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The *male sterility 8* mutation of maize disrupts the temporal progression of the transcriptome and results in mis-regulation of metabolic functions

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SUMMARY

Maize anther ontogeny is complex with expression of more than 30,000 genes over four days of cell proliferation, cell fate acquisition, and the start of meiosis. Although many male-sterile mutants disrupt these key steps, few have been investigated in detail. The terminal phenotypes of maize *male-sterile 8* (*ms8*) are small anthers exhibiting meiotic failure. Here we document much earlier defects: *ms8* epidermal cells are normal in number but fail to elongate, and there are fewer, larger tapetal cells that retain rather than secrete their contents. *ms8* meiocytes separate early, have extra space between them occupied by excess callose, and the meiotic dyads abort. Thousands of transcriptome changes occur in *ms8* including ectopic activation of genes not expressed in fertile siblings, failure to express some genes, differential expression compared to fertile siblings and about 40% of the differentially expressed transcripts appear precociously. There is a high correlation between mRNA accumulation assessed by microarray hybridization and qRT-PCR. Sixty-three differentially expressed proteins were identified after 2-D gel electrophoresis followed by LC/MS/MS (liquid chromatography tandem mass spectroscopy), including those involved in metabolism, plasmodesmatal remodeling, and cell division. The majority of these were not identified by differential RNA expression demonstrating the importance of proteomics to define developmental mutants.

Keywords

maize; *ms8*; anther; meiosis; microarray; proteomics

INTRODUCTION

In flowering plants, pollen production first requires cell proliferation and differentiation to produce an anther, then successful completion of meiosis in a subset of centrally-located anther locule cells, and finally the redifferentiation of anther somatic cells to support gametophyte maturation (Mascarenhas, 1990). These processes require coordinated gene expression among multiple cell types over 30 days in maize (Skibbe and Schnable, 2005). Maize anthers are bilaterally symmetrical with four lobes; cells divide and differentiate within each lobe to form four distinct somatic cell types (exterior epidermis, endothecium,

middle layer, and innermost tapetum (Ma *et al.*, 2008) surrounding the pollen mother cells (PMC). The tapetum plays a pivotal role in nutrient and enzyme secretion to the PMC (Goldberg *et al.*, 1993). Nuclear male sterility has been reported in many flowering plants, and in most cases mutations affect primarily tapetal or microspore development (Juan *et al.*, 2005; Xu *et al.*, 2006; Wang *et al.*, 2009).

Despite the existence of male-sterile mutants in *Arabidopsis*, tomato, rice, maize and other species, only a few genes that control the early phases of cell proliferation, cell fate setting, and initial differentiation have been identified. Furthermore, cloned genes represent diverse functions such as putative signaling pathways and metabolic enzymes rather than a cascade of transcription factors. For example, *Arabidopsis* *BAM1* (*BARELY ANY MERISTEM*) and *BAM2* encode Leu-rich repeat receptor-like kinases; *bam1* and *bam2* mutations affect cell division and differentiation patterns that result in the absence of endothelial, middle and tapetal layers (Hord *et al.* 2006). Similarly *Arabidopsis* *EXS* (*EXTRA SPOROGENOUS CELLS*) encodes a putative receptor thought to interact with a protein ligand encoded by *TPD1* (*TAPETUM DETERMINANT1*) Canales *et al.*, 2002; Yang *et al.*, 2003). *GhACS1* is a cotton anther-specific acyl-CoA synthetase (ACS) required for normal fatty acid metabolism in PMC and tapetal cells. Suppression of *GhACS1* expression severely affected the tapetal cells and microsporogenesis in early anther development (Wang and Li, 2009). *Male sterile converted anther 1* (*msca1*) encodes a maize (*Zea mays*) glutaredoxin (U. S. Patent # 20090038027) in which the anther has normal morphology, but all cells differentiate as leaf cell types (Chaubal *et al.*, 2003); normally, early leaf development genes are expressed in very immature anthers and then turned off, however, expression of these genes persists in *msca1* (Ma *et al.*, 2007). Additional genes involved in early *Arabidopsis* and maize anther development are summarized in Table S1.

Despite the importance of pollen in crop yield and hybrid seed production, molecular analysis of early anther development exploiting maize mutants was initiated only recently (Ma *et al.*, 2007). Maize has several advantages, primarily the large number of anthers per tassel and the lack of female floral parts in the tassel (Bedinger and Fowler, 2009). Relative to most angiosperms, maize anthers are larger, easier to dissect, and cellular events are highly synchronous within each anther. Male-sterile mutants that disrupt the normal pattern of cell proliferation and differentiation prior to meiosis can be highly instructive in charting steps in cell fate acquisition and function, because specification of anther cell types is completed by the onset of meiosis. *ms8* was first recognized for meiotic failure and lack of pollen (Beadle, 1931; Albertsen and Phillips, 1981), however, anther locule cellular organization, gene expression, and biochemical features have yet to be investigated. The current study was designed to quantify *ms8* defects cytologically and then to link the defects to transcriptome and proteome alterations with the additional goal of testing whether transcriptome changes were predictive of protein abundance changes.

RESULTS

ms8 anther development

ms8 plants are indistinguishable from fertile siblings during vegetative and ear development, however, *ms8* tassels have fewer branches, anthers do not exert, and no viable pollen is produced (Figure 1a). When fertile siblings shed pollen, *ms8* anthers are senescing. *ms8* anthers often adhere and cease elongating at 3.0 mm, about 60% of normal final length. Dwarfing of *ms8* could reflect early cessation of cell division and/or failure to sustain cell expansion. By scanning electron microscopy at 3.0 mm, *ms8* epidermal cells were well organized and the number of cells was similar to fertile siblings. Normal epidermal cells elongate longitudinally after meiosis and reach 85 μ m at the microspore stage, whereas *ms8* epidermal cells arrest at an average length of 52 μ m (Figure 2b). Therefore, smaller *ms8*

epidermal cells are one explanation for shorter anthers, but there is no substantial defect in longitudinal cell division.

Key stages of anther ontogeny relative to fertile anther length are summarized in Figure 1b. At 1.0 mm during rapid mitotic proliferation, there were no apparent cytological differences between *ms8* and fertile sibs (Figure 1b). At this stage there are four somatic wall layers of typical cell morphology. The endothecium and middle layers contain flattened cells elongated periclinally; neighboring tapetal cells are cuboidal with a denser cytoplasm. The presumptive pre-meiotic cells (PMC) at the center of each locule are encased in callose (Figure 1b). At the 1.5 mm stage, pollen mother cells partially separate and advance into meiotic prophase I in normal anthers (Figure 1b). Within *ms8* anthers, there was more space between the meiocytes, and this was occupied by excess callose (Figure 1b). Normal 2.0-2.5 mm anthers had meiocytes progressing through meiosis (Figure 1b); dyads were round and intact, with no gap between these two daughter cells (Figure 1c, 2a). In contrast, *ms8* meiocytes completed the first meiotic division but the dyads were smaller ellipsoids, with gaps between cells; the two daughter cells separated and collapsed soon after (Figure 1b, c, 2a). In subsequent stages, when fertile tetrads released and normal young microspore developed an exine, intine and germ pore (Figure 1c), there was only cellular debris in the center of *ms8* locules (Figure 1b, c), indicating that meiotic cells had disintegrated.

