A Conserved Mechanism of Bract Suppression in the Grass Family

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Suppression of inflorescence leaf, or bract, growth has evolved multiple times in diverse angiosperm lineages, including the Poaceae and Brassicaceae. Studies of Arabidopsis thaliana mutants have revealed several genes involved in bract suppression, but it is not known if these genes play a similar role in other plants with suppressed bracts. We identified maize (Zea mays) tassel sheath (tsh) mutants, characterized by the loss of bract suppression, that comprise five loci (tsh1-tsh5). We used map-based cloning to identify Tsh1 and found that it encodes a GATA zinc-finger protein, a close homolog of HANABA TARANU (HAN) of Arabidopsis. The bract suppression function of Tsh1 is conserved throughout the grass family, as we demonstrate that the rice (Oryza sativa) NECK LEAF1 (NL1) and barley (Hordeum vulgare) THIRD OUTER GLUME (TRD) genes are orthologous with Tsh1. Interestingly, NL1/Tsh1/TRD expression and function are not conserved with HAN. The existence of paralogous NL1/Tsh1/TRD-like genes in the grasses indicates that the NL1/Tsh1/TRD lineage was created by recent duplications that may have facilitated its neofunctionalization. A comparison with the Arabidopsis genes regulating bract suppression further supports the hypothesis that the convergent evolution of bract suppression in the Poaceae involved recruitment of a distinct genetic pathway.

INTRODUCTION

The modular nature of plant development facilitates an astonishing amount of morphological variation by modification of a simple repeating unit, called the phytomer (Gray, 1879). All the cells of the plant shoot are produced from groups of stem cells known as the shoot meristems, which initiate leaf primordia on their periphery and the tissues of the stem, including the vasculature and pith beneath. At the junction of the leaf and the stem, a new auxiliary meristem is often initiated that can itself grow out to reiterate this developmental pattern. Although it has been variously defined, the phytomer generally comprises the leaf, stem, and associated axillary meristem. Altering the balance of growth between different phytomer components generates morphological changes that characterize both life phase transitions as well as differences between species. A common phytomer modification after initiation of reproductive development is the suppression of leaf outgrowth in the inflorescence. Leaves that grow in the inflorescence are generally called bracts, and bract suppression is observed in many, but not all, angiosperm lineages.

The genetic mechanisms of bract suppression have received some attention in Arabidopsis thaliana, where bracts subtending flowers are suppressed. Interestingly, a group of cells that express markers of leaf development subtend Arabidopsis floral meristems, indicating the presence of a cryptic bract (Sawa et al., 1999; Byrne et al., 2000; Long and Barton, 2000), which is eventually subsumed by the initiating floral meristem (Heisler et al., 2005). Several genes that control this suppression have been identified, including the regulator of floral meristem identity LEAFY (LFY) (Weigel et al., 1992), which mediates bract suppression at least in part through UNUSUAL FLORAL ORGANS (UFO) (Hepworth et al., 2006). In addition, several genes with a general role in promoting leaf growth are also involved in bract suppression, including JAGGED (JAG), which is both necessary and sufficient for bract growth (Dinneny et al., 2004; Ohno et al., 2004), and the redundant BLADE-ON-PETIOLE1 (BOP1) and BOP2 genes, which are also required for bract suppression (Ha et al., 2003; Hepworth et al., 2005; Norberg et al., 2005). More recently, PUCHI, a gene initially characterized for its role in lateral root initiation (Hirota et al., 2007), has been shown to act in parallel with BOP1 and BOP2 to suppress bract growth, likely by promotion of LFY (Karim et al., 2009).

It is currently unclear what role, if any, these factors play in bract suppression more broadly in the angiosperms. Indeed, the independent evolution of bract suppression in diverse lineages raises important evolutionary questions regarding the molecular mechanisms underlying convergent morphologies. Are the same genes or networks repeatedly used to evolve similar morphologies of independent origin, or are new genes involved each time (Wray, 2002; Arendt and Reznick, 2006)? Bract suppression presents an
ideal system to investigate these important issues because it is a relatively simple morphological switch that is not required for viability and, thus, suitable for genetic analysis. Furthermore, bract suppression has evolved independently in the grass family (Poaceae) and the Brassicaceae. The grass family has many species amenable to a functional genetic characterization of bract suppression, including maize (Zea mays), rice (Oryza sativa), and barley (Hordeum vulgare). A careful comparison of bract suppression mechanisms in Arabidopsis and grass model species is likely to yield insight into the genetic constraints that result in convergent or parallel evolution (Yoon and Baum, 2004).

We have begun an analysis of the genetic mechanism of bract suppression in the grass family by isolating maize mutants, designated tassel sheath (tsh), that fail to suppress bract growth and thus produce leaves that sheath the basal tassel branches. Here, we describe the initial genetic characterization of five distinct tsh loci (tsh1-tsh5) and present the positional cloning of tsh1, which encodes a GATA domain transcription factor similar to HANABA TARANU (HAN) of Arabidopsis. Tsh1 is orthologous to third outer glume (trd) in barley and neck leaf1 (nl1) of rice; the latter was described recently by Wang et al. (2009). Because all the orthologs produce similar mutant phenotypes, we conclude that Tsh1-mediated bract suppression is conserved throughout the grass family. Our results indicate that Tsh1 was recruited to a novel bract suppression role in the grass family, suggesting that distinct genetic mechanisms underlie the convergent evolution of bract suppression in grasses compared with Arabidopsis.

