Honys and Twell 2003; Becker et al. 2003), provided analyses for mature pollen based on approximately one-third of the *Arabidopsis* genome. Both approaches, however, gave similar overall views of pollen gene expression. The microarray studies led to the identification of 992 (Honys and Twell 2003) and 1587 (Becker et al. 2003) genes expressed in mature pollen of which 39%/10% were considered pollen-specific. Based on these limited genome-wide studies, estimates of pollen-expressed genes in *Arabidopsis* were between 3500 and 5500. The classification of pollen-expressed and pollen-specific genes into functional categories revealed sev-

eral over-represented functional groups (cell wall, metabolism, cytoskeleton and signaling) among the pollen-specific genes. Moreover, pollen-specific genes were in general much more highly expressed than corresponding non-specific pollen-expressed genes (Honys and Twell 2003).

Further refinement was enabled by the availability of Affymetrix 23 K *Arabidopsis* ATH1 arrays. Currently, there are three publicly available independent data resources. The first contains microarray data covering four stages of male gametophyte development (uninucleate microspores, bicellular pollen, tricellular pollen and mature pollen) for ecotype Landsberg erecta (Honys and Twell 2004). The two remaining datasets were obtained from mature pollen grains from ecotype Columbia (Pina et al. 2005; Zimmermann et al. 2004). Apart from revealing the identity of the vast majority of male gametophyte-expressed genes, the major impact of these studies lies in the massively increased knowledge of the complexity and dynamics of haploid gene expression throughout single-cell development in plants (Honys and Twell 2004).

3.1
Quantification of Pollen-expressed Genes

Estimations of the number of genes active in the male gametophyte and their functional categorization are strictly dependent on data treatment, especially on the microarray normalisation algorithm[1] and the exclusive or inclusive treatment of detection calls[2] in replicates. We show how data treatment influences the outcome in Table 2. The use of the MAS4 algorithm in combination with the inclusive treatment of replicates can increase the number of expressed genes more than two-fold when compared to MAS5 using the exclusive approach. Therefore differentially normalised datasets cannot be reliably compared. Such variance however does not affect the correlation within differentially normalised data from one experiment; correlation coefficients were always above 0.99. This demonstrates that most of the variance is caused by low-abundance transcripts with uncertain expression. However, our analyses of four developmental stages showed that the MAS5 detection algorithm tended to eliminate a number of genes originally detected by MAS4 as expressed and which were experimentally confirmed as active. This was often the case even for abundant genes (B. Honysová and D. Honys unpublished results), highlighting the added value of the empirical MAS4 detection algorithm and comparative analysis.

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[1] Two detection algorithms were compared: empirical MAS4 and statistical MAS5

[2] Exclusive approach: a gene must have the detection call PRESENT in all replicates to be scored as expressed, it eliminates non-reliable genes, the final number of identified genes in always smaller than in any replicate; inclusive approach: a gene must be called PRESENT in at least one replicate to be scored as expressed, it keeps non-reliable genes, the final number of genes is always higher than in any replicate.

Table 2 The effect of the normalisation algorithm used to identify the number of genes expressed in mature pollen in three independent experiments involving two different ecotypes (Section X)

Dataset[a]	Exclusive MAS4	MAS5	Inclusive MAS4	MAS5	MAX/MIN[b] fold change
Honys	**7,264**	4,774	11,071	6,930	2.32
Pina	6,965	5,039	11,238	**6,558**[c]	2.23
AtGE73-1,2	8,566	6,396	11,939	8,195	1.87
AtGE73-1,3	8,321	6,192	11,748	7,924	1.90
AtGE73-2,3	8,394	6,283	11,764	7,916	1.87
AtGE73-1,2,3	7,671	5,938	12,756	8,520	2.15
MAX/MIN[b] fold change	1.23	1.34	1.08	1.25	

[a] Dataset labelling: Honys, Landsberg erecta (duplicate; Honys and Twell 2004); Pina, Columbia (duplicate; Pina et al. 2005); AtGE73, Columbia (triplicate; Zimmermann et al. 2004); AtGE73-1,2, AtGE73-1,3, AtGE73-2,3, all possible combinations of replicate pairs; AtGE73-1,2,3, all three replicates.
[b] Ratio of the maximum/minimum values in appropriate row/column; bold values, published estimates and normalisation algorithms used (Honys and Twell 2004; Pina et al. 2005).
[c] Only nuclear genes were included; the published value (6,587) also includes mitochondrial and plastid-encoded genes.

