

# Mutator transposon activity reprograms the transcriptomes and proteomes of developing maize anthers

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## SUMMARY

Despite the high conservation of anther gene expression patterns across maize lines, *Mu* transposition programmed by transcriptionally active *MuDR* results in a 25% change in the transcriptome, monitored over 90 h of immature anther development, without altering the morphology, anatomy or pace of development. Most transcriptome changes are stage specific: cases of suppression of normal transcripts and ectopic activation are equally represented. Protein abundance changes were validated for numerous metabolic enzymes, and highlight the increased carbon and reactive oxygen management in Mutator anthers. Active Mutator lines appear to experience chronic stress, on a par with abiotic treatments that stimulate early flowering. Despite the diversity of acclimation responses, anther development progresses normally, in contrast to male-sterile mutants that disrupt anther cell fate or function completely, and cause fewer transcriptome changes. The early flowering phenotype ultimately confers an advantage in *Mu* element transmission.

**Keywords:** transposon, Mutator, anther, maize, stress, microarray.

## INTRODUCTION

DNA transposable elements (TEs) contribute to genomic fluidity and gene expression both within an organism and over generations (Slotkin and Martienssen, 2007). Class-II TEs function via DNA intermediates, and are organized into families in which an autonomous element encodes a transposase that mobilizes non-autonomous family members. Transposases catalyze chromosome breakage during reactions that can increase local recombination, chromosome translocation and deletion: collectively, these activities are considered to lower the fitness of plants (Kidwell and Lisch, 2000). Nonetheless, TEs are recognized as major contributors to gene creation, to innovation in gene regulation and to genome evolution (Jordan *et al.*, 2003; Kazazian, 2004). For example, insertions (and subsequent excisions) into gene regulatory regions can alter gene expression patterns, whereas insertions into exons or introns can generate null mutations or alleles encoding novel transcripts and proteins (Girard and Freeling, 1999).

Although all organisms harbor TEs, most lack mobile elements, because TEs are typically silenced, and are rarely reactivated. Even when active TEs exist, host defenses such

as developmental restrictions may limit mobility to somatic or germinal cells (Sijen and Plasterk, 2003; Vagin *et al.*, 2006). In maize, the *MuDR/Mu* TE family is active only during terminal cell divisions in both somatic and germinal tissues; therefore, only small sectors harbor new insertion or excision alleles (Walbot and Rudenko, 2002). RNA-mediated silencing mechanisms are proposed to have evolved to suppress TEs: both global silencing and the host developmental defenses (Slotkin and Martienssen, 2007). TE silencing suppresses transposase transcription. Without transposase, in many eukaryotic examples, the dispersed TEs become highly methylated, which is a heritable mark of silencing (Slotkin and Martienssen, 2007). Interestingly, even when transposase is absent, methylated TEs continue to influence host genes. McClintock discovered that silenced *Spm* near a maize anthocyanin gene activates expression, restoring a uniform pale purple color. In contrast, when the *Spm* family is active, gene expression is suppressed, except after element excision, yielding a white kernel with purple dots (McClintock, 1984). Similarly, silenced maize *Mu1* plants contain weak readout promoters in

the terminal inverted repeats (TIRs) that permit the constitutive transcription of adjacent genes (Barkan and Martienssen, 1991).

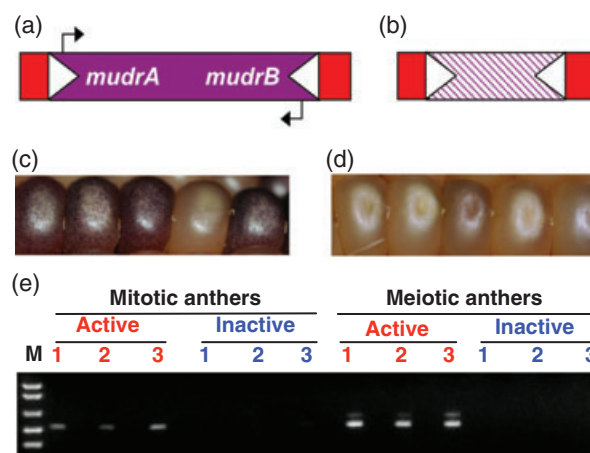
The impact of TEs on host physiology and development is typically studied by analyzing specific insertion alleles. Global properties have also been documented, such as the increased abundance of DNA repair transcripts in active compared with inactive lines (Casati and Walbot, 2003). In maize, the transposase-encoding *MuDR* element directs the movement of dozens of *Mu* elements: *MuDR/Mu* causes the highest mutation frequency of any TE family analyzed (Walbot and Rudenko, 2002). In highly active lines there are universal phenotypes, such as early flowering and missing tips on final leaves. These characteristics phenocopy classes of developmental mutations, and these traits disappear when *Mu* lines undergo spontaneous silencing. An unanswered question is whether such developmental defects, collectively called Mutator syndrome (Walbot, 1991), reflect the misregulation of individual genes or the more widespread, even global, reprogramming of maize gene expression.

The current study was designed to distinguish between two hypotheses: *Mu* insertion in or near specific genes results in developmental phenotypes or active Mutator lines reprogram host development. Pairs of active and derived inactive lines were examined using transcriptome and proteome profiling to resolve the developmental impact of active *MuDR/Mu*. Maize anthers were selected because they are readily staged, and because the progression through the stages is highly synchronous within each anther and among anthers of similar size (Ma *et al.*, 2008). Also, the resolution of transposition reactions switches during anther development. In strictly somatic cells such as the kernel and anther epidermis, *Mu* elements exhibit 'cut only' (excision without insertion) catalyzed by MURA823 transposase (Raizada and Walbot, 2000), whereas new heritable insertions are common in meiotic cells.

## RESULTS

### Biological materials and experimental design

*MuDR* encodes both a transposase and a helper protein gene (Figure 1a): both undergo alternative splicing to generate protein isoforms (Hershberger *et al.*, 1995). The multiple non-autonomous *Mu* elements generally only share ~215 bp of the TIR sequences with *MuDR* (Figure 1b). The 32-bp MURA binding site is the most highly conserved domain within the TIRs (Benito and Walbot, 1997). In active Mutator lines, the transposase programs *Mu* excision, resulting in very small purple dots when a pigment allele is monitored (Figure 1c). The visible mark of silencing is the loss of somatic excision (Figure 1d), an event that occurs in 10–30% of the progeny of an active Mutator plant (Walbot and Rudenko, 2002). At the molecular level, the loss of