From transverse sections (Figure S1), ~26 tapetal cells are present in 1.0 mm fertile locules, gradually increasing to ~40 (Figure 2c); only a subset of tapetal precursors present at 1.0 mm undergo a subsequent mitosis. In terms of morphology, both cell thickness (diameter in the radial dimension, Figure 2d) and width (diameter within the tapetal ring, Figure 2e) were relatively constant in early stages. After meiosis, however, the normal tapetum thins and begins to degenerate (Figure 1b, 2d). Much of the dark-staining contents are secreted to coat pollen, and subsequently tapetal cells appear nearly empty. In contrast, *ms8* had only ~20 tapetal cells at the 1.0 mm stage, increasing to ~28 at the post-mitotic 1.5 mm stage. Thus, there are fewer cell divisions and an early cessation of cell division (Figure 2c), indicating a different contributing factor to the dwarf size of *ms8* anthers in this cell layer compared to the epidermis.

At the 1.0 and 1.5 mm stages, the external morphologies of *ms8* and fertile anthers are virtually identical. Maintenance of anther girth appears to reflect excessive growth of the smaller number of *ms8* tapetal cells (Figure S1, a1 – a3). Cell length (radial diameter) was twice that of normal and width was slightly greater when anther length was 2.5-3.0 mm (Figure 2d, e, i, g). Consequently, *ms8* tapetal cells are very large, occupying much of the locule space, whereas the debris from degenerating meiotic cells occupies only a small area relative to meiotic cells in fertile siblings (Figure 1b). Unlike bi-nucleate normal tapetum (Figure 2a), a majority of *ms8* tapetal cells have a single nucleus (Figure 2a). Normal tapetal cells degenerate during microspore maturation (Figure 1b, 2a), whereas mutant tapetal cells did not shrink (Figure 1b) and few degraded nuclei were found at the 3.0 mm stage (Figure 2a).

Locule transverse area was approximated by multiplying two diameter measurements. Despite the abnormalities of *ms8*, locule size (Figure 2f - h) was equivalent to fertile siblings before meiosis. Thereafter, fertile locules continued growing in girth whereas the *ms8* mutant locule area decreased after the 2.0 mm stage. Reduced locule area was caused by extra thick tapetal cells and cell layers expansion failure. There is also a shape change: *ms8* locules are oval but fertile locules are round (Fig 1b).

Transcriptome diversity quantified by microarray hybridization

RNA samples from fertile and sterile anthers were hybridized to custom Agilent 4×44 microarrays containing 42,034 probes (excluding controls) to 39,162 genes (Skibbe *et al.*, 2009). Fertile anthers exhibit astonishing transcript complexity: there are 27,400 constitutively expressed genes, 2143 stage-specific, and 2484 expressed at two stages summing to 32,037 genes expressed over a 90 h period. This is more than 75% of the projected maize gene number. Highest diversity occurs at 1.5 mm (Figure 3a, b, S2a), confirming previous studies (Ma *et al.*, 2006; Ma *et al.*, 2008). Unlike these prior studies, we did not observe a 10% drop in transcript types at 2.0 mm. During the initial days of meiosis anther length is constant; hence size-based staging is not as accurate as cytology. We selected anthers at metaphase I of meiosis (Figure 1b); this narrower pooling allowed a greater depth of transcript detection than pooling 2.0 mm anthers.

As shown in Figures 3b and S2a, at 1.0 mm there are 197 stage-specific transcripts, 780 at 1.5 mm, and 1166 at 2.0 mm. During the 1.0 - 1.5 mm transition, which spans about 72 h (Ma *et al.*, 2008), there is a major transcriptome change: 1799 new transcripts (grey checked bar in Figure 3b) and 523 transcripts expressed in the 1.0 mm anther are not detectable at 1.5 mm (black checked bar in Figure 3b). At the subsequent 1.5 - 2.0 mm transition, which spans about 18 h (Ma *et al.*, 2008), 326 transcripts appeared (grey checked bar in Figure 3b), 780 transcripts (black checked bar in Figure 3b) found at 1.5 mm disappeared, and 359 transcripts (black bar in Figure 3b) present at both the 1.0 and 1.5 mm stages are no longer detectable.

The transcriptome of *ms8* anthers is also very large: 27,284 (72%) transcripts are shared across all three *ms8* anther stages, similar to fertile siblings (Figure 3a, b, S2b). Strikingly, the 1.0 mm *ms8* anther expresses nearly 2000 more genes than fertile, and many of these are stage-specific within the context of *ms8* development (Figure 3b). Although *ms8* anthers are defective, the transcriptome is highly dynamic because new transcription factors (5% [113/2224] of the stage-specific genes) and predicted nucleic acid binding and protein binding classes are expressed. Differential expression between *ms8* and fertile siblings was evaluated using a minimum fold change of 1.5 as calculated using the limma package in R (Smyth, 2005). As shown in Table S2, there are 3196 differentially regulated genes at 1.0 mm, representing reprogramming of 11% of the normal transcriptome (3196/29,124). The significant impact of the *ms8* mutation persists through the subsequent two stages with 3748 (12%) genes differentially regulated at the 1.5 mm stage, and 3328 (11%) differentially regulated at the 2.0 mm meiotic stage. Among the differentially regulated transcripts at the 1.0 mm stage, *ms8* anthers had fewer down-regulated (25%) than up-regulated (75%) genes whereas the opposite pattern occurs in the 1.5 and 2.0 mm stages (Table S2).

ms8 anthers develop precociously, achieving some developmental landmarks at a smaller size. Interestingly, among the 902 1.0 mm stage-specific transcripts of *ms8* anthers (grey bar in Figure 3b), 416 (46%) are expressed at later stages in normal anthers (Table S3); more than 41% (133/322) of the 1.5 mm stage-specific *ms8* transcripts are expressed at the 2.0 mm stage in fertile siblings (Table S4). Consequently, one aspect of differential gene expression in *ms8* is temporal acceleration of some transcriptome programs. This likely disrupts the temporally coordinated growth and differentiation of *ms8* anther cells resulting in the observed defects in cell division (tapetum) and cell elongation (epidermis).

Distinctive and shared features of transcriptome patterns of *ms8* and fertile anthers

The relationship between *ms8* and fertile was further explored by k-means clustering. For example, among fertile genes expressed constitutively at high levels (1000-fold above the median), 150 genes in *ms8* were down-regulated dramatically (average 4-fold) in 1.5 mm

anthers but expressed equivalently in the preceding and following stages (Figure 4a). Within this cluster are genes related to cell division, including cell division protein FtsH (TC286699) and T cytoplasm *male sterility restorer factor2* (*Rf2*). Aniline blue staining and quantitative assays detected more callose around the mutant meiocytes at the 2.0 mm stage than in fertile siblings (Figure 5, compare c and d to g and h, i). Ninety-five genes are down-regulated in the 2.0 mm *ms8* anther (Figure 4b), including β -D-glucosidase (Figure 5j), which is secreted by normal tapetum to dissolve callose (Bucciaglia and Smith, 1994). We hypothesize that excess callose accumulates because of a lack of degradation. There are 907 genes up-regulated in *ms8* during the 1.5 mm stage (Figure 4c). MYB transcription factors have been identified as important in cell fate setting (Pastore *et al.*, 2008), and 10 such genes show increased expression in 1.5 mm *ms8* anthers relative to fertile during the final step in cell fate setting in maize anther development (Table S5).