RESULTS

Isolation of tsh Mutants

Maize produces distinct male (tassel) and female (ear) inflorescences. Unlike Arabidopsis, the maize inflorescence undergoes a number of branching events before producing flowers (McSteen et al., 2000). All lateral branches arising from the inflorescence meristem are subtended by a suppressed bract (Figure 1C). In the tassel, these branching events generate branch meristems that give rise to long branches, and spikelet pair meristems (SPMs), which give rise to short branches also known as spikelet pairs (Figure 1C), while the ear lacks branch meristems and produces only SPMs. SPMs in turn produce the sessile and pedicellate spikelet meristems (SMs), although it is unclear whether both of these are produced by lateral branching events or if the pedicellate spikelet results from conversion of the apical SPM into an SM (Irish, 1997, 1998; Chuck et al., 1998; Wu and McSteen, 2007; Gallavotti et al., 2008). Under the lateral branching model, both pedicellate and sessile spikelets should be subtended by suppressed bracts, while under the conversion model, only the sessile spikelet should have a suppressed bract.

In the tsh1 mutant (Briggs, 1992; McSteen and Hake, 2001), the bracts of the tassel long branches and of the spikelet pairs of both the ear and tassel are not suppressed. However, bracts subtending either the sessile or pedicellate spikelet were not observed. We screened both transposon and ethyl methanesulfonate (EMS)-mutagenized maize populations and isolated seven other mutants with similar derepression of bract growth (Table 1). These mutants were all recessive, as all F1 progeny showed a wild-type phenotype. Phenotypic differences between several of these mutants suggested that distinct loci could be represented. Consequently, we performed complementation tests among the mutant alleles and found five distinct tsh loci (tsh1-tsh5). The phenotypes of tsh1, tsh2, and tsh4 were limited to bract growth and reduced branching, while tsh3 and tsh5 exhibited defects in floral development in addition to bract growth. As tsh2, tsh3, and tsh5 are each represented by a single allele, it is likely that additional tsh loci remain to be identified, indicating that maize bract suppression is under complex genetic control.

Since multiple alleles of tsh1 were isolated, we focused our efforts on its cloning and characterization. The reference allele (tsh1-ref) originated from a Mutator-active population, while three independent EMS-induced alleles came from random mutagenesis (tsh1-1 and tsh1-2) and a directed screen (tsh1-3) for new tsh1
alleles (see Methods). The \textit{tsh1} alleles differed in severity, with \textit{tsh1-ref} and \textit{tsh1-1} exhibiting strong expressivity, while \textit{tsh1-2} and \textit{tsh1-3} were weak and intermediate, respectively.

\textbf{tsh1 Phenotype}

The \textit{tsh} phenotype is named for the leaves that ensheath the tassel of \textit{tsh1} mutants (Figure 1). These leaves result from outgrowth of the normally suppressed bracts subtending the long branches at the base of the tassel. Unlike vegetative leaves, which consist of sheath, ligule, and a prominent blade region, the bracts of \textit{tsh1} are composed primarily of sheath, while occasionally bearing a highly reduced ligule and blade at the distal end. In addition, spikelet pairs are often subtended by bracts as well (Figure 1B). Bract growth was more frequent and the bracts were larger, toward the base of the tassel, while in the more apical regions, bract suppression appeared normal. In addition to bract growth, \textit{tsh1} mutants showed defects in branching, with a dramatic reduction in long tassel branches compared with their wild-type sibs and spikelets more frequently solitary, rather than paired (Table 2). In all cases, the solitary spikelets in \textit{tsh1} were subtended by bracts. The phenotype of the ear was similar to that of the tassel, with bracts subtending spikelet pairs more frequently at the base of the ear (Figures 1F and 1J), consistent with the predominately shared early developmental program of ear and tassel.

To investigate the early development of \textit{tsh1} mutants, we observed wild-type and \textit{tsh1-1} tassel primordia by scanning electron microscopy. Figure 1D shows the prominent bract growth subtending branches at the base of the tassel primordium and spikelet pairs of the central spike. Consistent with the mature phenotype, later arising SPMs were only occasionally subtended by growing bracts, indicating that \textit{Tsh1} suppresses early bracts, but other factors are sufficient for bract suppression as inflorescence development proceeds.

No obvious differences were seen during vegetative growth of \textit{tsh1} mutants compared with the wild type. \textit{tsh1} mutants appeared to flower at the same time as the wild type, and there was no obvious difference in plant height at maturity (data not shown). Thus, the phenotype of \textit{tsh1} appears to be limited to bract derepression and reduced branching.

\textbf{Positional Cloning of \textit{tsh1}}

Bulked segregant mapping (Michelmore et al., 1991) of 10 \textit{tsh1} plants from a \textit{tsh1-1}3\textit{B73} F2 population showed linkage with markers on the long arm of chromosome 6 (see Methods). \textit{tsh1} mutants appear to be limited to bract derepression and reduced branching.

\begin{table}
\caption{\textit{tsh} loci \label{tab:tsh_loci}}
\begin{tabular}{llll}
\textbf{Complementation Group} & \textbf{Alleles} & \textbf{Source} & \textbf{Phenotype} \\
\hline
\textit{tsh1} & \textit{tsh1-ref} & Unknown background, \textit{Mu} active & Bract growth \\
& \textit{tsh1-1} & A619, EMS & Reduced branching \\
& \textit{tsh1-2} & A619, EMS & \\
& \textit{tsh1-3} & B73, EMS & \\
\textit{tsh2} & \textit{tsh2-1} & Unknown background, \textit{RescueMu} & Bract growth \\
& & & Reduced branching \\
\textit{tsh3} & \textit{tsh3-1} & A619, EMS & Bract growth \\
& & & Reduced branching \\
& & & Late flowering \\
& & & Floral organ identity defects \\
\textit{tsh4} & \textit{tsh4-1} & W22, EMS & Bract growth \\
& \textit{tsh4-2} & W22, \textit{Ac-Ds} active & Reduced branching \\
\textit{tsh5} & \textit{tsh5-1} & A632 \times \textit{OH43}, EMS & Bract growth \\
& & & Reduced branching \\
& & & Floral organ identity defects \\
\end{tabular}
\end{table}

