The maize SBP-box transcription factor encoded by tasselsheath4 regulates bract development and the establishment of meristem boundaries

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In the second paragraph of the Discussion (on p. 1248), a figure was incorrectly cited.

The text should have read as follows:

“The derepressed bract growth also alters the apparent phyllotaxy, changing it from ordered rows to spiral by pushing the rows of spikelet pairs apart (Fig. 2F).”

The authors apologise to readers for this mistake.
The maize SBP-box transcription factor encoded by tasselsheath4 regulates bract development and the establishment of meristem boundaries

George Chuck1,*, Clinton Whipple2, David Jackson2 and Sarah Hake1

SUMMARY
Plant architecture consists of repeating units called phytomers, each containing an internode, leaf and axillary meristem. The formation of boundaries within the phytomer is necessary to differentiate and separate these three components, otherwise some will grow at the expense of others. The microRNA-targeted SBP-box transcription factor tasselsheath4 (tsh4) plays an essential role in establishing these boundaries within the inflorescence. tsh4 mutants display altered phyllotaxy, fewer lateral meristems and ectopic leaves that grow at the expense of the meristem. Double-mutant analyses of tsh4 and several highly branched mutants, such as ramosa1-3 and branched silkless1, demonstrated a requirement for tsh4 in branch meristem initiation and maintenance. TSH4 protein, however, was localized throughout the inflorescence stem and at the base of lateral meristems, but not within the meristem itself. Double labeling of TSH4 with the ramosa2, branched silkless1 and knotted1 meristem markers confirmed that TSH4 forms a boundary adjacent to all lateral meristems. Indeed, double labeling of miR156 showed a meristem-specific pattern complementary to that of TSH4, consistent with tsh4 being negatively regulated by this microRNA. Thus, downregulation of TSH4 by a combination of microRNAs and branching pathway genes allows the establishment of lateral meristems and the repression of leaf initiation, thereby playing a major role in defining meristem versus leaf boundaries.

KEY WORDS: Maize, Meristem, MicroRNA

INTRODUCTION
The shoot apical meristem forms at the apex of the plant and produces leaves at its flank. Lateral meristems form in the axils of leaves, and their number, rate of growth and determinacy pattern the general architecture of the plant. The leaf, lateral meristem and internode can collectively be considered as a unit, called a phytomer (Galinat, 1959). During the vegetative phase, the subtending leaf is large, whereas the axillary lateral meristem is small. Within the reproductive phase, however, the leaf portion of the phytomer, typically called the bract, is often repressed. Several studies indicate that control of cell fate within the phytomer plays a major role in the establishment of lateral organ architecture. For example, during the early stages of floral initiation in Arabidopsis there is a transient period during which cell fates are not fixed in lateral primordia. Such primordia can either form leaves or meristems depending on signals received from the environment and shoot apex (Hempel and Feldman, 1994; Hempel and Feldman, 1995). To date, the molecular nature of these signals and how they are perceived by the meristem to fix labile cell fates are unclear.

Bract suppression within the floral phase is a common feature found in dicotyledonous plants. For example, Arabidopsis bracts normally subtend floral meristems, but are later incorporated into the flower, leaving no morphological remnant (Heisler et al., 2005). Several Arabidopsis mutants, however, derepress bract development, including leafy (Weigel et al., 1992), jagged (Dinneny et al., 2004; Ohno et al., 2004), ufo (Hepworth et al., 2006) and bop (Hepworth et al., 2005; Norberg et al., 2005). Interestingly, all of these genes also function to control floral patterning, indicating that bract suppression and meristem function might be co-regulated.