**Figure 1.** Mutator-active and -inactive analysis.

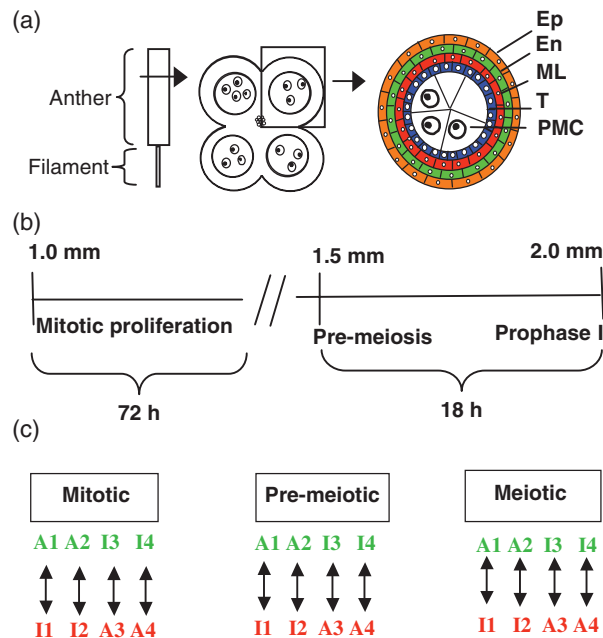
The autonomous *MuDR* element (a) and diverse non-autonomous *Mu* elements (b) share only ~215 bp of terminal inverted repeats (red). *MuDR* encodes transposase (MURA) and a helper protein (MURB). Genetic stocks heterozygous for a pigment reporter allele (*bz2/bz-mu*) monitor Mutator activity; *Mu* excision late in development yields densely purple spotted seed, proof of somatic transposon activity (c). Spontaneous inactivation occurs in ~10–30% of individuals, and results in the loss of kernel spotting (d). RT-PCR analyses of RNA from three replicates of *Mu*-active anthers at the mitotic and meiotic stages show that *mudrA* transcript accumulates in active samples, whereas the inactive samples display the hallmark silencing feature of diminution (nearly complete absence) of transposase transcripts after 30 amplification cycles (e).

excision is perfectly correlated with the absence of detectable transposase transcripts at two stages of anther development (Figure 1e).

Because Mutator silencing is enhanced by self-pollination, active lines are maintained by outcrossing. Therefore, the reporter allele and other *Mu* insertion sites are heterozygous, and nearly all are phenotypically cryptic as *Mu* insertion typically results in a recessive allele. In the Mutator family, new insertions are common but heritable excisions are very rare; therefore, existing TE insertion locations are inherited as Mendelian factors. Individual progeny consequently share about half of their insertions, plus in an active line each individual has a suite of new TE sites generated late in parental development. If TE silencing occurred in a sector of the parent, the resulting inactive progeny lack new insertion sites, but inherit segregating parental insertion sites. Given these properties of the *MuDR/Mu* elements in relation to the maize life cycle, individual active and inactive plants within one progeny share ~50% of the pre-existing parental insertion sites, but differ in *Mu* copy number, as the inactive individuals have no new insertions.

Anthers have four locules each comprised of four concentric cell layers that surround the developing meiotic cells (Figure 2a). Using size as a guide for staging, mitotic (1.0 mm), pre-meiotic (1.5 mm) and prophase-I meiotic (2.0 mm) anthers (Figure 2b) were dissected from a Mutator

active line and an epigenetically silenced (inactive) sister line (Takumi and Walbot, 2007) confirmed by RT-PCR (Figure 1e). Transcriptome profiling experiments were performed on a  $4 \times 44\text{K}$  platform, with 42 034 60-mer probes to 39 174 unique maize genes, representing about 80% of the current gene estimate based on a near-complete genome (Walbot, 2008), and enriched for anther-expressed genes (Ma *et al.*, 2008). The probe performance – the percentage of probes with signals  $>2.6$  times the standard deviation of the background (i.e. 99.5% confidence interval) – was 90.5%. The robustness of the signal detection for each spot on the array was tested by increasing the detection limit to 3.5 times the standard deviation of the background (i.e., 99.98% confidence interval). On average,  $\sim 3\%$  of the probes were eliminated by the higher confidence interval. Therefore, we consider the cut-off at 2.6 times the standard deviation to be robust and effective for classifying the expression status of probes (Figure S1). cRNAs from the Mutator-active and -inactive individuals were compared



**Figure 2.** Experimental materials for RNA profiling experiments.

Maize anthers at the completion of mitotic proliferation (a). The stamen is comprised of an anther and a filament. In transverse section a mitotic (1-mm stage) maize anther has a characteristic four-lobed structure. Each of the four lobes contains four concentric rings of somatic cells surrounding presumptive meiocytes: Ep, epidermis; En, endodermis; ML, middle layer; T, tapetum; PMC, pollen mother cell. Timeline of anther development (b).

A mitotic anther (1.0 mm) is temporally separated from a pre-meiotic anther (1.5 mm) by 72 h, whereas a pre-meiotic anther and meiotic anther (2.0 mm) are only 18 h apart. Microarray profiling experimental design (c).

Four biological replicates of *Mu*-active (a) or -inactive (I) maize anthers were collected for three stages. Within each stage, two samples were labeled with Cy3 (Green) or Cy5 (Red) and hybridized as shown.

using four biological replicates of each stage and a balanced dye swap design (Figure 2c).

### Mutator activity reprograms the transcriptome of developing anthers

Anthers from Mutator-active and -inactive lines from each stage express 27 000–31 000 transcripts, and over 32 000 transcripts are expressed during the 90 h of development examined (Table 1 and Table S1). In addition to the astonishing transcript diversity, immature anthers exhibit dynamic gene expression. Both the number of transcripts and overall patterns of expression (independent of differential expression) are similar for Mutator-active and -inactive anthers (Figure 3). The mitotic stages express approximately 10 times more stage-specific transcripts (active = 3077; inactive = 3006) as the subsequent two stages. Of the non-constitutive transcripts found in mitotic anthers, over half are expressed in the subsequent pre-meiotic stage (1386 out of 2542, and 1657 out of 2618, for active and inactive, respectively), whereas the remainder (1156 out of 2542, and 961 out of 2618, for active and inactive, respectively) are absent in the pre-meiotic stage, but are re-expressed during meiotic prophase I. As anther cells enter meiosis, there are far fewer stage-specific transcripts: the  $\sim 10\%$  decrease of transcript diversity at the start of meiosis confirms previous reports (Figure 3; Ma *et al.*, 2007, 2008).

