RESEARCH ARTICLE

MS23, a master basic helix-loop-helix factor, regulates the specification and development of the tapetum in maize

Guo-Ling Nan1,+, Jixian Zhai2,3, Siwaret Arikit2,+, Darren Morrow1, John Fernandes1, Lan Mai1,*, Nhi Nguyen1,§, Blake C. Meyers2,† and Virginia Walbot1,**

ABSTRACT

Successful male gametogenesis involves orchestration of sequential gene regulation for somatic differentiation in pre-meiotic anthers. We report here the cloning of Male Sterile23 (Ms23), encoding an anther-specific predicted basic helix-loop-helix (bHLH) transcription factor required for tapetal differentiation; transcripts localize initially to the normal four wall layers are observed. Microarray transcript profiling required for tapetal differentiation; transcripts localize initially to the specific predicted basic helix-loop-helix (bHLH) transcription factor than in ms32 and maturation. By contrast, MS32 is constitutive and independently upstream of bHLH51 and bHLH122, controlling tapetal specification and development. Among them, MS23 is the earliest-acting factor, upstream of bHLH51 and bHLH122, controlling tapetal specification and maturation. By contrast, MS32 is constitutive and independently regulated and is required later than MS23 in tapetal differentiation.

KEY WORDS: Pre-meiotic male reproduction, Regulatory hierarchy, Transcriptome, Proteome

INTRODUCTION

Maize is monoeccious, with separate inflorescences producing female-only ears and male-only tassels on the same plant. Tassels contain hundreds of florets, each with three stamens, compound organs consisting of a slender filament subtending an anther, a four-lobed organ in which meiosis occurs followed by pollen development. A relatively large size, the 30-day-long period of anther development, and the regularity of ontogeny in which anther length allows accurate developmental staging have facilitated dissection and confocal reconstruction of cell division and expansion patterns in maize anthers (Kellihier and Walbot, 2011). Additionally, there are hundreds of male-sterile mutants (Skibbe and Schnable, 2012). These maize resources were developed in part to facilitate hybrid seed production using male-sterile plants as ear parents with pollen supplied by a male-fertile partner line. These mutants are also a rich genetic resource to define steps in anther ontogeny.

The tapetum (TP) plays sequential, essential roles in anthers, ferrying nutrients to pre-meiotic cells and later the pollen mother cells (PMCs) and the meiocytes, remodeling the extracellular callose coating of the PMCs and tetrads, and secreting exine onto maturing pollen grains. As tapetal cells senesce, their collapsing walls form a landing pad to which the uninculeate pollen must attach (Kellihier and Walbot, 2011; Tsou et al., 2015). Mutations in numerous genes exhibit tapetal defects from pre- to post-meiosis (Timofejeva et al., 2013).

The bHLH transcription factors (TFs) in flowering plants consist of large families with 213 encoding genes annotated in maize (Lin et al., 2014), 178 in rice and 170 in Arabidopsis (Carretero-Paulet et al., 2010). Mutant analysis demonstrates that several tapetal bHLHs are crucial for (1) pre-meiotic to meiotic stages: Arabidopsis DYSFUNCTIONAL TAPETUM1 (DYT1) (Zhang et al., 2006; Feng et al., 2012), its rice homolog UNDEVELOPED TAPETUM1 (UDT1) (Jung et al., 2005), and the maize homolog, Male Sterility32 (Ms32) (Moon et al., 2013; rice ETERNAL TAPETUM1 (EAT1 – OS04G0599300, also named DELAYED TAPETUM DEGENERATION or DTD) (Niu et al., 2013; Li et al., 2013); rice TDR INTERACTING PARTNER2 (TIP2 – OS01T0293100; also named bHLH142 (Fu et al., 2014; Ko et al., 2014); Arabidopsis bHLH10, bHLH89 and bHLH91 (Zhu et al., 2015); as well as for (2) meiotic to post-meiotic stages: Arabidopsis ABORTED MICROSPORES (AMS) (Sorensen et al., 2003; Xu et al., 2010, 2014) and its rice homolog TAPETUM DEGENERATION RETARDATION (TDR) (Li et al., 2006; Zhu et al., 2008).

