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ORIGINAL ARTICLE

Feminized tassels of maize *mop1* and *ts1* mutants exhibit altered levels of miR156 and specific SBP-box genes

Judd F. Hultquist · Jane E. Dorweiler

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Abstract Development of the unisexual maize inflorescences requires the abortion of pistillate primordia in the florets of the developing tassel and the arrest of staminate primordia in the florets of the developing ears. Mutations of many genes that lie within this sexual differentiation pathway, such as *tasselseed1* (*ts1*), or that influence this pathway, such as *mediator of paramutation 1* (*mop1*), result in feminization of the normally male tassel. Here, we show the loss of *mop1* or *ts1* function results in increased mRNA levels for several members of the SBP-box gene family. Our analyses of this family expand the number of maize SBP-box genes from 9 to 31 members. Intron–exon structures as well as phylogenetic data support the division of these family members into six groups. The SBP-box genes upregulated in feminized tassels fall into two groups, share common structural motifs and include the presence of a target site for miR156. Small RNA blots show miR156 levels are decreased in both *mop1* and *ts1* mutants. While there is a correlation between miR156 levels and SBP-box gene transcript levels, this correlation is not absolute, and thus we hypothesize that decreased levels of miR156 may provide competency for SBP-box gene upregulation by other common factors yet to be identified. We present a model that provides a putative link between *ts1*, *ts2*, *ts4*, *Ts6*, and *mop1* in the sex-determination pathway.

Keywords Inflorescence development · Squamosa promoter binding protein · Tasselseed · RT-PCR · Sexual differentiation · Sex determination

Introduction

The evolution of angiosperms opened a new reproductive niche allowing for tremendous diversity, particularly in floral and inflorescence structure. Deviating widely from their ancestral forms, more derived flowers tend to be asymmetric, incomplete, and often imperfect with male and female structures in separate flowers. This can be seen in monoecious maize, where distinct male and female flowers (referred to as florets in grasses) occur in separate inflorescences. Each of the inflorescences initiates perfect florets containing both stamens and gynoecia, but specific structures arrest or abort during development to produce the distinct male and female inflorescences. Thus, a developmental switch during inflorescence development ultimately determines the sex of the maize inflorescences. The developmental switch is accompanied by the establishment of a suite of secondary sex characteristics specific to the ear or the tassel. The end result of the sex-determination developmental switch is a rigid, compact ear bearing hundreds of kernels, versus a flexible, branched tassel producing copious amounts of pollen.

The developmental changes that occur during sexual differentiation of maize inflorescences are reasonably well characterized, but our understanding of the genetic and molecular mechanisms underlying this differentiation is still largely incomplete. While there are a number of mutations in maize that disrupt this process (i.e. mutations of the *tasselseed1* through *tasselseed6* genes, which produce feminized tassels—Emerson 1920; Phipps 1928; Emerson et al.

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J. F. Hultquist · J. E. Dorweiler (✉)
Department of Biological Sciences, Marquette University,
P.O. Box 1881, Milwaukee, WI 53201-1881, USA
e-mail: jane.dorweiler@marquette.edu

1935; Nickerson and Dale 1955; Neuffer et al. 1997), most current models of sexual differentiation each involve relationships among only a small number of these genes. For instance, it has been shown that *tasselseed1* (*ts1*) either directly or indirectly works to activate the *ts2* gene, which facilitates the programmed cell death (PCD) pathway in the pistil primordia of the developing tassels (DeLong et al. 1993; Calderon-Urrea and Dellaporta 1999). Similarily, *ts4* encodes a member of the miR172 family that acts to inhibit *indeterminate spikelet1* (*ids1*), misexpression of which (in a dominant gain of function allele known as *Ts6*) results in increased meristem branching and inhibition of the pistil cell death pathway (Chuck et al. 2007b). Early genetic studies postulated that *ts1/ts2* and *ts4/Ts6* represent distinct classes of sex determination regulators (Irish et al. 1994), but it is still unknown exactly if and how these regulators of tassel development may be coordinated.

In addition to the above genes that affect sex determination, some genes affecting paramutation (an epigenetic interaction between specific alleles) also give rise to feminized tassels, specifically *mediator of paramutation1* (*mop1*) and *required to maintain repression6* (*rmr6*). In the case of homozygous *rmr6* mutants, the feminized tassel phenotype is highly penetrant (Parkinson et al. 2007), whereas in homozygous *mop1* mutants, this phenotype is stochastic, varies under different environmental conditions, and can range in severity (Dorweiler et al. 2000). For instance, some tassels of homozygous *mop1* mutants appear completely normal, while feminization on others may involve the entire tassel, be localized to a particular branch, or even involve only a small number of florets. *mop1* encodes a putative RNA-dependent RNA polymerase based on its homology to the RNA-Dependent RNA Polymerase 2 (RDR2) protein in *Arabidopsis* (Alleman et al. 2006). A combination of genetic, molecular and co-localization studies suggest that RDR2 functions in the small interfering RNA pathway and helps to maintain transcriptional silencing of heterochromatic regions (Xie et al. 2004; Pontes et al. 2006; Ronemus et al. 2006).

Despite the existence of several mutations altering sex determination, relatively little is known about the sexual differentiation pathway in maize. There are bound to be numerous genes involved in the dramatic phenotypic differences between the maize ear and tassel. We hypothesized that *Squamosa*-promoter Binding Protein encoding genes (SBP-box genes) in maize may play a role in inflorescence development and differentiation, especially given that data from other model systems demonstrate roles for SBP-box genes in flowering and floral development (Unte et al. 2003; Stone et al. 2005; Wu and Poethig 2006; Wijeratne et al. 2007; Zhang et al. 2007; Schwarz et al. 2008). To test this hypothesis, we sought to identify additional members of this gene family in maize and to determine whether any of the family members may be misregulated in abnormal tassel phenotypes.

The SBP-box gene family was first identified in *Antirrhinum majus* as a pair of genes hypothesized to activate transcription of the meristem identity gene *Squamosa* (Klein et al. 1996). This family encodes plant specific transcription factors involved in the maturation and reproductive transition of plant species including snapdragon, rice, *Arabidopsis*, tomato, and maize. All SBP-like genes encode a highly conserved SBP-domain (consensus—$CX_4CX_{13}HX_5HX_{15}C$ $QQCX_3HX_{11}C$; Birkenbihl et al. 2005). In *Arabidopsis*, as many as 19 *Squamosa*-promoter Binding Protein Like (SPL) genes have been identified (Cardon et al. 1999; Yang et al. 2008), and characterization of several reveals a wide array of functions (Cardon et al. 1997; Unte et al. 2003; Stone et al. 2005; Wu and Poethig 2006; Zhang et al. 2007; Schwarz et al. 2008). Some members of the *Arabidopsis SPL* gene family are specifically targeted by the microRNA family miR156 (Gandikota et al. 2007). The miR156 target site generally occurs within the coding region with the exception of *SPL3*, *SPL4*, and *SPL5*, which have it in their 3′ untranslated region (UTR).

Several *Arabidopsis SPL* genes have been shown to regulate floral timing and the development of floral architecture. *SPL3* mRNA increases in response to decreased miR156 levels (Wu and Poethig 2006), and binds to the promoter of the meristem identity gene *APETALA1* (Cardon et al. 1997) to modulate vegetative phase change. *SPL8* acts as a local, tissue-specific regulator of gibberellic acid signaling that promotes reproductive growth in anther development, but represses seed germination and root elongation (Unte et al. 2003; Zhang et al. 2007). *SPL14* plays a role in regulating programmed cell death, normal plant architecture, and flowering time (Stone et al. 2005). Finally, *SPL9* and *SPL15* act redundantly in promoting the juvenile-to-adult phase transition (Schwarz et al. 2008).

In *Zea mays*, relatively little is known about the SBP-box gene family. Initial characterization of the gene family identified four full-length and three additional partial mRNA sequences (named *sbp1* through *sbp7*; Cardon et al. 1999). Two functionally characterized maize genes, *teosinte glume architecture1* (*tga1*) and *liguleless1* (*lg1*), also encode an SBP-box (Moreno et al. 1997; Wang et al. 2005). *tga1* is most notably involved in glume development differentiating the ears of maize from those of teosinte (Dorweiler et al. 1993; Dorweiler and Doebley 1997). A single amino acid substitution has been shown to be responsible for the evolution of a retracted glume and subsequently exposed kernels (Wang et al. 2005). Reversion of this amino acid back to what is observed in teosinte, the direct ancestor of maize, leads to the hardening and partial extension of the glume. LG1 is required for ini-

tiation of ligule and auricle development, such that homozygous *lg1* mutant plants completely lack these structures (Sylvester et al. 1990; Becraft et al. 1990). As is observed in the SBP-families of other plants, only some of these genes contain a microRNA target sequence for miR156: *sbp5*, *sbp6*, *sbp7*, and *tga1* do, whereas *sbp1*, *sbp2*, *sbp3*, *sbp4*, and *lg1* do not. Recent studies in maize have suggested that miR156 may act as a master regulator promoting juvenile plant development through coordinated suppression of the SBP-box gene family (Chuck et al. 2007a). A decrease in miR156 and a coordinate increase in miR172, the antagonistic regulator that suppresses expression of genes essential for juvenile development, leads to the maturation and sexual transition of the plant (Chuck et al. 2007a).