On the contrary, when the same normalisation algorithm is applied to different datasets, the number of expressed genes is highly consistent, with a maximum difference of 25% (Table 2). This was also confirmed by comparison of correlation coefficients. All pairs of experiments except one, showed correlation coefficients above 0.94; the only exception was 0.91. This is significantly higher than in corresponding sporophytic experiments where values were within the range 0.78–0.99 (only experiments with more than one replicate were compared). Moreover, the differences observed between datasets did not correlate with ecotype for both gametophytic and sporophytic datasets. Therefore experimental conditions seemed to be more important than the ecotype itself and previously published speculations about significant pollen transcriptome differences between ecotypes (Pina et al. 2005) are not supported. Taken together, we can now estimate the total number of genes expressed in mature pollen to lie between 5000 and 7000 genes (see Table 2). We recommend analysis of expression data in pollen using both MAS4 and MAS5 algorithms. Normalized datasets based on both MAS4 and MAS5 can be accessed and downloaded at the Arabidopsis Gene Family Profiler (aGFP) site (http://aGFP.ueb.cas.cz).

3.2
Gametophytic-Sporophytic Overlap

Comparative analyses of the male gametophytic transcriptomes are being performed against an increasing number of sporophytic datasets. Unless otherwise stated, the following results were obtained using transcriptomic data from Landsberg erecta (four developmental stages, eight individual GeneChips; Honys and Twell 2004) as all corresponding samples originated from plants populations grown under identical conditions. Reference datasets were obtained from the NASCArrays database (Craigon et al. 2004), represent 62 experiments (154 individual chips) that provide transcriptomic data for seedlings, shoots, leaves, guard cells-enriched extracts, petioles, stems, hypocotyls, xylem, cork, root hair elongation zone, roots, inflorescences, young and old buds, siliques and cell suspension cultures[3]. All datasets were normalised together using the same algorithm[4].

Considering all developmental stages, 14 037 genes gave a positive expression signal in the male gametophyte. In individual stages the number of active genes gradually decreased from 11 615 (microspores, UNM) and 11 961 (bicellular pollen, BCP) through 8831 (tricellular pollen, TCP) to 7264 (mature pollen, MPG). Analysis and visualization of gametophytic-sporophytic overlap (Fig. 3A) demonstrates a relatively low number of strictly male gametophyte-specific genes (5.6% of all male gametophyte-expressed genes). This number is gradually decreasing as new sporophytic datasets emerge, especially those from more specialised tissues and individual cell types. The estimated number of strictly pollen-specific genes is therefore significantly lower than expected from previous studies (Tanksley et al. 1981; Pedersen et al. 1987; Stinson et al. 1987; Mascarenhas 1990; Honys and Twell 2003, 2004; Becker et al. 2003; Pina et al. 2005). However, those remaining male gametophyte-specific genes are characterised by very high expression signals, highlighting their importance and their potential as targets for functional analysis.

Male gametophyte gene expression can be divided into two major phases, early and late. 13 038 genes are expressed in microspores and bicellular pollen, while 9739 genes are expressed in tricellular and mature pollen. However there is significant overlap and the vast majority (8740 genes) are expressed in both phases (Fig. 3B). The division of genes into early and late groups enables more precise visualisation of possible overlaps of early and late male gametophytic genes with two subgroups of sporophytic genes, those expressed in above ground vegetative organs and those in roots. As an example of a deregulated tis-

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[3] The complete list of dataset codes is available from authors.