Zinc finger transcription factors are among the best studied plant DNA binding proteins, and several play important roles in floral development. In *Petunia hybrida*, seven family members were activated sequentially during anther development, and it was proposed that they might act as a regulatory cascade (Kobayashi *et al.*, 1998). Disruption of specific zinc finger genes causes floral abnormalities, i.e., mutations in *Arabidopsis rabbit ear* (*rbe*) (Takeda *et al.*, 2004) and rice *rid1* (Wu *et al.*, 2008). Previously, 253 unique zinc finger-related probes were examined from the maize 1.0 mm anther stage through mature pollen (Ma *et al.*, 2008), and both stage-specific and persistent expression patterns were observed. All but 28 of the 253 genes queried were expressed in at least 1 of the 3 stages examined here, and the majority ($186/253 = 74\%$) are expressed constitutively in both *ms8* and fertile anthers (cluster 1, Figure 6). A few family members are differentially expressed: TC314427 increases 10-fold from the 1.0 to 2.0 mm stages in both fertile and *ms8* anthers (purple line, cluster 3, Figure 6). In comparing *ms8* to fertile anthers, eight zinc finger family genes are differentially expressed in at least one stage (Table S6). Interestingly, zinc finger transcript abundances are very diverse; they range in expression from low to moderately abundant (~ 8 -fold above the median, cluster 1, Figure 6) to ~ 10 -12 fold above the median (clusters 2 and 3), and six express at exceptionally high levels (cluster 4).

Is there mis-expression of marker genes in specific anther cell types?

Previously *msc1*, *mac1* (*multiple archesporial cells1*), and *ms23* (*male sterile23*) were compared to fertile siblings in 1.0 to 2.0 mm anthers (Ma *et al.*, 2007). All three mutants lack a cytologically normal tapetum and were missing ten transcripts present in normal siblings; these 10 were designated tapetal marker genes. Six of these probes were queried in the current study: five had the 1.5 mm valley pattern, with a dramatic (>4 -fold) decrease in *ms8* compared to normal followed by an increase when meiosis began (Figure 7). Among them, only gene TC290478 was confirmed to be expressed in epidermal and tapetal cells by laser micro-dissection and microarray analysis (D. S. Skibbe, pers. communication); it decreased dramatically in *ms8* at the 1.5 mm stage. A sixth probe (TC314200) was low at the 1.0 mm stage in *ms8* relative to fertile sibs and increased at subsequent stages, exceeding wild type levels at the 2.0 mm stage. The *ms8* mutant has a recognizable tapetal layer albeit with fewer cells (Figure 2c), and these cells accumulate but never secrete dark-staining material (likely protein, Figure S1, a1 - a3). The array data indicate that tapetal cellular differentiation is disrupted, and the transcriptome pattern of 6 tapetal marker genes is abnormal at the 1.5 mm stage.

To address the contribution of an individual cell type to the distinctness of the *ms8* transcriptome, the sets of cell-type specific genes from three laser-microdissected cell types (i.e., the epidermis, tapetum and meiocytes) from 2.0 mm fertile anthers (D. S. Skibbe, pers. communication) were compared to the *ms8* transcriptome. The *ms8* mutant is 96 - 98% similar quantitatively to a normal anther for the cell type-specific transcripts assessed

(epidermis = 632 out of 659; tapetum = 451 out of 459; meiocytes = 1944 out of 2007); most differences between *ms8* and fertile siblings reflect mis-expression of transcripts expressed in more than one cell type at 2.0 mm rather than cell type-specific transcripts. Considered along with the measured impacts on growth in epidermal and tapetal cells it is clear that the *ms8* mutation affects multiple cell types.

qRT-PCR with primers for a panel of 22 genes was used to determine the validity of specific conclusions such as constitutive expression or differential regulation of *ms8* relative to fertile. Sixteen constitutive genes that maintained the same quantitative expression levels across all three stages were confirmed by qRT-PCR analysis of fertile and *ms8* samples. Six genes differentially expressed in at least one stage also were consistent with the microarray data (Figure S3, Table S7).

Identification of proteins with altered abundance by 2-D DIGE and LC/MS/MS

Anthers from the same family collected for array hybridization were used for 2D-DIGE, comparing *ms8* to normal siblings. For each stage, IEF was performed using two overlapping pH gradients, pH 4-7 and pH 6-11, analyzed on two different gels to increase the probability of separating proteins with similar isoelectric points. Typically, for pH 4-7 strips (24 cm), about 2700 spots were analyzed plus about 2200 spots for pH 6-11 strips (18 cm, data not shown). In the comparisons, virtually all resolved spots are yellow indicating a similar intensity (Figure S4). Eighty-five protein spots with differential intensities of more than 1.5-fold in *ms8* to fertile comparisons were detected across the three stages: 68 were higher in *ms8* anthers and 17 were lower (data not shown). For some spots, differential expression at several stages was detected (Table S8). After MS/MS peptide sequencing, 63 unique gene products were identified (Table S8) and functional annotations were summarized in Figure S5; proteins associated with meiosis and tapetal activities in other plants were found to be differentially expressed in *ms8* (Table S9).

As observed in transcriptome studies, the highest representation of differentially expressed proteins is in metabolism: 24 proteins are up-regulated and 5 are down-regulated in *ms8* anthers (Table S8). During microsporogenesis, the tapetum supplies nutrients to sporogenous tissue (Regan and Moffatt, 1990) preceded by synthesis of free fatty acids, polar lipids, and neutral esters stored in elaioplasts and lipid bodies (Piffanelli and Mudrph, 1998). ATP citrate lyase (Elshourbagy *et al.*, 1992) is critical in fatty acid synthesis and is elevated in *ms8* anthers. Two down-regulated proteins are involved in amino acid metabolism: homocysteine S-methyltransferase-3 and cysteine synthase. Several tapetum-specific markers are cysteine-rich proteins proposed to be components of a regulatory network in young anthers (Tzeng *et al.*, 2009); synthesis of such anther-specific regulators might be affected by altered amino acid levels. Starch is synthesized in anthers before meiosis and subsequently hydrolyzed to provide energy for lipid synthesis in both tapetum and microspores (Vizcay-Barrena and Wilson, 2006). Aldolase1 is also increased in *ms8* anthers, which could alter levels of sugar and starch, two molecules key to biosynthesis and energy balance. UDP-glucose-4-epimerase is higher in *ms8* anthers; this enzyme catalyzes the reversible epimerization of UDP-galactose and UDP-glucose. Elevated enzyme levels in *ms8* anthers might cause low UDP-galactose *in vivo* and affect the biosynthesis of galactose-containing cell-wall carbohydrates (Dormann and Benning, 1998). Phospholipase D increases 2.82 fold at the 1.5 mm stage in *ms8* anthers, when mutant meiocytes had already entered prophase I. Activation of this enzyme triggers plant microtubule reorganization in interphase and during preprophase of mitotic cells (Dhonukshe *et al.*, 2003), hence the up-regulation of this protein in *ms8* at the 1.5 mm stage could contribute to abnormal proliferation in the tapetum or aberrant features of meiosis.