Here we present an analysis of the SBP-box gene family in maize. Phylogenetic analyses of amino acid similarities and comparison of intron/exon structure supported the separation of these genes into six groups. RNA expression analyses were carried out using wild type versus feminized tassels from two different mutants (*ts1* and *mop1*). Increased mRNA levels for the same subset of SBP-box genes are detected in feminized tassels of both mutants. Furthermore, northern blots reveal decreased levels of miR156 in the feminized tassel tissue obtained from each mutant. These data suggest a potential role for miR156 and specific SBP-box target genes in proper inflorescence development.

Materials and methods

Plant stocks and growth conditions

Plant stocks consisted of several families segregating for *mop1-1* homozygous and *mop1-1/Mop1* heterozygous individuals as previously described (Dorweiler et al. 2000). These stocks are in a mixed W23/K55 genetic background. Plants were grown under a series of germination, water, and light conditions in an effort to determine whether specific conditions were more or less prone to elicit the feminized tassel phenotype, although no differences in frequency or phenotype were apparent. Seed were planted in individual plastic pots and were grown for approximately 7 weeks in a Percival growth chamber (PGC-15.5-AR). Alternatively, seed was germinated on MS-agar media in sterile Magenta boxes until shoots reached a height of nearly two inches prior to transplantation into individual plastic pots. Additional variables included growing plants in either short days (8 h light, 16 h dark) or long days (16 h light, 8 h dark), and watering with either distilled water or with a Peters® Excel® 15-5-15 Cal-Mag fertilized mix at 10% w/v. For all experiments, light in the chamber measured 700–900 μmol m^{-2} and the temperature was maintained at 26°C during light and dark conditions. Finally, a plant stock segregating 3:1 for wild-type versus *ts1/ts1* mutants in A188 background (kindly provided by Paula McSteen) was grown in long day conditions and watered with the fertilized mix.

Tissue collections and sample preparation

Upon confirming that plants had undergone the reproductive transition at the apical meristem, usually after about 7 weeks of growth, the developing tassel was dissected out, measured, and photographed. Tassels, ranging from 1.6 to 24 cm in length, were placed immediately on dry ice and stored at −80°C. Some smaller tassels were collected, but were not analyzed because they were of insufficient size to definitively score for feminization as evidenced by visible silks. Total RNA was extracted from each tissue sample using TRIzol® from Invitrogen (Cat. No. 15596-018) following manufacturer's protocol including the optional step for removal of excess polysaccharides.

Real-time PCR

Each RNA sample was treated with Ambion's TURBO DNA-free™ (Cat. No. 1907) following manufacturer's "Rigorous Treatment" protocol in an effort to remove any residual genomic DNA (gDNA). DNase treated RNA was subjected to reverse transcriptase reactions using oligo-dT primer and M-MLV Reverse Transcriptase (Promega Cat. No. M1705) as follows: 800 ng total RNA, 1 μL 10 mM oligo dT and dH_2O (to 17 μL) were mixed and incubated at 70°C for 5 min and flash cooled on ice. M-MLV Reaction buffer, 2 μL 2.5 mM dNTP and 200 units M-MLV enzyme were added to the reaction (25 μL total) and incubated at 37°C for 60 min followed by 15 min at 70°C. The final reaction mixture was diluted with 50 μL distilled water and stored at −20°C. All reverse transcriptase reactions were run in parallel with a negative control in which water was added instead of the M-MLV reverse transcriptase enzyme.

Quantitative Real-Time PCR was performed on a Bio-Rad MyIQ Single Color thermocycler with Bio-Rad SYBR Green Supermix (Cat. No.170-8884). Reaction volumes were 25 μL and consisted of 12.5 μL SYBR Green Supermix, 10 μL distilled water, 2 μL 5 mM primer mix (forward and reverse), and 0.5 μL cDNA template. Each reaction was performed in triplicate under the following conditions: 10 min activation at 95°C, 40 cycles of three-step amplification [10 s at 95°, 20 s at the determined optimal annealing temperature (varies by primer pair) and 20 s at 72°C], and a final extension of 1 min at 72°C followed

by the melt curve (80 cycles of 7 s at increasing temperatures starting at 55°C and increasing by 0.5°C at each cycle). Each template sample was originally run in parallel to its negative control from the reverse transcriptase reaction using a robust *ubiquitin1* primer (see below) to check for genomic DNA contamination before use in quantitative assays.

Primer design

Primers to 20 SBP-box genes were designed from contigs of EST sequences (the majority of which derive from maize inbred B73) using Gene Runner v. 3.05 (Hastings Software, Inc.), which facilitated in silico pre-screening to eliminate pairs with stable hairpin loops, dimers, bulge loops and internal loops. Experimentally, well-designed primers were identified based upon their ability to meet several standards: (1) specificity—generation of a single significant peak in the melt curve, (2) consistency—highly reproducible C_t (cycles to threshold) values within the reactions of a triplicate and (3) robustness—successful amplification over a range of annealing temperatures. Primers were always tested over a range of annealing temperatures and subsequently used at the determined optimal temperature identified by highest triplicate consistency and reaction efficiency. All of the primers used in this study are listed in Supplementary Table 1.

DART analysis

The results of Real-Time PCR were analyzed using DART-PCR (Peirson et al. 2003), a program that facilitates the analysis of the kinetics of each reaction performed. The average amplification efficiency of each primer pair was determined, and primers performing poorly were replaced. The average efficiency of all of the primer pairs discussed in this study ranged between 0.85 and 1.0. DART-PCR then uses the average amplification efficiency of the primer pair to calculate a value (R_0) representative of the initial template concentration. The R_0 values for *ubiquitin1* (*ubi1*) and experimental genes were used to calculate expression levels relative to *ubiquitin1*.

Sequence analysis

EST and genomic sequences were aligned by the multiple sequence comparison by log-expectation (MUSCLE) algorithm using the MUSCLE freeware (version 3.5; Edgar 2004) and visualization facilitated by the multiple sequence alignment editor program GeneDoc (http://www.nrbsc.org/gfx/genedoc/index.html). Where EST information was determined incomplete based on comparisons to the homologous rice and *Arabidopsis* SBP-box genes, those sequences in conjunction with probable splice sites in the genomic sequences identified by the plant splice site prediction program SplicePredictor (http://www.deepc2.psi.iastate.edu/cgi-bin/sp.cgi) were used to determine the most likely intron/exon structure of the full transcripts (See Fig. 2). Predicted translation of the transcripts and identification of start and stop codons was facilitated by the DNA analysis application Gene Runner v. 3.05 (Hastings Software, Inc.). Protein domains were positively identified using the domain database and functional analysis tool InterProScan (http://www.ebi.ac.uk/InterProScan/).

Phylogenetic analysis

Sequences were first aligned using the multiple sequence comparison by log-expectation (MUSCLE) algorithm using the MUSCLE freeware (version 3.5) designed to closely approximate a CLUSTALW alignment in a much shorter time frame. This original alignment was imported into the multiple alignment and phylogenetic tree analysis software Molecular Evolutionary Genetics Analysis (MEGA), version 4.0 (Tamura et al. 2007). The alignment was refined using the CLUSTALW algorithm (Thompson et al. 1994) within MEGA4 and subsequently used to infer phylogenetic relationships. Significance of the inferred relationships was determined by Bootstrap analysis (1000 replicates), as well as by testing reproducibility using varied algorithms and datasets. Trees were generated using either the full amino acid sequence, or using just the conserved SBP-domain (both zinc fingers through the NLS), and by using either Maximum Parsimony (Eck and Dayhoff 1966; Nei and Kumar 2000) or Neighbor Joining (Saitou and Nei 1987) methods in MEGA4.