Previous work on the dominant Corngrass1 (Cg1) mutant of maize showed that SBP-box transcription factors control lateral organ development and cell fate within the inflorescence. Cg1 is caused by the overexpression of miR156b/c, which results in inappropriate downregulation of several SBP-box genes during the floral phase (Chuck et al., 2007). Cg1 displays juvenile leaves throughout development, as well as an unbranched tassel with eutopic vegetative leaves during the floral phase (Poethig, 1988). These data suggest that the loss of one or more SBP-box genes is responsible for the Cg1 floral phenotype. Here, we provide evidence that tasselsheath4 (tsh4) is caused by mutation of zmSBP6, a target of Cg1 (Chuck et al., 2007). tsh4 primarily functions to repress lateral organ growth, but also affects phyllotaxy, axillary meristem initiation and meristem determinacy within the floral phase. Double-mutant analysis shows that tsh4 acts downstream of several genes responsible for the establishment of meristem determinacy, including ramosa1 (ra1) (Vollbrecht et al., 2005), ra2 (Bortiri et al., 2006), ra3 (Satoh et al., 2006) and branched silkless1 (bd1) (Chuck et al., 2002). Furthermore, because tsh4 is a target of miR156, the location of miR156 expression relative to that of tsh4 was analyzed, and found to be complementary. These results indicate that the establishment of lateral meristems leads to the expression of miR156, which in turn downregulates tsh4 within the meristem, thus restricting TSH4 activity to the subtending bract, where it suppresses growth.
MATERIALS AND METHODS

Isolation of tsh4 alleles

The tsh4-ref allele was isolated from EMS-mutagenized W22 lines from the Maize Tilling Project (http://genome.purdue.edu/maizetilling/). The tsh4-m1 allele was isolated from Ds-mutagenized W22 lines (http://www.plantgdb.org/prj/ACsTagging/). The tsh4-mum1 allele was isolated from Trait Utility System for Corn (TUSC) libraries (Bensen et al., 1995). Crosses between tsh4-mum1 and either tsh4-ref or tsh4-m1 resulted in a tsh4 phenotype, demonstrating that they are allelic.

Phenotypic analysis

Double mutants were made between the tsh4-mum1 allele and ra1-ref, ra2-DM and ra3-ref, and bd1-glu mutants by self-crossing the F1. tsh4-mum1 homozygotes were selected by PCR using oligos 6F (5’-GAGCGG-TTGACTACTAATGTTGCTCT-3’) and 6R (5’-AACGACATGGAACTCGAATGTTCT-3’). ra2-DM mutants were selected using oligos full ra2 F (5’-AAAAGAATTCGAGTGTAACCCGTGAGCACCA-3’) and full ra2 R (5’-TTTTCTCAGGCTGCTGTCTCCGCTCTC-3’). ra1-x mutants were selected using a linked glossy mutant marker, and by sequencing the ra1 gene to identify homozygotes using primers full F (5’-GCTCTCCATTCTTGCTTCTCT-3’) and full R (5’-GTTGCTGTCC-TGTCGTCCTGTC-3’). Double mutants for ra2-ref were made by self-crossing ra2-ref homozygous plants that were heterozygous for tsh4-mum1 and screening for tsh4-mum1 homozygotes by PCR. bd1-glu mutants were screened using oligos bd1-7 (5’-ATGATGAAATCCCGGACCTGG-3’) and bd1-9 (5’-CGGTGCCTGTAGAAGCAGTG-3’).

All double mutants were repeated using different allelic combinations. The tsh4-m1 allele was used to make doubles with ra1-ref, ra2-DM and ra3-ref, and bd1-ref. Similar phenotypic results were derived with both tsh4 alleles.

Samples were fixed in formalin/acetic acid/alcohol (FAA) overnight, critical-point dried, sputter coated and examined as previously described (Chuck et al., 1998).

Molecular analysis of tsh4

The full-length tsh4 cDNA was originally cloned from maize ear cDNA using primers corresponding to SBP-box clone TC305894 (Chuck et al., 2007). Full-length genomic sequence was derived from the Maize Sequencing Project (maizesequence.org). Northern blot analysis was performed as previously described (Kerssetter et al., 1994), probing with the 3’ end of the tsh4 cDNA outside the SBP domain. MicroRNA northern blots were performed as described (Chuck et al., 2007).

Cleavage assay

Polyadenylated RNA was isolated from 0.5 cm tassel primordia and 200 ng used directly for ligation to the GeneRacer 5’ RACE oligo and reverse transcribed (Invitrogen). One microliter of the reverse transcription reaction was used for RT-PCR using the GeneRacer 5’ oligo in combination with the SBP6 R1 oligo (5’-GCTGGTGGTGAATGGTT-3’), which is located 320 bases from the microRNA binding site. The cleaved PCR product was cloned into pGEM-T Easy (Promega) and sequenced using M13 primers.