Besides metabolic pathways, proper anther development requires diverse regulatory processes. 14-3-3 proteins are phosphoserine-binding proteins that regulate targets via direct protein-protein interactions, modulating among other processes plasma membrane H-ATPase, nitrate reductase, sucrose phosphate synthase, and diverse cytoplasmic enzymes (Chung *et al.*, 1999). A 14-3-3 like protein (GI: 162458469) is down-regulated in *ms8* 1.5-mm-stage anthers (Table S8), and another 14-3-3 like protein (GI: 226499592) was up-regulated from the 1.0-mm to 1.5-mm stage (Table S8, Figure S6). The aberrant abundances of such 14-3-3 factors could contribute directly to *ms8* defects. In *ms8* anthers, methyl binding domain protein expression was increased before and during meiotic prophase I, and this may be related to the incipient dissolution of the PMC as developmentally-regulated DNA methylation is crucial in plants (Zemach and Grafi, 2007). Prohibitins are evolutionarily conserved proteins (Takahashi *et al.*, 2003) that play key roles in cell cycle regulation, receptor-mediated signaling at the cell surface, senescence, tumor suppression, mitochondrial function and morphology (Narasimhan *et al.*, 1997; Nadimpalli *et al.*, 2000; Chen *et al.*, 2005). There are 4 distinct full-length prohibitin-like genes in maize (Nadimpalli *et al.*, 2000) and both prohibitin 1 and prohibitin 4 are up-regulated at the meiosis stage in *ms8* anthers (Table S8), another hallmark of dysfunction.

In eukaryotes, the spindle assembly checkpoint monitors the interaction between chromosomes and microtubules at the kinetochores, an elaborate mechanism required for correct chromosome segregation during mitosis (Caillaud *et al.*, 2009). We speculate that increased accumulation of mitotic checkpoint protein BUB3 (Table S8) in young *ms8* anthers subsequently affects spindle morphology/function in meiocytes and leads to abnormal meiosis. Phragmoplastin is a dynamin-like protein associated with cell plate formation in plants, and it is encoded by 8 types of maize ESTs (data not shown); in addition to mis-expression of mitotic checkpoint protein BUB3 that might affect phragmoplast expansion, accumulation of excess phragmoplastin (Table S8) may also disrupt meiotic progression because these cells are normally interconnected.

Congruence of *ms8* anthers array data to proteome analysis

We compared the *ms8*-regulated proteins identified by 2-D DIGE with the *ms8*-regulated RNAs pinpointed by microarray studies (Figure S3b). Probes corresponding to only eight of the 63 identified differentially expressed proteins had differential RNA accumulation: 14-3-3 like protein (226499592), aspartate aminotransferase, translation initiation factor 5A, protein disulfide isomerase, adenosylhomocysteinase, GTP-binding protein SAR1A, histone H2A, and elongation factor 1-alpha. For the remaining proteins, there was no significant change in RNA abundance. The small overlap between proteomics and microarray data highlights the inability of transcriptome profiling to identify changes in translational regulation, protein turnover kinetics, and post-translational modification. Therefore, the proteomics assessment yielded critical additional information augmenting understanding of the *ms8* phenotypes.

DISCUSSION

Previous studies classified *ms8* as defective in tetrad formation (Albertsen and Phillips, 1981). Based on cytological and morphometric analysis we find that *ms8* causes a dwarf anther with a cell expansion defect in the epidermis and cell proliferation and differentiation defects in the tapetum evident before meiosis initiates. Because excess callose is retained around the PMC and tapetal cells remain filled with material, it is likely that defective tapetal secretion starves meiotic cells resulting in abortive meiosis. A striking feature of anther development is high transcriptome diversity, observed in maize (Ma *et al.*, 2006; this study), *Arabidopsis* (Wijeratne *et al.*, 2007), and rice (Jung *et al.*, 2005). The progression of

gene expression programs is profoundly disrupted in *ms8*, because mutants express genes atypical of normal anthers and express many genes precociously.

Analysis of cell type-specific expression indicates that most epidermal, tapetal, and meiotic cell marker genes are expressed at quantitatively normal levels in *ms8* at the 2.0 mm stage. Therefore, the majority of differences between *ms8* and normal reflect mis-expression of transcripts expressed in more than one cell type at the 2.0 mm stage. *ms23* and *ms32* are defective because presumptive tapetal cells undergo a periclinal division forming two aberrant layers (Chaubal *et al.*, 2000); *ms8* is very distinctive from these strictly tapetal mutants in that a much higher fraction (>10%) of the *ms8* transcriptome is altered than in either tapetal mutant (~1%, Ma *et al.*, 2007; C. A. Young and V. Walbot, unpublished data). Furthermore, more than 40% of mis-expressed genes in *ms8* represent acceleration of developmental programs relative to fertile siblings. This insight indicates that precise temporal modulation is key to the progression of early anther development.

The 1.5 mm stage of *ms8* had the most significant changes compared to fertile in that transcriptome diversity was decreased, and there were the largest number of differentially expressed genes. *ms8* meiocytes cease meiosis and the tapetum is highly abnormal by the 2.0 mm stage -- phenotypic consequences of mis-expression at the 1.5 mm stage 18 h earlier. We propose that the 1.5 mm stage represents a decision point for anther developmental processes, which if aberrant result in meiotic failure.

Mutations affecting tapetal cell size and number have been reported across a broad spectrum of flowering plants (Ma, 2005), and almost all lead to an abortive meiosis and male sterility. *Arabidopsis TDF1* (*defective in tapetal development and function 1*) expresses an anther-specific transcript that encodes a putative R2R3 MYB transcription factor, which is indirectly required for callose breakdown. From the tetrad stage, *tdf1* tapetum cells are abnormally vacuolated and enlarged with excessive cell division (Zhu *et al.*, 2008). Mutation in rice *TDR* (*tapetum degeneration retardation*) encoding a putative basic helix-loop-helix protein caused tapetal cells to grow abnormally large, ultimately occupying the majority of the locule (Li *et al.*, 2006) as found in maize *ms8*. Both *tdf1* and *tdr* mutants have small locules of irregular shape (Li *et al.*, 2006; Zhu *et al.*, 2008), a feature shared by many other mutants (Sorensen *et al.*, 2003; Yang *et al.*, 2003; Albrecht *et al.*, 2005; Zhang *et al.*, 2006); however, locule dimension and cell count assays are available only for *ms8*, pinpointing specific defects. Meiotic failure is a routine feature among *Arabidopsis* mutants with tapetum developmental defects (Sorensen *et al.*, 2003; Yang *et al.*, 2003; Albrecht *et al.*, 2005; Zhang *et al.*, 2006; Zhu *et al.*, 2008); *ms8* dyads broke down and meiosis aborted, another example connecting tapetal abnormality to meiotic failure. Transcription profiling and proteomics data are not available for *tdf* and *tdr*, or other maize anther mutants in early stages for comparison to *ms8*. Therefore, this study provides a compilation of in-depth transcriptome and proteome changes during early anther development and will be a reference for future studies of additional mutants of maize and other species.

Callose, a β -1, 3-glucan polymer with β -1, 6-branches, is an insoluble polysaccharide, deposited onto the PMC at the start of meiotic prophase I. It reaches a maximum at the tetrad stage (Popova *et al.*, 2008). By aniline blue staining and quantitative analysis, *ms8* anthers accumulate more callose at 1.5 mm, which occupied the extra space between PMC, while at the 2.0 mm stage callose is so excessive that meiocytes are often crowded to one side of the locule. Callose accumulation appears to reflect failure of the tapetum to degrade and remodel this polysaccharide, because β -D-glucosidase is down-regulated in *ms8* mutants and no differentially regulated callose synthase genes were detected. Based on sequence homology, twelve *CALLOSE SYNTHASE* (*CalS*) genes have been identified in *Arabidopsis*, and five of them are required for microgametogenesis (Chen *et al.*, 2009). For

example, *CalS5* encodes a callose synthase protein responsible for the temporary deposition of the callose wall surrounding microspores. *CalS5* gene knockouts severely disrupted callose deposition and reduced fertility (Dong *et al.*, 2005). AtMYB103 is an R2R3 MYB protein that controls callose dissolution in *Arabidopsis*. In knockout mutants, callose persisted into the final stages of pollen maturation; such lines are male-sterile (Zhang *et al.*, 2007). Several studies have also described mutants in callose wall formation and dissolution in petunia (Izhar and Frankel, 1971; Warmke and Overman, 1972) and tobacco (Worrall *et al.*, 1992) that disrupt fertility. Collectively, the evidence indicates that the timing of callose formation and dissolution are critical for normal fertility, and *ms8* is a new example in maize of a callose defect.