Considering that active Mutator anthers have normal morphology, anatomy and fertility, it is surprising that  $\sim 3000$  genes are differentially expressed at each stage compared with an inactive line (Table 1). Genes up- and down-regulated are evenly distributed at the mitotic and meiotic stages: at the pre-meiotic stage, approximately 50% more transcripts are down-regulated in Mutator-active individuals than in their inactive counterparts. Summing all three stages, 8223 transcripts (25% of the total transcripts expressed over three stages) are differentially expressed. Over 85% of these differentially expressed genes are stage specific (Figure 4a,b), indicating that Mutator activity has a significant impact at each stage. At the mitotic stage, *Mu* elements have not yet begun to transpose, because most

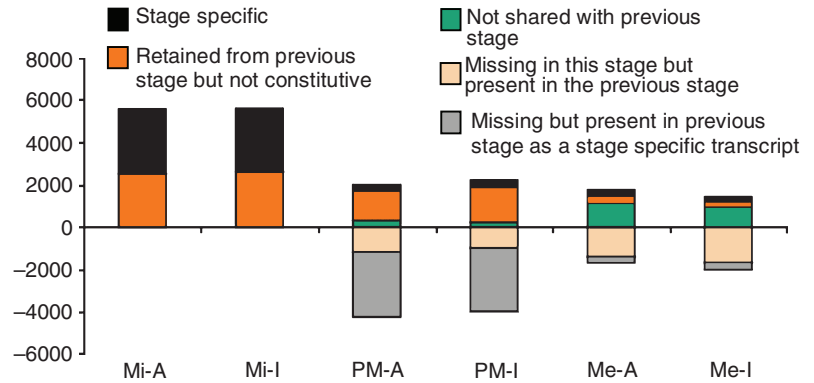
**Table 1.** Genes differentially expressed between *Mu*-active versus -inactive lines

Stage	Total no. genes expressed	No. of genes differentially expressed*	Stage-specific differential expression
Mitotic	31521	2917 (9.2%)	92.3%
Pre-meiotic	28247	3230 (11.4%)	68.0%
Meiotic	27672	3460 (12.5%)	71.3%

\* $P < 0.05$ : fold change  $>1.5$  or expression level 2.6 times the standard deviation of the background in four out of four arrays, or above the median expression level if expressed on three out of four arrays. The complete list of genes is given in Table S1.

**Figure 3.** Transcript composition in Mutator-active and -inactive anthers.

The approximately 25 000 transcripts shared by all three stages are not shown. The numbers above the x-axis represent transcripts that are: stage specific (black); retained from the previous stage but not constitutive (dark orange); or not shared with the previous stage (green). The numbers below the x-axis represent transcripts that are: missing in this stage but present in the previous stage (light orange); and missing but present in the previous stage as a stage-specific transcript (gray). The stage abbreviations are: Mi, mitotic; PM, pre-meiotic; Me, prophase-I meiotic. A, active; I, inactive.



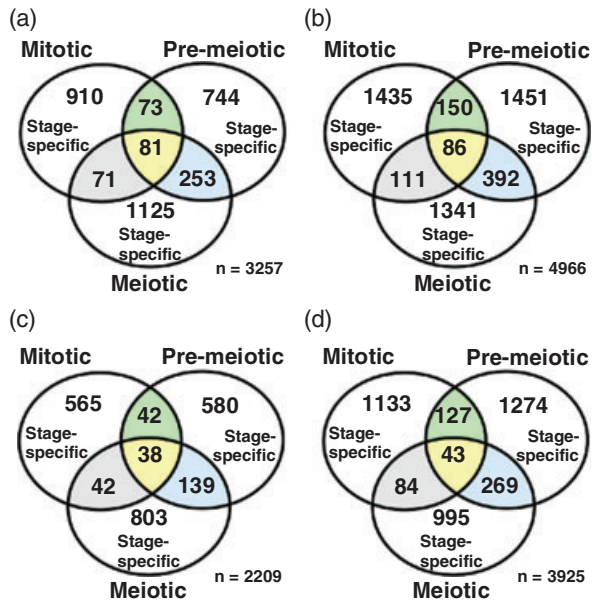
excisions are just prior to or after final cell divisions (Raizada and Walbot, 2000), corresponding to the pre-meiotic stage. Heritable insertion events are calculated to start equally late in diploid precursor, meiotic or haploid cells, resulting in individual pollen grains or small clusters of pollen sharing new *Mu* insertions (Robertson, 1980). Thus, Mutator activity alters the host transcriptome prior to and continuing through the period of active transposition.

Two classes of differentially expressed transcripts exist: up/downregulated or expressed only in Mutator-active or only in inactive lines. Over 67% of the differences are in the 'on/off' category, and the vast majority of these are stage-

specific (Figure 4c,d). Therefore, Mutator activity results in the *de novo* activation of genes typically silent during anther development, as well as in the silencing of genes that are characteristic of each stage.

**Confirmation of the microarray results using quantitative RT-PCR**

Quantitative RT-PCR was performed to verify a subset of the genes that were up-regulated or switched 'on' in the mitotic stage Mutator-active individuals. In total, 21 differentially expressed genes were tested in three biological replicates of Mutator-active and -inactive individuals (Table S2). The normalized  $R_0$  values (compared with the ubiquitin conjugase control gene) were analyzed using the Wilcoxon rank-sum test (Wilcoxon, 1945). The fold change was significantly higher ( $0.005 < P < 0.1$ ) in Mutator-active samples for 14 of the 21 genes tested. The fold change was higher in Mutator-active individuals for four additional genes (but above the *P*-value cut-off), and the fold change was higher in Mutator-inactive individuals for the three remaining genes (but above the *P*-value cut-off). The high level of correlation between the qRT-PCR experiments and the microarray results support the reported differences. Furthermore, these verification data add to a previous set of genes validated by qRT-PCR from the same microarray platform (Ma *et al.*, 2008).



**Figure 4.** Analysis by developmental stage of differentially expressed genes in *Mu*-active versus -inactive anthers.

The sets of stage-specific (white), common to all stages (yellow) and shared between stages transcripts (green, mitotic and pre-meiotic; blue, pre-meiotic and meiotic; and gray, mitotic and meiotic) are displayed in Venn diagrams. Four classes of transcripts are defined: up-regulated in *Mu*-active anthers (a); down-regulated in *Mu*-active anthers (b); 'on' in *Mu*-active anthers and not expressed detectably in *Mu*-inactive anthers (c), and 'off' in *Mu*-active anthers and expressed in *Mu*-inactive anthers (d). The number of genes in each class is listed in the bottom right of each panel.

**Mutator elements are not present in or near master regulatory genes**

When active Mutator lines are self-pollinated, deleterious mutations are uncovered as a consequence of homozygosity of recessive, defective alleles. *MuDR/Mu* element copies in active and sister inactive plants range from fewer than 10 to more than 50 (Walbot and Warren, 1988). As *MuDR/Mu* elements preferentially insert into RNA polymerase-II transcription units (Fernandes *et al.*, 2004), to avoid deleterious phenotypes Mutator lines were maintained by crossing with an inbred line: a functional allele is present for each gene, except in tiny sectors with new insertions. The pairs of lines used here should share one-quarter of parental *MuDR/Mu*



sites, based on pedigree. To determine if elements are in or near master regulatory genes that might cause gain-of-function phenotypes, or which show dosage sensitivity, inverse PCR was used to identify the *Mu* locations in an active line. A total of 46 *Mu* insertion sites were recovered from 200 clones. The flanking sequences of 15 out of 46 corresponded to predicted maize genes within 1 kb of an annotated exon. Only one insertion was in a predicted transcription factor, but its expression was unaffected by Mutator activity status, as monitored by microarray hybridization (Tables S3 and S4). Therefore, *Mu* element insertions are unlikely to be driving the thousands of gene expression differences observed, particularly as one normal allele is present for each insertion site.