Generation of a TSH4 antibody, immunolocalization and double labeling

Sequence encoding the full-length TSH4 protein coupled to a His tag was cloned into the pET21d expression vector (Novagen). Recombinant protein was isolated under denaturing conditions, dialyzed and injected into rabbits (ProSci). The serum was affinity purified using either full-length TSH4 protein coupled to a GST tag (Novagen), or the C-terminus of TSH4 outside the SBP-box domain coupled to the GST tag. Similar results on western blots and immunolocalizations were obtained using both antibodies. Using the antibody to the full-length protein, a single band was detected in wild-type nuclear extracts but not in those from the tsh4 mutant, indicating that the antibody is specific (see Fig. S1B in the supplementary material).

Immunolocalization was performed using previously described protocols (Jackson, 1991). To perform double labeling using RNA in situ probes, the in situ hybridization was performed first using previously described probes for kn1 (Jackson, 1991), ra2 (Bortiri et al., 2006), bd1 (Chuck et al., 2002) and miR156 (Chuck et al., 2007) (Exiqon). After the alkaline phosphatase development step, the reaction was stopped by immersion in 1×PBS and further blocked in 0.1% BSA in PBS. A 1/100 dilution of TSH4 antibody was added to the slides and incubated overnight at 4°C. After three washes in blocking solution to remove primary antibody, an anti-rabbit biotinylated secondary antibody was added (Vector Labs) and incubated at room temperature for 1 hour. After washing as above, an anti-biotin tertiary antibody was added (Vector Labs) and incubated for 1 hour at room temperature. After washing as above, the slides were developed using the DAB staining system (Vector Labs), mounted in aqueous mounting media and photographed.

RESULTS

Normal maize development

Maize produces two types of inflorescence: the tassel and the ear (Fig. 1A,E). Upon the floral transition, the shoot apical meristem converts into a tassel primordium, which is located in a terminal position and contains male flowers, whereas ears are located in the axils of vegetative leaves and contain female flowers. The inflorescence meristem (IM) initiates a series of lateral meristems in a sequential ordered pattern. Only the tassel IM initiates branch meristems (BMs), whereas both the tassel and ear initiate spikelet pair meristems (SPMs) in a spiral phyllotaxy (Fig. 2A,C,E). Each SPM initiates a pair of spikelet meristems (SMs) in a distichous phyllotaxy (Fig. 2C,E). The SM initiates two sterile leaves, called glumes, followed by two lemmas, each of which contains a floral meristem in its axil (Cheng et al., 1983).

The tsh4 mutant phenotype

A screen for mutants that cause derepression of bract growth within the maize inflorescence uncovered several alleles of tsh4. The mutants were all analyzed in a W22 background. tsh4 mutant tassels typically had one to five long tassel branches, instead of ~13 as in wild type (Fig. 1A; Table 1). In place of tassel branches, long bract leaves were present, clustered at the base of the tassel (Fig. 1B; Table 1). Removal of these leaves revealed compression of the internodes, as if bract growth occurred at the expense of internode growth (Fig. 1C). Bract growth often occurred at the expense of lateral meristems of the tassel branch as well, as axillary tassel branches were often absent or highly reduced, consisting of only a few spikelets (Fig. 1D). In addition, solitary spikelets often appeared instead of spikelet pairs along the main rachis. Both solitary and paired spikelets were frequently subtended by bracts (Fig. 1F; Table 1). Since the maize ear does not have branches, these branching defects were not detectable in the ear. Derepressed bracts, however, were still visible at the base of the ear (Fig. 1E). In addition, the phyllotaxy of the kernel rows was different in tsh4, showing a near spiral phyllotaxy, instead of straight ordered rows of kernels as in wild type (Fig. 1E). These defects resulted from growth of the derepressed bracts subtending the spikelet pairs in the ear. The decrease in the number of BMs and spikelet pairs, and increase in the growth of lateral organs, suggested that TSH4 might regulate the decision as to whether lateral primordia are partitioned into either determinate or meristemmatic cells.