A tapetal cell arises from periclinal division of a bipotent precursor, the secondary parietal cell (SPC). In the ms23-ref mutant, pre-tapetal initials conduct an extra periclinal division generating a defective double layer (Chaubal et al., 2000). This complete bilayer is unique, because other mutants exhibit irregular periclinal divisions. The epidermis (EP), endothecium (EN) and middle layer (ML) appear normal cytologically in ms32-ref, whereas the tapetal cells lack a dense cytoplasm and are not binucleate, two characteristics of normal TP during meiosis; the meiocytes in ms23-ref fail to progress beyond meiotic prophase I. We report here the cloning of the bHLH encoded by Ms23 and demonstrate that its transcript accumulates in the SPC before TP formation and then reaches much higher levels as tapetal cells differentiate. We elucidate the distinct and overlapping processes regulated by MS23 and a second bHLH, MS32, and propose a sequential deployment and hierarchy of four tapetal bHLHs. Finally, we contrast the maize pattern with that of related bHLHs in rice and Arabidopsis.

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RESULTS
Confocal analysis of cytological defects in ms23-ref mutant anthers

Using confocal images with an established stage numbering convention as a guide (Kelliher et al., 2014), the changing cytology of the ms23-ref mutant was compared with its fertile counterpart across key stages of anther development (Fig. 1A). We examined three axes: x (width), y (length) and z (depth) (Fig. 1B). At Stage 4 – the three-wall-layer stage shortly after a periclinal division of the primary parietal cells (PPCs) generated EN and the SPC – ms23-ref and fertile siblings were indistinguishable. At Stage 7, about half of the bipotent SPCs had divided periclinally, with a complete four-wall-layered lobe formed by Stage 8. The periclinal divisions of both PPCs and SPCs appeared normal in ms23-ref anthers, in terms of initiation timing and completion of a four-layer anther wall.

In fertile anthers, the pre-meiotic archesporial cells completed mitotic divisions before Stage 9, then matured into the PMCs; these synthesized DNA and organized the meiotic chromosomes then entered prophase I, progressed through zygotene, and entered meiosis II at Stages 8, 9 and 10, respectively (Nan et al., 2011). During the pre-meiotic and meiotic stages, somatic wall layer cells continued to divide anticlinally to add anther width and length.

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periclinal division; these cells were vacuolated and often irregularly shaped with oblique walls rather than the regular architecture of cuboidal, densely cytoplasmic cells found in fertile TP (Kelliher and Walbot, 2011). The aberrant fourth and fifth innermost layers are termed t1 and t2 (Fig. 1C) as previously described (Chaubal et al., 2000). At Stage 10, both t1 and t2 cells had similar shapes and sizes viewed in the xy plane (Fig. S1). However, the outer ring of t1 cells were thinner in the z-dimension, resembling the ML in fertile anthers, whereas the inner t2 layer had a greater z-dimension, closer to the TP in fertile anthers (Fig. S1).

Using cell count data, we further analyzed the division patterns in \textit{ms23-ref} (Fig. 2). Because mutant anthers continued to elongate and lobes expanded, it was not surprising to see continuous length- and girth-adding anticlinal divisions. The divisions along the y axis of EP* (the outermost, EP-like first layer), EN* (the EN-like second layer), and ML* (the ML-like third layer) in \textit{ms23-ref} were similar to EP, EN and ML in fertile anthers, respectively (Fig. 1C; Fig. 2A). The cell count data of the t1 layer resembled those of the TP until Stage 8 when t2 appeared. To examine the girth-adding anticlinal divisions, we viewed the xz plane and observed similar cell counts before Stage 8 across all four wall-layers but some cell types soon deviated (Fig. 2B,C). Although anticlinal divisions of fertile tapetal cells along the x axis slowed after Stage 8, the aberrant t1 cells maintained the same pace of cell division and t2 cells appeared from ectopic periclinal divisions (Fig. 2C). By Stage 10, the t1 layer had conducted significantly more anticlinal divisions than normal TP ($P=0.049$). In \textit{ms23-ref}, the cell counts along the x axis of EP*, SPC* (the SPC-like third layer during the three-wall-layer stage) and ML* did not show significant differences, but the mutant EN* exhibited a significantly reduced rate of anticlinal divisions, particularly at Stage 9 ($P=0.041$) (Fig. 2B,C).