Post-meiotic tapetal cells nourish microspores and deposit complex lipoidal mixtures in tryphine or pollenkit onto pollen (Bedinger, 1992). Several genes involved in lipid metabolism play important roles in *Arabidopsis* pollen development (Aarts *et al.*, 1997; Mou *et al.*, 2000). As was found in *ms8*, this class of proteins was also up-regulated in the tomato *7B-1* male-sterile mutant (Sheoran *et al.*, 2009). Aberrant regulation of enzymes involved in polysaccharide (plastid starch synthase I precursor), sugar (UDP-glucose-4-epimerase, Aldolase1), and lipid metabolism are hallmarks of *ms8* and other early-acting male-sterile mutants in flowering plants. Given the significant impact of the *ms8* mutation on the transcriptome and proteome during early maize anther development, *ms8* appears to be involved in the regulation of multiple metabolic pathways.

Experimental procedures

Plant material and microscopy

ms8 was obtained from P. Bedinger (Colorado State University, Ft. Collins CO) in the ND101 inbred and backcrossed four times into W23 and maintained in 1:1 segregating families (*ms8/ms8* × *ms8/+*) in summer fields at Stanford University or in greenhouses equipped with lights to replicate 50% of summer solar fluence. Samples were removed from 3–6 cm immature tassels by cutting through the leaf whorl; at maturity, dissected plants were scored for fertility. Only upper floret anthers were pooled from several spikelets after careful developmental staging and then sections prepared (Wang *et al.*, 2009) for bright field microscopy (Axioskop 40 Pol, Carl Zeiss MicroImaging, Inc, Germany) or stained with aniline blue (Aldrich Chemical Company, Milwaukee, WI) to visualize callose under UV illumination (Axioskop 40 Pol, DAPI channel). Callose was also quantified fluorimetrically in anther extracts (Kohle *et al.*, 1985). Meiocytes were observed by hematoxylin-iron-aceto-carmine staining (Chang and Neuffer, 1989). Scanning electron microscopy (FEI Quanta 200 scanning electron microscope, FEI company, Hillsboro, OR) images were obtained using a published protocol (Ohashi-Ito and Bergmann, 2006). Morphometric data were collected from four independent samples; four locules for each stage were examined, averaged, and the data analyzed in Excel (Microsoft, Redmond, WA). Propidium iodide (CalBiochem, San Diego, CA) was used at 20 µg/mL in PBS solution (pH 7.4) for 30 min, with 3 infiltrations for 1–2 min each to stain anther nuclei for laser-scanning confocal microscopy using a Leica SP5 (Leica Microsystem Inc, U.S.). Anther samples were imaged in water on a glass slide with a cover slip using 520 to 550 nm excitation and 600 to 650 nm emission. Cell dimension measurements were obtained from propidium iodide images and calculated by volocity software (PerkinElmer, MA, U.S.A.).

Microarray analysis and qRT-PCR assays

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) from pools of staged anthers, and labeled cRNA was prepared as described previously (Ma *et al.*, 2006) for hybridization on slides with 4 arrays each of 44K elements (Agilent Technologies, Santa

Clara, CA) using the Two-Color Microarray-Based Gene Expression Analysis protocol (Agilent). Four independent biological replicates at each developmental stage were analyzed with balanced dye labeling to minimize systematic variances (Table S10, Ma *et al.*, 2006; Kerr and Churchill, 2001). After hybridization according to the manufacturer's protocols, slide scanning, feature extraction, signal criterion, statistical and graphical criteria, and GO annotation were performed as described previously (Skibbe *et al.*, 2009). Normalized intensities for each probe were averaged for the 4 biological replicates and the resulting final expression data and gene annotation were summarized in Table S11. The log base 2 of the ratio of *ms8* versus fertile sibling expression was used for clustering differentially-expressed genes as described previously (Ma *et al.*, 2008). The log base 2 of the normalized expression values was used for clustering the zinc finger genes. Primer pairs (Table S7) were designed for selected genes, then qRT-PCR (quantitative real-time reverse transcriptase polymerase chain reaction) reactions and amplification conditions followed published protocols (Ma *et al.*, 2008).

2D-DIGE

Protein extraction and quality assessment were performed as reported previously (Skibbe *et al.*, 2009). Three biological replicates of each developmental stage were used to identify differentially abundant spots between *ms8* and fertile siblings after labeling protein aliquots with either Cy3 or Cy5 (GE Healthcare, NJ). Four replicates were used for microarrays, because a balanced dye swap design is required for maximal statistical power; this design feature is not required for the proteomics analysis because an internal reference containing the same proteins was included in every gel. A mixture of all samples was labeled with Cy2 (GE Healthcare) as this internal reference on all gels to improve the accuracy of quantitative comparisons (Alban *et al.*, 2003). An example of reproducibility is shown in Figure S4, utilizing a dye swap experiment of pairs of biological replicates. Gels were scanned using a Typhoon 8600 (GE Healthcare) with appropriate wavelengths for excitation and emission (Sheoran *et al.*, 2009). Images were processed with PROGENESIS PG220 software (Non-linear USA, <http://www.nonlinear.com>) with statistical analyses in this software. Spots with fold change ≥ 1.5 or ≤ -1.5 and *p* value < 0.05 were considered to be differentially expressed. 200 μ g aliquots of protein from *ms8* and its fertile counterpart plus 25 μ g of each sample labeled with Cy3 or Cy5 DIGE fluor were separated on a 2D-DIGE preparative gel. The gel was fixed and stained with Deep Purple (Fluorotechnics, <http://www.fluorotechnics.com>), spots of interest were edited with DeCyder software (GE Healthcare) and picked by an Ettan spot picker (GE Healthcare). In-gel tryptic digestion was done as described previously (Rosenfeld *et al.*, 1992, donatello.ucsf.edu/ingel.html).