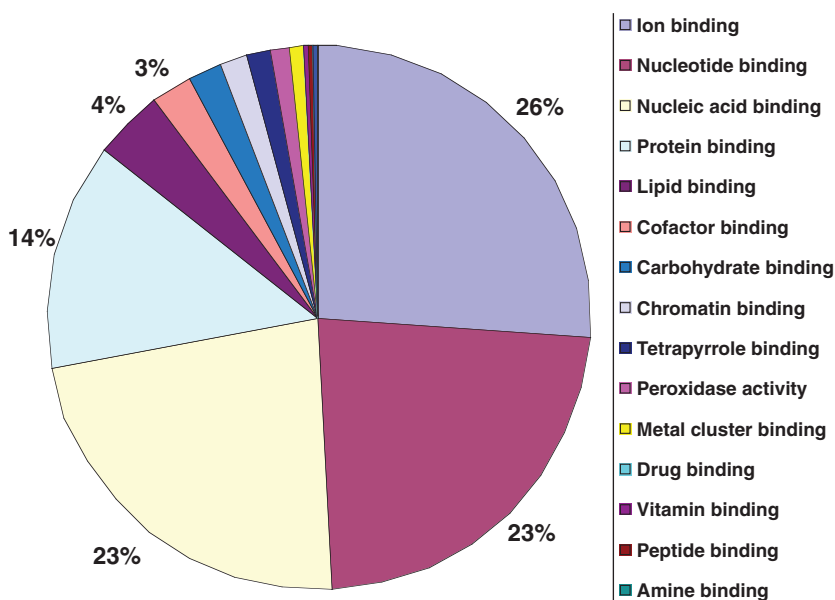
### *MuDR/Mu* biology and MURA binding sites

Although *MuDR/Mu* transpose late, the TIR promoters are active in stem cells (Benito and Walbot, 1997; Raizada and Walbot, 2000), and MURA and MURB transcripts and proteins are ubiquitous (Rudenko and Walbot, 2001). All mobile *Mu* share conserved (~28/32) MURA binding motifs in their TIRs; non-motile *Mu* have degenerate motifs that may still bind MURA, but with less avidity (Benito and Walbot, 1997). Could Mutator activity reprogram the transcriptome through such degenerate MURA binding sites? The B73 maize genome sequence was queried to identify MURA binding sites so as to discover whether any exist in promoters. Only 26 putative MURA binding sites of >90% similarity were found on 17 bacterial artificial chromosomes (BACs) dispersed throughout the genome (data not shown). Half of these were within 1 kb of a gene lacking a predicted function, and half were next to genes with a predicted function, but with no similarity to master regulatory genes that control

flowering. The relative paucity of MURA binding sites and their absence in or near master floral genes suggests that the global gene expression differences result from a more general response to Mutator activity.

### Mutator activity alters chromatin factors

Gene ontology (GO) terms were assigned to each differentially expressed probe through AgBase (<http://www.agbase.msstate.edu>), and terms for binding ion, nucleotide, nucleic acid and protein comprised 26, 23, 23 and 14% (collectively 86%), respectively (Figure 5; Table S5). Of particular interest is the repair of double-stranded DNA breaks caused by *Mu* transposition. Mobile *MuDR/Mu* increase leaf transcripts with roles in DNA repair to an extent similar to that observed under UV-B, a DNA damaging agent (Casati and Walbot, 2003). Several DNA repair and chromatin-related proteins were differentially regulated in our comparisons, and we predict that these compensate for the direct effects of Mutator activity. Unique combinations of histones H1, H2A, H2B and H4 were differentially expressed in a stage-specific manner in active Mutator anthers, and a single histone H2A probe was up-regulated at all stages. Several other genes that are likely to be associated with the direct action of MURA transposase were up-regulated at the mitotic stage, including: a DNA damage-binding protein 1b ('on-off'); histone deacetylases HDT1 and HDT3 (up-regulated); and a ubiquitin-activating enzyme ('on-off'). Active Mutator anthers also down-regulated a histone H4 at the mitotic and meiotic stages, as well as histones H1 and H2A. At meiosis, the down-regulated genes for Mutator-active lines included a DNA mismatch repair protein, a histone-lysine-*N*-methyltransferase and a DNA methyltransferase. At the mitotic and meiotic stages, a homolog to RAD51A, which functions in



**Figure 5.** Gene ontology term assignment to the 8223 differentially expressed probes.

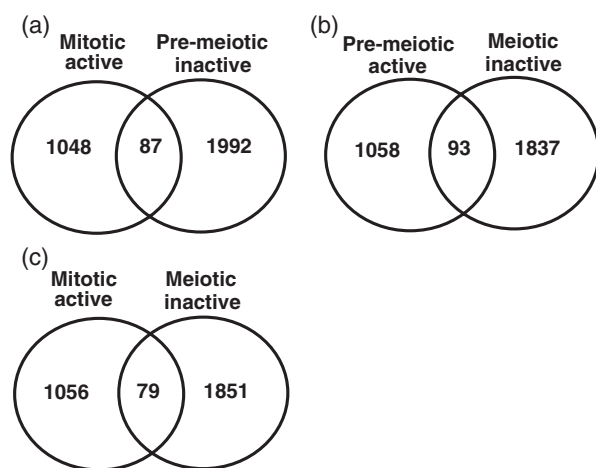
Genes in classes A and B (Figure 4) were evaluated. The percentage of each term is shown next to its corresponding pie sector. The unknown category (~50%) was omitted.

double-strand break repair during meiosis (Li *et al.*, 2007), was not detectably expressed.

### Does Mutator activity accelerate development?

Active and inactive Mutator lines are phenotypically identical, except that active lines have upright leaves, lack leaf tips and flower several days earlier. The difference in the days to flowering observed is general: many pairs of sister active and inactive Mutator lines in different inbred lines share this property (Robertson, 1986; Walbot, 1986). Does Mutator accelerate anther development? If true, genes up-regulated in the active line at an early stage should overlap with genes up-regulated in the inactive line at the next stage(s). As shown in Figure 6, fewer than 10% of the genes up-regulated in the active Mutator individuals fall into the accelerated development class. Therefore, the early flowering characteristic cannot be explained solely by accelerated development. Reinforcing this, the cytology of active and inactive anthers is identical at each stage, suggesting that the progression of anther development is normal.