Map-based cloning of the \textit{Ms23} gene

\textit{ms23-ref} was a historic, unmapped recessive male-sterile mutant. Using bulk segregation analysis, \textit{Ms23} was initially placed on both chromosomes 6 and 8 (Fig. S2). PCR markers flanking the two implicated regions were tested and confirmed the male sterility phenotype to co-segregate with polymorphic markers near bins 8.00–8.01 on chromosome 8. Thirty recombinants were found after surveying $>250$ sterile plants from several 1:1 homozygous male-sterile/heterozygous fertile populations segregating the \textit{ms23-ref}, \textit{ms23-6027} or \textit{ms23-6059} alleles. \textit{Ms23} placement was within 0.75 Mb of the end of chromosome 8S. RNA-seq data for the 15 gene models in this region were retrieved from qTeller (http://qTeller.com); only GRMZM2G021276, spanning positions 95,823 to 98,367 and encoding a predicted bHLH protein, showed tassel-specific expression (Fig. S3). Tiling PCR primer pairs were employed to identify allele-specific lesions (Fig. 3; Fig. S4). In \textit{ms23-ref}, the entire gene is deleted; this deletion encompasses up to 96.5 kb defined by retention of the two closest annotated genes and their transcripts: GRMZM2G122850 at 67.4–71.3 kb and GRMZM2G081127 at 167.8–168.8 kb (data not shown). \textit{ms23-6027} is a frameshift allele with a 2-bp (AT) insertion at position 1076, causing an early termination codon at position 1091 immediately upstream of the predicted bHLH region (Fig. 3; Fig. S4A). The \textit{ms23-6059} allele has a 4-bp (GCTC) insertion, 13 bases after the predicted translation initiation site (Fig. 3).

As annotated in Maize B73 RefGen_v3, the 2361 bp \textit{Ms23} gene is confirmed to have four exons; cDNA prepared from fertile W23 anthers validated a 1619-nt primary transcript with an initiation

\begin{figure}
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\caption{Anther cell count data from confocal images. (A) Cell counts in a column along the y axis. (B) Cell counts of EP/EP*, EN/EN* and SPC/SPC* in the xz plane (transverse section). (C) Cell counts of ML/ML* and TP, t1 and t2 in the xz plane. In fertile anthers: EP, epidermis; EN, endothecium; ML, middle layer; TP, tapetum. In \textit{ms23-ref} anthers: EP*, the presumptive epidermis; EN*, the presumptive endothecium; ML*, the presumptive middle layer; t1, the fourth layer; t2, the fifth layer. Fertile data points are plotted as a solid line with sterile sibling data as dotted lines. Error bars represent s.d.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{fig3}
\caption{Structure of \textit{Ms23} alleles. There are four exons; the start codon (ATG) at nucleotide 775 is in exon 2, and the stop codon is in exon 4 at nucleotide 2100. The bHLH domain (striped box) encompasses amino acid positions 168 to 226 spanning the second intron (1278 to 1548). The \textit{ms23-ref} allele is null, because the entire gene is deleted; the \textit{ms23-6027} and \textit{ms23-6059} alleles contain frameshift mutations caused by insertions at cDNA positions 1077 (orange triangle, AT) and 769 (green triangle, GCTC), respectively.}
\end{figure}
reached its peak at Stage 10 (Fig. 4A); it was undetectable (or near the limit of detection) in all other samples (Fig. 4B). By RNA-seq, Ms23 transcript was detectable in anthers before Stage 4 at 200 and 400 μm (Zhai et al., 2015), and the levels rose steadily, peaking around Stage 10, after which it gradually decreased.

By in situ hybridization (Fig. 5), Ms23 transcript was detected in the SPCs and both daughter cells (pre-ML and pre-TP) immediately after the periclinal division (Stage 7). However, the PMCs had a patchy signal; the callose coat appeared to trap probe, and we considered the signal to be an artifact (Zhang et al., 2014). Within a day, when SPC daughters diverge morphologically into distinctive cell shapes, Ms23 signal intensity was reduced in the maturing ML (Fig. 5). From Stages 8 to 9, Ms23 transcripts predominated in the TP (Fig. 5). Loss of Ms23 signal in the ML, later enrichment in the TP, and a 10-fold increase at Stage 10 compared with Stage 7 indicate that although the SPC expresses Ms23, there was a substantial increase specifically in the differentiating TP (Fig. 5). Either transcript stability or transcription or both were reduced in the ML. No antisense transcript was detected with a sense probe in the control (Fig. 5A, inset).

Many bHLH proteins are associated with tapetal development

Using the MS23 protein sequence, many related genes were identified in angiosperms (Fig. S5; http://www.gramene.org/). Phylogenetic relationships of these bHLHs in maize, rice and Arabidopsis (Fig. 6) and a multiple sequence alignment of bHLH domains (Fig. S6) suggest that there has been conservation of regulatory networks for tapetal development.

