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Floret-specific differences in gene expression and support for the hypothesis that tapetal degeneration of *Zea mays* L. occurs *via* programmed cell death

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Abstract

The maize (*Zea mays*) spikelet consists of two florets, each of which contains three developmentally synchronized anthers. Morphologically, the anthers in the upper and lower florets proceed through apparently similar developmental programs. To test for global differences in gene expression and to identify genes that are coordinately regulated during maize anther development, RNA samples isolated from upper and lower floret anthers at six developmental stages were hybridized to cDNA microarrays. Approximately 9% of the tested genes exhibited statistically significant differences in expression between anthers in the upper and lower florets. This finding indicates that several basic biological processes are differentially regulated between upper and lower floret anthers, including metabolism, protein synthesis and signal transduction. Genes that are coordinately regulated across anther development were identified *via* cluster analysis. Analysis of these results identified stage-specific, early in development, late in development and bi-phasic expression profiles. Quantitative RT-PCR analysis revealed that four genes whose homologs in other plant species are involved in programmed cell death are up-regulated just prior to the time the tapetum begins to visibly degenerate (i.e., the mid-microspore stage). This finding supports the hypothesis that developmentally normal tapetal degeneration occurs *via* programmed cell death.

Keywords: anther development; programmed cell death; microarray; maize

Introduction

Male gametophyte development is a highly coordinated process that requires interactions between sporophytic and gametophytic anther tissues. In maize (*Zea mays* L. ssp. *mays*) anthers, these interactions have been extensively studied at the morphological level, and anther development has been shown to proceed through a series of well-defined

developmental stages consisting of two processes, microsporogenesis and microgametogenesis (Horner and Palmer, 1995; Wise et al., 1999; Ma, 2005). Microsporogenesis is divided into seven stages and begins with the sporogenous mass stage, which is defined as the first stage in which all of the anther tissues become distinguishable. This stage is followed by the meiocyte, dyad and tetrad meiosis-encompassing stages. During these stages, the innermost cell layer in the anther wall (i.e., the tapetal cells) undergoes a number of striking changes, including increases in cytoplasmic RNA and proteins (Horner and Palmer, 1995). At the meiocyte stage, the 2C sporogenous mass cells secrete callose walls interior to their primary walls, which disrupts the direct connection between the

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developing microspores and the tapetum. This is followed by the dyad (meiosis I) and tetrad (meiosis II) stages, ultimately producing four haploid microspores. At the mid-microspore stage, the tapetum begins to degenerate (Warmke and Lee, 1977), a process that has been hypothesized to be regulated by programmed cell death (PCD) (Wang et al., 1999). During the mid microspore stage the microspores continue to enlarge, their walls become even thicker, and their small vacuoles fuse into a large vacuole. The late-microspore stage marks the end of microsporogenesis. The remaining three stages (early, mid, and late pollen) encompass microgametogenesis and culminate in the production of mature pollen complete with energy reserves to be consumed during pollination.

Recent research on PCD has identified several key genes required for the establishment of PCD. For example, the rice *TDR* gene encodes a bHLH protein that acts as a positive regulator of PCD at the tetrad stage (Li et al., 2006). In petunia, the *TAZ1* gene encodes a tapetum-specific zinc finger protein whose tapetal cell layer expression is required to prevent precocious PCD (Kapoor et al., 2002). The similarity of expression profile and mutant phenotype of *TAZ1* and the *Arabidopsis* homeodomain protein MS1 suggests that these two putative transcription factors may act together to regulate tapetal cell development and prevent PCD (Wilson et al., 2001; Vizcay-Barrena and Wilson, 2006).

Genes whose steady-state transcript or protein levels accumulate in anthers in a stage-specific fashion have been identified in several species. For example, in maize a β -glucanase that accumulates after the tetrad stage liberates the developing microspores from their callose walls (Steiglitz, 1977), pectin levels in developing *Lilium* anthers increase after meiosis and become maximal at the early microspore stage (Aouali et al., 2001), and serine protease activity protease peaks in developing *Lilium* anthers at the tetrad stage (Taylor et al., 1997; DeGuzman and Riggs, 2000). Similarly, a tobacco calcium/calmodulindependent protein kinase increases in accumulation during meiosis and peaks at the tetrad stage (Poovaiah et al., 1999).

In grasses, anthers develop within a reproductive structure called a spikelet. A maize spikelet consists of two floral compartments, the upper and lower floret, each of which contains three anthers (Fig. 1). Anthers in the upper and lower florets proceed through the same developmental stages and are identical at the gross anatomical level, with the exception that the lower floret is developmentally delayed by two to three days with respect to the upper floret (Hsu et al., 1988; Hsu and Peterson, 1991). Even so, two reports suggest that anthers in the upper and lower florets of maize express different sets of genes. First, transcripts from two MADS box genes, *ZMM8* and *ZMM14*, were only detected in the upper floret during spikelet develop-



Fig. 1. A maize spikelet. Maize spikelets consist of upper (right) and lower (left) florets, each of which contains three anthers. The anthers in the upper and lower florets are at approximately the late and early pollen stages, respectively.

ment (Cacharrón et al., 1999). Second, in plants that are homozygous for rf2a mutant alleles, lower floret anthers exhibit developmental arrest, while anthers in the upper floret develop normally (Liu et al., 2001).