Several maize mutants display phenotypes opposite to those of tsh4, exhibiting an enhanced number of tassel branches. These include ra1, ra2 and ra3 (McSteen, 2006). All ramosa mutants display SPM indeterminacy, replacing spikelet pairs with branches or spikelet multimers containing extra spikelets. Thus, the ramosa genes function to impose determinate identities on the SPM (McSteen, 2006). ra2 encodes a LOB-domain transcription factor (Bortiri et al., 2006) that controls the expression of RA1, a C2H2
The branched silksless1 (bd1) gene, which encodes an ERF-type AP2 transcription factor (Chuck et al., 2002), affects meristem identity. bd1 mutants display an indeterminate SM in the tassel, whereas in the ear the SM look morphologically like BM (Chuck et al., 2002; Colombo et al., 1998). Double mutants between bd1 and tsh4 showed an additive phenotype in the tassel (Fig. 1K), where the number of long branches was reduced as in tsh4, but the SMs were indeterminate as in bd1. In the ear, however, the bd1 phenotype was suppressed in double mutants, as the long branches were dramatically shortened and subtended by large bract leaves (Fig. 1L). Thus, the tsh4 mutant suppresses all BM development, whether in the tassel, as in the ramosa mutants, or in the ear, as in bd1 mutants.

Each double-mutant combination was repeated using the tsh4-m1 allele, and similar results were observed (data not shown).

**Scanning electron microscopy (SEM) analysis**

Analysis of tsh4 development by SEM highlighted differences in meristem initiation and lateral organ suppression in the mutant as compared with the wild type. Normally, in the tassel, the first lateral primordia initiated by the IM are BMs, which have a rounded morphology (Fig. 2A). By contrast, in tsh4 mutants the first lateral primordia were flattened and later became bract leaves (Fig. 2B). Later, in wild-type tassels, the SPMs are initiated in ordered rows (Fig. 2C), but in tsh4 the bracts initiated first, and later the SPMs were seen in the axils of the bracts (Fig. 2D). In wild-type ear tips, the bract primordia could be seen prior to the SPMs, but were soon overtaken by growth of the SPMs in the axils, such that no sign of the bract remained by the time SMs were formed (Fig. 2E). In tsh4 mutants, however, the bract continued to grow, covering the SPM and forcing the rows of SPMs apart, better revealing that the spiral phyllotaxy normally present in wild type was absent due to the lack of internode elongation (Fig. 2F). Together with the double-mutant data, these results demonstrate that tsh4 is required for BM initiation as well as bract suppression.

**Molecular analysis of tsh4**

Previous work identified a minimum of seven maize SBP-box genes responsible for all aspects of the Cg1 mutant phenotype (Chuck et al., 2007). Using reverse genetics (Bensen et al., 1995), a Mutator transposon insertion was recovered in the last exon of one particular gene, TC305894 (or zmsSBP6), that was responsible for the tsh4-mum1 mutant phenotype. Analysis of two other tasselsheath mutants with similar phenotypes identified additional lesions in the same gene. tsh4-m1 contains a Dissociation (Dx) transposon insertion within the last exon, whereas the tsh4-r allele derived by EMS mutagenesis has a G-to-A transition within the SBP domain that is not present in the progenitor W22 sequence (Fig. 3A). Alignment of the amino acid sequence of the SBP DNA-binding domains of TSH4 and related homologs from maize and other grasses showed a valine-to-methionine transition mutation in the product at a highly conserved amino acid (see Fig. S1A in the supplementary material). Analysis of tsh4 transcripts in the three mutant alleles showed reduced transcript levels in both transposon-induced alleles (Fig. 3B). Together with the fact that all three mutations are allelic (see Materials and methods), these results demonstrate that the tsh4 mutant phenotype is in fact caused by loss-of-function of TC305894. A neighbor-joining tree of related SBP-box genes in maize, rice, Brachypodium, sorghum and Arabidopsis showed two duplications in the grass family that produced the tsh4 gene lineage. The first duplication occurred near the base of the grasses, creating two grass clades, whereas a more recent duplication resulted in two maize copies in each of the grass clades (Fig. 3C).

zinc-finger transcription factor (Vollbrecht et al., 2005). ra3 encodes a trehalose-6-phosphate phosphatase that may also act upstream of ra1 (Satoh et al., 2006). To determine whether tsh4 functions within the ramosa pathway, double mutants between tsh4 and ra1, ra2 and ra3 were made. In all cases, the double mutants appeared tsh4-like, displaying fewer tassel branches, indicating that tsh4 is epistatic to the ramosa genes (Fig. 1G-J). ra2 displayed an additional phenotype of an elongated pedicel, which was not suppressed in the double mutant (Fig. 1I). This might indicate that tsh4 operates with ra2 to affect meristem determinacy, rather than stem growth of the pedicel.