MS23 and its paralog bHLH122 fall in clade A along with two rice proteins, TIP2 and EAT1 (Fig. 6). TIP2 is the rice ortholog of MS23, sharing 72.68% similarity in the full-length protein and 91.11% within the bHLH domain (Table S1). Maize bHLH122 is the ortholog of rice EAT1. In Arabidopsis, there are three clade A orthologs, bHLH10, bHLH89 and bHLH91, which are stamen specific and appear to act redundantly (Zhu et al., 2015). Maize bHLH51, rice TDR and Arabidopsis AMS, all in the B clade, are larger proteins, ranging from 551 to 625 amino acids, with 74.6% similarity in the bHLH domain (Fig. S6). Ms32, the other historical maize mutant with tapetal periclinal divisions (Chaubal et al., 2000) and high expression in the TP (Moon et al., 2013), encodes a protein that falls into clade C along with rice UDT1 and Arabidopsis DYT1 (Fig. 6).
Microarray profiling of **ms23-ref** and **ms32** anthers

The **ms23-ref** and **ms32** transcriptomes were compared with those of fertile siblings to quantify gene expression patterns. To analyze the differentially expressed genes, only the probes detected at Stage 9 in both mutant and fertile anthers were further analyzed (n = 24,682) at the criteria of 1.5-fold difference and \( P \leq 0.05 \). More genes were affected in **ms23-ref** (1236 up, 1293 down) compared with **ms32** (520 up, 866 down) (Fig. 7). The shared, differentially regulated set accounted for a smaller portion (24%) in **ms23-ref** compared with those (44%) in **ms32** (Fig. 7). The severely downregulated class showed greater fold change than those upregulated (Fig. S7). For example, there were over three times (217 down versus 61 up) and seven times (88 down versus 12 up) more downregulated genes in the **ms23-ref** anthers at the 4-fold and 8-fold change criteria, respectively (Fig. S7). The **ms32** transcriptome showed a similar trend with over four times (126 down/26 up) and approximately six times (59 down/10 up) more downregulated genes at the 4- and 8-fold change criteria, respectively. We conclude that MS23 and MS32 are transcriptional activators in most contexts because there are more substantial defects in gene activation than in repression.

MS23 is a master regulator in maize anthers

Normalized abundances of RNA-seq reads were obtained from published data spanning ten stages from 200 to 5000 \( \mu \)m of W23 anthers (Zhai et al., 2015). Selecting the ten most abundant bHLH transcripts at each stage yielded 23 genes. **Ms23**, **bHLH51** and **bHLH122** were in the top ten across the 1500 to 3000 \( \mu \)m stage whereas **Ms32** expression was lower with less fluctuation (Table S2; Fig. 8). **Ms23** transcript peaked at 2000 \( \mu \)m, nestled between two bursts of **bHLH122** at 1500 and 2500 \( \mu \)m; **bHLH51** was the most abundant bHLH transcript type in meiotic (2000 \( \mu \)m) through uninucleate microspore (3000 \( \mu \)m) stages. As most other bHLH transcripts subsided post-meiotically, **Ms32** exceeded all but **bHLH51** in anthers at the 5000 \( \mu \)m stage, shortly before pollen shed (Fig. 8). To explore global transcripts in **ms23-ref** anthers, we conducted deep sequencing (RNA-seq) at 400-, 700-, 1000-, 1500- and 2000-\( \mu \)m stages in the original ND101 background. The near invariant cyanase (GRMZM2G134747) transcript (Ma et al., 2007) was used...
as an internal normalization standard to compare the RNA-seq data between the W23 fertile and ms23-ref libraries. Many TFs were misregulated in ms23-ref anthers (Table S4). Using a 2-fold criterion, 28% of the bHLH class (n=58), 30% of MYB (n=50), 30% of bZIP (n=39) and 16% of MADS (n=12) TFs were affected (Table S4). About 40% (n=63) showed a 4-fold or greater difference (log2≥−2 or log2≥2). The data not only validated ms23-ref as a true null allele but also expanded the list of bHLHs either positively or negatively regulated by Ms23. In 1500-µm ms23-ref anthers, bHLH122 was almost undetectable and bHLH151 was down 12-fold, indicating that their expressions were dependent on MS23 (Fig. S9). These differences were validated with qRT-PCR (data not shown).