We propose that *MuDR/Mu* globally reprogram gene expression, and accelerate the transition to flowering without perturbing anther development. It is possible that earlier flowering reflects chronic stress, impacting the timing of the switch from vegetative to floral development. Several abiotic stresses have been shown to accelerate flowering in plants, including water deficit (Angus and Moncur, 1977), UV-C (Martinez *et al.*, 2004) and chronic ionizing radiation (Sax, 1955; Kovalchuk *et al.*, 2007).



**Figure 6.** Mutator-active and -inactive anthers express unique sets of differentially regulated transcripts.

A developmental advancement hypothesis was tested by comparing the genes up-regulated in Mutator-active lines at an early stage with genes up-regulated later in Mutator-inactive lines. Three comparisons were performed: (a) up-regulated in Mutator-active lines at the mitotic anther stage versus up-regulated in Mutator-inactive lines at the pre-meiotic stage; (b) up-regulated in Mutator-active lines at the pre-meiotic anther stage versus up-regulated in Mutator-inactive lines at the meiotic stage; and (c) up-regulated in Mutator-active lines at the mitotic anther stage versus up-regulated in Mutator-inactive lines at the meiotic stage.

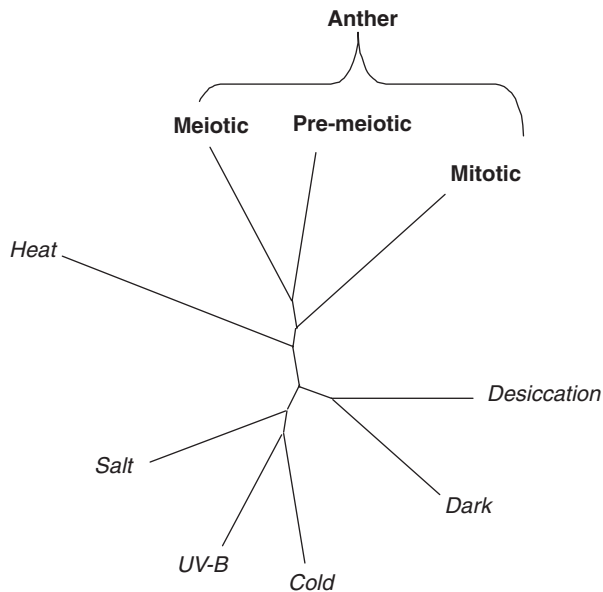
### Mutator activity elicits atypical expression of metabolic genes

To maintain normal growth, plants must acclimate efficiently to stress, which often generates reactive oxygen species (ROS). Plants combat ROS through the small redox molecules glutathione, thioredoxin and ascorbate (de Pinto and De Gara, 2004; Potters *et al.*, 2004), and secondary metabolites, particularly phenylpropanoids. In active Mutator plants, several genes with roles in central metabolism and ROS management were up-regulated in mitotic anthers, including glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase,  $\beta$ -amylase and cytochrome P450s. Genes with roles in the production of phenylpropanoids were up-regulated in pre-meiotic Mutator active anthers: anthocyanin transcription factors *C1* and *R1*, a flavonoid 3',5'-hydrolase, cytosolic aldehyde dehydrogenase RF2C, *O*-methyltransferase ZRP4, chalcone synthase (*C2*), 4-coumarate CoA ligase and cinnamoyl CoA reductase. Surprisingly, all of these genes are normally expressed at maximal levels post-meiotically, 7–10 days later in development (Skibbe *et al.*, 2008). Therefore, Mutator activity elicits the atypical early expression of genes with roles in oxidative stress and secondary metabolism. Thus, the host responds to Mutator activity in a manner suggesting that cellular redox sensors or ROS defenses are activated.

To determine if active *Mu* elements elicit cellular responses similar to any particular stress, the expression profiles were compared with six abiotic seedling treatments (Figure 7). In this unrooted tree based on Mutator-active compared with -inactive anthers, or stress with control seedlings, the three anther stages are clustered distinctly from the seedlings. This branch is equidistant from all six abiotic stress conditions. In particular, the genes expressed in anthers with active *MuDR/Mu* do not cluster with UV-B. In looking at the complete stress data set, the percentage of genes differentially expressed between treatment and control ranges from 5 to 15%, similar to the ~10% differential expression found at any single anther stage. With the total differential expression of ~25% for three anther stages, the TE-associated responses are more profound than a classic abiotic treatment.

### Overlap of differentially expressed transcripts with stress treatments

Despite the distinctiveness of the active Mutator responses, some genes associated with specific stress treatments were shared. Plants respond to biotic and abiotic stresses via a number of complex signaling pathways. In wounding, genes in the pathways controlling the hormones jasmonic acid and ethylene are up-regulated, whereas those producing auxin are down-regulated (Cheong *et al.*, 2002). These findings are significant because they demonstrate that transcriptome changes can be mediated by pathways independent of



**Figure 7.** Phylogenetic grouping of Mutator-active versus -inactive differentially expressed transcripts with groups of genes differentially expressed in six abiotic stress treatments.

mitogen-activated protein (MAP) kinases. In this study, the genes involved in ethylene and jasmonic acid production showed no discernable pattern – several genes were up- and down-regulated in Mutator-active anthers at all three stages. For auxin, 12 out of 13 genes were down-regulated in Mutator-active anthers, with one gene at the mitotic stage, seven genes at the pre-meiotic stage, four genes at the meiotic stage, and one gene in both the pre-meiotic and meiotic stages. These data suggest an intriguing relationship between suites of genes responding to a mechanical stress and the stress introduced by a mobile transposon. For example, mechanical stress (touch genes) could be triggered by delayed cell division, and a delay in cell division resulting from DNA damage could cause the stalling of replication forks: these delays could lead to a slight asynchrony in cellular proliferation within and between the anther rings of cells without disrupting the final anatomy (Figure 2).

#### Mutator activity reprograms the anther proteome

To complement the transcriptome profiling, two-dimensional difference gel electrophoresis (2D-DIGE) was used to analyze the ~2500 most abundant proteome constituents. Proteins from active or inactive mitotic anthers were isolated and labeled with either Cy3-DIGE or Cy5-DIGE dyes. Two protein samples were mixed and separated in the first dimension based on pI (pH 4–7), and then by mass. A composite of six biological replicates was generated and analyzed for statistically significant differences. The 2D-DIGE procedure is highly sensitive: as little as 125 pg can be detected in an individual spot, and a spot can contain

between one and six different proteins (Figure S2). In mitotic anthers over 2500 spots were visualized for both active and inactive samples: 48 were differentially expressed ( $P < 0.05$ ; fold change  $> 1.5$ ), with fold changes ranging from 1.5 to 8.3, with a median of 2.2. In contrast to the transcriptome where the up- and down-regulated differences were evenly divided between the active and inactive samples, 47 out of 48 significant protein differences were more abundant in the active Mutator individuals.