Fig. 1. The maize tsh4 mutant phenotype and genetic interactions. (A) Mature tsh4-m1 tassels (left) showing fewer branches than in wild-type W22 (right). (B) Base of tsh4-mum1 tassel showing ectopic bract leaves. (C) Base of tsh4-mum1 tassels with bract leaves removed showing compressed nodes (arrowheads). (D) Magnification of reduced axillary branch (arrowhead) in the axil of a bract leaf (leaf has been removed). (E) Comparison of mature ears of tsh4-m1 (left) and wild type (right). (F) Comparison of mature spikelet pairs of wild-type W22 (left) and tsh4-r (right) with ectopic bract. (G) Comparison of tassels of ra1-r (left) and ra1-r; tsh4-mum1 (right) showing suppression of the ra1 phenotype in the double mutant. (H) Comparison of tassels of ra2-DM (left) and ra2-DM; tsh4-mum1 (right) showing suppression of the ra2 phenotype in the double mutant. (I) Comparison of spikelet pairs of ra2-DM (left) and ra2-DM; tsh4-mum1 (middle) with wild-type W22 (right). (J) Comparison of tassels of ra3-r (left) and ra3-r; tsh4-mum1 (right) showing the suppressed ra3 phenotype in the double mutant. (K) Comparison of tassels of bd1-glu (left) and bd1-glu; tsh4-mum1 (right) showing the additive phenotype in the double mutant. (L) Comparison of transverse sections of ears of bd1-glu (left) and bd1-glu; tsh4-mum1 (right) showing the suppressed bd1 phenotype in the double mutant.
Table 1. Phenotypic analysis of tsh4 inflorescences

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of branches</th>
<th>Branches with bracts (%)</th>
<th>Solitary spikelets on central spike (%)</th>
<th>Nodes with bracts on central spike (%)</th>
<th>Empty nodes on central spike (%)</th>
<th>Solitary spikelets with bracts on central spike (%)</th>
<th>Paired spikelets with bracts on central spike (%)</th>
<th>Nodes with bracts on branches (%)</th>
<th>Solitary spikelets with bracts on branches (%)</th>
<th>Paired spikelets with bracts on branches (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W22</td>
<td>13.8 (0.68)</td>
<td>0 (0)</td>
<td>2.77 (0.63)</td>
<td>0.09 (0.09)</td>
<td>0 (0)</td>
<td>1.67 (1.67)</td>
<td>0 (0)</td>
<td>16.6 (0.19)</td>
<td>0.82 (0.71)</td>
<td>3.17 (0.18)</td>
</tr>
<tr>
<td>tsh4-Ds</td>
<td>5.1 (0.52)</td>
<td>8.17 (3.07)</td>
<td>22.8 (3.26)</td>
<td>18.1 (3.66)</td>
<td>0.12 (0.12)</td>
<td>29.9 (4.27)</td>
<td>15.0 (3.48)</td>
<td>72.8 (1.28)</td>
<td>78.6 (5.11)</td>
<td>72.6 (3.60)</td>
</tr>
<tr>
<td>tsh4-ems</td>
<td>1.5 (0.48)</td>
<td>2.50 (2.26)</td>
<td>53.9 (3.17)</td>
<td>37.4 (10.2)</td>
<td>5.39 (1.8)</td>
<td>38.5 (11.0)</td>
<td>30.6 (9.02)</td>
<td>96.4 (1.50)</td>
<td>93.9 (2.76)</td>
<td>96.6 (1.76)</td>
</tr>
</tbody>
</table>

Plants were grown to anthesis, then tassels collected and dried. Ten tassels were counted for each genotype (n=10). The mean is shown, with the s.e.m. in parentheses. As the tsh4 phenotype was consistently stronger in the long tassel branches, as compared with the central spike, these are reported separately.

`tsh4` contains an miR156 binding site at the 3' end of the coding region (Fig. 3A), indicating that it is a target of repression by microRNAs. Although miR156 is temporally regulated and is generally absent during the adult phase of vegetative development (Chuck et al., 2007; Wu and Poethig, 2006), expression reappears within the inflorescence (Fig. 3D). Eleven MIR156 genes are present in the maize genome (Fig. 3E), but all are more highly expressed in juvenile shoots than inflorescences (Zhang et al., 2009). Recently, a similar microRNA gene, MIR529, was described that shares 14-16 bp of homology with MIR156 and is more highly expressed in tassels than shoots (Zhang et al., 2009). Cleavage analysis of `tsh4` transcripts from wild-type tassels showed that the majority of cleavage (68%) occurred between base pairs 10 and 11 of the predicted microRNA binding site for miR529, whereas 31% occurred near the predicted site for miR156 (Fig. 3F). Thus, miR156 and miR529 function together to repress `tsh4` within inflorescences.