By contrast, Ms32 expression was slightly up (~3.7-fold) in ms23-ref at 2000 µm when there were about twice as many TP-like cells (t1+t2) in ms23-ref, indicating that Ms32 could be expressed in these aberrant cells in the absence of MS23. qRT-PCR results substantiated these findings and further illustrated that expression of Ms32 was somewhat delayed in ms23-ref (Fig. S10A). Although bHLH122 expression was lower in ms32 anthers at Stage 9, neither Ms23 nor bHLH51 was affected (Fig. S10B).

The expression patterns of all 213 maize bHLHs across these five anther stages in both fertile and ms23-ref plants were analyzed with algorithms from the Cluster 3.0 program (http://bonsai.hgc.jp/~mdehoon/software/cluster/). An average linkage heat map of hierarchical gene clustering based on Euclidean distance showed a tight correlation (0.96) between W23 and bHLH51 (Fig. S11). bHLH122 was also associated with Ms23 and bHLH51 (0.28) whereas Ms32 was dissimilar (0.00).

Pathway analysis (http://pathway.gramene.org/maizecyc.html) further illustrated many downstream cellular processes, including secondary metabolism, hormone biosynthesis/degradation, transporters, respiration/energy alteration, and lipid/fatty acid metabolism, were affected across Stages 4-10 in the ms23-ref anther (Fig. S12). In particular, the hormone pathways were notably correlated (Table S5), also validating the array data of a tassel-specific dihydroflavonol-4-reductase gene (GRMZM2G168893) in the brassinosteroid pathway (Table S2).

### Distinct protein expression patterns

Trypsin-digested protein samples from W23 fertile anthers at Stages 8 and 10 plus pollen were analyzed by HPLC-MS/MS. MS23 and bHLH51 were abundant proteins at Stage 10 but not at Stage 8 (Table 1). bHLH122 protein was below the detection limit at both stages, consistent with the RNA-seq result that the transcript levels were low at both stages, bracketing a spike at Stage 9. These results suggested that there could be stage-specific deployment and rapid turnover across a 3-day span. As expected from its constitutive RNA expression, MS32 was found in both Stage 8 and 10 anthers and was highly abundant in pollen (Table 1). MS32 is a widely expressed protein detected in leaves (Facette et al., 2013) and transcripts are found in multiple organs (Fig. S13; https://mpss.danforthcenter.org/dbs/index.php?SITE=maize_RNAseq).

### Protein and genetic interactions

Using yeast two-hybrid assays, the interactions among the bHLH domains of MS23, MS32, bHLH51 and bHLH122 were tested. All possible combinations of interactions, including all negative controls, were examined. Strong interactions occurred with co-translation of MS23+bHLH51, MS32+bHLH122, bHLH51+ bHLH122, and bHLH51+bHLH151 (Fig. S14). Other homodimers and heterodimers were either weak or not detected consistently.

We generated ms23-ref, ms32 double-mutant plants for cytological studies. Confocal images showed additivity with the first extra periclinal division observed around Stage 8, similar to the timing of the ms23-ref single mutant, and multiple extra layers, a typical phenotype of ms32; were found in the double mutant at Stage 11 (Fig. S15). Both the protein interactions and the genetic data support the model of sequential actions of MS23 and MS33 in separate pathways during tapetal differentiation, with a later common cascade during TP degeneration.

### Impact of TP defects on small RNA biogenesis

There is a drastic decrease in Dcl5 (formerly Dcl3b) transcripts in ms23-ref; DCL5 is required to generate 24-nt phasedRNAs and, as expected, these are virtually eliminated in ms23-ref (Fig. S8; Zhai et al., 2015). In mac1 and msc1a mutants, both lacking the TP (Wang et al., 2012; Chaubal et al., 2003), the miR2275 trigger molecules for 24-phasiRNA biogenesis are nearly undetectable (Zhai et al., 2015). miR2275 is present in ms23-ref; however, the 24-PHAS precursor transcripts are drastically reduced (Zhai et al., 2015). A data re-analysis indicated that ms23-ref accumulated an abnormal distribution of miR2275 types (Fig. S16). In fertile anthers, there was a single dominant member of the family (zma-miR2275b-5p), representing ~40% of all the subtypes and about 50 times more abundant than zma-miR2275b-3p in 1000-µm anthers (Fig. S16). In ms23-ref, diverse miR2275 species were present: in