Small RNA gel blots

Total RNA (20 μg/lane) was subject to gel electrophoresis on 12.5% denaturing urea polyacrylamide gels prepared by the Sequagel® Sequencing System Kit (EC-833) and transferred to GeneScreen Plus-R membranes by electrophoresis using Biorad's Mini Trans-Blot® Electrophoretic Transfer Cell as described (Lee and Ambros 2001). After transfer, they were cross-linked at 1,200 mJ and baked for 1 h at 70°C. Oligonucleotide probes were labeled using Integrated DNA Technologies' Starfire™ Oligonucleotide Labeling System and Perkin Elmer/NEN 6000 Ci/mmol alpha-^{32}P ATP. Hybridization to the membranes at 35°C in 7% SDS, 0.2 M Na_2PO_4 pH 7.0 was allowed to proceed overnight. Membranes were washed at 42°C three times with 2× SSPE, 0.1% SDS. The blots were exposed on Molecular Dynamics Phosphorimager screens and signals were quantified using ImageQuant Software.

Results

The maize genome contains at least 31 SBP-box genes

Publicly curated databases (PlantGDB, http://www.plantgdb.org/, NCBI, http://www.ncbi.nlm.nih.gov/, TIGR, http://www.tigr.org/, and MAGI http://www.magi.plantgenomics.iastate.edu/) were searched for maize sequences of high amino acid similarity to the conserved SBP-domain. These searches identified an assortment of EST contigs, EST singletons, as well as complete mRNA. These searches suggested an initial set of 25 unique EST contigs containing an SBP-box domain, some of which corresponded to the previously identified maize SBP-box genes (eg. *lg1*, *tga1*, and *sbp1* through *sbp7*). Each putative unique transcript was consequently used to search the same public databases to identify corresponding genomic sequence. Given that maize contains a large number of nearly identical paralogs, a series of reiterative BLAST searches between genomic and EST sequences were used to differentiate ESTs originating from nearly identical paralogs. As a final check, the full-length amino acid sequences of the 18 rice SBP-box genes were used to search the EST and genomic databases at NCBI. The combination of mRNA/EST and genomic sequences enabled us to determine that there are at least 31 unique SBP-box genes in the maize genome.

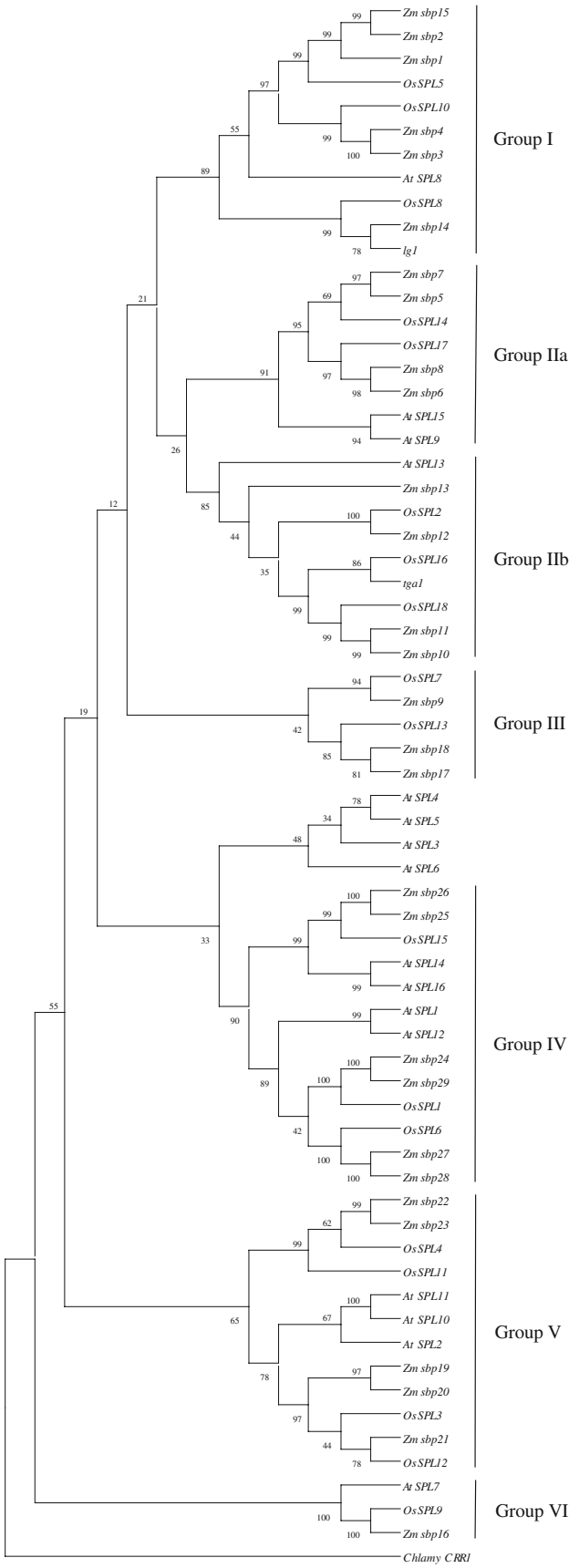
With two exceptions, we present paired genomic and mRNA/EST sequences for each of these 31 unique genes (Supplementary Table 2). One of the original maize SBP-box genes, *sbp2*, is one of the exceptions. The full-length cDNA is available in GenBank (AJ011615; Cardon et al. 1999), but we have been unable to find matching EST or genomic sequences thus far. The second (*sbp14*) is a genomic sequence for which no EST data was available, but rather SplicePredictor (http://deepc2.psi.iastate.edu/cgi-bin/sp.cgi) and reiterative tBLASTn searches using amino acid sequences of closely related genes (*Os SPL8* & *lg1*) were used to infer a putative amino acid sequence. Excluding *tga1* and *lg1*, we have followed the nomenclature first established with the cloning of maize *sbp1* through *sbp7*, and thus named the remaining SBP-box genes *sbp8* through *sbp29* (Supplementary Table 2). Our searches also identified a small number of additional genomic sequences containing the conserved SBP-box, but these were either of insufficient length to identify complete genes or contained a stop codon within or immediately following the SBP-box, suggesting they were pseudogenes. Such sequences were not pursued further. Although the maize genome is not yet fully sequenced, it is notable that this tentative total of 31 is nearly twice as many SBP-box genes as are present in the rice (18) and *Arabidopsis* (19) genomes, consistent with the allotetraploid origin of maize (Gaut and Doebley 1997).

Phylogenetic analyses support six groups of maize SBP-box genes

In order to best compare the maize SBP-box genes to one another and to their *Arabidopsis* and rice homologs, phylogenetic relationships among the members of all three species were examined concurrently with the intron/exon structure of the maize gene family. Predicted amino acid sequences for the 31 maize SBP-box genes were determined, aligned relative to rice and *Arabidopsis* genes, and used to infer phylogenetic relationships (Fig. 1). Multiple trees were generated by varying the algorithm (Neighbor Joining versus Maximum Parsimony), or the sequence composition (entire protein vs. SBP-box only), and the major cladistic groups were highly reproducible. All variations among trees related to deep relationships among the groups rather than the genes comprising each group or their relative relationships to one another within the group.

The maize SBP-box genes fell into six distinct clades, referred to as Group I through Group VI. Consistent with observations that genomic structure is a well-conserved evolutionary character state (Hardison 1996; Paquette et al. 2000; Rogozin et al. 2003), each group shares a characteristic intron/exon structure with the exception of Group III as explained below (Fig. 2). All genes maintain the defining SBP-box within the first couple exons, which is interrupted by the presence of a positionally conserved intron falling directly after the CQQCX motif. The final $XHX_{11}C$ portion of the SBP-box domain, along with a conserved nuclear localization sequence (NLS), is contained in the next exon. Other introns tend to be positionally conserved within a group, and sometimes between related groups. Consistent with observations for SBP-box genes in other species, some (17 of 31) maize SBP-box transcripts contain a target site for members of the miR156 family of microRNAs, either in the coding region or in the 3′ untranslated region (UTR). The presence and position of this putative target site also tends to be conserved within a group, although the position varies among the diverse genes in Group III.