**TSH4 expression**

To analyze the accumulation TSH4, an antibody was generated against full-length TSH4 protein. This antibody identified a protein present in nuclear extracts of wild-type inflorescences, but not against full-length TSH4 protein. This antibody identified a protein present in nuclear extracts of wild-type inflorescences, but not against full-length TSH4 protein. Overall, the location of TSH4 appeared to mark both stem and repressed bract, but not meristem.

It is curious that TSH4 marked the position of lateral primordia and was required for the normal number of branches and spikelet pairs, but was never found in meristems. To determine whether tsh4 does in fact play a role in marking and establishing meristem boundaries, double labeling of TSH4 and several meristem markers was performed. Expression of the knotted1 (`kn1`) homeobox gene marked all shoot meristems (Jackson et al., 1994), and double labeling of `kn1` RNA and TSH4 showed that `kn1` and TSH4 did not overlap in the IM and BM (Fig. 4F,G). Radial sections through the tassel tip at the point of SPM initiation showed that `kn1` was expressed in an internal domain in the stem, whereas TSH4 was found adjacent to it, external to the `kn1` domain. Interestingly, `kn1` was absent from the anlagen of the SPM, but present later as the SPM developed (Fig. 4G). This is consistent with a role for `kn1` in maintaining the growth of meristems, rather than in their initiation (Hake et al., 2004).
To assay the overlap between the initiating SPM and TSH4 accumulation, double labeling with the lateral meristem marker ra2 was performed (Fig. 4I). ra2 was expressed in the SPM anlagen, transiently in the SPM and SM, but not in the FM or bracts (Bortiri et al., 2006). Unlike the result with kn1, a clear overlap between ra2 expression and TSH4 occurred in the SPM anlagen, but later resolved into complementary domains (Fig. 4J), pointing to an early role for TSH4 in controlling primordium fate. Finally, to understand the role of TSH4 in the establishment of the SM, double labeling with the SM-specific marker bd1 (Chuck et al., 2002) and TSH4 was assayed. In normal plants, bd1 transcripts first appear in the axil of the initiating outer glume at the base of the SM. TSH4 protein did not overlap with bd1 in this domain (Fig. 4K), and may be excluded from this area in wild type (Fig. 4D). Together, these results show that TSH4 is expressed in a pattern that is complementary to lateral meristems such as the BM and SM, but which transiently overlaps in the early SPM.

Factors that negatively regulate tsh4

Meristem-specific factors are likely to downregulate tsh4, as downregulation of TSH4 was not observed in mutants that fail to initiate lateral meristems, such as barren1 (ba1) (Fig. 5A) (Ritter et al., 2006).
wild type. TSH4 protein was detected on the abaxial side of the indeterminate SPM in the ra1-ref allele (Fig. 5E). As the ra1 mutant SPM grew, ectopic TSH4 protein was also detected on the adaxial side of the indeterminate SPM (Fig. 5F), co-incident with ra1 expression (Vollbrecht et al., 2005). In a ra2 mutant background, ectopic TSH4 protein was also observed in indeterminate SPMs (Fig. 5G), although the localization was mostly abaxial (Fig. 5H). Abaxial ectopic TSH4 in indeterminate SPMs was also seen in ra3 backgrounds (Fig. 5L). Finally, ectopic TSH4 was observed in bd1 mutant SMs that morphologically resemble BMs (Fig. 5K). Abaxial ectopic localization of TSH4 was observed in the region of the bd1 SM that would normally form glumes and lack TSH4 in wild type (Fig. 5L). Taken together, these results might indicate that ramosa and bd1 genes directly or indirectly negatively regulate TSH4 protein accumulation to regulate branch growth.

To determine whether the converse is true, i.e. whether TSH4 negatively regulates the ramosa genes, we carried out in situ hybridizations on tsh4 mutants. As shown in Fig. 5M, ra2 transcript was ectopically expressed in tsh4-m1 bracts, indicating mutual negative regulation between ra2 and tsh4. By contrast, no ectopic expression of ra1 or ra3 was observed in tsh4 backgrounds (G.C., unpublished).