Spots corresponding to 39 out of 47 of the differentially expressed spots were identified on a preparative gel. Protein identities were obtained by LC-MS/MS for 30 out of the 39 spots. Similar to the transcriptome profiling, many metabolic enzymes were identified (Table S6): aconitate hydratase (spots 829 and 871), phosphoglucomutase (spots 1240 and 1245),  $\beta$ -glucosidase (spot 1346), pyruvate kinase (spots 1336 and 1346), fructokinase (spot 2021) and aldehyde dehydrogenase (spots 1346 and 1457). Two genes with roles in redox status (NADPH-thioredoxin reductase, spots 2085 and 2133) and a glutathione-S-transferase III (spot 2464) were also identified. The consistent identification of metabolic proteins in both the transcriptome and proteome profiling strengthens the argument that Mutator-active lines are responding to stress induced by altered metabolism.

#### The transcriptome and proteome data are highly congruent

The bias for protein abundance increases in active Mutator samples raised the question of whether the RNA and protein results are congruent. Twenty-eight probes corresponding to peptides from 21 out of 30 protein spots were identified on the microarray. A total of 20 out of 21 genes were up-regulated in both experiments, although the fold change was less in the transcriptome. All but two of the 28 microarray probes were in the upper quartile of the hybridization signal (Figure S3; Table S7). Furthermore, 14 of the probes were between two and eight times the median intensity value, seven were 32–128 times higher and five probes were  $> 500$  times higher. As the spot intensity approaches the upper limit the resolution of the abundance difference decreases. Therefore, the proteome analysis was highly predictive of transcript changes for corresponding genes, although protein increases were larger either from a post-transcriptional mechanism or from the increased resolution of proteomics for highly abundant species.

#### DISCUSSION

Maize geneticists have used *MuDR/Mu* for over 30 years because of the high forward-mutation frequency. The deleterious effects associated with the insertion of Mutator elements into RNA polymerase-II transcriptional units are overcome by maintaining active lines as heterozygotes: dominant mutations are rare and heterozygotes are usually indistinguishable from the wild type (Birchler *et al.*, 2005). Although transposition is restricted to terminal cell divi-

sions, or are post-mitotic, *MuDR*-encoded transcripts and proteins are found ubiquitously, even in stem cells. A consistent phenotype of active Mutator plants is that they shed pollen several days earlier than inactive sisters. This study was designed to distinguish between two hypotheses proposed to explain the impact on flowering time: *Mu* insertion in or near specific genes results in developmental phenotypes, or active *MuDR/Mu* reprogram host development. Most *Mu* elements in an active line were located by cloning a flanking sequence. Of the 46 insertion sites defined via iPCR, none were in or near a master regulatory gene, and only one was in or near a transcription factor-like gene. Therefore, we conclude that *MuDR/Mu*-element insertion sites are not responsible for the observed transcriptome and proteome differences. This conclusion is reinforced by the long-standing observations that diverse active Mutator lines – with TE insertions in different locations – share the common phenotype of early flowering (Robertson, 1986; Walbot, 1986).

#### Mutator activity substantially alters the anther transcriptome and proteome

Maize anther development, from the initiation of a primordium through to the shedding of pollen, takes 29 days (Ma *et al.*, 2008). During the first 3 days the two founding cell types (L1 epidermal and L2 internal) undergo rapid mitotic divisions, followed by cellular differentiation, to establish anther locules containing all the cells and cell types. In the subsequent 18 h, there is a transition from the pre-meiotic anther, in which mitosis has ceased, to the 2.0-mm anther, in which the central cells are in meiotic prophase I. Because progression is identical in active and inactive Mutator plants, the modification of the developmental program – such as fewer cell divisions or precocious cellular differentiation – does not occur.

To evaluate the impact of an active Mutator transposon family on anther development, both the transcriptome and proteome of developing maize anthers were assessed with the goal of finding an explanation for accelerated flowering time. A general finding is that immature anthers are even more transcriptionally complex than has previously been reported (Ma *et al.*, 2008). At each stage examined, anthers express between 27 000 and 31 000 transcripts, and over 32 000 transcripts in total (>70% of the estimated maize genes) during the 90-h period examined. Gene expression is dynamic, because large suites of genes are stage specific or are expressed at only two of the three stages. The impact of Mutator activity is very large: about 25% of all probes tested are differentially expressed during the surveyed 90-h period; 85% of the gene expression differences between active and inactive lines are stage specific; and three quarters of the differences are represented by the 'on-off' category. An active Mutator system causes profound changes in the transcriptome, primarily

by activating and suppressing the transcription of genes in a stage-specific manner. The magnitude of the differences was surprising, considering that the Mutator active and epigenetically silenced lines are closely related, and share many *Mu* insertion sites. The magnitude of changes caused by an active Mutator system is greater than that observed in early acting male-sterility mutants of maize, which cause the mis-differentiation of cell types (*ms23* and *mac1*) during the 1.0–1.5-mm stages of development (Ma *et al.*, 2007). The normal ontogeny and function of anthers, and subsequently pollen, from active Mutator plants demonstrates the robustness of anther development: despite enormous differences in the panel of genes expressed, the pace and outcome of anther development are normal in active Mutator plants.

The hypothesis that the large number of stage-specific differences reflects an advancement in the developmental program, i.e. that active lines express part of the pre-meiotic anther program precociously at mitotic or normal meiotic anther genes at pre-meiosis, was tested. Overall, fewer than 10% of the genes differentially expressed in an active line fell into this category of subsequent expression in the inactive line. Therefore, the underlying biological phenomena require another explanation.

To test whether transcriptome changes were carried through to alterations in the protein composition of anthers, the mitotic anther stage of active and inactive lines were assessed by 2D-DIGE. In this method, only the most abundant proteins were visualized; despite the limitation of examining only ~2500 protein spots, 48 differences were documented between active and inactive lines. In contrast to the transcriptome profiling, in which differentially expressed transcripts were approximately divided equally as up- or downregulated in active lines, 47/48 protein differences represented cases of increased abundance in the active Mutator samples. Nearly all of these differentially expressed proteins are metabolic enzymes.

#### Precocious flowering as a stress response

Early flowering has long been observed as a stress-escape strategy to ensure the completion of reproduction (Sax, 1955; Angus and Moncur, 1977; Martinez *et al.*, 2004; Kovalchuk *et al.*, 2007). Several pathways have been reported to control flowering time, including MAP kinase pathways and other master gene networks. In this study these pathways were not up-regulated. Instead, the genes found to be up-regulated at both the RNA and protein levels had functions in general metabolism, or in the production of secondary metabolites with the ability to scavenge ROS or maintain cellular redox status. The types of processes impacted by Mutator activity are similar to those identified in abiotic stress responses in maize seedlings (Fernandes *et al.*, 2008). The 8200 transcriptome differences between active and inactive Mutator lines were compared with six



abiotic stresses. Clustering demonstrated that the anther stages were represented on one branch, and that the sets of differentially expressed genes in the Mutator experiment were equidistant to each of the stress treatments. This result indicates that Mutator activity is a unique condition (Figure 7), but is of equal magnitude to abiotic stresses.