\begin{table}
\caption{\textit{tsh1} phenotypic characterization \label{tab:tsh1_phenotypes}}
\begin{tabular}{llllll}
\textbf{Genotype} & \textbf{No. of Long Branches} & \textbf{Percentage of Solitary Spikelets on the Central Rachis} & \textbf{Percentage of Long Branch Nodes with Subtending Bracts} & \textbf{Percentage of Spikelet Pairs with Subtending Bracts} & \textbf{Percentage of Solitary Spikelets with Subtending Bracts} \\
\hline
A619 \textit{n = 4} & 5 (1.472) & 5.03\% (1.72) & 14\% (5.49)\textsuperscript{a} & 0.061\% (0.061) & 0\% (0) \\
t\textit{sh1-1} \textit{n = 4} & 0 (0) & 17.0\% (3.62) & 95\% (5.00) & 53.2\% (3.18) & 93.8\% (1.18) \\
\end{tabular}
\end{table}

Phenotypic analysis of the \textit{tsh1} mutant from measurement of tassels collected after emergence but before anthesis. Due to the variability in number of branches and spikelet pairs between individual tassels, the percentage that was subtended by bracts was calculated for each. The percentage of solitary spikelets was calculated for the central spike rather than the branches as it was less variable than the branches. The average of these percentages for four tassels is reported here with the standard error of the mean in parentheses. Long branch nodes are the nodes at the base of the tassel below the central spike. Long branches are present at these nodes in the wild type, while \textit{tsh1} always had empty nodes, almost always subtended by a bract.

\textsuperscript{a}While wild-type A619 tassels do not have conspicuous bracts, occasionally small filamentous enations were observed. Since these enations were seen frequently in \textit{tsh1} mutants, they were counted as bracts for this analysis.
was subsequently localized between the simple sequence repeat (SSR) markers umc2059 and bnlg1740; next, a larger F2 mapping population was generated, and these markers were used to screen 2340 recombinants for consanbants. As the maize genome was not yet sequenced, we used synteny with rice to place tsh1 between the maize orthologs of Os05g50220 and Os05g50340, a region spanning 10 annotated genes in the rice genome. Of these genes, two (Os05g50310 and Os05g50270) were predicted transcription factors and possible candidates for tsh1 (Figure 2A). Sequencing of the maize ortholog of Os05g50270 from three independent EMS alleles (tsh1-1, tsh1-2, and tsh1-3) revealed mutations consistent with EMS mutagenesis (G->A or C>T transitions) likely to disrupt protein function (Figure 2C). These independent alleles had known progenitors (see Methods), and sequencing showed the progenitors did not carry the mutations, confirming that we had identified the correct Tsh1 gene.

Tsh1 encodes a GATA-domain zinc-finger transcription factor similar to HAN of Arabidopsis. A protein alignment in Figure 2D shows the conserved GATA domain in addition to a conserved HAN motif of unknown function in the N-terminal region found only in a subgroup of GATA domain proteins closely related to HAN (Reyes et al., 2004). tsh1-1 contains an early stop codon (W142Stop), while tsh1-2 has a missense mutation (P140S) in a conserved residue of the P-pattern domain, consistent with the phenotypic strength of these alleles (strong and weak, respectively). tsh1-3 revealed the correct Tsh1 gene.

A phylogenetic analysis of Tsh1 and HAN-like genes from both eudicots and monocots showed that most grasses contain two parallel lineages to Tsh1 that we have named Ntt-like1 and Ntt-like2 for NECKLEAF1/Tassel sheath1/THIRD OUTER GLUME-like (see below). Ntt-like1 and Ntt-like2 together with the grass Tsh1 orthologs form a clade sister to the eudicot HAN orthologs (Figure 2B). Bayesian posterior probability was strong for the Ntt-like1 and Tsh1 clades. However, the group we have designated Ntt-like2 was paraphyletic in this analysis. This is likely an artifact of limited sampling as the genes we have grouped in Ntt-like1 and Ntt-like2 are syntenic in rice, maize, and sorghum (Sorghum bicolor). In addition to these three lineages, the maize genome contains a duplicate of Tsh1 (Tsh1dup) on chromosome 8 that appears to be the result of a recent duplication event. While there are ESTs for Tsh1 from meristematic tissues, there are none for Tsh1dup, and several attempts to detect expression of this gene by RT-PCR failed, suggesting that it is either a pseudogene (although there were no obvious deleterious mutations) or expressed at very low levels in the inflorescence. Two other HAN-like genes in Arabidopsis appear to have arisen from relatively recent duplications before the divergence of Arabidopsis and Brassica. The presence of these independent duplicates in Arabidopsis and the grass family mean that Tsh1 is not a simple ortholog of HAN, but rather belongs to a clade sister to that containing HAN.

### Tsh1 Is Expressed in the Cryptic Bract Primordium

We examined localization of Tsh1 transcripts by RNA in situ hybridization on young tassel primordia. We observed weak Tsh1 expression on the periphery of the inflorescence meristem in a stripe of cells that appeared to correspond to the cryptic bract anlagen (Figure 3A). Expression became more intense and localized to an adaxial domain in the suppressed bract as it became a ridge subtending an emerging branch meristem (Figure 3B). In older tassel primordia, Tsh1 showed a similar pattern, with expression in a group of cells corresponding to the suppressed bract subtending SPMs. Prolonged exposure indicated that Tsh1 expression was maintained as the SPM developed and eventually incorporated into the pedicel, indicating that the bract cells are ultimately co-opted by the pedicel of the spikelet pair (Figure 3D). Tsh1 expression was also observed transiently in the SPM (Figure 3C). Expression of Tsh1 in the cells of the cryptic bract is consistent with a direct role in bract suppression, as suggested by the tsh1 mutant phenotype.