To test the hypothesis that upper and lower floret anthers exhibit different patterns of gene expression at the same stage of development and to better understand biological processes that occur during anther development, gene expression profiles from upper and lower floret anthers at six developmental stages were compared using cDNA microarrays. Statistical analyses comparing upper versus lower floret anthers identified 701 genes from 13 functional categories that are differentially expressed. In addition, cluster analyses of developmental expression patterns identified sets of genes with specific profiles, including those that are up-regulated early in microsporogenesis, and those that are up-regulated late in microsporogenesis. Four genes involved in PCD were up-regulated at the early microspore stage, just prior to the time the tapetum begins to visibly degenerate (i.e., the midmicrospore stage). This finding strongly supports the hypothesis that tapetal degeneration occurs via PCD. In addition, many genes involved in protein synthesis exhibited differential expression patterns during anther development.

Materials and methods

Plant materials and anther collection

Maize plants of the inbred line Ky21 were grown at the Curtiss Research Farm in Ames, IA, USA. The plants used in this experiment had the pedigree numbers 01-4524, 01-6021, and 01-6024. After harvesting, the bases of tassel stems were immediately submerged in water. The tassels

were then transported to the laboratory (approximately 10 min travel time) in an air-conditioned automobile. Upon arrival at the laboratory anthers were dissected, staged, and sorted using the following strategy: one anther from each floret was squashed and staged using a compound microscope, and the two remaining anthers from a floret were immediately frozen in liquid nitrogen. Eight to twenty anthers per individual per stage per floret were collected and stored at -80° C until RNA isolation. Anther collection and dissection were performed between approximately 9:00 AM and 11:00 AM each morning. The collection dates were 7/23/2001 and 7/26/2001 for 01-4524, 7/31/2001 and 8/7/ 2001 for 01-6021, and 8/5/2001 and 8/9/2001 for 01-6024.

RNA isolation and amplification

RNA was isolated from eight to twenty anthers per stage per floret type (i.e., upper or lower) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Equal amounts of RNA from one to four individuals per stage per floret type were pooled randomly to generate one biological replicate. In total, 24 biological replicates were generated. Approximately 100 ng of total RNA from each biological replicate were used as starting material for T7-based linear RNA amplification, performed as described by Nakazono et al. (2003). Each biological replicate yielded between 30 µg and 50 µg of amplified RNA (aRNA).

Microarray procedures

A 12,160 element cDNA microarray (ISU Maize 12 K cDNA Generation II Version B-IG) was generated at the Iowa State University Center for Plant Genomics (details at http://www.plantgenomics.iastate.edu/maizechip/). Of the 12,000 ESTs printed on the slide, 8,832 cDNA clones were from the Stanford Unigene Set 1 developed by the National Science Foundation-funded Maize Gene Discovery Project (http://www.maizegdb.org). Post-analysis, each of the EST clones used as template to generate the spotted probe was sequenced to confirm the sequence identity. All of the sets that were confirmed are referred to as high confidence probes. In total, 7,450 high confidence probes were tested in this experiment. Fluorescent targets were synthesized and hybridized as described (Skibbe et al., 2006) and only targets that contained more than 3,000 picomoles of cDNA, more than 60 picomoles of Cy dye, and more than one dye molecule per 50 bases were used for hybridizations.

Microarray analyses

Microarray analyses procedures are outlined briefly be-

low; detailed procedures are provided in Skibbe et al. (2006). Each microarray chip (48 total) was scanned six times in ascending amounts of laser power and PMT gain with a ScanArray 5000 (Packard, Meriden, CT, USA). Three scans from each chip were selected on the basis of the median value of the natural log of the signal median for all probes on the slide. The values for the low, medium, and high scan intensity data sets were 5.7, 7.2 and 8.7, respectively. An R implementation of the *lowess* normalization method (Dudoit and Fridlyand, 2002) was used to normalize the two channels for each combination of slide and scan intensity. The *lowess* normalized data from each scan was used to conduct a mixed linear model analysis separately for each of 12,160 probes using a strategy similar to that of Wolfinger et al. (2001).

Clustering method

One set of clusters was generated for each data set (low, medium, and high) using the ratio of expression between adjacent stages (five comparisons) and the loop stage (one comparison), where a total of 4,438 (2,944 high confidence) probes with *P*-values less than 0.05 for at least one of the six comparisons were included in the clustering process.

Initial clustering revealed that the loop comparison yielded a very large differential expression value that dominated the clustering process (data not shown). Therefore, this comparison was not included in subsequent clustering experiments. The expression ratios for the adjacent time point comparisons from the low, medium, and high data sets were gathered into data records, where each data record is a 15 number vector of the form: (Lo1:2, Lo2:3, Lo3:4, Lo4:5, Lo5:6, Med1:2, Med2:3, Med3:4, Med4:5, Med5:6, Hi1:2, Hi2:3, Hi3:4, Hi4:5, Hi5:6). Because these data records were used to generate parallel coordinate plots of the clusters of gene expression ratios in each clustering, even the data not participating in the clustering are still displayed to aid in analysis.

The K-means clustering technique (Baldi and Brunak, 2001) was used to generate 40 clusters, a number selected as approximately 1% of the number of statistically significant differences. The K-means algorithm functions as follows: a set of randomly selected K data items represent the initial cluster centers, and the remaining data items are assigned to a cluster with the closest cluster center. Then, the algorithm enters a repeated correction phase, which proceeds as follows: the members of each cluster are averaged to locate a new cluster center. In this computation the *i*th value of the cluster center is the average of the *i*th values of its members. The assignment to cluster is then recomputed, and each data record is either moved to the cluster whose center it is closest to or left in its current cluster if it is still closest to the center of that cluster. This iterative step is repeated until no data records change clusters. In this experiment, the K=40 clustering for the microarray data under analysis terminated within a few hundred iterations for each clustering.