Amino acid similarity among Group I genes supported a highly reproducible clade (Fig. 1), which is also supported by genomic structure (Fig. 2). Both Group I and Group II genes are similar in typically having three exons, but Group II can be distinguished from Group I by the presence of a miR156 target site located in the coding region of the last exon, directly following the exon containing the NLS. Translation of the miR156 target site always encodes a similar string of amino acids ALSLLS. The phylogenetic tree suggests early bifurcation of Group II to yield two subgroups, labeled Group IIa and Group IIb (Fig. 1), which also correlates with subtle differences in genomic structure (Fig. 2). Group III contains three maize genes with relatively diverse intron/exon structures. *sbp17* and *sbp18*



◀ **Fig. 1** Evolutionary relationships among maize, rice and *Arabidopsis* SBP-box genes. This Bootstrap consensus tree was inferred from 1,000 replicates (Felsenstein 1985) in MEGA4 using the Maximum Parsimony method (Eck and Dayhoff 1966; Nei and Kumar 2000) with the full amino acid sequences. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together are shown next to the branches. Amino acid sequences of 31 maize *sbp* genes, 18 rice *SPL* genes, and 16 *Arabidopsis SPL* genes were aligned using MUSCLE, and refined by ClustalW in MEGA4 (Tamura et al. 2007). The full alignment is provided as part of the supplementary online data. Multiple additional trees were generated using either the full amino acid sequence, or using just the conserved SBP-domain (both zinc fingers through the NLS), and using either Maximum Parsimony or Neighbor Joining (Saitou and Nei 1987) methods in MEGA4. All major clades/groups, as well as relationships within each of these groups, were well supported regardless of the sequence dataset or method used. Amino acid sequences of 16 *Arabidopsis* SPL genes were obtained from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/), the 18 SBP-box genes from rice were obtained from the Database of Rice Transcription Factors (DRTF, http://drtf.cbi.pku.edu.cn/). The *Chlamydomonas* SBP-box containing *Copper Responsive Regulator* (*CRR1*) gene was obtained from the Sanger Institute (http://www.sanger.ac.uk/bioinfo/), and included as a putative outgroup

encode very small proteins, contain the nuclear localization sequence in the final exon, and have a miR156 site that falls in their 3′ UTR. In contrast, *sbp9* is structurally more similar to Group II, but is distinct in the size of the first intron and location of the miR156 target site (Fig. 2). Phylogenetic analyses of the amino acid sequences also routinely place *sbp9* as distinct from Group II, perhaps suggesting that the genomic structural features shared with Group II are relatively ancient character states (plesiomorphic). Group IV was well supported by bootstrap analyses (Fig. 1). These genes have four coding exons, although ESTs for *sbp19* and *sbp21* exhibited one or more non-coding 5′UTR exons with some varied splicing (Fig. 2). A distinctive characteristic of this group relative to the rest was the presence of an additional exon between the two exons containing the NLS and miR156 target site (Fig. 2). Group V genes are particularly unique in that they have a large number of exons (10–12), and all lack a miR156 site (Fig. 2). All six maize Group V genes, as well as the orthologous *Arabidopsis* and rice genes, contain a highly conserved set of ankyrin repeats, a common protein–protein interaction motif, near the C-terminus. The ankyrin repeats and several conserved amino acid residues within and around the SBP-domain provide strong support for this clade. Group V clades as a weak sister group to several *Arabidopsis* genes that have no clear monocot orthologs (this work and Gandikota et al. 2007). Group VI contains a single maize gene, *sbp16*, that appears unique relative to the rest, containing ten exons structurally distinct from the pattern seen among Group V, no ankyrin repeats, and no

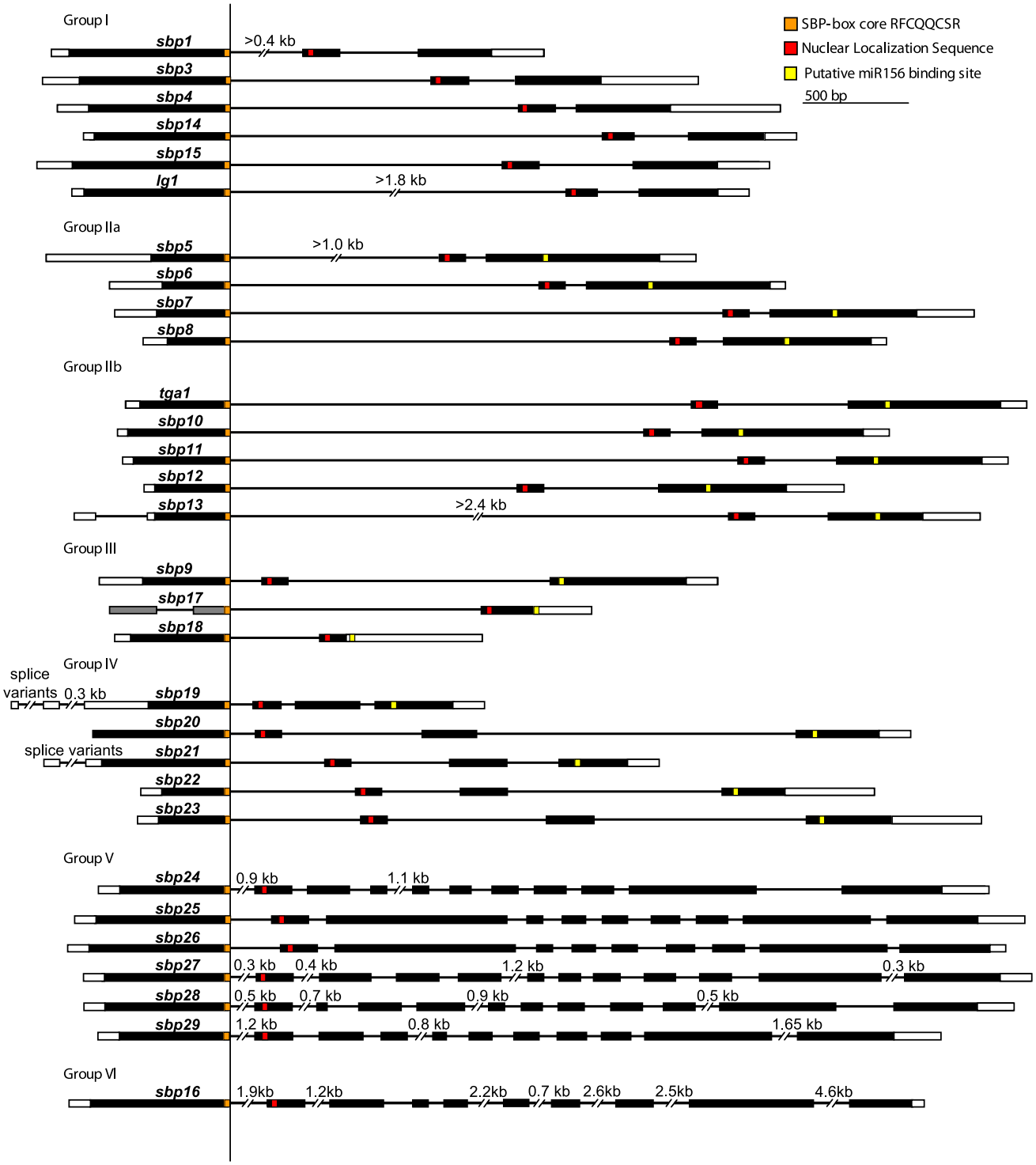


Fig. 2 Intron/Exon structure of Maize SBP-box genes. Schematic representation of the maize SBP-box gene family demonstrates the structural similarities within specific groups. Introns are shown as *black lines*, exons as *black boxes*, and UTR regions as *open boxes*. The RFCQQCSR core of the SBP-domain is shown as an *orange box*, the NLS as a *red box*, and the miR156 target site as a *yellow box*. The region upstream of the SBP-domain of *sbp17* is shown in *grey* to reflect uncertainty regarding the coding region: current EST sequence data suggests that the translation start may reside within the conserved SBP-domain, rather than farther upstream, suggesting either that the sequence data is incomplete, or that this gene may represent a pseudogene. For the sake of space, some of the longer introns were condensed as signified by a double slash (//) within an intron and the length it represents above (X kb). In some cases, the genomic sequences failed to span the entire length of the intron, in which case the minimal length is indicated (>X kb). The Ankyrin repeats are not specified, but span about 300 nucleotides in the final two exons of all Group V genes

miR156 target site. Thus, separation of the SBP-box gene family into six groups is well supported by both amino acid similarity and genomic structure.