[4] MAS4 detection algorithm, normalisation of all arrays to the median probe intensity level, calculation of model-based gene-expression values using Perfect Match-only model (dChip 1.3 software, http://www.dchip.org; Li and Wong 2001), exclusive treatment of replicates for identification of expressed genes
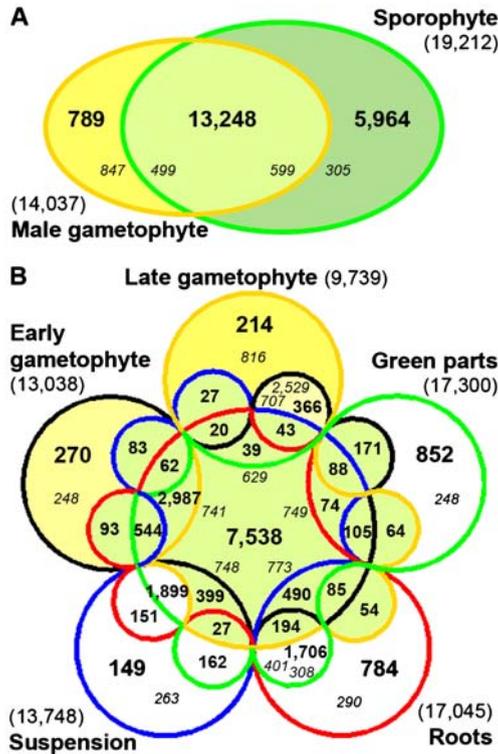
Fig. 3 Venn diagrams showing the quantitative overlap between male gametophytic and sporophytic transcriptome profiles. **A** Male gametophytic-sporophytic overlap. **B** Overlap of early pollen and late pollen transcriptomes with different sporophytic datasets represented by green parts (leaves, stems, petioles), roots and suspension cultures. The number of genes forming each segment is shown in bold. Numbers in italics show the average relative expression signal for genes in specific tissues within the particular segment

sue as well as another single cell type, suspension-cultured cells were included (Fig. 3B). From this analysis we can conclude that the late genes make a significantly greater contribution to the group of highly expressed pollen-specific genes. Late genes also represent the only dataset where the mean relative expression is higher for specific genes than for constitutive genes. The mean signal for gametophytic genes in the two late developmental stages (2529) is far higher than the mean signal within other genesets (mostly 300–500). These data also suggest that the early male gametophytic gene expression program is much more similar to that of the sporophyte.

Because of the gradually decreasing number of putative male gametophyte-specific genes, a new expression profile must be considered. This comprises non-specific pollen-expressed genes that show enhanced expression in the male gametophyte. Such pollen-enhanced genes were defined as genes with

maximum male gametophytic expression at least fivefold higher than the maximum expression in the sporophyte. We identified 1364 pollen-enhanced genes (9.7% of all male gametophytic genes), that were similarly distributed among late-pollen genes (1014 genes, 10.4% of late genes) and early-pollen genes (1084 genes, 8.3% of early genes). Within the late group these numbers resemble and thus confirm previously published estimates of preferential gene expression (Stinson et al. 1987; Mascarenhas 1990; Becker et al. 2003). Importantly, our analysis significantly extends this knowledge to include four stages of male gametophyte development.

3.3
Two Global Male Gametophytic Gene Expression Programs

Analysis of the overlap between gametophytic and sporophytic expression and the relative levels of expression in both generations clearly demonstrated marked differences. Moreover, there were striking differences between early and late male gametophytic transcriptomes that highlight the uniqueness of the late pollen transcriptome. The extent of such differences is most apparent when the expression profiles of all male gametophyte-expressed genes are visualised (Fig. 4A). Male gametophyte development is under control of at least two successive global gene expression programs, early and late, that should be precisely controlled at the transcriptional level. Out of approximately 1350 predicted *Arabidopsis* transcription factors (AGRIS web page: http://arabidopsis.med.ohio-state.edu/; Davuluri et al. 2003; Riechmann et al. 2000; Parenicova et al. 2003; Toledo-Ortiz et al. 2003), 612 were expressed in developing male gametophytes (542 early, 405 late). Of these, 49 were pollen-enhanced and only 27 were pollen-specific. These genes represent strong candidates for transcriptional regulators of male gametophyte development.
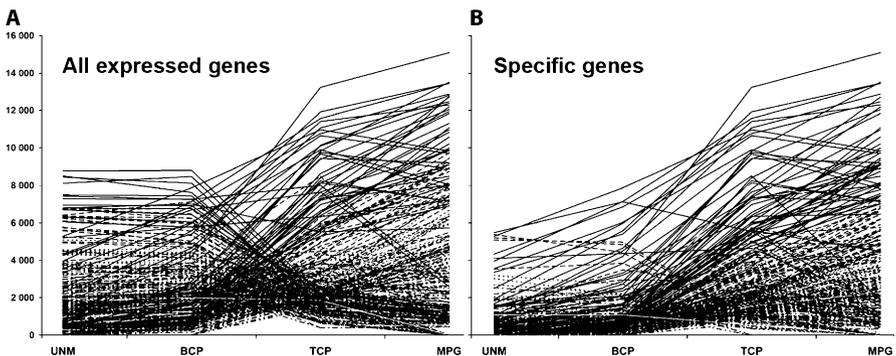