**DISCUSSION**

Plant architecture can be viewed as a repeating series of phytomers, each of which contains three components: a leaf, axillary meristem and internode. Cell lineage analyses have shown that the phytomer can be considered a compartment, as clonal sectors transverse the entire phytomer and end at phytomer boundaries (Johri and Coe, 1983). It follows that the cells within the phytomer may share a common origin, and that subsequent cell fate decisions fix the cells into their separate identities. By altering the balance of cells within the phytomer unit and controlling how they are partitioned to each component, it is possible to create the wide range of morphological variation found in grass inflorescences.

We show that the tsh4 gene plays a major role in establishing domains within the phytomer. Floral bracts are derepressed at the base of both long branches and spikelet pairs, resulting in fewer of each in the mutant (Fig. 1; Table 1). This derepressed bract growth occurs at the expense of the axillary meristem, as more cells are allocated for lateral organ growth than meristem growth, thus reducing meristem potential. The ectopic expression of lateral meristem markers, such as ra2, in the bracts of the tsh4-m1 mutant (Fig. 5M) supports the idea that meristems with reduced growth potential result from misallocation of SPM cells into the growing bract. Since TSH4 is found adjacent to meristem boundaries (Fig. 4F-K), misallocation of cells might result from the lack of establishment of a boundary between meristem and lateral organ. The derepressed bract growth also alters the apparent phyllotaxy, changing it from ordered rows to spiral by pushing the rows of spikelet pairs apart (Fig. 3F). This suite of phenotypes is found in the dominant Cg1 mutant and suggests that loss of expression of tsh4-related genes is likely to be the primary cause of the diverse floral defects observed in Cg1 inflorescences (Chuck et al., 2007).

Genes related to tsh4 in Arabidopsis regulate the duration of leaf initiation (plastochron) (Schwarz et al., 2008; Wang et al., 2008). SPL9 and SPL15 are functionally redundant and display an increased rate of vegetative leaf initiation in the double mutant. In addition, the double mutants have more branches (Schwarz et al., 2008), a phenotype also seen in miR156 overexpressers in Arabidopsis (Schwab et al., 2005). The opposite phenotype, a decrease in the rate of leaf initiation, is obtained when a microRNA-
resistant version of SPL9 is expressed from various promoters (Wang et al., 2008). Similar to tsh4, the SPL9 transcript is found in the stem and young leaf primordia and is absent from meristems (Wang et al., 2008). Using different domain-specific promoters to drive SPL9 expression, it was found that the effects on plastochron occurred when driven by lateral organ-specific promoters. These results suggested to the authors that the SPL9 effects on plastochron occur non-cell-autonomously (Wang et al., 2008). Our results, however, indicate that it is unlikely that TSH4 itself functions non-cell-autonomously, as the protein and RNA coincide in the anlagen of lateral organ primordia.

Although both the maize tsh4 mutant and the Arabidopsis spl9;spl13 double mutant have more leaves than wild type, there are striking differences in function. Axillary branch number increased with the increase in leaf number in the spl9;spl15 plants (Schwarz et al., 2008), but tsh4 mutants, by contrast, exhibit a loss of axillary branch growth concomitant with growth of the depressed bract. Despite initiating many extra leaves, spl9;15 mutants do not display an overall difference in meristem size (Schwarz et al., 2008), whereas tsh4 mutants initiate fewer lateral meristems with reduced growth potential. In Arabidopsis, there is no derepression of cryptic bract growth or compensatory growth in the components of the phytomer in the double mutant, whereas in maize derepressed bract leaves grow at the expense of the axillary meristem. It appears that the miR156/SPL cassette was recruited in maize to function specifically to balance the decision to be leaf versus meristem in axillary locations, whereas the same cassette functions in the shoot apex of Arabidopsis to regulate the timing of leaf initiation.