Several SBP-box genes exhibit upregulation in feminized tassels of homozygous *mop1-1* plants

In order to test the hypothesis that some of the variations in tassel phenotype observed in *mop1-1* individuals may be due to the altered expression of one or more SBP-box genes, quantitative Real-Time RT-PCR (qRT-PCR) was performed for a broad sampling of genes representing each of the different groups of SBP-box genes (Table S1). Furthermore, given that the feminization phenotype is variable and appears to be influenced by environment (Dorweiler et al. 2000), we sought to identify environmental conditions with the greatest effect on the homozygous *mop1-1* mutants. Individual tassels were collected from *mop1-1* plants and their wild type siblings grown under varied growth chamber conditions, and collected tassels were examined for evidence of feminization. Tassel length was also noted as a rough indicator of developmental stage, although each tassel bears florets along a continuum of developmental stages, with the most mature florets present near the base of the tassel. Tassels ranged from 1.6 cm (when the phenotypic differences between normal and feminized tassels first become readily apparent under a dissecting microscope) to greater than 14.0 cm (when tassels are fully differentiated, with well developed hairy glumes enclosing the anthers, or long protruding silks on the feminized tassels). Each experimental condition produced one or more feminized tassels among the homozygous *mop1-1* plants, and never among the wild-type siblings, but no single condition resulted in a majority of the homozygous mutants being feminized. qRT-PCR expression analyses within each of these experiments suggested a specific subset of SBP-box genes exhibit increased RNA levels among the feminized tassels of homozygous *mop1* mutants. Figure 3 and Supplementary Fig. 2 report the data from a technical replicate of qRT-PCR assays for RNA samples from several of the varied growth conditions. Samples included in this replicate were chosen to provide a series of three comparable length tassels, where each group of three included a feminized tassel, a normal tassel from a homozygous *mop1-1* mutant, and a tassel from a wild-type plant. Figure 3 presents this data averaged according to genotypic/phenotypic category, whereas Supplementary Fig. 2 presents the data for each individual RNA sample according to tassel length.

Expression levels between the phenotypically normal tassels from homozygous *mop1* mutants and those from wild type plants were not statistically different for any of the 16 SBP-box genes tested (Fig. 3 and supplementary

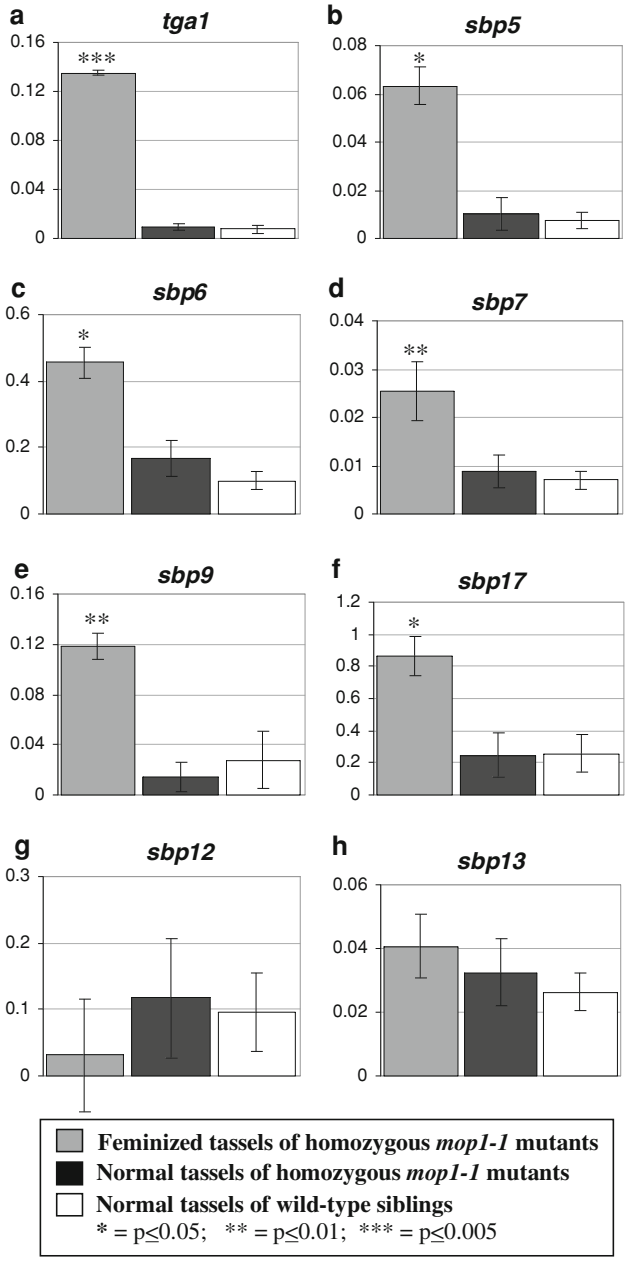


Fig. 3 Expression of six Group II and two Group III SBP-box genes in tassels of plants segregating for homozygous *mop1-1* and heterozygous *Mop1/mop1-1* genotypes. Each chart presents average qRT-PCR expression data relative to *ubiquitin* in developing tassels (≤10 cm, $N = 6$ for each category ± S.E.). Supplementary Fig. 2 presents this data with each individual tassel plotted according to tassel length, as well as data for eight additional SBP-box genes that appear to be unaffected in the same set of RNA samples

Fig. 2). The expression of many SBP-box genes was also unaffected by *mop1* genotype or feminization phenotype (Supplementary Fig. 2). However, feminized tassels from homozygous *mop1-1* plants exhibited higher relative mRNA levels for several Group II genes (*tga1*, *sbp5*, *sbp6*, and *sbp7*) and two Group III genes (*sbp9* and *sbp17*) when compared to normal tassels from either wild-type siblings

or homozygous *mop1-1* tassels (Fig. 3a–f). We also observed that increased levels of mRNA were often most pronounced in the youngest feminized tassels (~1.6–3 cm), but levels decreased until there was often no statistical difference relative to expression levels in wild type or in homozygous *mop1* mutants with a normal tassel phenotype (>8 cm; Supplementary Fig. 2a–c, e, f). *sbp7* displayed a distinct expression pattern relative to the other upregulated genes, maintaining higher relative expression levels in feminized tassels of all lengths tested (Supplementary Fig. 2d). Two Group II genes, *sbp12* and *sbp13,* displayed no significant expression differences among feminized, normal or wildtype tassels (Fig. 3g, h, and Supplementary Fig. 2g, h).

Several group II genes and *sbp17* exhibit upregulation in feminized tassels of *ts1* individuals

Based on the differential expression of a specific subset of SBP-box genes in only the feminized tassels of homozygous *mop1-1* mutants, we hypothesized that this expression pattern is characteristic of the feminized tassel phenotype and not a direct consequence of the *mop1-1* genotype. To test this hypothesis, qRT-PCR was performed for the same subset of SBP-box genes in tassels from a family segregating for the *tasselseed1* (*ts1*) mutant allele. Given that most of the genes upregulated in feminized tassels of homozygous *mop1* mutants were from Group II, we also assayed additional Group II genes in these samples. Group II genes *tga1*, *sbp5* through *sbp8*, and *sbp11* all exhibited higher mRNA levels in feminized tassels of homozygous *ts1* plants as compared to wild type siblings (Fig. 4a–f). Expression data for the Group III genes *sbp9* and *sbp17* suggested a similar trend of higher mRNA levels in feminized tassels compared with wild type siblings (Fig. 4g, h), although the *sbp9* difference was not statistically significant (Fig. 4g). Tassels collected from homozygous *ts1* mutants covered a narrower range of lengths (3.6–7 cm), so no definitive pattern relative to length could be ascertained. The Group II genes *sbp12* and *sbp13* (as well as ten other SBP-box genes assayed) displayed no significant differential expression between feminized and wild type tassels (Supplementary Fig. 3). Thus all maize SBP-box genes for which increased mRNA levels were observed in feminized tassels of homozygous *mop1* mutants also exhibit increased mRNA levels in feminized tassels of homozygous *ts1* mutants, with the possible exception of *sbp9*. The above *ts1* data also extend this trend to include two additional Group II genes; *sbp8*, and *sbp11*.

In the case of a few genes (for example, *sbp7*, *sbp9*, and *sbp17*), comparisons among normal tassels in the genetic backgrounds segregating for the *mop1* versus *ts1* mutations suggest differences in relative levels of RNA (*cf.* Figs. 3, 4).