To ensure that the Tsh1 expression domain we observed corresponds to the suppressed bract, we examined expression of Tsh1 and Z. mays yabby15 (Zyb15) on adjacent sections of young tassel primordia (Figures 3E and 3F). Zyb15 is an ortholog of FILAMENTOUS FLOWER (FIL) (Juarez et al., 2004), a marker of leaves and other lateral organs and a robust marker of the Arabidopsis cryptic bract (Siegfried et al., 1999; Dinneny et al., 2004; Heisler et al., 2005). Zyb15 was observed in a group of cells on the margin of the inflorescence meristem in the cryptic bract similar to Tsh1. However, Zyb15 expression was not maintained in the suppressed bract, but rather decreased in intensity as its associated meristem began to develop (Figure 3E). Adjacent sections hybridized with Tsh1 show that the Zyb15 domain overlapped with the Tsh1 domain, although Zyb15 was restricted to outermost layers, while Tsh1 expression extended several cell layers inward. The coexpression of Tsh1 and Zyb15 confirms that Tsh1 is indeed expressed in the cryptic bract.

We also observed that the transient Tsh1 expression in the SPM overlapped with Zyb15, raising the possibility that these cells correspond to a cryptic bract subtending the branching event that produces the sessile and/or pedicellate spikelets (Figure 3F). To better resolve the expression in the SM, we hybridized Tsh1 and Zyb15 to transverse sections of an elongating branch meristem, which produces SPMs distichously (Figures 3G to 3I). Neither Tsh1 nor Zyb15 expression was observed during the initial stages of SPM development when the SPM is a uniform mound of cells (data not shown). However, as the SPM began to branch, Tsh1 expression was observed in the smaller of the meristems, corresponding to the sessile spikelet primordium (Figure 3G). Similarly, Zyb15 was expressed in the emerging sessile spikelet primordium (Figure 3H). Unlike Tsh1, which was expressed in most of the sessile spikelet primordium, Zyb15 was restricted to the cells in a lateral domain (Figure 3H). Serial sections revealed that Zyb15 and Tsh1 were maintained in the emerging sessile SMs for three to four nodes but were absent from the sessile spikelet in later nodes (Figures 3N and 3O). While Tsh1 was never observed in the pedicellate SM (data not shown), Zyb15 eventually was (Figures 3I and 3O).

These results suggest that the branching events by which the SPM produces the sessile and pedicellate spikelets are distinct, at least with respect to the presence of Tsh1. The coexpression of Tsh1 and Zyb15 in the sessile spikelet primordium indicates that the sessile spikelet is initiated in the axil of a cryptic bract.
Figure 2. Positional Cloning, Gene Structure, and Phylogenetic Analysis of Tsh1.

(A) Schematized summary of the positional cloning of Tsh1. On top is maize chromosome 6 with the position of SSR markers flanking tsh1 used to identify recombinants for fine-scale mapping. Below this is the alignment of maize chromosome 6 and rice chromosome 5, showing syntenic genes in the region containing Tsh1. Recombination frequency is indicated above the maize markers. Below the synteny alignment is the region in rice that was ultimately used to identify potential candidates to sequence tsh1 alleles. Again, recombination is indicated, this time above the rice genes that were used to identify orthologous maize genes.

(B) Bayesian phylogenetic analysis of NL1/Tsh1/TRD and Ntt-like genes from the grasses and HAN-like genes from eudicots. Posterior probability
However, since Tsh1 and Zyb15 were not coexpressed in the pedicellate spikelet primordium, the eventual expression of Zyb15 in the pedicellate spikelet may not mark a cryptic bract, but rather the initiating glume of the pedicellate SM. Consequently, it is still unclear if the pedicellate spikelet is also produced by an axillary branching event or a conversion of the indeterminate SPM into a determinate pedicellate SM (Irish, 1997, 1998; Chuck et al., 1998; Wu and Mcsteen, 2007; Gallavotti et al., 2008). If Zyb15 in the pedicellate spikelet marks a cryptic bract that does not express Tsh1, this would be consistent with the axillary branching model. On the other hand, if Zyb15 is simply marking the initiating glume of the pedicellate spikelet, this would explain the absence of Tsh1 and support the conversion model.

To visualize TSH1 protein localization, we created an N-terminal yellow fluorescent protein (YFP) fusion to TSH1 (YFP-TSH1) under the control of the native Tsh1 promoter, and maize plants were transformed with this construct. In the YFP-TSH1 lines examined, fluorescence was strongest in the most basal bracts subtending tassel branch primordia (Figures 3J to 3M). YFP-TSH1 was localized to both the nucleus and the cytoplasm, consistent with a role as a transcription factor (Figures 3K to 3M). Interestingly, localization appeared to be more enriched in nuclei toward the base of branch primordia (Figure 3K). The YFP-TSH1 protein localization pattern is the same as the one we observed for the Tsh1 transcript, indicating that TSH1 protein does not traffic from cell to cell.

**Conservation of Tsh1 Function in Diverse Grasses**

Most grasses have suppressed bracts, although the closest nongrass outgroups (Ecdyiocolea, Joinvillea, and the Restionaceae) found by phylogenetic studies (Briggs et al., 2000; Bremer, 2002; Michelangeli et al., 2003) have prominent bract growth in their inflorescences. These observations suggest that a bract suppression mechanism evolved early in the evolution of the grass family. If Tsh1 was involved in the evolution of bract suppression in the grasses, we reasoned that it would have a similar bract suppression function in diverse grass lineages. Rice, barley, and maize span most of the phylogenetic diversity of the grass family (Grass Phylogeny Working Group, 2001). Interestingly, classical mutants in rice, nl1 (Morinaga and Fukushima, 1943), and barley, trd (Ivanova, 1937; Koznak, 1953), have a similar phenotype to tsh1, and both map to syntenic genomic locations (Kishimoto et al., 1992; Pozzi et al., 2003).

nl1 mutants of rice have prominent growth of the suppressed bracts at the base of the panicle. We sequenced the rice ortholog of Tsh1 (Os05g50270) from two nl1 alleles listed in the rice mutant database Oryzabase (see Methods). Both alleles had deletions, one removing a highly conserved amino acid of the GATA domain, and the other with an 8-bp deletion causing a frame shift (Figure 4J). In situ localization of NL1 revealed expression in the suppressed bracts at the base of the panicle (Figures 4G and 4H). This expression pattern was similar to Tsh1, although rice produces suppressed bracts with no axillary meristems in this region, which represent the last leaves produced as the meristem transitions to reproductive development. NL1 was absent from vegetative leaf primordia (Figure 4I). Similar to Tsh1, NL1 transcript was localized to the adaxial surface of these suppressed leaves. These results confirm a recent report by Wang et al. (2009), describing the 8-bp deletion allele, which they named nl1-1, although the second allele described here (which we designated nl1-4) was not included in their study.