Fig. 4 Transcriptome profiles of all expressed genes (**A**) and male-gametophyte-specific genes (**B**) during male gametophyte development. There is a clear over-representation of highly-expressed late genes within the male-gametophyte-specific subset

Table 3 Correlation coefficients resulting from comparison of gametophytic and selected sporophytic transcriptomic datasets

Tissue	UNM[b]	BCP[b]	TCP[b]	MPG[b]	Chips[a]
UNM[b]	—	0.961	0.419	0.220	2
BCP[b]	0.961	—	0.565	0.333	2
TCP[b]	0.419	0.565	—	0.865	2
MPG[b]	0.220	0.333	0.865	—	2
Siliques	0.587	0.572	0.238	0.127	21
Embryo	0.593	0.558	0.166	0.073	18
Seedlings	0.468	0.470	0.214	0.117	10
Rosette leaves	0.449	0.446	0.185	0.093	28
Roots	0.549	0.550	0.166	0.116	10
Inflorescence	0.630	0.629	0.287	0.164	4
Young buds	0.576	0.576	0.256	0.143	3
Old buds	0.544	0.247	0.262	0.155	3
Suspension	0.545	0.541	0.278	0.164	5

[a] Number of individual GeneChips used for each analyses.
[b] uninucleate microspores, bicellular pollen, tricellular pollen, mature pollen.

The switch point between both developmental programs occurs prior to PMII (Fig. 4A). Taking into account the gradual decrease in the number of male-gametophyte-expressed genes, early genes, follow the general trend of reduction in complexity. The absence of most abundant early male gametophyte-specific genes in tricellular pollen datasets clearly demonstrates this switch. On the contrary, a number of very abundant late genes are activated after PMII and the majority of highly expressed late genes show pollen-specific expression patterns. This switch to late program genes supports the previously published uniqueness of the late male gametophytic transcriptome (Honys and Twell 2003; 2004). This uniqueness can be semi-quantified by comparison of correlation coefficients between gametophytic and sporophytic transcriptome datasets (Table 3). Moreover, gene expression early in gametophyte development is significantly more similar to that in the sporophyte than to late male gametophytic developmental stages. This could suggest a significant contribution of pre-meiotic gene expression to the early gametophyte expression profiles.

3.4
Functional Characterisation of Male Gametophytic Genes

Male gametophyte development is under the control of two very different developmental programs. To evaluate more thoroughly the divergence of these programs, the dynamics of the distribution of male gametophyte-

expressed mRNAs between gene function categories was analysed. Twelve gene function categories were defined as described previously (Honys and Twell 2003).

3.4.1
Transcription in Microspores

The distribution of microspore-expressed genes among functional categories was similar to that of rosette leaves and all genes on the ATH1 microarray (Fig. 5). Among rare exceptions were genes involved in signaling and stress-responses ($\sim$ 1% under-represented) and genes involved in protein synthesis and transport ($\sim$ 1% over-representation). More striking differences were found when the distribution of expressed genes was represented according to their relative expression levels. This led to a dramatic expansion in the contribution of genes involved in protein synthesis from 5% to 14%. This highlights the importance of the protein synthesis program initiated early during male gametophyte development.