Reversed-phase LC-electrospray tandem mass spectrometry (LC-MS/MS) analysis

Digests were separated by nanoflow LC as described (Oses-Prieto *et al.*, 2007) with minor revisions. LTQ-FT and LTQ-Orbitrap raw data were converted to ASCII peak lists by Mascot Distiller software v2.1.0.0 (Matrix Science, Boston, MA); the parameters for MS processing were used as described previously (Oses-Prieto *et al.*, 2007). The peak lists were searched in ProteinProspector version 5.3.2 (<http://prospector2.ucsf.edu/prospector/html/misc/revhist.htm>) (public version at <http://prospector.ucsf.edu>) using an existing protocol with minor updates (Deng *et al.*, 2007). The peak lists were searched using the NCBIInr database as of July 10, 2009, using all entries for *Zea*, *Oryza*, *Hordeum*, *Triticum*, *Secale*, and *Arabidopsis* (249,067 entries searched). Because the maize genome is not yet completely annotated, in some cases we relied on identification of peptides conserved in predicted proteins of plants with completely sequenced genomes (*Arabidopsis*, rice) or in cereals (*Triticum*, *Hordeum* and *Secale*) (Table S9). The acceptance criteria were described previously (Skibbe *et al.*, 2009); only proteins with at least 2 peptides identified were considered further. When several accessions matched

the same set of peptides, the entries with the most descriptive name were reported. Individual protein isoforms were reported when unique peptides were found; if isoforms shared the peptides, they were all reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Aarts MGM, Hodge R, Kalantidis K, Florack D, Wilson ZA, Mulligan BJ, Stiekema WJ, Scott R, Pereira A. The *Arabidopsis* MALE STERILITY 2 protein shares similarity with reductases in elongation/condensation complexes. *Plant J.* 1997; 12:615–623. [PubMed: 9351246]
- Alban A, David SO, Bjorkestén L, Andersson C, Sloge E, Lewis S, Currie I. A novel experimental design for comparative two-dimensional gel analysis: two dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics.* 2003; 3:36–44. [PubMed: 12548632]
- Albrecht C, Russinova E, Hecht V, Baaijens E, Vries S. The *Arabidopsis thaliana* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES 1 and 2 control male sporogenesis. *Plant Cell.* 2005; 17:3337–3349. [PubMed: 16284305]
- Albertsen MC, Phillips RL. Developmental cytology of 13 genetic male sterile loci in maize. *Genome.* 1981; 23:195–208.
- Beadle GW. Genes in maize for pollen sterility. *Genetics.* 1931; 17:413–431. [PubMed: 17246660]
- Bedinger P. The remarkable biology of pollen. *Plant Cell.* 1992; 4:879–887. [PubMed: 1392600]
- Bedinger, PA.; Fowler, JE. The maize male gametophyte. In: Bennetzen, JL.; Hake, SC., editors. *Handbook of Maize: Its Biology.* New York: Springer; 2009. p. 57-77.
- Bucciaglia PA, Smith AG. Cloning and characterization of *Tag 1*, a tobacco anther beta-1,3-glucanase expressed during tetrad dissolution. *Plant Mol Biol.* 1994; 24:903–914. [PubMed: 8204827]
- Caillaud MC, Paganelli L, Lecomte P, Deslandes L, Quentin M, Pecrix Y, Bris ML, Marfaing N, Abad P, Favory B. Spindle assembly checkpoint protein dynamics reveal conserved and unsuspected roles in plant cell division. *PLoS ONE.* 2009; 4:1–9.
- Canales C, Bhatt AM, Scott R, Dickinson H. *EXS*, a putative LRR receptor kinase, regulates male germline cell number and tapetal identity and promotes seed development in *Arabidopsis*. *Current Biol.* 2002; 12:1718–1727.
- Chang MT, Neuffer MG. A simple method for staining nuclei of mature and germinated maize pollen. *Biotechnic Histochemistry.* 1989; 64:181–184.
- Chaubal R, Anderson JR, Trimmell MR, Fox TW, Albertsen MC, Bedinger P. The transformation of anthers in the *msc1* mutant of maize. *Planta.* 2003; 216:778–788. [PubMed: 12624765]
- Chaubal R, Zanella C, Trimmell MR, Fox TW, Albertsen MC, Bedinger P. Two male-sterile mutants of *Zea mays* (Poaceae) with an extra cell division in the anther wall. *Am J Bot.* 2000; 87:1193–1201. [PubMed: 10948005]
- Chen JC, Jiang CZ, Reid MS. Silencing a prohibitin alters plant development and senescence. *Plant J.* 2005; 44:16–24. [PubMed: 16167892]
- Chen XY, Liu L, Lee E, Han X, Rim Y, Chu H, Kim SW, Sack F, Kim JY. The *Arabidopsis* callose synthase gene *GSL8* is required for cytokinesis and cell patterning. *Plant Physiol.* 2009; 150:105–113. [PubMed: 19286936]

- Chung HJ, Sehnke PC, Ferl RJ. The 14-3-3 proteins: cellular regulators of plant metabolism. *Trends Plant Sci.* 1999; 4:367–371. [PubMed: 10462770]
- Deng Z, Zhang X, Tang W, Osés-Prieto JA, Suzuki N, Gendron JM, Chen H, Guan S, Chalkley RJ, Peterman TK, Burlingame AL, Wang Z. A proteomics study of brassinosteroid response in *Arabidopsis*. *Mol Cell Proteomics.* 2007; 6:2058–2071. [PubMed: 17848588]
- Dhonukshe P, Laxalt AM, Goedhart J, Gadella TWJ, Munnik T. Phospholipase D activation correlates with microtubule reorganization in living plant cells. *Plant Cell.* 2003; 15:2666–2679. [PubMed: 14508002]
- Dong X, Hong Z, Sivaramakrishnan M, Mahfouz M, Verma DPS. Callose synthase (CalS5) is required for exine formation during microgametogenesis and for pollen viability in *Arabidopsis*. *Plant J.* 2005; 42:315–328. [PubMed: 15842618]
- Dormann P, Benning C. The role of UDP-glucose epimerase in carbohydrate metabolism of *Arabidopsis*. *Plant J.* 1998; 13:641–652. [PubMed: 9681006]
- Elshourbagy NA, Near JC, Kmetz PJ, Wells TN, Groot PH, Saxty BA, Hughes SA, Franklin M, Gloger IS. Cloning and expression of a human ATP-citrate lyase cDNA. *Eur J Biochem.* 1992; 204:491–499. [PubMed: 1371749]
- Goldberg RB, Beals TP, Sanders PM. Anther development: basic principles and practical applications. *Plant Cell.* 1993; 5:1217–1229. [PubMed: 8281038]
- Hord CLH, Chen C, DeYoung BJ, Clark SE, Ma H. The BAM1/BAM2 receptor-like kinases are important regulators of *Arabidopsis* early anther development. *Plant Cell.* 2006; 18:1667–1680. [PubMed: 16751349]
- Izhar S, Frankel R. Mechanism of male sterility in *Petunia*: The relationship between pH, callase activity in the anthers, and the breakdown of the microsporogenesis. *Theoret Appl Gen.* 1971; 41:104–108.
- Juan KH, Han MJ, Lee YS, Kim YW, Hwang I, Kim MJ, Kim Y, Nahm BH, An G. Rice *Undeveloped Tapetum1* is a major regulator of early tapetum development. *Plant Cell.* 2005; 17:2705–2722. [PubMed: 16141453]
- Kerr MK, Churchill GA. Statistical design and the analysis of gene expression microarray data. *Genet Res.* 2001; 77:123–128. [PubMed: 11355567]
- Kobayashi A, Sakamoto A, Kubo K, Rybka Z, Kanno Y, Takatsuji H. Seven zinc-finger transcription factors are expressed sequentially during the development of anthers in *petunia*. *Plant J.* 1998; 13:571–576. [PubMed: 9680999]
- Kohle H, Jeblick W, Poten F, Blaschek W, Kauss H. Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+} -dependent process. *Plant Physiol.* 1985; 77:544–551. [PubMed: 16664095]
- Li N, Zhang DS, Liu HS, Yin CS, Li XX, Liang WQ, Yuan Z, Xu B, Chu HW, Wang J, Wen TQ, Huang H, Luo D, Ma H, Zhang DB. The rice *tapetum degeneration retardation* gene is required for tapetum degradation and anther development. *Plant Cell.* 2006; 18:2999–3014. [PubMed: 17138695]
- Ma H. Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. *Annu Rev Plant Biol.* 2005; 56:393–434. [PubMed: 15862102]
- Ma J, Duncan D, Morrow DJ, Fernandes J, Walbot V. Transcriptome profiling of maize anthers using genetic ablation to analyze pre-meiotic and tapetal cell type. *Plant J.* 2007; 50:637–648. [PubMed: 17419846]
- Ma J, Morrow DJ, Fernandes J, Walbot V. Comparative profiling of the sense and antisense transcriptome of maize lines. *Genome Biol.* 2006; 7:1186/gb-2006-7-3-r22
- Ma J, Skibbe DS, Fernandes J, Walbot V. Male reproductive development: gene expression profiling of maize anther and pollen ontogeny. *Genome Biol.* 2008; 9:1186/gb-2008-9-12-r181
- Mascarenhas JP. Gene activity during pollen development. *Annu Rev Plant Physiol.* 1990; 41:317–338.
- Mou Z, He Y, Dai Y, Liu X, Li J. Deficiency in fatty acid synthase leads to premature cell death and dramatic alterations in plant morphology. *Plant Cell.* 2000; 12:405–417. [PubMed: 10715326]
- Nadimpalli R, Yalpani N, Johal GS, Simmons CR. Prohibitins, stomatins, and plant disease response genes compose a protein superfamily that controls cell proliferation, ion channel regulation, and death. *J Biol Chem.* 2000; 275:29579–29586. [PubMed: 10862763]