Several barley trd mutants, all mapping to the same location on chromosome 1H (5) (Franckowiak and Lundqvist, 1997), were obtained and grown to observe the phenotype. Whereas the wild-type barley inflorescence produces a triplet of spikelets in the axil of a suppressed bract (Figure 4A), the trd mutants had prominent bract growth (Figures 4B and 4C). As in tsh1, these bracts were larger and more common near the base of the inflorescence. Since no barley Tsh1 ortholog was found from BLAST searches of EST collections, we used degenerate PCR to isolate the orthologous barley sequence. Phylogenetic analysis of this sequence clearly showed that it belongs to the Tsh1 clade and is not one of the duplicate Ntt-like genes (Figure 2B). Sequencing of this gene from the trd-2061 and trd-1695 alleles revealed a 7-bp deletion in the first exon causing a frame shift in trd-2061 and a Trp>Tyr substitution in a highly conserved amino acid of the GATA domain in trd-1695 (Figure 4J). Multiple attempts with different primer pairs failed to amplify this gene from the trd-227 allele, suggesting that it may be caused by a deletion. We concluded from the distinct lesions in the independent trd alleles, its synteny with tsh1 and nl1, and their similar bract growth phenotype that Trd is the Tsh1 ortholog.

In situ hybridization of Trd showed a similar expression pattern to that observed for NL1 and Tsh1, with expression in the cells of the suppressed bract. Trd expression was observed in the suppressed bract anlagen and was maintained strongly in the bracts at the base of the inflorescence (Figures 4D to 4F). This may reflect the delayed maturation of spikelets at the base relative to those in the middle of the barley inflorescence (Bonnett, 1935). However, unlike NL1 and Tsh1, Trd expression was found throughout the suppressed bract and not restricted to an adaxial domain (Figure 4F).

In summary, the bract suppression function of the maize, rice, and barley Tsh1 orthologs appears to be largely conserved. Expression of NL1 (Wang et al., 2009; this study) and TRD are also very similar to Tsh1. This conservation of function and
Figure 3. Expression of Tsh1.

(A) to (D) In situ localization of Tsh1 transcript. (A) and (B) show early tassel primordia with Tsh1 localization in the inflorescence meristem and marking the cryptic bract (arrowheads); adaxial localization of the repressed bracts is clear in (B). Branch primordium of an older-stage tassel showing Tsh1 in the cryptic bract (arrowheads) and the dividing SPM (asterisks) is shown in (C). After the initiation of glume development and elongation of the spikelet pair, Tsh1-expressing cells were incorporated into the pedicel (arrowheads in [D]).

(E) and (F) Adjacent sections hybridized with Tsh1 (left) and Zyb15 (right), demonstrating that their expression domains overlap in the cryptic bract (E) and the dividing SPM (F).

(G) to (I) The dividing SPM with Tsh1 (G) localized to a broad domain throughout the emerging sessile spikelet (ss) and Zyb15 (H) localized to a much smaller domain in a lateral region of the emerging sessile spikelet. Colocalization of Tsh1 and Zyb15 in the sessile spikelet indicates that it is likely initiating in the axil of a cryptic bract. In older spikelet pairs, Zyb15 was eventually detected in the pedicellate spikelet (ps) (I), although this may correspond to glume initiation rather than a cryptic bract subtending the pedicellate spikelet.

(J) to (M) Transgenic expression of Tsh1pro::YPFP-Tsh1. (J) shows a scanning electron micrograph of a young tassel primordium, indicating the region where YFP-TSH1 expression was observed in (K). YFP-TSH1 is most easily detected in the suppressed basal bracts (sb) and subtending branch meristems (bm) (K) and [L]. YFP-TSH1 is localized in both the cytoplasm and nucleus (K) to [M], although YFP-TSH1 becomes more strongly nuclear in the proximal region of the suppressed bract (K). Green represents YFP-TSH1 fluorescence, while red represents FM464 fluorescence marking the plasma membrane.

(N) A false color summary of the Tsh1 expression domain in the tassel. Expression is seen in the suppressed bract subtending the SPM and in the sessile spikelet marking a presumed cryptic bract in this region but was not detected in the pedicellate spikelet.

(O) Summary of Zyb15 expression in the tassel. Expression is similar to Tsh1, although there is also expression in the pedicellate spikelet (asterisks) later in development that may correspond to glume initiation rather than a cryptic bract.
expression strongly suggests that the majority of grass diversity inherited a common genetic mechanism for bract suppression involving NL1/Tsh1/TRD and is consistent with the evolution of a distinct bract suppression mechanism in a common ancestor of the grasses.

DISCUSSION

Here, we report the isolation of maize mutants that fail to suppress bract development and the characterization and cloning of Tsh1, which encodes a GATA zinc-finger protein similar to HAN. The bract suppression function of Tsh1 appears to be conserved in diverse grasses, as the rice NL1 and barley TRD genes are orthologs of Tsh1. Tsh1 and its orthologs in rice and barley are expressed in a domain corresponding to the suppressed bract, suggesting that these genes function there to repress bract growth.