Our interpretation is that *MuDR*-encoded products act as a low, but chronic, stress that alters cellular homeostasis via redox status and/or the production of ROS. In developing anthers, such altered homeostasis is sufficient to elicit substantial transcriptome changes as part of the host's acclimation responses. Plant development can be profoundly modulated by environmental conditions: for example, large leaves in the shade and smaller leaves in bright light can be present on the same plant. Indeed, the leaf transcriptomes of maize lines were found to differ by up to 15% (Ma *et al.*, 2006). In contrast, differences in immature anthers (mitotic and pre-meiotic stages) from the same study were insignificant, and the pollen transcriptomes were virtually identical (Ma *et al.*, 2006). Therefore, during reproduction the program of gene expression is highly conserved and becomes progressively more canalized as anthers progress. Despite this trend, major modulations in the expression program during the initial 90 h of anther development – the period of active mitosis and cell fate setting – does not disrupt the normal pace of development, nor result in any measurable phenotype in an active Mutator line. In contrast, male-sterile mutants with single gene defects can lack one or more cell layers, but have far fewer transcriptome changes (Ma *et al.*, 2007). The buffering of normal development despite transcriptome and proteome changes in active Mutator lines appears to be much more similar to the acclimation responses during mild stress. As sessile plants live in a variable environment, successful acclimation can involve diverse metabolic responses that permit normal development despite perturbation. In the case of active Mutator plants, we conclude that an earlier than normal switch to flowering occurs that is then followed by the normal progression of anther development.

#### Advantages of Mutator activity

Is there any host advantage to an active Mutator system? This question is usually answered in terms of the diversification of new alleles caused by insertions. In a developmental context, an active Mutator system may be a type of 'fitness test' for the robustness of anther development. That is, despite the profound changes in metabolic processes, can a normal anther develop, will meiosis proceed and will viable pollen be produced? There is also a distinctive reward for the early shedding of pollen: with separate male and female flowers, maize is typically outcrossed through the wind dispersal of pollen. Early flowering individuals are more likely to pollinate others, and hence the Mutator stress syndrome may be one mechanism to explain the persistence

of active *MuDR/Mu* despite the deleterious nature of most *Mu* insertion alleles.

All maize lines examined have multiple methylated *Mu* elements, including derivatives of *MuDR*, in or near genes (Rudenko and Walbot, 2001). Once silenced, inactive Mutator lines are considered to be standard lines, and the transcriptomes are similar to standard maize inbred lines (D.S. Skibbe, D. Wang and V. Walbot, unpublished data). Recently, a naturally occurring locus (*Mu killer*) capable of silencing *MuDR* transcription via small RNAs was reported (Slotkin *et al.*, 2003, 2005). Preliminary experiments comparing the mitotic and meiotic transcriptomes of Mutator-active lines with *Mu killer* Mutator-silenced lines have yielded fascinating results. The lines silenced by *Mu killer* show minimal overlap with spontaneously silenced, inactive lines, or with standard inbred lines, suggesting that distinct pathways are responsible. Nonetheless, the *Mu killer* lines also have normal anther development.

## EXPERIMENTAL PROCEDURES

### Transcriptome profiling

Three Mutator-active and three silenced (inactive) sister lines with *bz2-mu2*, *bz2-mu3* or *bz2-mu4* reporter alleles were used. Spotted (active) or non-spotted (inactive) kernels from appropriate ears were selected and grown in Molokai, Hawaii (Winter 2005) or San Luis Obispo, CA (Summer 2006). Anthers were dissected from the upper florets and transferred onto dry ice and stored at  $-80^{\circ}\text{C}$ . For four biological replicates of active and inactive lines, RNA was isolated from 15 to 20 anthers using Trizol (Invitrogen, <http://www.invitrogen.com>), according to the manufacturer's instructions. RNA was assessed with the Quant-iT Ribogreen RNA Assay Kit (Invitrogen) and agarose gel electrophoresis, and treated with DNase (PicoPure RNA kit; Molecular Devices, <http://www.moleculardevices.com>). Total RNA (500 ng) was amplified and labeled with either Cy3 or Cy5 using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, <http://www.agilent.com>). A 750-ng portion of each labeled cRNA was hybridized for 17 h at  $60^{\circ}\text{C}$  on a  $4 \times 44\text{K}$  Agilent *in situ* synthesized oligonucleotide platform, along with spike-in controls for calculating target RNA concentrations. Slides were scanned on an Agilent G2565AA, and images were processed with the Agilent Feature Extraction software. After the removal of the few ( $<0.001\%$ ) spots flagged as being below background, saturated or outliers, dye-normalized values for each channel from Feature Extraction were normalized again to the median array intensity. Probes were considered to be 'on' if all four intensities were  $>2.6$  standard deviations above the average background. Probes with three of four intensities  $>2.6$  standard deviations above the average background were considered to be 'on' if the average intensity was at or above the median of the class of probes with three good intensities. Using the R package limma, median intensities for each channel were normalized within each array using the 'loess' method, and between arrays using a quantile method. All microarray data associated with these experiments are available at GEO (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE13118.

### Quantitative real-time PCR

Approximately 2  $\mu\text{g}$  of DNase-treated total RNA from three biological replicates of Mutator-active and -inactive mitotic anthers was reverse transcribed with an oligo-dT primer using the

SuperScript-III first-strand synthesis system for RT-PCR, as recommended by the manufacturer (Invitrogen). Primer pairs were designed using PRIMER3, and were synthesized by ILLUMINA (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed on an OPTICON2 sequencing detection system (MJ Research, a part of Bio-Rad, <http://www.bio-rad.com>). A 50- $\mu$ l reaction mixture, containing 25  $\mu$ l of iQ SYBR Green Supermix, 0.25  $\mu$ M of each primer and approximately 10 ng of cDNA, was amplified using the following cycling parameters: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min and a plate read. Upon completion of the program, a melting curve analysis was performed from 55 to 95°C, with a read every 0.5°C with a 10-sec hold. Of the 26 genes tested, 21 were confirmed to amplify a single band, via melting curve analysis and agarose gel electrophoresis (complete primer list in Table S2). The threshold cycle numbers ( $C_t$ ), at which each sample reached the threshold fluorescence level for each type of PCR product, and the primer efficiency values were determined for all samples using the PCR Miner algorithm (Zhao and Fernald, 2005). All  $C_t$  values were normalized to ubiquitin conjugase, a gene constitutively expressed in maize anthers (Ma *et al.*, 2008). Nine normalized values per gene per activity state were analyzed using the Wilcoxon rank-sum test (Wilcoxon, 1945).