Functional assignment of microarray probes

One of 13 functional classes was manually assigned to each of the 701 probes that exhibited highly significant statistical differences from the floret comparison $(P \le 0.001)$ and to each of the 2,944 probes $(P \le 0.05)$ used in the cluster analysis. These functional classes are those used by the EU Arabidopsis Genome Project (Bevan et al., 1998, 1999) and are based on the yeast categories (Mewes et al., 1997) which were originally devised for Escherichia coli (Riley, 1993). The categories were metabolism, energy, cell growth and development, transcription and post-transcription, protein synthesis, protein destination and storage, transporters, intracellular traffic, cell structure, signal transduction, disease and defense, transposons, secondary metabolism, and unknown or unclassified function. The cDNA sequence associated with each probe was compared with the non-redundant protein (BLASTX-NR) and nucleic acid (BLASTN-NR) databases using the basic alignment search tool (Altschul et al., 1990; Altschul et al., 1997). Functions were inferred based first on similarity to proteins with known or predicted functions. For probes without similarity to proteins of known function, function was assigned based on similarity to nucleic acid. Probes not meeting these criteria were assigned to the unknown or unclassified function category. Probes with predicted function based on protein or nucleic acid similarity were assigned into one of the 12 known function categories by first determining the biological pathway based on the gene name and reference searches and then using the category breakdown described in Bevan et al. (1998) as a placement guide.

Gene selection and primer design

Gene-specific primers were designed for 12 ESTs that exhibited significantly different expression for the floret comparisons in at least one of the scan settings and exhibited fold changes of approximately two or greater were selected for expression validation *via* quantitative RT-PCR. Each primer pair was selected based on the following criteria: 1) predicted melting temperatures were between 58°C and 61°C; 2) a primer length between 18 and 24 bases; 3) the guanine-cytosine content ranged from 40% to 60%; and 4) a predicted amplicon length between 80 bp and 200 bp. When possible, primer pairs were selected that spanned one or more exon-intron junctions. Primer pair specificity was confirmed computationally by BLAST analyses against the MAGI (http:// magi.plantgenomics.iastate.edu/) and GenBank databases and molecularly by confirming the presence of a single band when amplifying genomic DNA.

Reverse transcription

Two stage-specific aRNA replicates were generated for each stage (excluding the tetrad stage) by pooling equal amounts of aRNA from the upper and lower floret samples. Two floret-specific aRNA replicates were generated by pooling equal amounts of aRNA from each stage (excluding the tetrad) sample. For each replicate, reverse transcription was performed on 800 ng of aRNA spiked with 1 ng of *in vitro* transcribed RNA from a human gene (GenBank accession no. AA418251) as described by Nakazono et al. (2003) except that both poly dT and random hexamers were used as primers.

Quantitative RT-PCR

All tested primer pairs were confirmed to yield a single band upon PCR amplification as assayed *via* agarose gel electrophoresis (on a 3% gel) and dissociation curve analysis. Seven serial five-fold dilutions (1, 1:5, 1:25, 1:125, 1:625, 1:3125, and 1:15625) of cDNA template were made to obtain the PCR efficiencies for each primer pair. The dilution curve and correlation coefficient R^2 for each primer pair were obtained according to the method described in ABI user bulletin #2 (4). All primer pairs exhibited primer efficiencies with a correlation coefficient greater than 0.99.

Quantitative RT-PCR (qRT-PCR) was performed on an ABI GeneAmp 5700 sequencing detection system using SYBR Green I master mix (Applied Biosystems, Foster City, CA, USA). All PCR experiments were conducted using forty cycles with an annealing temperature of 60°C in a reaction mixture containing 200 nmol/L each primer and 1 mmol/L magnesium chloride and a 1:200 dilution of each cDNA pool (per biological replicate) as template; all reactions were performed in triplicate.

The *E*-value (i.e., 1+PCR efficiency) was obtained from the dilution curve and the mean C_t values of each gene was calculated and used for fold change calculations using the method described by Pfaffl (2001). The spiked human gene (GenBank accession no. AA418251) was not detected in any non-spiked samples, so it was used as the reference gene for all comparisons.

Data availability

All microarray data associated with these experiments (e.g., raw microarray data, statistically analyzed data sets, and clustering results) are available at NCBI's GEO (http://www.ncbi.nlm.nih.gov/geo/) under GEO accession no. GSE3017.

Results

The finding that in maize rf2a mutants anther arrest occurs in the lower floret but not in the upper floret (Liu et al., 2001) led us to hypothesize that gene expression differs in anthers from upper and lower florets, even at the same stage of development. To test this hypothesis and to gain a better understanding of processes that occur during anther development, anthers from both florets at six developmental stages were collected. Two gene expression comparisons were performed: 1) a direct comparison between the upper and lower floret at each of the six developmental stages, and 2) a time-course experiment across the six developmental stages for both florets (Fig. 2).

To conduct these experiments, it was necessary to isolate RNA from anthers at defined stages of development. Unfortunately, the process of staging an anther is a destructive process. To determine whether anthers within a floret exhibit synchronized development, whole spikelets were fixed, embedded, and sectioned through both the upper and lower floret. These analyses established that all three anthers within a floret were synchronized during the meiocyte, dyad/tetrad mixture, tetrad, early microspore, mid microspore, and mid/late microspore stages (data not shown) (staging system reviewed in Skibbe and Schnable, 2005). This synchrony allowed us to stage one anther from a floret and isolate RNA from the two remaining (unsquashed) anthers. These RNA samples were amplified (Nakazono et al., 2003), labeled and used for microarray experiments.

Experimental design

The outline for the cDNA microarray comparisons for one biological replicate is shown in Fig. 2. The arrows between the upper and lower floret samples at the same stage represent comparison 1 (floret comparison experiment) and the arrows between the six stages represent the comparison 2 (time-course experiment). The design for biological replicate 2 was identical to that illustrated in Fig. 2, with the exception that the dye labeling was reversed for the time course experiments. Therefore, in this 48-chip cDNA microarray experiment, each RNA sample was hybridized four times per biological replicate, and each stage was hybridized a total of eight times.