In addition to the expression in bract primordia, Tsh1 was also expressed in an apparent cryptic bract subtending the sessile spikelet in the maize spikelet pair. The function of this expression is unclear, as we did not observe ectopic bract growth subtending the sessile spikelet in tsh1 mutants. However, it is possible that another factor acts redundantly with Tsh1 to suppress bracts during this branching event. Indeed, even in a putative null tsh1 mutant, the bracts subtending a large percentage of the spikelet pairs are still suppressed, indicating either redundancy.

Figure 4. Functional Conservation of Tsh1 Orthologs in Rice and Barley.

(A) to (C) The phenotype of barley trd mutants. Wild-type spike of barley (A) produces spikelets distichously, with no subtending bracts. trd mutants (trd-227 [B] and trd-2060 [C]) initiate fewer spikelets and have prominent bracts subtending spikelet primordia.

(D) to (F) In situ hybridization of TRD shows expression in suppressed bracts. Initiating spike primordium (D) expresses TRD in suppressed bracts that subtend spikelet primordia. In later-stage spike primordium, TRD is expressed in suppressed bracts near the inflorescence meristem but is not maintained (E). However, TRD expression is maintained throughout suppressed bracts at the base of the spike (F).

(G) to (I) In situ hybridization of NL1 shows expression in the suppressed bract at the base of the panicle (G) that is localized to an adaxial domain (H). Expression was not observed in the vegetative meristem or leaf primordia (I).

(J) Gene structure of TRD (above) and NL1 (below), with the location of mutations indicated. Bars = 500 μm in (A) and (B) and 250 μm in (C).
in the suppression pathway or the existence of distinct pathway(s) regulating bract suppression later in inflorescence development. Possible candidates for such genes include the other Tsh loci as well as the Tsh1 duplicate locus or one of the Ntt-like genes. We were unable to detect expression of Tsh1dup, although this could be an effect of the genetic background, and Tsh1dup might be more highly expressed in other backgrounds and thus could function in a partially redundant manner with Tsh1.

**NL1/Tsh1/Trd Are Homologs of HAN but Have Divergent Phenotypes**

Our phylogenetic analysis clearly demonstrates that Tsh1, NL1, and TRD belong to a clade sister to the one that includes HAN of Arabidopsis. Despite their shared ancestry, Tsh1 and HAN do not appear to share an obvious conserved developmental role. han mutants are pleiotropic, with defects in floral development and embryo patterning (W. Lukowitz and T. Navy, personal communication; Zhao et al., 2004). However, han mutants do not show derepressed bract growth, the primary phenotype seen in tsh1, nl1, and trd. Furthermore, the expression of HAN, in a narrow strip of cells between the apical meristem and seen in tsh1, nl1, and trd, is distinct from that of Tsh1, NL1, and TRD, which are strongly expressed in suppressed bracts. This apparent divergence in function and expression could be a consequence of the duplications in the HAN/HAN-like or Tsh1/Ntt-like lineages and subsequent neo- or subfunctionalization. Neither the han-like (W. Lukowitz and T. Navy, personal communication) nor han mutants have a bract growth phenotype, suggesting that bract suppression is either a neo-functionalization in the Tsh1/Ntt-like lineage that occurred specifically in the grasses or that Tsh1 subfunctionalized a bract suppression function that was lost from the HAN/HAN-like lineage. While it is possible that the common ancestor of Tsh1 and HAN had a bract suppression function that was partitioned to Tsh1 following a duplication (subfunctionalization), this would require that grass outgroups lacking bracts had lost this ancestral function, and it was subsequently regained and subfunctionalized in the grass Tsh1 lineage. We feel the more likely scenario is that HAN maintains the ancestral function, and bract suppression by Tsh1 is a neo-functionalization. If this is the case, then we would expect one or both of the Ntt-like genes to have retained a shared ancestral function with HAN. Identification of mutants in these paralogous genes should help clarify this issue.

If indeed the NL1/Tsh1/TRD lineage subfunctionalized a bract suppression function that was lost from the HAN/HAN-like lineage, while it is possible that the common ancestor of Tsh1 and HAN had a bract suppression function that was partitioned to Tsh1 following a duplication (subfunctionalization), this would require that grass outgroups lacking bracts had lost this ancestral function, and it was subsequently regained and subfunctionalized in the grass Tsh1 lineage. We feel the more likely scenario is that HAN maintains the ancestral function, and bract suppression by Tsh1 is a neo-functionalization. If this is the case, then we would expect one or both of the Ntt-like genes to have retained a shared ancestral function with HAN. Identification of mutants in these paralogous genes should help clarify this issue.

**Bract Suppression in the Brassicaceae and Poaceae**

Comparison of the bract suppression mechanisms in Arabidopsis and in the grass family will shed light on the ways evolution converges on similar morphologies. Leaf initiation in Arabidopsis begins with establishment of a local auxin maximum (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005), and this mechanism appears to be conserved in maize (Gallavotti et al., 2008; Lee et al., 2009). This is closely followed by downregulation of the meristematic genes STM/Kn1 (Jackson et al., 1994; Long et al., 1996), which is maintained by the ARP genes (for ASYMMETRIC LEAVES1/Rough sheath2/PHANTASTICA) (Schneeberger et al., 1998; Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000). Next, genes important for abaxial/adaxial polarity (Chitwood et al., 2007) and blade expansion, including the YABBY gene FIL, are expressed (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 2004). Upon the floral transition, Arabidopsis bract growth is inhibited by multiple factors, including LFY and its cofactors UFO, BOP1, BOP2, and PUCHI. Mutations in each of these genes results in the growth of bracts subtending flowers and pleiotropic effects on floral development (lfy, ufo, and bop), leaf development (bop), or lateral root development (puchi). Interestingly, the early molecular events of leaf initiation occur in the suppressed bract of Arabidopsis, indicating they are not sufficient for bract outgrowth; thus, other factor(s) are likely the targets of the bract suppression pathway. In Arabidopsis, suppression of JAG appears to control, at least in part, inhibition of bract growth, as it is both necessary and sufficient for bract development (Dinnery et al., 2004; Ohno et al., 2004). Taken together, these findings suggest an emerging pathway for bract suppression in Arabidopsis (Figure 5).