With some exceptions (e.g., some transcription factors and receptor proteins), we can expect many gametophytic genes with important functions to be relatively highly expressed. The contribution of such genes is more easily visualized when only mRNAs forming the high-abundance class[5] are considered (2197 genes). Within the high abundance class, genes involved in protein synthesis are again the most numerous (15% of all 2197 genes) and the most highly expressed (25% of overall signal intensity). The increase of other over-represented functional categories (metabolism, protein fate) was less dramatic with only a 1–3% increase in gene number and signal intensity. A different figure was obtained when only genes specific to the male gametophyte were analysed (423 genes). Highly expressed, but mostly constitutive genes involved in protein synthesis disappeared (only 2% genes and signal) and were replaced by newly emerging gene categories of cell wall metabolism (massive increase to 6% gene number and 12% signal) and transport (7% genes, 11% signal). Both of these categories were even more dominant among highly expressed genes, together comprising nearly one third of the overall expression signal.

3.4.2
Transcription in Developing Pollen

The transcriptome of mature pollen grains differs markedly from all other tissues and is accompanied by a significant reduction in complexity (Honys and Twell 2003, 2004). Moreover, although the down-regulation of most microspore-expressed genes is a general trend, a significant group of genes

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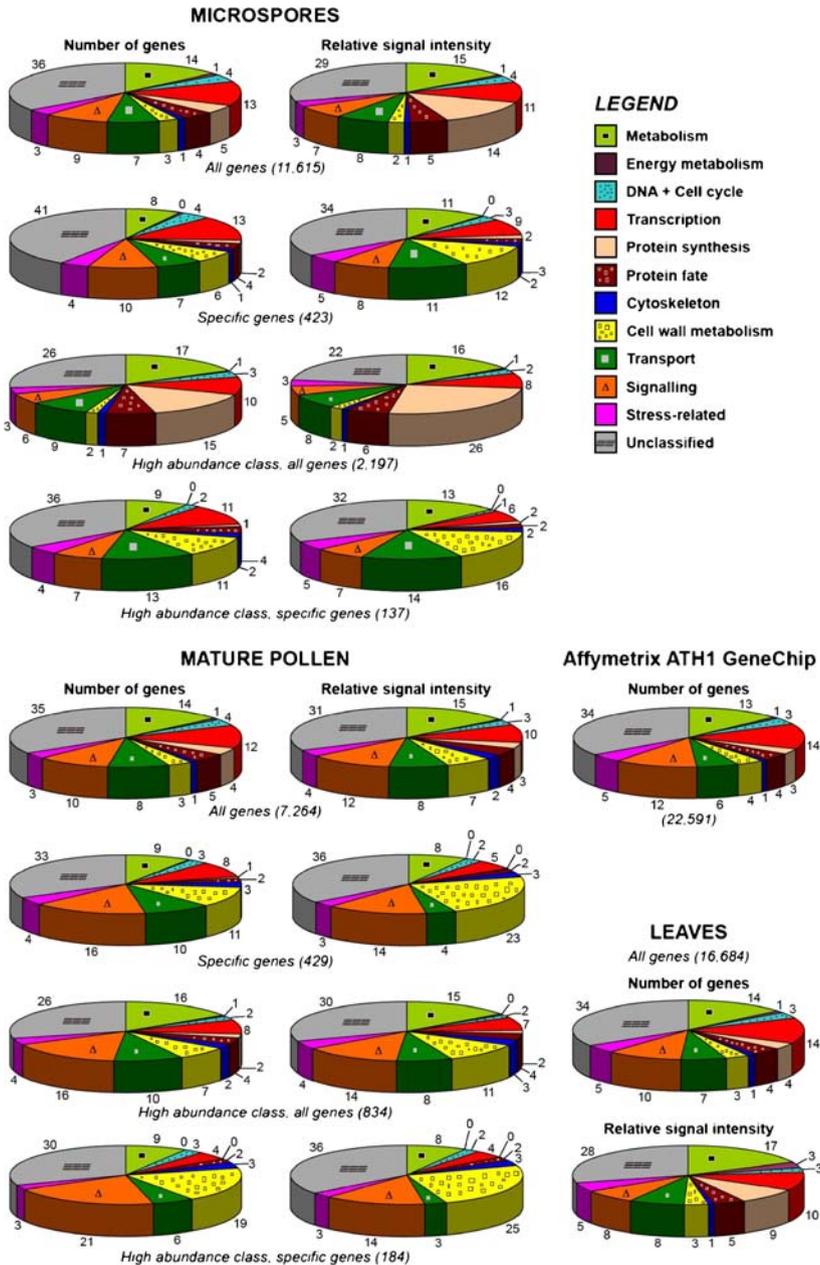[5] Genes with the expression signal up to tenfold less than the maximum signal.