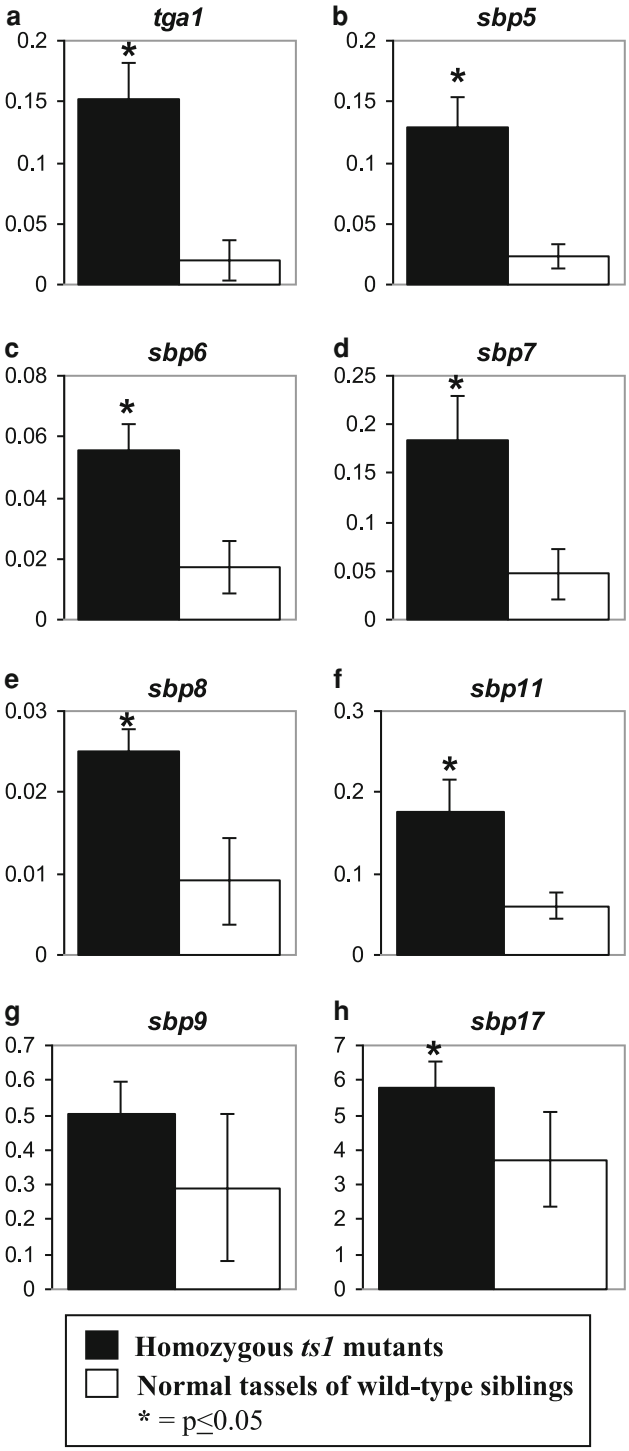


Fig. 4 Expression of six Group II and two Group III SBP-box genes in tassels from a family of plants segregating for the recessive *ts1* mutation. Each chart presents average qRT-PCR expression data relative to *ubiquitin1* in developing tassels (≤10 cm, $N = 4$ for each category ± S.E.). Supplementary Fig. 3 reports data for additional SBP-box genes that appear to be unaffected in the same set of RNA samples. *Black bars* = *ts1* mutant (feminized) tassels (*ts1/ts1*, $n = 4$, 3.6–7.0 cm), *white bars* = tassels from wild-type siblings (*Ts1/Ts1* or *Ts1/ts1*, $n = 4$, 4.9–10 cm)

However, such differences are most likely due to slight differences in amplification efficiencies between these two experiments. Consistent with this interpretation, comparison of the qRT-PCR profiles for these genes also reveal subtle shifts in the melt peak of the resulting amplicons, consistent with possible sequence polymorphisms between the genetic backgrounds used in the two experiments (A188 for *ts1* vs. mixed W23/K55 background for *mop1*). Nevertheless, melt curves and amplification efficiencies are consistent within a given genetic background, such that the comparisons of relative RNA abundance within each experiment remain valid.

miR156 levels are decreased in the feminized tassels of both *ts1* and *mop1-1* mutants

One of the defining characteristics of Group II and III SBP-box genes is the presence of a miR156 target site. While all miR156a-k family members contain the seed sequence necessary to bind the SBP-box family member target sites, Group II genes have a perfect match for the mature miR156j family member, whereas the Group III genes, *sbp9* and *sbp17*, have one and two nucleotide mismatches respectively at the 3′ end of the microRNA (Fig. 5a). To test the hypothesis that increased RNA levels of the specific subset of SBP-box genes are due to the down-regulation of miR156, small RNA gel blots were performed using several of the same RNA samples as were tested for SBP-box gene expression (Fig. 5b, c). The smallest tassels (≤3 cm) did not yield sufficient RNA for gel blot analyses, but larger tassels that exhibited statistically higher expression of the above SBP-box genes were available for analysis. Comparing the feminized tassels of *tasselseed1* homozygous mutants with the tassels of their wild type siblings of similar length, there is a clear decrease in the level of miR156 (Fig. 5b). This differential expression appears as though it may be more pronounced at later developmental stages, with the longer wild-type tassels exhibiting higher levels of miR156 than shorter tassels from sibling plants. These differences in the level of miR156 coincide with phenotypic changes observed in the corresponding tassels, i.e. smaller tassels are predominately unisexual, with only the most basal (and thus older) florets having undergone a sexual differentiation such that the presence or absence of silks is evident, whereas the longer tassels are more fully differentiated with prominent ovules and elongated silks on feminized tassels or prominent hairy glumes covering the developing stamens on wild-type tassels.

A comparable experiment assayed miR156 levels in feminized versus normal tassels from homozygous *mop1-1* plants and their wild-type siblings (Fig. 5c). Similar to the results for *ts1*, feminized tassels from homozygous *mop1-1*

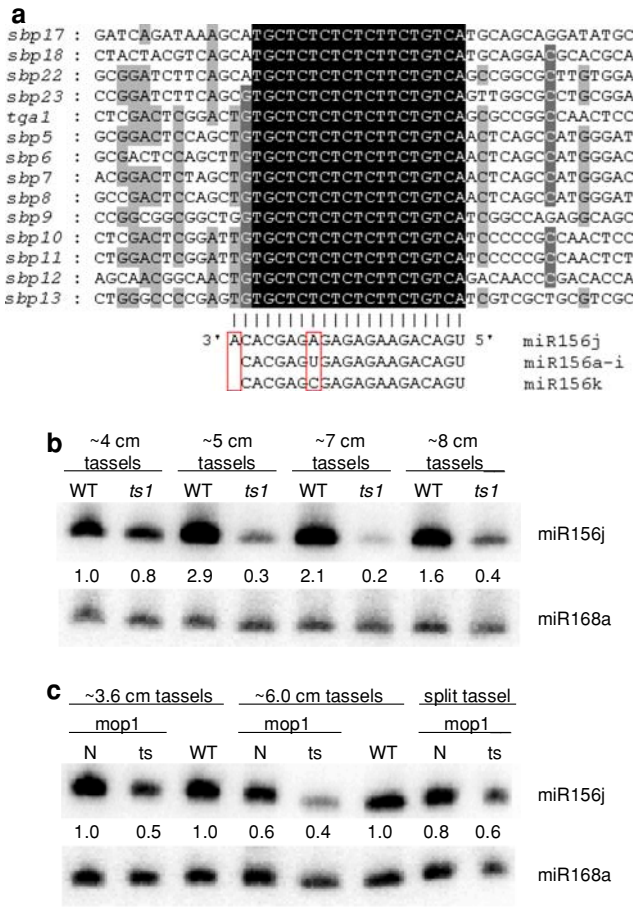


Fig. 5 Reduced levels of miR156 in feminized tassels of *mop1* and *ts1* genotypes. **a** The microRNA target sites for several maize SBP-box genes, including all Group II and Group III genes and representatives from Group IV, are aligned with the sequences for all mature maize miR156s. All Group II genes are a perfect match to miR156j, whereas miR156a-i and k include one or more mismatches relative to the target sites of the maize SBP-box genes. **b** Small RNA Northern blots demonstrate reduced levels of miR156 in the feminized tassels of homozygous *ts1* mutants relative to the normal tassels from wild-type (WT) plants. Samples were loaded with RNA from the smallest tassels on the *left*, and larger tassels on the *right*. Probes complementary to miR156j and miR168a were hybridized sequentially to the same blot, with a control exposure after the first to ensure complete stripping of the blot. For both probes, the bands corresponded with size markers at ~20–21nt. miR156 signals were normalized relative to miR168 as a loading control, and scaled relative to the smallest WT tassel. miR168 serves as a control as it is ubiquitously expressed and also demonstrates that the microRNA processing machinery is functional (Chuck et al. 2007b). **c** An experiment similar to that in (b) reveals reduced levels of miR156 in tassels from homozygous *mop1-1* plants. Tassel samples were grouped according to size (the three on the *left* were ~3.5 cm, and those on the right were ~6 cm), each group containing one wild-type (WT) and two homozygous *mop1* tassels. The phenotypes of *mop1* tassels include normal (N), feminized (ts), and a mixed tassel (~N) that exhibited normal, feminized and barrenized spikelets over various parts of the tassel. The 'split tassel' represents a single tassel that exhibited feminization localized to a limited number of tassel branches, and thus the tissue sample was split accordingly with the 'ts' sample being largely feminized and the N sample being entirely normal. Normalization is relative to miR168, as in (b)

plants exhibited lower miR156 levels relative to tassels from wild-type plants. Perhaps consistent with our observation that the feminization phenotype of homozygous *mop1-1* plants exhibits variable penetrance, such that some tassels appear largely normal and others are feminized to varying degrees, the miR156 levels from homozygous *mop1-1* plants are variable relative to those seen in wild-type tassels. For example, the normal (~3.6 cm) tassel from a homozygous *mop1-1* plant (first lane, Fig. 5c) exhibits comparable levels to the wild-type tassel (third lane, Fig. 5c), whereas other tassels from homozygous *mop1-1* mutants, including the normal (~6 cm) tassel (fourth lane, Fig. 5c) exhibit lower levels relative to wild-type (sixth lane, Fig. 5c).