Several of the genes in the Arabidopsis bract suppression pathway have been characterized in either maize or rice and thus provide an opportunity to assess functional conservation. In maize, the redundant zfa and zfb genes are largely conserved in function with LFY; however, no ectopic bract growth is observed in zfa zfb double mutants (Bomblies et al., 2003). In rice, ABERRANT PANICLE ORGANIZATION1 (APO1) is the likely ortholog of UFO (Ikeda et al., 2007), but apo and ufo mutants differ significantly in phenotype, and there is no obvious effect on bract growth in apo (Ikeda et al., 2005, 2007). The closest related genes to PUCHI, Branched silkless1 (maize) and FRIZZY PANICLE (rice), are also significantly diverged in function from their Arabidopsis homologs and have no bract growth phenotype (Komatsu et al., 2001, 2003; Chuck et al., 2002). Mutants in the rice JAG ortholog OPEN BEAK (OPB) have been described, although it is not yet clear if OPB plays any role in bract suppression (Horigome et al., 2009). In sum, in spite of numerous functional studies on orthologous genes in the grass family, there is no evidence of a conserved bract suppression pathway between Arabidopsis and rice or maize. Furthermore, our results indicate that a conserved regulator of bract suppression in the grasses, Tsh1/NL1/TRD, has evolved a novel expression domain and function compared with its Arabidopsis ortholog.

It is perhaps not surprising that the grass family has evolved a novel mechanism of bract suppression. Both Brassicaceae (Saunders, 1923; Iltis, 1957; Al-Shehbaz et al., 2006) and the grass family (Poaceae) (Watson and Dallwitz, 1992; Grass
Phylogeny Working Group, 2001; Decraene et al., 2002; Rudall et al., 2005) clearly evolved from ancestors that had prominent bracts. Thus, convergent evolution of bract suppression in these distant angiosperm groups is not controlled by a common genetic mechanism. This is in contrast with several examples from both plant and animal organ development, where similar genetic mechanisms have been involved in morphological convergence. For example, repeated evolution of compound leaves appears to involve the cooption of class I KNOX gene expression (Bharathan et al., 2002). Similarly, the independent pelvic reduction in threespine and ninespine sticklebacks is regulated by Pitx1 (Shapiro et al., 2006). Since so few examples of convergent development have been examined at a molecular level in plants or animals, it is not yet clear whether independent recruitment of either the same or distinct genetic mechanism is more common. Thus, it will be interesting to investigate if Tsh1 orthologs or other bract suppression gene candidates from Arabidopsis control bract suppression in other angiosperm taxa that have independently evolved bract suppression.

Possible Utility of Bract Suppression

Bract suppression is seen frequently in the angiosperms. Even in species where bracts are not completely suppressed as they are in maize and Arabidopsis, reduction of bract size relative to vegetative leaves is common. It is not immediately clear why bract suppression and reduction has evolved so frequently. One possible explanation, in the case of insect pollinated plants, is that bract growth obscures the flower; thus, individuals that suppress bract growth have more conspicuous flowers, giving them a selective advantage. This, however, is not a satisfying explanation for wind-pollinated plants, such as the grasses. Another possibility takes into account that bract development compromises the size and/or speed with which the meristem it subtends can develop, possibly by using energy resources (Coen and Nugent, 1994). Thus, halting bract growth would facilitate meristem growth and favor the complex branching events seen in many grass inflorescences or the rapid floral development of Arabidopsis. This idea is consistent with the mutant phenotypes of tsh1 and other tsh mutants, where derepression of bract growth is accompanied by reduced branching and fewer spikelets. Tsh1 is expressed on the periphery of the meristem in the cells of the suppressed bract anlagen, suggesting that its effects on meristem branching are an indirect consequence of bract growth. Several other lines of evidence also point to a close association between growth of the meristem and its subtending bract. Morphologically, the association of bract and floral meristem is so tight in Arabidopsis that they are generally considered to form a common floral meristem/bract primordium, although this primordium is divided into distinct molecular domains corresponding to bract and meristem (Heisler et al., 2005). Ablation of the floral meristem portion of this common primordium by diptheria toxin results in Arabidopsis bract growth (Nilsson et al., 1998). Similarly, maize mutants with compromised meristem initiation or growth also have increased bract development (Ritter et al., 2002; Phillips et al., 2009). PUCHI, LFY, and UFO are
each expressed in the floral meristem of Arabidopsis but are excluded from the cryptic bract; hence, they repress bract growth non-cell autonomously (Hepworth et al., 2006; Karim et al., 2009). These observations support a model in which the leaf and its subtending meristem are in a competition for growth factors and resources, and bract suppression is likely to be a consequence of developmental constraints that accompany increased meristem size or proliferation after the floral transition.

METHODS

Origin of tsh, nl1, and trd Mutants

Seed for tsh1-ref was provided by Paula McSteen who obtained it from Pioneer Hi-Bred International, where it was isolated from a Mutator transposon active population (Briggs, 1992). tsh1-1, tsh1-2, and tsh3 were isolated from a screen of EMS-treated M2 families in the A619 background. tsh1-3 was isolated by screening for noncomplementation of 6000 M1 progeny from tsh1-ref ears pollinated with EMS-treated B73 pollen, as described (Neuffer, 1994). tsh2 was found in a screen of Mutator RescueMu (Raizada et al., 2001) families. tsh4-1 was isolated from a screen of Ac-Ds active families in W22 (Kolkman et al., 2005; Ahern et al., 2009), while tsh4-2 came from a phenotypic screen of EMS W22 M2 families generated for TILLING (Till et al., 2004). tsh5 was identified from an M2 screen of EMS OH43 × A632 families.