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**Table 1. Proteomic data corroborate the RNA-seq results**

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<th>Protein and genetic interactions</th>
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<tr>
<td><strong>A. Proteomics – spectral counts</strong></td>
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<tr>
<td>Protein</td>
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<tr>
<td>MS23</td>
</tr>
<tr>
<td>MS32</td>
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<tr>
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<td>bHLH122</td>
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<tr>
<th>B. RNA-seq transcript abundance (normalized)</th>
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<tr>
<td>Ms32</td>
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<td>494</td>
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<tr>
<td>bHLH122</td>
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The relative spectral abundance of each protein analyzed by HPLC-MS/MS was normalized as described by Facette et al. (2013). RNA-seq data are from W23 fertile anthers (Zhai et al., 2015). n.d., not detectable.
order of abundance, the expected zma-miR2275b-5p, zma-miR2275a-3p, zma-miR2275b-3p, zma-miR2275c-3p and zma-miR2775a-5p (Fig. S16). We conclude that the mature miRNAs of the miR2275 family are not accumulating normally in the t1 and t2 of ms23-ref anthers. With altered miR2275 composition and near elimination of both 24-nt pre-miRNAs and mature miR2275a-3p, zma-miR2275b-3p, zma-miR2275c-3p and ms23-ref mutants exhibit sterility (Zhu et al., 2015). Maize Ms23 (rice Ms23), is mainly expressed in the EN from Stages 4 to 7, presumably through a mobile signal (Wang et al., 2012). We hypothesize that this outer epidermal arc might also act instructively to promote cell commitment and differentiation in the ML, some aspect of which is counter-regulated by Ms23.

No mutants for maize bHLH122 or bHLH51 are available; however, bHLH122 transcription is highly dependent on Ms23, placing it downstream of Ms23, similar to its rice ortholog EAT1 (Niu et al., 2013). bHLH51 is likely to be more important later during pollen development similar to its rice TDR and Arabidopsis AMS orthologs based on its peak expression after Stage 11 (Fig. 8).

Maize ms32 and the orthologous rice udt1 mutants share the phenotype of ectopic periclinal divisions; however, there are no extra periclinal divisions observed in the orthologous Arabidopsis dvt1 mutant. In all three species, the cells in the anomalous ‘TP’ expand tremendously in the z-dimension and may crush the arrested meiocytes; the post-meiotic terminal phenotypes of meiocyte failure and pollen abortion are likely consequences of earlier defects in lobe ontogeny arising from aberrant periclinal divisions of tapetal cells. Because maize anthers are much larger and develop more slowly (Kellihier et al., 2014), we determined that Ms32 is constitutively expressed starting in early lobe ontogeny, but there are no obvious cytological defects until Stage 9. Arabidopsis DYT1 is expressed across the Arabidopsis anther stages 4-7 (Feng et al., 2012), which corresponds to Stages 4-11 in maize and the rice anther stages 4-8 (Table S6; Sanders et al., 1999; Zhang and Wilson, 2009; Kellihier et al., 2014). Furthermore, Ms32 is expressed in many maize organs (Fig. S13), although there are no mutant phenotypes noted outside of anthers. Therefore, Ms32 is likely to be functionally redundant with other bHLHs in most contexts. Similarly, rice UDT1 is found in diverse organs whereas TIP2, the ortholog of Ms23, is mainly restricted to the inflorescence (http://ensembl.gramene.org/genome_browser/index.html).

Despite conservation of bHLHs important in tapetal development, mutant studies demonstrate distinctive features of the gene hierarchy, time of action, and mutant phenotypes of four bHLHs (Fig. 9). In maize and rice, the two clade A genes (Ms23 and bHLH122, and TIP2 and EAT1) are functionally distinctive (and in rice both are required for fertility) whereas in Arabidopsis, there are three copies of clade A genes (bHLH10, bHLH9 and bHLH91) and only triple mutants exhibit sterility (Zhu et al., 2015). Maize Ms23 (rice TIP2) controls expression of both bHLH51 (rice TDR) and bHLH122 (rice TIP1).
EAT1) (Fig. S10). By contrast, rice EAT1 positively regulates the expression of TIP2 (Ko et al., 2014). Arabidopsis AMS is regulated by bHLH10, bHLH89 and bHLH91. Ms32 is upstream of bHLH122 but does not directly regulate the expression of either Ms23 or bHLH151. In rice, the ortholog of Ms32 (UDT1) is upstream of both EAT1 and TDR. In Arabidopsis, the ortholog of Ms32 (DYT1) is also upstream of bHLH10, bHLH89, bHLH91 and AMS. Interestingly, Ms32, UDT1 and DYT1 are all upregulated in the mutants corresponding to ms23-ref (rice tip2 and Arabidopsis bhlh10/bhlh89/bhlh91) (Fu et al., 2014; Ko et al., 2014; Zhu et al., 2015).