Discussion

Our sequence analyses indicate the presence of at least 31 SBP-box genes in the maize genome. Phylogenetic analyses of amino acid sequences and analyses of genomic intron/exon structure support the division of the family into six groups with distinctive characteristics. Our expression analyses demonstrate that a specific subset of SBP-box genes exhibited increased mRNA levels in feminized tassels of both *mop1-1* and *ts1* mutants. Moreover, reduced levels of miR156 in feminized tassels, as shown by small RNA blots, may allow for the up-regulation of this subset of SBP-box genes.

Phylogenetic groupings

The majority of our groupings of SBP-box genes based upon both amino acid similarities and genomic structure are in close agreement with the identification of six groups of SBP-box genes in rice (termed S1 through S6, Xie et al. 2006). In most cases, subtle differences were among nodes with weaker statistical support in one or both analyses and can be attributed to differences in both the length and number of sequences used for the phylogenetic analyses. For example, one notable difference is the grouping of genes with conserved ankyrin repeats. We identified several such ankyrin repeat containing genes that consistently fall into Group V based upon both genomic structure and amino acid similarity. Xie and colleagues, having focused their alignment and phylogenetic analysis on the conserved SBP-domain, place one of the ankyrin-repeat-containing genes, *Os SPL1*, in their S6 group (analogous to Group IIb presented above) and thus with several genes that lack these repeats, although its position is very basal and only moderately statistically supported (Xie et al. 2006). Beyond these subtle differences, our major cladistic groups are all highly reproducible and correspond well to the cladistic groups presented in rice by Xie and colleagues.

These phylogenetic relationships are further supported by commonalities in tissue-specific regulation. RT-PCR expression analyses by Xie and colleagues identified a subset of genes (*Os SPL7*, *Os SPL14*, *Os SPL16*, *Os SPL17*, and *Os SPL18*) that are heavily expressed in young inflorescences (panicles) of rice (Xie et al. 2006). Each of these rice genes represent putative orthologs of Group II and Group III maize genes that exhibit increased mRNA levels in feminized tassels (*sbp9*, *sbp5* and *7*, *tga1*, *sbp6* and *8*, and *sbp11*, respectively; Fig. 1). When Xie and colleagues assayed expression levels of rice *SPL* genes in transgenic plants overexpressing miR156, several of the same genes (*Os SPL14*, *Os SPL16*, and *Os SPL18*) have reduced transcript levels, but the levels of *Os SPL2*, the putative ortholog of *sbp12* and *sbp13*, remain unaffected. These commonalities in expression profiles among specific putative orthologs, including the observation that an orthologous subset of miR156 target site containing genes (*Os SPL2*, *sbp12*, and *sbp13*) are not altered in coordination with altered miR156 levels, argue for the presence of additional conserved regulatory mechanisms.

Correlations between miR156 levels and SBP-box gene expression

The question of what establishes the observed differential expression among specific SBP-box genes still remains. Feminized tassel tissue exhibited reduced levels of miR156 (Fig. 5), but the correlation between the levels of miR156 and SBP-box targets was not absolute. First, not every SBP-box gene with the miR156 target site was affected. Second, if miR156 were the only or primary regulatory factor acting upon the SBP-box targets, one would expect expression levels to exhibit a nearly perfect inverse correlation. Thus, based on the observed levels of miR156, the expected expression pattern for the SBP-box target genes would be higher in feminized than wild type or normal tassels at all developmental stages with the difference more pronounced at later stages. While we did observe increased levels of SBP-targets in feminized relative to wild type and normal tassels, this difference was more pronounced in shorter tassels and disappeared in longer tassels.

The observation that the levels of miR156 and SBP-box targets were not perfectly correlated supports the hypothesis that other shared regulators may be principally responsible for SBP-box gene upregulation and that reduction of miR156 may be necessary but is not sufficient. A wealth of data in *C. elegans* supports this hypothesis. Systematic knockouts of numerous individual microRNA genes demonstrate that the majority are not critical for viability or normal development (Miska et al. 2007), and that double or triple knockouts are sometimes required to disrupt normal developmental pathways (Abbott et al. 2005). Furthermore,

accumulation of the microRNA alone appears to be insufficient to cause a downregulation of the target in all cases (Moss 2007) suggesting that other mechanisms and factors are needed. Reciprocally, reduction of the microRNA alone may not be sufficient to cause upregulation of the target without the necessary activators or transcription factors. Taken together, these studies suggest that regulatory redundancies, either between microRNAs or among microRNAs and other regulatory factors, tend to maintain expression levels of key targets to ensure normal development (Moss 2007; Miska et al. 2007). There are both sequence and genomic structural similarities among the specific maize SBP-box genes whose mRNA levels are altered in feminized tassels. Decreased levels of miR156 may provide competency for SBP-box gene upregulation, but only a specific subset may be affected through sequence specific interactions with a yet to be identified transcription factor or other shared regulator.

Maize inflorescence development

Current knowledge concerning the differentiation of ear and tassel is based upon several genetic mutations, as well as specific genetic or molecular interactions (e.g. Dellaporta and Calderon-Urrea 1994; Parkinson et al. 2007; Chuck et al. 2007b). In addition to developmental differentiation affecting the reproductive structures directly, differentiation also affects sterile bracts (e.g. glumes or the palea and lemma) that subtend the reproductive organs of each floret and take on unique secondary sex traits depending upon whether they are developing in the ear or the tassel. For instance, glumes that subtend each spikelet (pair of florets) are leaf-like, green and photosynthetic in the tassel, whereas these same structures are short, thick, and lignified in the ear. Prior work suggests that some of the maize SBP-box genes for which we observed differential expression in feminized versus normal tassels are expressed predominantly within such vegetative structures of the ear or tassel. One example is *teosinte glume architecture1* (*tga1*), which has been characterized with respect to its role in the evolution of maize from teosinte (Dorweiler et al. 1993; Dorweiler and Doebley 1997; Wang et al. 2005). *tga1* plays a prominent role in glume development in the ear, and its transcript is not typically abundant in the tassel (Wang et al. 2005). Similarly, *sbp5* and *sbp6* RNAs appear to be more abundant in ear than tassel, but their normal expression in tassels appears to be restricted to vegetative structures relatively early in the development of the lateral meristems of the tassel (Chuck et al. 2007a). Consistent with the fact that each of these genes (*tga1*, *sbp5* and *sbp6*) is typically more abundant in the ear, we found increased transcript levels in feminized tassels relative to wild type. Taken together, these data suggest that *tga1*, *sbp5* and *sbp6* function in vegetative structures of the inflorescence and play a more prominent role in ear development. Thus, one possibility is that these genes, and possibly other differentially expressed Group II and Group III SBP-box genes, influence secondary sex traits in these sterile bracts.

Nevertheless, many of the remaining Group II and Group III SBP-box genes for which we observe differential expression are previously uncharacterized, and thus the possibility remains that they may function in other tissues of the inflorescence. Development of true unisexual maize inflorescences requires the abortion of pistils in the tassel and the arrest of stamens in the ears (Cheng et al. 1983). Several studies have proposed a central role of *tasselseed1* (*ts1*) and *tasselseed2* (*ts2*) in establishing the maize monoecious pattern primarily by promoting a PCD pathway within the pistil primordia of the tassel (DeLong et al. 1993; Calderon-Urrea and Dellaporta 1999). At least one member of the SBP-box gene family, *At SPL14*, has been postulated to play a role in regulating PCD (Stone et al. 2005), leaving open a possible role for one or more SBP-box genes in the PCD pathway of sex determination. Future research is needed to test these and other possibilities.