To extract additional information from the microarray experiment, a multiple scan strategy was utilized (Skibbe et al., 2006). Briefly, data from three quantitatively defined scan levels (low, medium, and high) were acquired, quantified, normalized, and statistically analyzed using a mixed model approach (Wolfinger et al., 2001). When the statistically significant data sets were combined across scan intensities, 30%–40% more statistically significant differences were detected as compared with a single scan approach (Skibbe et al., 2006).

The upper and lower floret anthers exhibit differential patterns of gene expression

To identify genes with statistically significant differential expression based on floret (i.e., floret main effect), the data from all stages and both florets were analyzed jointly and a P-value was calculated for the test whose null hypothesis is that gene expression is the same in both florets when averaging over the developmental stages. This approach draws strength from both the number of arrays (48) and the total number of measurements (96) to provide a precise P-value for an individual probe in this comparison. Conducting a single set of tests for floret main effects, rather than conducting separate floret comparisons for each time point, was appropriate because differences between florets were consistent across developmental stages (i.e., no significant evidence of interaction between florets and developmental stage was detected). At the P=0.001 level of significance, 701 genes exhibited significantly different levels of expression in the upper and lower florets (with an estimated false-positive discovery rate of less than 2%); up-regulated genes were evenly distributed between the upper and lower florets (359 vs. 342) (Table 1; Supplemental Table 1).



Fig. 2. Experimental design. Six developmental stages were selected for each floret. Arrows between samples denote performed microarray comparisons, where the tail represents the sample labeled with the Cy3 and the arrowhead represents the sample labeled with Cy5. Stages: M, meiocyte; D/T, dyad/tetrad; T, tetrad; EM, early microspore; MM, mid microspore; M/LM, mid/late microspore.

Table 1 Distribution of the significantly different probes among the 13 functional categories based on the up-regulated floret

Functional classification	Lower floret	Upper floret	
Metabolism	14 (4.1)	50 (13.9)	
Energy	6 (1.8)	2 (0.6)	
Cell growth and development	3 (0.9)	3 (0.8)	
Transcription and post-transcription	12 (3.5)	17 (4.7)	
Protein synthesis	12 (3.5)	7 (1.9)	
Protein destination and storage	10 (2.9)	20 (5.6)	
Transporters	5 (1.5)	9 (2.5)	
Intracellular traffic	3 (0.9)	5 (1.4)	
Cell structure	17 (5.0)	13 (3.6)	
Signal transduction	16 (4.7)	26 (7.2)	
Disease and defense	7 (2.0)	5 (1.4)	
Transposons	1 (0.3)	7 (1.9)	
Secondary metabolism	3 (0.9)	3 (0.8)	
Unknown or unclassified function	233 (68.1)	192 (53.5)	
Totals	342 (100)	359 (100)	

The percentages of probes per functional class are shown in the parentheses.

Table 2

Summary of genes tested via qRT-PCR that are differentially regulated by floret

Validation of microarray results using qRT-PCR

Quantitative RT-PCR was conducted on amplified RNA from upper and lower florets to verify a subset of the statistically significant differences detected in the upper versus lower floret anther gene expression comparison. In total, twelve differentially expressed genes were tested for validation via qRT-PCR (Table 2). The correlation between the log₂ fold change estimated from qRT-PCR versus the log₂ fold change estimated from the microarray experiment was 0.646. There were two outlying points where the qRT-PCR and microarray estimates differed substantially. Without these points, the correlation was 0.851. Both correlations were statistically significant at well below the 0.05 level. For both of the outlying points (probes 2213 and 10817), the direction of the fold change according qRT-PCR was reversed from that estimated by the microarray experiment. These results are consistent with a separate analysis where 16 of 17 genes tested exhibited fold change directional differences that were consistent with the microarray results (correlation was 0.899, P<0.001) (Skibbe et al., 2006).

Probe ID	GenBank accession no.	Functional classification	Putative gene identification [organism]	Up-regulated floret	P-stage scan(s)	Fold change ^a		
						Microarray	qRT-PCR repl. 1	qRT-PCR repl. 2
2213	BG873869	Disease and defense	Putative wound inducive gene [Oryza sativa]	Lower	Low-Medium-High	0.21	1.33	2.44
3665	DV492812	Unknown or unclassified	N/A	Lower	Low-Medium-High	0.12	0.44	0.52
3767	BM073443	Transcription and post-transcription	Putative MADS-domain transcription factor [Zea mays]	Upper	Low-Medium-High	2.08	2.80	4.99
4852	BG842452	Unknown or unclassified	N/A	Upper	Low-Medium-High	2.63	2.71	3.08
5291	BM074272	Unknown or unclassified	N/A	Upper	Low	2.04	1.48	1.53
6366	BG841418	Metabolism	Putative aminotransferase [Oryza sativa]	Upper	Low	2.04	2.50	3.59
6753	BG840993	Unknown or unclassified	N/A	Lower	Medium-High	0.45	0.91	1.39
8144	DV490599	Unknown or unclassified	N/A	Lower	Low	0.28	0.46	0.47
8455	DV550444	Unknown or unclassified	N/A	Lower	Low	0.62	0.61	1.43
8949	DV942497	Protein synthesis	Phenylalanyl-tRNA synthetase [Oryza sativa]	Lower	High	0.43	0.76	0.90
10311	DV491620	Energy	PSI J-protein [Nicotiana sylvestris]	Lower	Low-Medium-High	0.36	0.20	0.23
10817	DV490479	Cell structure	Beta-fructosidase [Zea mays]	Lower	Low-Medium-High	0.20	1.61	1.67

^a Upper versus lower floret.