At the protein interaction level, there are similarities as well as distinctions. Ms32 interacts with bHLH51 but not bHLH122 (Fig. S14) paralleling its counterpart in rice (Fu et al., 2014; Ko et al., 2014). Additionally, Ms32 only forms strong dimers with bHLH122 in maize whereas bHLH51 forms both homodimers and strong dimers with bHLH122. In Arabidopsis, interactions are less specific because all possible combinations tested positive among bHLH10, bHLH89, bHLH91, DYT1 and AMS (Xu et al., 2010; Feng et al., 2012). Neither the rice nor Arabidopsis anther proteomes has been investigated to determine which proteins are present simultaneously; however, we predict that bHLH partner exchange will be a common theme during anther development.

The timely deployment of various bHLH complexes regulates the sequential progression of TP into several distinctive roles over 2 weeks of anther development, ultimately leading to successful pollen production. Ms32 is the earliest regulator identified, controlling a suite of TFs important for early TP development. It is present in the SPC at Stage 7 and continues to direct pre-tapetal cells into young TP at Stage 8. Because Ms32 does not form homodimers, we hypothesize that during Stages 7 to 8, Ms32 must form a heterodimer with an unknown partner (Fig. S17) to prevent periclinal divisions in pre-tapetal cells. By contrast, Ms32 is not required until Stage 9 when periclinal divisions are initially observed in the ms23 mutant anthers. This timing of the ms32 phenotype coincides with the first expression peak of bHLH122; therefore, the bHLH122-MS32 complexes are potentially the key for terminating periclinal divisions of TP beyond Stage 9. Next, at Stage 10, when Ms32 is at its highest expression level and bHLH51 expression reaches its first peak, the MS23-bHLH51 heterodimers might serve crucial roles for re-differentiation of the TP to nourish meiotic cells and accumulate materials utilized after meiosis. When bHLH122 transcripts reappear and Ms32 levels off at Stage 11, we propose that bHLH51 breaks away from Ms32 to bind with bHLH122 and also forms homodimers, both of which are likely to be essential for the next phase of TP development as secretory cells to dissolve tetrad callose and deposit exine onto pollen. There is support for this model in rice, in which competition between TIP2 and EAT1 has been suggested (Fu et al., 2014; Ko et al., 2014). Ultimately, we propose that bHLH51 homodimers initiate programmed cell death, as in rice and Arabidopsis (Zhang et al., 2008; Xu et al., 2010, 2014).

Each network in maize, rice and Arabidopsis is unique but includes the same protein types. We demonstrate that MS23 is the master factor during the 5-day period of tapetal specification and early differentiation in maize. Ms32 has a more general role in multiple maize organs, and it is only indispensable at Stage 9 when it is required to slow further cell divisions and prevent periclinal divisions. In rice, a feedback relationship between TIP2 and UDT1 in parallel pathways is also proposed (Fu et al., 2014). A second report in rice, however, proposes a different hierarchy and puts TIP2 downstream of UDT1 (Ko et al., 2014), similar to the proposed network in Arabidopsis. Our maize data are more extensive in capturing stage-specific events with more in-depth quantitative data analysis substantiated with proteomics, permitting clearer justification for the timing and deployment of bHLH complexes in the maize TP.

**MATERIALS AND METHODS**

**Plant material**

ms23-ref and ms32 seeds were provided by Pat Bedinger (Colorado State University); ms23-6027 and ms23-6059 were obtained from the Maize Genetics COOP Stock Center (Urbana, IL, USA). Stocks were maintained by pollinating ears on male-sterile plants by pollen from a fertile, heterozygous sibling (ms×F) to maintain 1:1 segregating lines. To increase the visibility of recombinants in the mapping populations, ms23-6027 and ms23-6059 plants were introgressed once by crossing with B73 and A619 inbred lines followed by selfing (F1) then an msF cross (F2).