- Narasimhan S, Armstrong M, McClung JK, Richards FF, Spicer EK. Prohibitin, a putative negative control element present in *Pneumocystis carinii*. *Infect Immun*. 1997; 65:5125–5130. [PubMed: 9393805]
- Ohashi-Ito K, Bergmann DC. *Arabidopsis* FAMA controls the final proliferation/differentiation switch during stomatal development. *Plant Cell*. 2006; 18:2493–2505. [PubMed: 17088607]
- Oses-Prieto JA, Zhang X, Burlingame AL. Formation of ϵ -formyllysine on silver-stained proteins. *Mol Cell Proteomics*. 2007; 6:181–192. [PubMed: 17138591]
- Pastore J, Chavdaroff N, Wagner D. LMI2, a MYB transcription factor involved in the vegetative to reproductive transition in *Arabidopsis thaliana*. *Dev Biol*. 2008; 319:565–575.
- Piffanelli P, Murphy DJ. Novel organelles and targeting mechanisms in the anther tapetum. *Trends Plant Sci*. 1998; 3:250–253.
- Popova AF, Ivanenko GF, Ustinova AY, Zaslavsky VA. Localization of callose in microspores and pollen grains in *Sium latifolium* L. plants in different water regimes. *Cytol Gen*. 2008; 42:363–368.
- Regan SM, Moffatt BA. Cytochemical analysis of pollen development in wild-type *Arabidopsis* and a male-sterile mutant. *Plant Cell*. 1990; 2:877–889. [PubMed: 12354970]
- Rosenfeld J, Capdeville J, Guillemot JC, Ferrara P. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal Biochem*. 1992; 203:173–179. [PubMed: 1524213]
- Sheoran IS, Ross ARS, Olson DJH, Sawhney VK. Differential expression of proteins in the wild type and *7B-1* male-sterile mutant anthers of tomato (*Solanum lycopersicum*): A proteomic analysis. *J Proteomics*. 2009; 71:624–636. [PubMed: 19032992]
- Skibbe DS, Schnable PS. Male sterility in maize. *Maydica*. 2005; 50:367–376.
- Skibbe DS, Fernandes JF, Medzihradszky KF, Burlingame AL, Walbot V. Mutator transposon activity reprograms the transcriptomes and proteomes of developing maize anthers. *Plant J*. 2009; 59:622–633. [PubMed: 19453454]
- Smyth, GK. Limma: linear models for microarray data. In: Gentleman, R.; Carey, V.; Dudoit, S.; Irizarry, R.; Huber, W., editors. *Bioinformatics and Computational Biology Solutions using R and Bioconductor*. New York: Springer; 2005. p. 397–420.
- Sorensen AM, Kröber S, Unte US, Huijser P, Dekker K, Saedler H. The *Arabidopsis* *ABORTED MICROSPORES (AMS)* gene encodes a MYC class transcription factor. *Plant J*. 2003; 33:413–423. [PubMed: 12535353]
- Takahashi A, Kawasaki T, Wong HL, Suharsono U, Hirano H, Shimamoto K. Hyperphosphorylation of a mitochondrial protein, prohibitin, is induced by calyculin A in a rice lesion-mimic mutant *cdr1*. *Plant Physiol*. 2003; 132:1861–1869. [PubMed: 12913143]
- Takeda S, Matsumoto N, Okada K. *RABBIT EARS*, encoding a superman-like zinc finger protein, regulates petal development in *Arabidopsis thaliana*. *Development*. 2004; 131:425–434. [PubMed: 14681191]
- Tzeng JD, Hsu SW, Chung MC, Yeh FL, Yang CY, Liu MC, Hsu YF, Wang CS. Expression and regulation of two novel anther-specific genes in *Lilium longiflorum*. *J Plant Physiol*. 2009; 166:417–427. [PubMed: 19013663]
- Vizcay-Barrena G, Wilson ZA. Altered tapetal PCD and pollen wall development in the *Arabidopsis ms1* mutant. *J Exp Bot*. 2006; 57:2709–2717. [PubMed: 16908508]
- Wang D, Li C, Zhao Q, Zhao L, Wang M, Zhu D, Ao G, Yu J. *Zm401p10*, encoded by an anther-specific gene with short open reading frames, is essential for tapetum degeneration and anther development in maize. *Functional Plant Biol*. 2009; 36:73–85.
- Wang XL, Li XB. The GhACS1 gene encodes an acyl-CoA synthetase which is essential for normal microsporogenesis in early anther development of cotton. *Plant J*. 2009; 57:473–486. [PubMed: 18826432]
- Warmke HE, Overman MA. Cytoplasmic male sterility in sorghum. I. Callose behavior in fertile and sterile anthers. *J Hered*. 1972; 63:103–108.
- Wijeratne AJ, Zhang W, Sun Y, Liu W, Albert R, Zheng Z, Oppenheimer D, Zhao D, Ma H. Differential gene expression in *Arabidopsis* wild-type and mutant anthers: insights into anther cell differentiation and regulatory networks. *Plant J*. 2007; 52:14–29. [PubMed: 17666023]