Several functional categories are differentially regulated by floret

To examine functional differences identified in the floret comparison, the cDNA sequence from each of the 701 statistically significant genes was compared against protein and nucleotide databases using the basic local alignment search tool (Altschul et al., 1990, 1997). Each gene was then classified into one of 13 functional categories (Supplemental Table 1; Materials and methods). All thirteen categories were represented in both florets. Eight categories (metabolism, energy, protein synthesis, protein destination and storage, transporters, intracellular traffic, signal transduction, and transposons) have 1.5 or more times the number of up-regulated probes in one floret as compared with the other floret. Furthermore, the metabolism category contains a statistically significant number of differentially expressed genes between these two morphologically similar floral compartments. Some of these differentially regulated genes and their possible relationships to anther development are discussed below.

Basic biological processes are differentially regulated by floret

The photosynthetic conversion of solar energy into chemical energy occurs via a series of reduction-oxidation reactions controlled by Photosystem I (PSI) and Photosystem II (PSII) multi-protein complexes. In eukaryotes, the PSI core complex contains 14 subunits and a lightharvesting complex (LHCI) with four subunits (reviewed in Chitnis, 2001; Scheller et al., 2001). While much is known about the structures and functions of many of the subunits, relatively little is known about the developmental or environmental conditions that regulate their expression. In this study, different sets of photosynthesis-related genes were up-regulated in each floret. Three proteins with various functions in photosynthesis were found to be upregulated in the lower floret, relative to the upper floret (Supplemental Table 1). These included a PSI J-protein, a putative light-harvesting chlorophyll a/b binding protein and cytochrome $b_6 f$ complex iron-sulfur subunit 1.

Another functional class of genes up-regulated in the upper floret was metabolism. Eleven genes involved in sugar metabolism, four involved in lipid metabolism, and three involved in nitrogen metabolism were preferentially expressed in the upper floret (Supplemental Table 1).

A delicate balance exists in cells between protein synthesis and degradation, and the proper coordination of these events is essential for survival. Proteolysis can occur as a result of protein misfolding, the inability of a protein to function properly, the need for carbon and nitrogen recycling, or developmental or environmental signaling events (reviewed in Sullivan et al., 2003). In plants, the ubiquitin/26S proteasome pathway plays an important role in controlling proteolysis and plant development (Vierstra, 1996; Hellmann and Estelle, 2002; Vierstra, 2003). In *Arabidopsis*, the accumulation of the *AP3* and *PI* floral development transcription factors is controlled by the *UFO* gene, which encodes an F-box protein hypothesized to form the ubiquitin-E3 component of the proteasome pathway (Samach et al., 1999; Zhao et al., 2001). In the upper floret, many components involved in the ubiquitin/26S proteasome pathway were up-regulated, including four putative ubiquitin conjugating enzymes and several 26S proteasome protein subunits (Supplemental Table 1).

Genes involved in protein synthesis were also differentially regulated between the upper and lower floret anthers. More specifically, five ribosomal proteins (RPs) were differentially expressed, with four being preferentially expressed in the lower floret and one in the upper floret (Supplemental Table 1). This finding suggests that protein translation is differentially regulated between the upper and lower florets.

The most highly represented class of up-regulated genes for both the upper and lower florets was that with unknown or unclassified function (Table 1). These genes represented 54% and 68% of the total statistically significant differences in the upper and lower florets, respectively, a frequency that is similar to the frequency of genes with unknown or unclassified function on the chip (i.e., approximately 60%). The observation that these genes of unknown/unclassified function are differentially regulated in the anthers of the upper and lower florets may provide a clue to help elucidate their function(s) *in vivo*.

Identification of coordinately expressed genes through cluster analysis

Gene expression profiling was also used to identify genes that differ in expression during anther development. Many genes (n=275) were differentially regulated across stage even at the P=0.001 level of significance. To identify sets of genes with similar expression patterns across development, a P=0.05 level of significance was used. While the larger P-value somewhat increases the estimated false discovery rate (FDR) relative to a P-value of 0.001 (6.9 vs. 1.9, 3.2 vs. 0.8, and 5.0 vs. 1.4% for the low, medium and high scans, respectively), it also significantly increases the size of the data set. We consider this an acceptable trade-off, where the goal was to identify genes with similar expression patterns during anther development, rather than to test the hypothesis that gene expression varies during anther development as was the goal of the floret comparison discussed earlier.

To identify genes that exhibited similar patterns of gene expression during microsporogenesis, genes with *P*-values less than 0.05 for at least one of the five adjacent stage to stage comparisons (n=2,944) were clustered independently

for the low and high scan intensity data sets using the *K*-means technique (Baldi and Brunak, 2001). For each scan intensity (i.e., low and high) 40 clusters were generated (80 clusters total), and cluster types were readily identifiable upon visual examination, including stage-specific, down-regulation at the early microspore stage, increasing expression throughout development and decreasing expression throughout development (Fig. 3; Supplemental Table 2).

Early microspore up-regulated genes

At the beginning of the early microspore stage, a thin microspore wall forms beneath the callose wall, which is subsequently digested. A single pore forms in each developing microspore and orients towards the tapetum, which contains both uni- and bi-nucleate cells. Several clusters with peak expression at the early microspore stage were identified (Fig. 3B; Supplemental Table 2; Clusters 3L, 5H,



Fig. 3. Examples of clusters of coordinately expressed genes. A: genes up-regulated at the tetrad stage (Cluster 15L); B: genes up-regulated at the early microspore stage (Cluster 5H); C: genes up-regulated at the mid-microspore stage (Cluster 4H); D: genes down-regulated at the early microspore stage (Cluster 11H); E: genes up-regulated in early microsporesis (Cluster 0L); F: genes up-regulated in late microsporegenesis (Cluster 24H). Stages: M, meiocyte; D/T, dyad/tetrad; T, tetrad; EM, early microspore; MM, mid microspore; M/LM, mid/late microspore.