**Map-based cloning**

Total leaf genomic DNA was extracted from 23 male-sterile and 23 fertile sibling individuals in a 1:1 family for the ms23-ref allele. Each DNA sample was adjusted to 25 ng/µl before combining to make one sterile and one fertile pool for bulk segregation analysis at the Genomic Technologies Facility at Iowa State (Liu et al., 2010). PCR primers were tested to find polymorphic markers distinguishing fertile from sterile plants (Table S7). By scoring the presence/absence of recombinants at diverse marker locations, the interval containing the gene was narrowed to the tip of chromosome 8S and eventually to gene model GRMZM2G021276.

**Phylogenetic analysis**

Protein sequences of the putative orthologs of MS23 were obtained from Gramene (http://www.gramene.org) and aligned using Cobalt (http://www.st-va.ncbi.nlm.nih.gov/tools/cobalt/cgi?CMD=Web). A maximum likelihood tree using a Poisson model with the highest likelihood was generated using MEGA 6.06 (Tamura et al., 2013; http://www.megasoftware.net/).

**RNA extraction, microarray hybridization, qRT-PCR, in situ RNA hybridization and RNA-seq**

Various organs, including anthers, were collected directly into tubes chilled in liquid nitrogen. Total maize RNA was isolated using the PureLink RNA Mini Kit (Invitrogen) incorporating TRizol Reagent (Invitrogen) with an on-column DNase I (Qiagen) digestion according to the manufacturer’s instructions. Microarray experiments were conducted as previously described (Nan et al., 2011). qRT-PCR was performed as previously described with three biological samples and three technical replicates unless stated otherwise (Zhang et al., 2014). Table S8 lists the PCR primer sequences used in this study. For in situ hybridization, immature 3–5 cm tassels were fixed and embedded in paraffin following a standard protocol (Jackson et al., 1994). Sections (7 µm) were hybridized as previously described (Zhang et al., 2014) using probes synthesized from PCR-generated fragments with 5′-ATTAGGTGACACTATAGGAGAACCCTTGTGAATCTTGGTG-3′ and 5′-TAATACGACTCATATAGAGGAGAAACCCCTTGTGAATCTTGGTG-3′ primer pairs.

RNA-seq libraries were constructed using TruSeq RNA Sample Prep kits (Illumina). PARE libraries were constructed as previously described (Zhai et al., 2014). All libraries were sequenced on an Illumina HiSeq 2500 instrument at the Delaware Biotechnology Institute.

**Confocal microscopy**

Anthers were collected directly into 100% ethanol and stored at 4°C until staining and imaging utilizing protocols described previously (Kellher and Wallbot, 2011).

**Yeast two-hybrid assay**

The BHLH domains of MS23, MS32, bHLH122 and bHLH51 were PCR amplified (Table S9) and cloned into pCR2.1-TOPO vectors (Invitrogen). Clones with correct, in-frame sequences were subcloned into both the bait (pGBK7T7) and/or prey (pGAD7T) vectors (Clontech). Co-transformation
bait and prey vectors into competent AH109 yeast cells (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4A, gal80A, lys2::GALUAS::GAL1TATA-HIS3, GAL2TATA::GAL2TATA-ADE2, URA3::MEL1UAS::GAL1TATA-lacZ) was performed using a modified lithium acetate-mediated method (Clontech). Yeast clones containing both a bait and a prey were selected on low stringency SD plate (SD/–Leu/–Trp; Clontech). Interactions were further verified by screening on high stringency SD plates (SD/–Adc/–His/–Leu/–Trp; Clontech).

**Proteomics**

Fertile anthers from the W23 inbred were used. Approximately 300 anthers at 1 μm and 100 anthers at 2000 μm were collected directly into 1.5-mL screw-cap tubes chilled in liquid nitrogen. Pollen was collected from an actively shedding tassel and immediately stored in liquid nitrogen. Trypsin-digested protein samples were analyzed by HPLC-MS/MS as described by Facette et al. (2013) using duplicate samples.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

G.-L.N. and V.W. conceived and designed the experiments; G.-L.N. conducted the experiments; D.M., L.M. and N.N. performed the microarray experiments; J.F. analyzed the array data; G.-L.N., B.C.M. and V.W. wrote the manuscript; all authors read and approved the manuscript.

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**Data availability**

Microarray and RNA-seq data have been deposited in http://www.ncbi.nlm.gov/geo under accession numbers GSE90968 and GSE90849, respectively.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.140673.supplemental

**References**