Fig. 5 Distribution of expressed mRNAs among gene function categories. Proportional representation of both the number of genes and their relative expression signals among gene function categories is presented for early (microspore) and late (mature pollen) developmental stages. The equivalent distribution of all genes on the ATH1 array and genes expressed in leaves are shown as controls

are specifically up-regulated late during pollen development. However, this reduction in complexity and parallel activation of different gene sets is not uniform across gene function categories (Fig. 5). Compared to microspores, there was a massive reduction in the protein synthesis group. On the contrary, the most over-represented gene categories were cell wall metabolism, cytoskeleton and signaling. For example genes involved in cell wall synthesis and metabolism comprise more than 19% of highly expressed specific mRNAs (25% signal). Other important functional categories such as transport and stress-related, mostly contained less abundant non-pollen-specific genes. From this perspective, 23 K GeneChip experiments were confirmatory for mature pollen, since these gene function categories were found to be the most massively up-regulated based on analysis of 8K GeneChip data (Honys and Twell 2003).

What was new and surprising was the extent of transcription of mRNAs forming those up-regulated gene function categories. Since we knew that the majority of the most abundant pollen-expressed mRNAs were also pollen-specific (Fig. 4), they represent a relatively narrow set of genes. Moreover, there was an enormous increase in the average signal per gene in these categories. In sporophyte and early male gametophyte, the average signal/gene was within the range 300–500, whereas in late male gametophyte, this value increased to approximately 1000. With the exception of the protein synthesis category all other gene function groups were more abundantly represented among pollen-specific mRNAs. In particular, the average signal/gene for pollen-specific genes involved in cell wall metabolism reached nearly 6000. All these findings unequivocally confirm the bias of male gametophytic gene expression towards functional specialization required for the proceeding progamic phase involving storage, signaling and rapid pollen tube growth.

3.4.3
Post-transcriptional Regulation

Pollen germination in many species has been shown to be largely independent of transcription but vitally dependent on translation (see Twell 1994, 2002). *Arabidopsis* was shown to follow this general trend (Honys and Twell 2004) and there is compelling evidence that many mRNAs are stored in preparation for translation during tube growth (reviewed in Twell 2002). The late accumulation of pollen mRNAs and the presence of full-length transcripts in pollen tubes demonstrate that many mRNAs survive pollen dehydration and rehydration, some of which are stored as unique mRNP particles, EPPs (Honys et al. 2000).

Developmental transcriptomic studies prove the existence of a large number of stored mRNAs in mature pollen. Most abundant late pollen-expressed transcripts up-regulated after pollen mitosis II fall into this category and represent a stored mRNA charge. These studies also revealed that it is not only

mRNA that are stored but also the pre-formed protein synthesis machinery. Most protein synthesis genes are down-regulated in mature pollen and *Arabidopsis* pollen tubes are strictly dependent on ongoing translation (Honys and Twell 2004).