5L, 9L, 19L, 20H, 21H, 21L, 28H, 37H, and 39L). In combination, these clusters contained 504 probes, 279 of which had unknown or unclassified function (Supplemental Table 2).

During the early microspore developmental stage, by-products of phenylpropanoid metabolism (e.g., yellow flavonoids) begin to accumulate in the developing anthers (Bedinger and Russell, 1994). Many genes involved in this pathway reached their peak accumulation levels at the early microspore stage, including 4-coumarate CoA:ligase, *O*-methyl transferase (three different probes), cinnamic acid 4-hydroxylase and chorismate synthase (Supplemental Table 2).

During the early microspore stage of anther development, mitochondria are 40 times more abundant in tapetal cells than in the surrounding tissue (Lee and Warmke, 1979). The abundance of mitochondria would suggest that anthers at this stage are metabolically active. This expectation is supported by the observation that 67 probes in the metabolism class were up-regulated at the early microspore stage (Supplemental Table 2). These probes represent a number of basic metabolic processes, including carbon and nitrogen metabolism (e.g., carbonic anhydrase 3, glutamine synthetase, alanine aminotransferase), and sugar metabolism (e.g., sucrose synthase 2, hexokinase, citrate synthase, triosephosphate isomerase, malate dehydrogenase and fructokinase 1) (Supplemental Table 2).

Since developing microspores are metabolically active, it is possible that oxygen (or sugar) could become limiting. Under these conditions, the ethanolic fermentation pathway is up-regulated to recycle NAD⁺ for glycolysis (op den Camp and Kuhlemeier, 1997; Tadege and Kuhlemeier, 1997; Tadege et al., 1999). Ethanolic fermentation requires three enzyme activities: pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and aldehyde dehydrogenase (ALDH). In the first step, pyruvate is converted into acetaldehyde by PDC. The fate of acetaldehyde is determined by the NAD⁺/NADH ratio within the cell, where high ratios result in acetaldehyde being oxidized to acetate by ALDH and low levels result in acetaldehyde being reduced to ethanol by ADH. When NAD⁺ is available, acetate is produced by the sequential oxidation of ethanol to acetaldehyde via ADH and acetaldehyde to acetate via ALDH. The ALDH component of ethanolic fermentation (RF2B) was up-regulated approximately four-fold at the early microspore stage (Supplemental Table 2). Previously, in vitro kinetic analyses demonstrated that acetaldehyde is a substrate for RF2B (Liu and Schnable, 2002). Furthermore, the expression of the rice RF2B ortholog, was induced by hypoxic treatment (Tsuji et al., 2003). An intriguing possibility is that either oxygen or sugar may be limited during the early microspore stage of development. Alternatively, RF2B function may become necessary to detoxify an aldehyde substrate that preferentially accumulates at this stage of development.

Genes implicated in programmed cell death are up-regulated at the early microspore stage

During normal anther development, tapetal cells begin to degenerate during the mid-microspore stage (Warmke and Lee, 1977). Due to the highly coordinated timing involved in the degeneration of the tapetal cell layer, it has been proposed by Wang et al. (1999) that this process is mediated by programmed cell death (PCD), an active process in response to developmental or environmental cues. While genes regulating PCD have been extensively studied in animal systems (reviewed in Reed, 2000), identification of their counterparts in plants has been challenging. Swidzinski et al. (2004) used a proteomic approach to identify 11 proteins encoded by eight genes [carbonic anhydrase (=aconitate hydratase), mitochondrial lipoamide dehydrogenase, catalase 1, catalase 3 (peroxisomal), a putative EP1-like glycoprotein, voltage dependent anion selective channel (VDAC) protein, putative superoxide dismutase, and an unknown protein] that increased in abundance in Arabidopsis after PCD-inducing treatments. Four of these eight genes (carbonic anhydrase, mitochondrial lipoamide dehydrogenase, catalase, and VDAC protein) were included in the clustering data set. Maize orthologs to two of the Arabidopsis genes (VDAC and carbonic anhydrase) were up-regulated ~11 and 100-fold at the early microspore stage, respectively (Supplemental Table 2).

The expression levels of the maize orthologs for five candidate PCD genes (carbonic anhydrase, mitochondrial lipoamide dehydrogenase, catalase (peroxisomal), VDAC, and superoxide dismutase) at five of the six tested developmental stages were analyzed *via* qRT-PCR (Fig. 4). These analyses revealed that the transcript levels for four of the five genes are up-regulated at the early microspore stage. This high degree of overlap between the Swidzinski et al. (2004) data set and the genes identified in this study support the hypothesis that the degeneration of the tapetum, which is first detectable cytologically at the mid-microspore stage of maize anther development, occurs *via* PCD.