### Inverse PCR

Insertion sites for a subset of the *MuDR/Mu* family (*Mu1*, *Mu2*, *Mu3*, *Mu4* and *Mu8*, which collectively constitute >90% of the mobile *Mu* elements (Walbot and Rudenko, 2002), were determined using an inverse PCR (iPCR) procedure. A 500-ng portion of *bz2-mu2* (active) genomic DNA was digested with one unit of *Bam*HI and *Bgl*II restriction enzymes (Invitrogen) in a 25- $\mu$ l reaction volume: the reference TE sequences lack these sites. After incubation at 37°C for 2 h, the samples were phenol:chloroform purified, DNA was ethanol precipitated, washed with 70% ethanol, resuspended in 319  $\mu$ l water and then ligated at 22°C for 1 h under dilute conditions that favor circularization. The ligated DNA was precipitated in ethanol, washed with 70% ethanol and resuspended in 20  $\mu$ l of 10 mM Tris-HCl, pH 8.0. Circularized DNA was recovered using the QiaQuick Plasmid DNA Purification Column in 150  $\mu$ l buffer EB, as recommended by the manufacturer (Qiagen, <http://www.qiagen.com>).

The genomic DNA flanking the *Mu* insertion sites was PCR amplified in a 50- $\mu$ l reaction using 5  $\mu$ l of the ligated DNA as template, 1  $\mu$ M *Mu*-TIR primer (5'-AGAGAAGCCAACGCCAWCG-CCTCYATTCGTC-3'), 0.2 mM dNTPs, 1X *Pfu*Ultra Buffer and 2.5 U *Pfu*Ultra per reaction (Stratagene, <http://www.stratagene.com>) under the following conditions: 94°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 5 min, plus a final extension at 72°C for 10 min. For each reaction, the PCR products were purified using the Qiagen PCR Purification Kit, as recommended by the manufacturer. iPCR products were subcloned into the pCR4Blunt-TOPO vector using the manufacturer's longest recommended incubation times (Invitrogen). A total of 192 colonies were picked and amplified using the rolling circle method, and then bidirectionally sequenced using T3- and T7-promoter primers.

### 2D-DIGE

Protein was extracted from approximately 100 anthers per biological replicate (Casati *et al.*, 2005). Protein quality was assessed on 4–15% SDS-PAGE gels after staining with Coomassie R-250. Twelve biological replicates representing two alleles (*bz2-mu3* and *bz2-mu4*), two activity states (active or inactive) and three biological replicates per allele per activity state were used to identify spots differentially abundant between Mutator-active and -inactive

proteomes. For each allele, an active biological replicate was compared with an inactive replicate on a single gel for a total of three comparisons per allele and six comparisons for the two paired lines.

Images were processed with PROGENESIS PG220 software (Non-linear USA, <http://www.nonlinear.com>). For statistical analyses, images from either the Mutator-active or -inactive gels were combined to create 'average' gels. For within-allele comparisons, spots were required to be present on two of the three gels to be included on the average gel. For the active versus inactive comparisons spots were required to be present on five of the six gels to be included. Using the average gels, *P* values and fold change differences were calculated for each spot. *P* values of <0.05 were considered to be statistically significant.

### Preparative gel analysis and spot picking

A 400- $\mu$ g portion of protein plus 25  $\mu$ g of the sample labeled with Cy5 DIGE fluor from the *bz2-mu3* Mutator active line was separated on a 2D-DIGE preparative gel. The gel was fixed overnight, washed and stained with Deep Purple (Fluorotechnics, <http://www.fluorotechnics.com>). Deep purple bulk proteins were visualized with an excitation wavelength of 532 nm, and the emission was viewed through a 560LP or 610BP filter. Alignment of this preparative sample image with the Cy5 analytical image identified 35 of the 47 differentially regulated spots. Each spot was excised using an Ettan spot picker (GE Healthcare, <http://www.gehealthcare.com>) fitted with a 1.5-mm cutter head.

### In-gel digestion, mass spectrometry and spot identifications

Excised spots were trypsin-digested and prepared for LC/MS/MS as described by Casati *et al.* (2005). The peptides were fractionated on a reversed-phase column (C18, 75  $\mu$ m  $\times$  150 mm) at a flow rate of  $\sim$ 300 nL min<sup>-1</sup>. Typically the column was equilibrated at 2% solvent B, then a linear gradient was developed to 35% solvent B over 30 min. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The eluent was directly introduced into a quadrupole-orthogonal acceleration time-of-flight mass spectrometer (QSTAR XL, MDS Sciex, <http://www.sciex.com>), and data acquisition was performed in an information-dependent manner: 1-s survey scans were followed by 3-s collision-induced dissociation experiments on computer-selected multiply charged ions.

Peak picking was performed using the MASCOT script embedded in ANALYST using the default parameters. Maize, rice and Arabidopsis sequences of UniProt.2006.10.21, and all sequences in reverse, were searched using an in-house version of PROTEINPROSPECTOR (v4.25.3) with the following parameters: only tryptic peptides were considered; cleavage in front of Pro; and one missed cleavage were permitted; the carbamidomethylation of Cys was considered to be a fixed modification; and protein N-acetylation, Met oxidation and the cyclization of N-terminal Gln residues were permitted as variable modifications. The mass accuracy for precursor and fragment ions was 200 and 300 ppm, respectively. The acceptance criteria were a minimum peptide score of 15, a minimum protein score of 22 and a maximum expectation value of 0.1. Single-peptide-based identifications were manually validated. If the peptides identified matched multiple proteins, a single entry number was selected as follows: (i) whenever a maize protein was in the list, it was selected; (ii) if multiple entries shared the same sequence, only the top hit was reported, unless unique sequences were identified for the lower ranking maize proteins – such multiple entries are not reported for rice or Arabidopsis because the corresponding maize protein may contain each sequence; (iii) 'full

proteins' were selected over fragments; and (iv) entries with descriptive names were selected over hypothetical proteins.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Distribution of the signal intensity for probes expressed on between zero and four arrays in Mutator-active and -inactive individuals.

**Figure S2.** Visualization of 2D-DIGE separation of mitotic anther protein from a Mutator-active and -inactive individual.

**Figure S3.** Microarray spot intensity versus the proteomics fold change.

**Table S1.** Expression data for all 42 034 probes on the microarray.

**Table S2.** A detailed description of the qRT-PCR data from the 21 genes tested to verify the microarray data.

**Table S3.** Mutator insertion sites recovered by inverse PCR that correspond to spots on the microarray.

**Table S4.** Inverse PCR BLASTX-NR results for clones not represented on the microarray.

**Table S5.** Gene ontology term assignment to all probes on the microarray as well as the relevant expression data for each probe.

**Table S6.** Summary of the 30 protein spots identified as up-regulated in the Mutator-active mitotic anthers.

**Table S7.** Proteomics versus microarray data for congruent spots. Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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