Three nl1 mutants were obtained from Oryzabase (www.shigen.nig.ac.jp/rice/oryzabase). Accessions HO708 and HO709 contained the same lesion, which we designated as nl1-4, while HO716 corresponds to nl1-1. Three trd alleles were obtained from the National Plant Germplasm System (www.ars-grin.gov/npgs), with the following accession numbers: GSHO227 (trd-227), GSHO2061 (trd-2061), and GSHO1695 (trd-1695). The Bowman cultivar was used to isolate the wild-type sequence.

Positional Cloning of Tsh1

SSR markers for bulked segregant analysis were obtained from the Maize SSR Primer Set (Sigma-Aldrich). These were screened for polymorphism between our parental lines (A619 mutant × B73 wild type) using the online maize (Zea mays) SSR database (www.maizegdb.org) to identify a core set of 96 markers spanning the 10 maize chromosomes. To generate new markers for fine mapping of tsh1, rice genes in the interval between Os05g50120 and Os05g50380, which have orthologous maize genes anchored to the physical map in a syntenic location, were BLASTed against the maize MAGI database (magi.plantgenomics.iastate.edu) of nonrepetitive high-throughput genomic sequences to identify putative maize orthologs. Primers were designed to these maize genes, and they were sequenced from the parental lines of our mapping populations. If the parental sequences were polymorphic, cleaved amplified polymorphic sequence or derived cleaved amplified polymorphic sequence (Neff et al., 1998) markers were then designed and used to genotype recombinant F2 individuals and narrow the interval containing tsh1. PCR amplification of Tsh1 was difficult with standard Taq polymerase and standard PCR conditions, likely due to its high GC content. However, Phusion (Finnzymes) used with the provided GC buffer allowed consistent and robust Tsh1 amplification from both cDNA and genomic DNA. The full-length coding sequence of Tsh1 was amplified with the Tsh1 F1 primer (5′-ATGGCGTGCAACCACCTGCTC-3′) and the Tsh1 R1 primer (5′-CTGTACCAATACTTTTCGAGCC-3′).

Isolation of TRD

Degenerate primers were designed for the HAN motif (HAN-like F1, 5′-GTBGAATGCCKCTBC5YTSSG-3′) and the GATA domain (GATA R1, 5′-CKYKCYTCYCTCCTCCTCCTCCT5-3′) and were used to amplify a genomic fragment corresponding to the barley (Hordeum vulgare) Tsh1 ortholog. A gene-specific primer from this sequence (HvTsh1 F3 3′RACE, 5′-TGCTACTACAAACGGATGAGTG-3′) was then used in a 3′ rapid amplification of cDNA ends (RACE) on young Bowman inflorescence cDNA synthesized with the SuperscriptIII kit (Invitrogen) and a polyT primer. The 5′ RACE using First Choice RLM RACE kit (Applied Biosystems) was then used according to manufacturer’s protocol to isolate the complete TRD cDNA.

In Situ Hybridization

PCR products for 5′ and 3′ regions of the NL1, TRD, and Tsh1 cDNAs were amplified using the following primers: Tsh1 5′ untranslated region (UTR) 5′-CTATAAATCCCTGAATCTGC-3′, Tsh1 5′ UTR R (5′-ATGCTCTGGGATGGCGATGCC-3′), Tsh1 3′ UTR F (5′-ACCTTGTTCCAGTACAACTAATC-3′), and Tsh1 3′ UTR R (5′-GTACTACGGAATAGTAATTG-3′). These PCR fragments were then cloned into TOPO Zero Blunt (Invitrogen) and linearized, and then digoxigenin-labeled RNA was synthesized using T7 RNA polymerase. The respective 5′ and 3′ probes were combined in the hybridization for NL1, TRD, or Tsh1. For Zyb15, the complete cDNA was used rather than a combination of shorter fragments. Hybridization was performed largely as described by Jackson et al. (1994), with the exception that Tsh1 hybridization was incubated overnight at 65°C, rather than the standard 55°C.

Phylogenetic Analysis

HAN and Tsh1 orthologs were obtained by BLAST searches of EST and genomic databases (see Supplemental Table 1 online for accession numbers). Genomic sequences were annotated to remove the intron, alignment was created using ClustalX, and this alignment further adjusted by hand using MacClade. MODELTEST (Posada and Crandall, 1998) was used to determine the model of evolution (GTR+G+I) to be used in the Bayesian phylogenetic analysis. MRBAYES (Huelsenbeck and Ronquist, 2001) was used to estimate the phylogeny, with 2.4 million generations, a sample frequency of 100, and a burn-in of 60,000. The tree was rooted with outgroup GATA ZF sequences lacking the HAN motif.

Scanning Electron Microscopy

Freshly dissected samples of maize tassels and barley inflorescences were mounted live to stubs with silver adhesive (Electron Microscopy Sciences) and kept cold on ice until viewing in a Hitachi S-3500N Scanning Electron Microscopy (SEM) instrument. Images were taken quickly (within 15 min) to minimize damage by the electron beam on the live sample, using an accelerating voltage of 5.0 kV and a working distance of 10 to 20 mm.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers GU722205 (TRD genomic) and GU722206 (TRD cDNA). Accession numbers for Tsh1, NL1, TRD, and HAN-like family members are listed in Supplemental Table 1 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Tsh1 Phylogeny.

Supplemental Methods. Creation of YFP-TSH1 Transgenic Maize Construct and Lines.
Supplemental Data Set 1. Alignment Used to Generate the Phylogenetic Tree in Figure 2B.

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NOTE ADDED IN PROOF
Isolation of the tsh4 locus has been reported recently by Chuck et al. (2010).

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