Transcription of protein synthesis genes is differentially regulated throughout development

Several classes of transcription factors exhibit sequential expression during anther development, including seven zinc-finger proteins (Kobayashi et al., 1998). These observations led to the expectation that genes involved in transcription and/or translation would be highly represented in this cluster type. Several clusters were visually identified that exhibited a decrease in expression at the early microspore stage of development (i.e., early microspore valley) (Fig. 3D; Supplemental Table 2; Clusters 8H, 8L, 11H, 11L, 22H, 22L and 35L) or high expression in the early stages and low expression in the late stages (Fig. 3, E and F;



Fig. 4. Four of five PCD candidate genes are up-regulated at the early microspore developmental stage. Quantitative RT-PCR performed on both biological replications yielded similar results; results from only one biological replicate are shown. M, meiocyte; D/T, dyad/tetrad; EM, early microspore; MM, mid microspore; M/LM, mid/late microspore. Probe 6742, carbonic anhydrase; Probe 7067, catalase; Probe 2931, putative CuZn-superoxide dismutase; Probe 4564, voltage-dependent anion channel protein 2; Probe 3500, dihydrolipoamide dehydrogenase.

Supplemental Table 2; Clusters 0L and 27H). The early microspore valley clusters contained 476 probes, 213 of which had unknown or unclassified function (Supplemental Table 2). While twelve of the thirteen functional classes were identified for these clusters, the majority of the known function genes belonged to the protein synthesis class. In the early microspore valley cluster (Fig. 3D), 128 probes represented 77 different genes involved in protein synthesis (Supplemental Table 3). Although all of these genes were involved in various aspects of protein synthesis, most of the genes were 40S and 60S ribosomal subunit proteins, as well as other miscellaneous ribosomal proteins.

Genes up-regulated late in anther development

As anther development nears the mid and late microspore stages, new metabolic and developmental functions are required to meet the needs of the developing microspores. For example, the mid and late microspore stages mark a critical developmental transition point between microsporogenesis and microgametogenesis, as demonstrated by the pronounced changes in the RNA and protein populations (Bedinger and Edgerton, 1990) and *in vitro* protein synthesis patterns (Mandaron et al., 1990) immediately following microsporogenesis.

Several clusters of genes were identified which increased expression late in microsporogenesis (Fig. 3F; Clusters 2L, 16H, 16L, 18L, 24H, 24L, 26H, 31H and 36H). These up-regulated late in microsporogenesis clusters contained 579 probes, 325 of which had unknown or unclassified function (Supplemental Table 2). All 13 functional classes were represented, and a number of biological processes were identified, including water transport, pectin modification, phenylpropanoid metabolism, and anthocyanin accumulation.

The phenylpropanoid pathway is a secondary metabolic pathway with several branch points that give rise to a variety of products, including lignins, flavones, flavanols, tannins, anthocyanins, and isoflavones (Buchanan et al., 2000). In maize, chalcone synthase (CHS) performs the first committed step in anthocyanin production by condensing one molecule of *p*-coumaryl-CoA with three molecules of malonyl-CoA to produce tetrahydroxychalcone (naringenin chalcone) (Grotewold, 2006). Mutant analyses of CHS in maize and petunia demonstrated that CHS plays an important role in flavonol synthesis and pollen development (Coe et al., 1981). Mo et al. (1992) demonstrated that normal pollen tube germination could be restored when germination media was supplemented with micromolar amounts of flavonol aglycones. Furthermore, the ms26, ms*-5126, and ms*-bs7 genic male sterile mutants in maize encode a cytochrome P450-like protein similar to flavonone-5'-hydroxylase (GenBank accession no. AF366297, US Patent no. 7098388), a nari-geninchalcone synthase-like gene (GenBank accession no. BM500617, US Patent no. 5689048), and a dihydroflavonoid-reductase like protein (GenBank accession no. AF366295, US Patent no. 6956118), respectively. In this experiment, genes similar to chalcone synthase (three different probes), cytochrome P450 (four probes) and flavonol synthase were up-regulated in a manner consistent with those reported for the cloned male sterile genes (Table 3).

Table 3 Genes involved in phenylpropanoid biosynthesis identified in the up-regulated late in microsporogenesis clusters^a

Probe ID	GenBank accession no.	Cluster no. ^b	P-stage ^c	Putative Gene Identification [organism]	BLASTX <i>E</i> -value
138	DV493382	36H	0.0121	Putative cytochrome P450 [Oryza sativa]	2E-49
344	BQ538817	16H	0.0001	Cytosolic aldehyde dehydrogenase RF2D [Zea mays]	8E-42
1108	BM074489	2L	0.0975	Putative cytochrome P450 [Oryza sativa]	9E-48
2062	DV490164	36H	0.0096	4-coumarate coenzyme A ligase [Zea mays]	1E-52
6058	BG873965	18L	0.0002	Putative flavonol synthase [Oryza sativa]	5E-52
7005	DV489527	2L	0.0096	Putative cytochrome P450 [Oryza sativa]	3E-19
7348	DV491912	2L	0.0294	Chalcone and stilbene synthases, C-terminal domain, putative [<i>Oryza sativa</i>]	1E-72
7960	BM076073	36H	0.1750	Chalcone synthase [Zea mays]	8E-16
9444	BM348583	18L	0.0016	Putative cytochrome P450 [Oryza sativa]	2E-06
9461	BM073205	24H	0.0128	Putative o-methyltransferase ZRP4 [Oryza sativa]	1E-36
9463	BM073115	24L	0.0003	Putative cinnamoyl-CoA reductase [Oryza sativa]	1E-30
10186	BM080220	36H	0.3948	Chalcone synthase [Zea mays]	3E-27
10250	BM378275	31H	0.0110	Putative cinnamoyl-CoA reductase [Oryza sativa]	2E-14
10443	DV942948	2L	0.0208	Putative o-methyltransferase ZRP4 [Oryza sativa]	9E-75

^a The full data set is available in Supplemental Table 2. ^b Clusters are numbered from 0 to 39 followed by the scan intensity used for clustering, where L is Low and H is High. ^c *P*-stage is the *P*-value for the test whose null hypothesis is that expression is the same in all stages studied.