In rice, the NECK LEAF1 (NL1) gene affects both plastochron and phytomer. nl1 mutants display a shortened plastochron, are slightly dwarfed, have overgrown bracts at the base of primary branches, smaller panicles and fewer branches (Wang et al., 2009). NL1 encodes a GATA transcription factor similar to that encoded by HANABA TARANU (HAN) (Zhao et al., 2004). NL1 is expressed within inflorescence bract primordia and is likely to regulate PLA1, which encodes a cytochrome P450 monooxygenase that controls plastochron (Miyoshi et al., 2004). The fact that HAN mutants in Arabidopsis display no bract phenotypes indicates that monocotyledonous plants have evolved a unique set of genes to control bract suppression during the floral phase. tasselsheath1 is the maize ortholog of NL1 (C.W., unpublished), but is likely to operate genetically in a parallel pathway to tsh4 (data not shown).

Although tsh4 is not expressed in meristems, the double-mutant data clearly indicate that tsh4 plays a role in lateral meristem initiation. Plants that are mutant for both tsh4 and ra1, ra2, ra3 and bd1, which alone are highly branched maize mutants, exhibit a tsh4 phenotype (Fig. 1G-L), demonstrating that BM growth requires tsh4 gene function. The ectopic expression of tsh4 in the abaxial domain of the BM-like SPM of ra1, ra2, ra3 (Fig. 5E-J), or in the BM-like SM of bd1 (Fig. 5K,L), provides insight into the mechanism by which tsh4 may indirectly affect BM activity. Indeterminate growth of BMs in highly branched mutants or wild-type tassel branches might require tsh4 gene activity to suppress bract development. Otherwise, misallocation of cells into derepressed bracts would decrease meristem potential of the branch, thus inhibiting its growth. When tsh4 is active, the cells of the repressed bract are incorporated into the growing stem, allowing extended branch elongation. This is shown by the discrete TSH4 accumulation on the faster growing, abaxial side of the indeterminate branches of all ramosa mutants as well as of bd1 (Fig. 5F,H,J,L). tsh4, however, is not required for growth of the stem itself, as tsh4 mutants still have stems and do not suppress the elongated pedicel stem phenotype of the ra2-DM mutant (Fig. 11). Thus, the primary function of tsh4 is to repress outgrowth of bract primordia within the inflorescence, causing the cells that express it to be incorporated into the stem, therefore allowing extended branch growth.

Different factors are required for the regulation of tsh4 during early and late SPM development. A major early factor negatively regulating tsh4 function is miR156 and the closely related miR529. Arabidopsis SPL transcription factors are known to be regulated by miR156 at the level of translation (Gandikota et al., 2007), thus necessitating the need for antibodies to analyze SPL gene expression. TSH4 protein was excluded from all meristems, whereas expression of miR156 was complementary, being specific to meristems, strongly suggesting that miR156 and miR529 function to restrict TSH4 from meristems. Thus, miR156 and miR529 play a major role in the establishment of inflorescence architecture by downregulating tsh4 within the SPM anlagen where tsh4 and ra2 expression coincide (Fig. 6), and by limiting tsh4 activity to the stem and bract, where it suppresses development. Once the SPM boundary has been established and the meristem begins to grow, knl expression initiates to maintain the meristem (Fig. 6). Mutual negative regulation between ra2 and tsh4 occurs in adjacent domains, as demonstrated by the ectopic expression of ra2 in the derepressed bracts of tsh4 mutants (Fig. 5M). ra2, however, also represses tsh4, as tsh4 is epistatic to ra2 (Fig. 1H) and TSH4 is ectopically expressed in ra2 mutants (Fig. 5H). Mutual negative regulatory loops are a common feature of transcription factor biology (Crews and Pearson, 2009) and might serve as a mechanism to reinforce developmental boundaries.

The effects of tsh4 on plant development demonstrate that the phytomer behaves as a single unit that is difficult to subdivide into individual components. Although tsh4 is expressed in only a subset of the phytomer, it affects growth, initiation and phyllotaxy of every aspect of the phytomer by repressing cell growth and...
differentiation in a specific domain, thus allowing compensatory growth in other parts. Since at least three closely related tsh4-like genes are present in the maize genome (Fig. 3C; see Fig. S1A in the supplementary material), it is likely that they also have phytomer functions. One of these genes, zmsSBP8, is likely to be a duplicate of tsh4, although it is expressed at lower levels (data not shown), which might explain why tsh4 alone displays a phenotype. Nevertheless, the isolation of mutations in these genes will provide a clearer picture of how growth of the various phytomer components is coordinated.

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Competing interests statement
The authors declare no competing financial interests.

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