- Worrall D, Hird DL, Hodge R, Paul W, Draper J, Scott R. Premature dissolution of the microsporocyte callose wall causes male sterility in transgenic tobacco. *Plant Cell*. 1992; 4:759–771. [PubMed: 1392594]
- Wu C, You C, Li C, Long T, Chen G, Byrne ME, Zhang Q. *RID1*, encoding a Cys2/His2-type zinc finger transcription factor, acts as a master switch from vegetative to floral development in rice. *Proc Nat Acad Sci USA*. 2008; 105:12915–12920. [PubMed: 18725639]
- Xu SX, Liu GS, Chen RD. Characterization of an anther- and tapetum-specific gene and its highly specific promoter isolated from tomato. *Plant Cell Rep*. 2006; 25:231–240. [PubMed: 16491381]
- Yang SL, Xie LF, Mao HZ, Puah CS, Yang WC, Jiang L, Sundaresan V, Ye D. The *TAPETUM DETERMINANT1* gene is required for cell specialization in the *Arabidopsis* anther. *Plant Cell*. 2003; 15:2792–2804. [PubMed: 14615601]
- Zemach A, Grafi G. Methyl-CpG-binding domain proteins in plants: interpreters of DNA methylation. *Trends Plant Sci*. 2007; 12:1380–1385.
- Zhang W, Sun Y, Timofejeva L, Chen C, Grossniklaus U, Ma H. Regulation of *Arabidopsis* tapetum development and function by *DYSFUNCTIONAL TAPETUM1* (*DYT1*) encoding a putative bHLH transcription factor. *Development*. 2006; 133:3085–3095. [PubMed: 16831835]
- Zhang ZB, Zhu J, Gao JF, Wang C, Li H, Li H, Zhang HQ, Zhang S, Wang DM, Wang QX, Huang H, Xia HJ, Yang ZN. Transcription factor *AtMYB103* is required for anther development by regulating tapetum development, callose dissolution and exine formation in *Arabidopsis*. *Plant J*. 2007; 52:528–538. [PubMed: 17727613]
- Zhu J, Chen H, Li H, Gao JF, Jiang H, Wang C, Guan YF, Yang ZN. *Defective in tapetal development and function 1* is essential for anther development and tapetal function for microspore maturation in *Arabidopsis*. *Plant J*. 2008; 55:266–277. [PubMed: 18397379]

Figure 1.

Phenotypic analysis of *ms8* anther locule and cell morphology. (a) External morphology. Samples were collected from families segregating 1:1 for fertility (*ms8*/+): sterility (*ms8*/*ms8*). A typical *ms8* tassel is thinner and has fewer tassel branches at maturity. Dissected sterile anthers are shriveled and brown, compared to fertile anthers, which are plump and pale yellow-green. Scanning electron microscopy was used to assess epidermal cell shape and number at the 3 mm stage of *ms8* and 4 mm stage of fertile individuals. Bar = 1 mm (white), bar = 100 μm (black). (b) Each spikelet encloses two florets with three anthers each; lower floret anthers develop more slowly. There are four anther locule wall layers: epidermis, endothecium, middle layer and tapetum. PMCs undergo meiosis and proceed through the dyad (two cells) and tetrad (four cells) stages. During meiosis, the PMCs and product cells are normally connected by plasmodesmata. After meiosis, haploid cells normally undergo two mitotic divisions to form pollen, the microgametophyte. Anther progression is assessed by two measures: anther length and differentiation of the innermost (pre-meiotic, meiotic, and then post-meiotic) cells. Cytological staging relative to maize anther length is shown above the locule photographs. Transverse sections of locules from

ms8 and from fertile plants at five developmental stages were assessed. Bar = 30 μ m for 1.0 mm and 1.5 mm anther locules; bar = 75 μ m for 2.0 – 3.0 mm anther locules. Arrows highlight the features of *ms8* and its fertile counterpart. (c) Meiocytes from *ms8* and from fertile anthers at different developmental stages were stained by hematoxylin-iron-aceto-carmin. *ms8* dyads were smaller ellipsoid, separated from each other and collapsed soon after. Bar = 30 μ m.

Figure 2.

Morphometric analysis of *ms8* and fertile anthers. (a) Propidium iodide stains nuclei in meiocytes (upper panels) and tapetal cells (lower panels). *ms8* anther at 2.2 mm length, ellipsoid dyads with gaps between them (arrow); fertile locules were filled with round dyads and tetrads. A majority of *ms8* tapetal cells have a single nucleus at 2.2 mm (arrow); they remain intact and reach maximum size when anthers are 3 mm. Most fertile tapetal cells are bi-nucleate at 2.5 mm, and they have collapsed and nuclear staining was faint at 3 mm. Bar = 50 μm. (b1) Epidermal cell dimension comparison of *ms8* and fertile anthers from 1.0 mm to 3.0 mm. (b2) Longitudinal and latitude directions are shown by arrows. (c – g) Morphometric comparison of *ms8* and fertile anthers. (c) The number of tapetal cells. (d) Tapetal cell thickness. (e) Tapetal cell width. (f) Locule area. (g) Dimensions of the central zone internal to the tapetum. (h) Illustrates the two measurements taken for the x (shorter diameter) and y (longer diameter) at five stages of *ms8* and fertile siblings. The measurement of tapetal cell thickness and width are shown in (i) and (g), respectively. Error bars indicate standard deviation.

Figure 3.

Transcriptome analysis of *ms8* and fertile siblings anthers. (a) Transcriptome sizes at three stages of *ms8* and fertile siblings anthers. (b) Analysis of transcriptome changes during fertile (left part) and sterile (right part) anther development; 27,400 transcripts shared by all stages of fertile anthers and 27,284 transcripts shared by all stages of *ms8* anthers are not shown. The y-axis of (a) shows the number of probes expressed, the y-axes of (b) show the number of probes; the x-axes list the developmental stages.

Figure 4. Differentially expressed transcripts grouped by k-means clustering. Transcripts constitutively expressed in fertile plants were clustered, resulting in the *ms8*/Fertile fold change patterns shown. (a) 150 probes were down-regulated at the 1.5 mm stage; (b) 95 probes were down-regulated at the 2.0 mm stage; (c) 907 probes were up-regulated at 1.5 mm. The y-axes are the log_2 ratio fold change of *ms8*/Fertile. The x-axes show the developmental stages.

Figure 5.

Aniline blue staining for callose in *ms8* and fertile sibs. (a – d) Fluorescence in *ms8* anthers under UV illumination. (e – h) Fertile counterparts under the same UV intensity. (a), (c), (e) and (g) Bright field and UV illumination. (b), (d), (f) and (h) UV only. (a), (b), (e) and (f) Anthers at 1.5 mm. More fluorescence was detected in *ms8* locules (a, b) than in fertile counterparts (e, f), which means excess callose surrounds *ms8* PMC. The callose occupied the extra space between separated *ms8* cells. (c), (d), (g) and (h) Anthers at 2.0 mm. More callose surrounds the multinucleated syncytium in *ms8* locules, and no separate meiocytes were observed (c and d). The normal meiocytes were well separated and covered with a thin coat of callose (g and h). (i) Callose comparison between *ms8* anther and fertile counterpart. The y-axis shows the callose amount, and the x-axis shows two developmental stages. (j) The expression pattern of β -D-glucosidase, an enzyme that is secreted by the tapetum to dissolve callose and release the tetrads. The y-axis is the fold change of *ms8*/Fertile in log₂ ratio, the x-axis shows the three developmental stages named by anther length.

Figure 6.

Clustering of zinc finger related gene expression. Genes are sorted after k-means clustering ($k = 4$). Arrows in cluster 3 show TC314427 increases 10-fold from the 1.0 to 2.0 mm stages in both fertile and *ms8* anthers. M1.0, 1.0 mm stage *ms8* anther; M1.5, 1.5 mm stage *ms8* anther; M2.0, 2.0 mm stage *ms8* anther. F1.0, 1.0 mm stage fertile anther; F1.5, 1.5 mm stage fertile anther; F2.0, 2.0 mm stage fertile anther.

Figure 7. Expression comparison of potential cell layer markers. Fold change pattern of previously published potential tapetum markers (Ma *et al.*, 2007), N=6. The y-axis is log₂ (fold change). The x-axis shows the anther developmental stages.