The phenylpropanoid biosynthetic pathway was also represented in the up-regulated late in microsporogenesis clusters. Lignin biosynthesis requires a coordinated set of reactions including phenylalanine ring modification, side-chain modification, and polymerization (Buchanan et al., 2000). Studies by Nair et al. (2004) have raised the possibility that the order of reactions is different from the traditional depiction, but the enzymes required to catalyze the reactions are the same. One gene, REF1, encodes a cytosolic aldehyde dehydrogenase (cALDH) that converts coniferaldehyde and sinapaldehyde to their corresponding carboxylic acids. Maize contains two ALDHs (rf2c and rf2d) predicted to accumulate in the cytosol (Skibbe et al., 2002). In the up-regulated late in microsporogenesis clusters, the rf2d gene was up-regulated. In addition, three other genes involved in lignin biosynthesis (4-coumarate CoA-ligase, cinnamoyl-CoA reductase, and o-methyltransferase ZRP4) were up-regulated (Table 3).

Discussion

In this study, cDNA microarrays were used to examine gene expression in developing anthers from two floral compartments at six developmental stages. To extract additional biological information, each array was scanned six times, and three quantitatively defined signal strengths (low, medium, and high) were selected for statistical analysis. This approach identified an additional 30% to 40% of statistically significant differences as compared to a single scan strategy. Analyses of the distribution of the statistically significant differences demonstrated that gene expression differs between the upper and lower florets. In addition, cluster analysis of these data identified large numbers of genes that are coordinately regulated during development.

Anthers in the upper and lower florets exhibit preferential differences in gene expression

Differential gene expression between upper and lower florets was tested using a mixed model statistical method (Wolfinger et al., 2001) and combined with a calculation to determine the estimated false discovery rate (FDR) (Storey and Tibshirani, 2003). At a P=0.001 level of statistical significance, the 701 statistically significant differences, representing approximately 9% of the genes tested, were predicted to contain less than 2% false positives, and represented each of 13 functional categories (Table 1). We conclude that although the upper and lower florets are morphologically similar, they differ significantly with respect to their gene expression profiles.

While only the metabolism category exhibited a significantly disproportionate number of differences between upper and lower floret anthers, several other functional categories (energy, protein synthesis, protein destination and storage, transporters, signal transduction, and transposons) included a disproportionate number of differentially regulated members for one floret. Closer analyses of four of these categories (metabolism, energy, protein synthesis, and protein destination and storage) revealed interesting differential regulation of basic biological processes. Two of these differences were the opposing processes of protein translation and proteolysis. The failure to properly coordinate protein translation and proteolysis is likely to have drastic consequences on plant development. But because the upper and lower florets both produce viable pollen, the coordination appears to be sufficient for this purpose. It is, however, possible that deficiencies in the proteolysis machinery could predispose the lower floret to physiological perturbations caused by mutations in critical genes (e.g., rf2a).

Molecular mapping, and more recently sequencing of the maize genome has established that large segments of the maize genome are duplicated (Helentjaris et al., 1988). This observation raises the question of whether different homologs of the same functional class are expressed in the upper and lower floret anthers. In this study a spotted cDNA array was used to measure gene expression. cDNA arrays may not be able to distinguish the expression of closely related homologs. Hence, it is likely that the observed differences underestimate the true difference in expression between upper and lower floret anthers.

Cluster analyses of microarray data

To identify genes with similar expression patterns throughout development, the microarray data from the developmental time course were clustered using the *K*-means clustering technique (Baldi and Brunak, 2001), and various cluster types were identified, including stage-specific, up-regulated early in anther development, and up-regulated late in anther development.

Selection of the number of output clusters is a difficult problem because there is not an agreed upon method of finding a "right" number of clusters. To illustrate this problem, if too few clusters are used, objects that clearly do not belong together will be placed in the same cluster and result in a confusingly loose cluster. Conversely, if too many clusters are used, objects that belong together will be placed in separate clusters. The latter situation can be overcome by visual inspection of the clusters because clusters with similar parallel coordinate plots are easy to identify and manually unite. For this reason, n=40 clusters was selected in an *ad hoc* manner because it yielded clusters with similar objects and coordinate plots.

Selection of an appropriate *P*-value is an important consideration in microarray experiments. The upper versus lower floret study used a very stringent *P*-value (P < 0.001) to test whether the upper and lower florets exhibit similar patterns of gene expression, while the cluster analyses used a more relaxed *P*-value (P < 0.05) because the purpose of clustering was to identify genes with similar expression patterns, rather than test a specific hypothesis. Even with the less stringent *P*-value, the false-positive rate was still less than 7% for both the high and low scan intensity data sets. We therefore consider that the less stringent *P*-value used for clustering is a reasonable and fair compromise to increase the number of genes participating in the clustering process.

The majority (approximately 65%) of the clustered genes had either an unknown or unclassified function even using a very relaxed BLAST *E*-value cut-off of e^{-5} . The data from the clustering experiments represent a valuable resource for not only better understanding anther development but may also facilitate the assignment of functions to these genes with unknown functions. We have, however, not yet tested whether this method will prove successful.

Broader impacts on anther biology

The production of viable pollen is essential for propagation of genetic information through the male reproductive structure in maize. While much has been published describing morphological features and consequences of mutants affecting male fertility, a firm understanding of the underlying biological processes that contribute to normal anther development in maize does not exist. By analyzing steady state gene expression among various stages of anther development and between two floral structures, a benchmark describing some of the biological processes and metabolic networks has been established. These data represent a valuable resource and an excellent starting point for focusing on target genes in the postgenomic era.

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Supplemental Data

Supplemental Tables 1–3 associated with the article can be found in the online version at www.jgenetgenomics.org.

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