Transcriptomes and proteomes often differ and the absence of certain genes in transcriptomic data does not necessarily mean the absence of their protein products; transcriptomic data also demand experimental proof. For example a recent hypothesis of inactivation of the small RNA pathway in late male gametophyte (Pina et al. 2005) already deserves revision. This hypothesis was formulated on the absence of mRNAs encoding proteins involved in the small RNA pathway in mature pollen. Those mRNAs were found to be present earlier in male gametophyte development and to follow the general trend of down-regulation (Honys and Twell 2004). Our re-analysis[4] of all available mature pollen datasets revealed that one gene (At2g32940) was expressed in Landsberg pollen (Honys and Twell 2004), two genes (At1g48410, At2g27040) were active in the AtGE73 dataset from Columbia pollen (Zimmermann et al. 2004) and At5g21150 was expressed in all three datasets. Surprisingly, application of the normalisation algorithm[6] used by Pina et al. (2005) still identified three of these genes (At1g48410 (Argonaute 1), At2g32940 At5g21150) as active in the AtGE73 datasets from the same ecotype (Zimmermann et al. 2004). Such inconsistencies illustrate the importance of inclusion of all publicly available datasets in such analyses. Moreover, there are examples of the successful application of RNAi approaches in the male gametophyte (Gupta et al. 2002; S-A. Oh, M. Das, J.A. Johnson and D. Twell unpublished results). Taken together, available experimental and developmental transcriptomic data favor the function of the small RNA pathway at earlier stages in development and its persistent activity analogous to the presynthesis and utilization of the protein synthesis machinery in pollen tubes.

3.5
Conclusions from Transcriptomic Studies

Pollen transcriptomic studies provide the first comprehensive genome-wide view of the complexity of gene expression and its dynamics during single cell development in plants. Male gametogenesis is accompanied by large-scale repression of gene expression associated with the termination of cell proliferation and the selective activation of new groups of genes involved in maturation and post-pollination events. Development is also associated with major early and late transcriptional programs and the expression of around 600 putative transcription factors as potential regulators. These data

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[6] MAS5 detection algorithm, dChip 1.3 software (http://www.dchip.org; Li and Wong 2001), inclusive treatment of replicates for identification of expressed genes

also highlight a diminished role for transcription and the important role of mRNA and protein storage in mature pollen. Over representation of genes involved in cell wall metabolism, cytoskeleton and signaling highlight the functional specialization of pollen in preparation for key progamic functions including recognition of target tissues and rapid directional pollen tube growth.

4
Perspectives – Integrating Genetic and Transcriptomic Data

Genetic screens for male gametophytic mutations are far from reaching saturation given that almost all mutations are represented by single alleles. Estimates of the number of genes that are essential for male gametophyte development including the progamic phase can be based on equivalent segregation ratio distortion screens in which all mutants affecting transmission were characterized (Lalanne et al. 2004; Johnson et al. 2004). Based on the assumption that approximately 180 000 T-DNA insertions are required to achieve saturation, Johnson et al. (2004) estimated that $\sim$ 330 genes would be identified. Similar calculations for the transposon-based Ds screen (Lalanne et al. 2004) would predict $\sim$ 575 genes. However the proportion of mutants affecting development in these two screens varied from 10% (T-DNA) to 30% (Ds) corresponding to $\sim$ 30 to 170 genes respectively. In stark contrast, estimates of the total number of genes expressed throughout male gametophyte development based on microarray data are $\sim$ 11 000 to 14 000 (Honys and Twell 2004). Moreover, because of the frequent co-expression of gene family members in developing pollen (Honys and Twell 2004), genetic redundancy is likely to be a major feature and will be a significant limitation to genetic screens. To combat this deficiency, comprehensive transcriptome data can now be used to accelerate and target functional studies using reverse genetics. Targeted genetic approaches include assembly of selected multiple knockout mutants based on co-expression data. An alternative solution for closely related genes likely to be redundant is to down regulate multiple family members using antisense or RNAi approaches. This will be made more effective if cell-and stage-specific promoters can be identified based on microarray expression profiles. Promoters that are specifically active in generative and sperm cells and at late stages in pollen development have now been identified (Engel et al. 2005; Rotman et al. 2005), and microarray data analysis has enabled the isolation of promoters specifically active at microspore stage (D. Honys, S-A Oh, D. Twell, unpublished). In conclusion, the integration of established genetic approaches and resources with recent trancriptomic data analysis heralds an exciting new era in which the comprehensive identification of male gametophytic gene functions is a realistic goal.

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