Levels of miR156 were also altered between wild type and feminized tassels of *mop1* and *ts1* mutants. Although miR156 is most noted for its role in prolonging the maize juvenile phase, high levels of miR156 in wild type tassel were not entirely unanticipated given that miR156 is detected in the developing inflorescence (Chuck et al. 2007a). Chuck et al. (2007a) demonstrated that the prolonged juvenile phase of the maize *Corngrass* (*Cg1*) mutant can be attributed to ectopic expression of the tandem miR156b/c transcript. In wild type plants, miR156 is readily detectable in the leaf primordia of juvenile plants, undetectable in the shoot apical meristem after the juvenile to adult transition has occurred, and then reappears in the spikelet pair meristems and spikelet meristems of the developing tassel inflorescence (Chuck et al. 2007a). There are a total of 11 predicted miR156 family members in maize (miRbase; http://microrna.sanger.ac.uk), many of which produce mature miR156 identical to miR156b and miR156c (See Fig. 5a), leaving the potential that one or more distinct family members may be responsible for miR156 production in specific tissues. Our hybridization conditions with miR156 did not distinguish between different mature members of the family, and thus leaves the possibility that distinct levels of miR156 could have been driven by any of the 11 family members. In fact, hybridization of our small RNA blots with a probe complementary to miR156a gave identical results to those presented for the miR156j probe (data not shown). If more than 1 of the 11 *miR156* genes are expressed in the tassel, the magnitude of the observed effect between normal and feminized tassels

suggests that multiple *miR156* genes may be affected by feminization (Figs. 5b, c).

However, the majority of *sbp* genes showing increased levels of mRNA in feminized tassels contain a perfect match for miR156j (Fig. 5a). Thus, one possibility is that perhaps miR156j is principally responsible for regulating the SBP-box genes in the inflorescence whereas miR156b/c are principally responsible for their control during the juvenile to adult transition. While both family members would have high enough sequence complementarity to target the SBP-box genes, perhaps the difference in complementarity could lead to a difference in the manner in which they are regulated (Millar and Waterhouse 2005). While most plant miRNA targets are subject to cleavage, there are clear examples involving translational repression, including *SPL3* repression by miR156 (Gandikota et al. 2007). The observation that a specific subset of related SBP-box genes were the only ones for which altered mRNA levels were detected could be explained by the possibility that only those genes are subject to cleavage due to perfect complementarity of miR156j; the other SBP-box genes containing a target site may get translationally repressed, but remain intact leading to more stable RNA levels (e.g. Olsen and Ambros 1999).

Beyond its role in regulating the expression of SBP-box genes, miR156 is also postulated to function antagonistically with miR172 in the juvenile-to-adult transition. While ectopic expression of miR156b/c prolongs the juvenile phase in *Cg1* mutant plants, expression of miR172 is required for the transition to adulthood through repression of *glossy15*, an APETALA2 (AP2)—like transcription factor that maintains the juvenile phase (Lauter et al. 2005). Chuck and colleagues demonstrated an inverse correlation in the levels of miR156 and miR172 throughout the developmental transition, and thus suggested that these microRNAs play antagonistic roles in regulating this transition (Chuck et al. 2007a). It is worth noting that miR172 plays a role in both the juvenile-to-adult transition and the sexual differentiation pathway. The miR172 encoding gene, *tasselseed4* (*ts4*), controls proper development and sex determination within the tassel by repressing the AP2-like gene, *ids1* (Chuck et al. 2007b). *ts4* produces miR172e, which is distinct from other miR172 family members by a single nucleotide. If a similar antagonistic relationship exists between miR156 and miR172 family members during inflorescence development, the relative levels of miR156 and miR172 might function in regulating sex determination within the tassel, establishing the male versus female identity of young inflorescence primordia. Alternatively, given that developing inflorescences produce axillary meristems that transition through a series of developmental states from Branch Meristem (BM), Spikelet Meristem (SM) and finally Floral Meristem (FM; McSteen et al. 2000), the relative levels of miR156 and miR172 could influence the determinacy of branch primordia within the inflorescence, such that high levels of miR156 are required to maintain branch primordia in indeterminate states, whereas a gradual transition toward higher levels of miR172 are required to ultimately transition each of the branch primordia to the determinate floral meristem state. Future experiments will be directed toward testing predictions of these alternative models.

Ultimately, the sex determination process is critical for establishing the maize monoecious pattern. Early maize inflorescences (i.e. immature ears and tassels) are bisexual, having perfect florets containing both pistils and stamens. Pistils are aborted within the tassel through the action of *ts1* and *ts2*, and disruption of this pathway appears to induce a full sex-determination switch such that affected florets retain their pistils and arrest their stamens (Calderon-Urrea and Dellaporta 1999; Irish et al. 1994). In the pistils of the ear, *silkless1* (*sk1*) acts to inhibit this pathway and allows for development of female tissue (Jones 1932, 1934). Recent genetic experiments suggest that *required to maintain repression6* (*rmr6*) may regulate the spatial pattern of *sk1* expression, inhibiting it from acting in the early tassel thus allowing for *ts1* and *ts2* induced PCD in pistils (Parkinson et al. 2007). Parkinson and colleagues suggested that *mop1* and *rmr6* may feed into this regulatory pathway at a similar point, namely by influencing *sk1* expression, based upon their shared effects on paramutation and tassel feminization. Consistent with this position, *mop1* does not influence levels of *ts2* RNA (J. F. Hultquist and J. E. Dorweiler, unpublished data). The data presented herein demonstrate that feminized tassels of both *mop1* and *ts1* mutants exhibit distinct levels of miR156 and an evolutionarily related group of SBP-box genes. Given current knowledge of sex determination within the tassel, we do not suspect that *ts1* or *mop1* are acting directly upon these genes, but rather that additional, yet to be identified regulatory factors are involved (Fig. 6). Investigating SBP-box gene expression and miR156 levels in some of the other sex-determination mutants, such as the additional *tasselseed* mutants, as well as looking at *sbp* gene expression in the ear, should provide useful data for testing and expanding this model. This may be particularly true for the two additional dominant *tasselseed* mutants, *Ts3* and *Ts5*, for which very little is known.

Conclusion

Our analyses expand the maize SBP-box gene family from 9 to 31 members. Intron–exon structures as well as phylogenetic data support the division of these family members into six groups. We show that the mRNA levels of specific

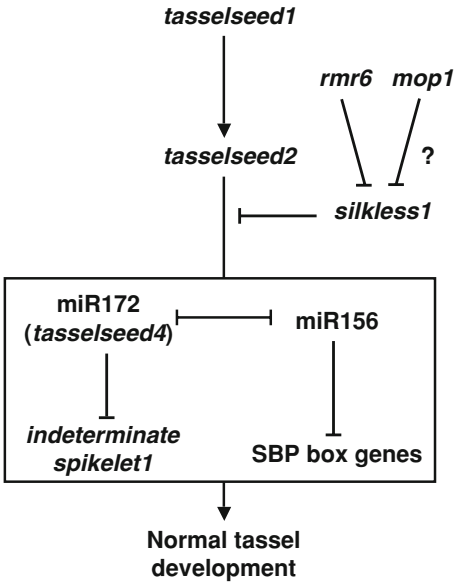


Fig. 6 Model for sex determination in maize tassels. Hypothetical relationships among several genes affecting tassel development. Relationships among *ts1*, *ts2*, *sk1*, *rmr6* and *mop1* are as proposed previously (Dellaporta and Calderon-Urrea 1994; Parkinson et al. 2007). While the effects of miR172 (*ts4*) on *ids1* (including failure to regulate the dominant *Ts6* allele), and miR156 on the SBP-box genes are previously established, the suggestion that miR172 and miR156 may act antagonistically is based upon a similar relationship proposed during juvenile to adult transition. These factors are placed downstream of *ts1* and *mop1*, but the number of steps and nature of the relationships involved remain unclear

SBP-box genes are increased in the feminized tassels of two different mutants, whereas miR156 levels are decreased. While there is a correlation between decreased miR156 and increased SBP-box gene transcript levels, we hypothesize that reduction of miR156 may be necessary but not sufficient for the upregulation of specific SBP-box targets. The sequence and structural similarity among the Group II and Group III SBP-box genes would support the idea of other shared regulators, such that downregulation of miR156 may provide competency for SBP-box gene upregulation by other common factors.

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