MORE SPIKELETS1 Is Required for Spikelet Fate in the Inflorescence of Brachypodium

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Grasses produce florets on a structure called a spikelet, and variation in the number and arrangement of both branches and spikelets contributes to the great diversity of grass inflorescence architecture. In Brachypodium (Brachypodium distachyon), the inflorescence is an unbranched spike with a terminal spikelet and a limited number of lateral spikelets. Spikelets are indeterminate and give rise to a variable number of florets. Here, we provide a detailed description of the stages of inflorescence development in Brachypodium. To gain insight into the genetic regulation of Brachypodium inflorescence development, we generated fast neutron mutant populations and screened for phenotypic mutants. Among the mutants identified, the more spikelets1 (mos1) mutant had an increased number of axillary meristems produced from inflorescence meristem compared with the wild type. These axillary meristems developed as branches with production of higher order spikelets. Using a candidate gene approach, mos1 was found to have a genomic rearrangement disrupting the expression of an ethylene response factor class of APETALA2 transcription factor related to the spikelet meristem identity genes branched silkless1 (bd1) in maize (Zea mays) and FRIZZY PANICLE (FZP) in rice (Oryza sativa). We propose MOS1 likely corresponds to the Brachypodium bd1 and FZP ortholog and that the function of this gene in determining spikelet meristem fate is conserved with distantly related grass species. However, MOS1 also appears to be involved in the timing of initiation of the terminal spikelet. As such, MOS1 may regulate the transition to terminal spikelet development in other closely related and agriculturally important species, particularly wheat (Triticum aestivum).

Plant shoots develop from the shoot apical meristem, which comprises a central region of pluripotent cells and a peripheral region where cells are recruited to form lateral organs. The shoot apical meristem is established early in embryogenesis and gives rise to all aerial portions of the plant. Following a reproductive transition, the shoot apical meristem converts to an inflorescence meristem, which may produce axillary meristems that form branches and flowers. The production, arrangement, and fate of meristems determine inflorescence architecture.

Plant inflorescence architecture is vastly diverse, and one of the most dramatic reflections of this diversity is in the major cereal crop species. In maize (Zea mays), the tassel and ear inflorescence is indeterminate. Axillary meristems produced by the inflorescence in the tassel are initially indeterminate branches. Both the tassel and ear inflorescence, as well as branches of the tassel, produce spikelet pair meristems that then form determinate spikelet meristems, which terminate with production of two flowers or florets (Bortiri and Hake, 2007; Kellogg, 2007). In the rice (Oryza sativa) inflorescence or panicle, branch meristems initially produce secondary branch meristems. Both primary and secondary branches produce lateral spikelet meristems and a terminal spikelet meristem. The spikelet meristem gives rise to a single floral meristem and a single floret (Bommert et al., 2005; Wang and Li, 2008). By comparison, the inflorescence, or spike, of barley (Hordeum vulgare) and wheat (Triticum aestivum) is unbranched (Bonnett, 1935, 1936). In barley, the inflorescence meristem is indeterminate and produces lateral spikelet meristems that form three florets. In wheat, the inflorescence meristem is determinate and produces a limited number of lateral spikelet meristems and a terminal spikelet meristem, with each spikelet meristem producing multiple florets.

Genes that are necessary for inflorescence development have been identified through analysis of mutants in maize and rice, and many of these genes control meristem initiation and fate (Bommert et al., 2005; Sreenivasulu and Schnurbusch, 2012). One group of mutants fails to produce branches and spikelets. barren stalk1 (ba1) in maize results in a radical decrease in the production of axillary meristems and forms an unbranched inflorescence without spikelets (Ritter et al., 2002; Gallavotti et al., 2004). Mutation in LAX PANICLE1...
(LAX1), the rice ortholog of ba1, also results in fewer spikelets (Komatsu et al., 2001, 2003b). The rice MONOCULM1 (MOC1) gene (also known as SMALL PANICLE) is required for initiation of axillary meristems throughout vegetative and reproductive development. moc1 mutants lack tillers and have few inflorescence branches and spikelets (Li et al., 2003; Oikawa and Kyozuka, 2009). barren inflorescence2 (bif2) in maize does not produce branches or spikelets in the inflorescence and is required for the maintenance of all types of axillary meristems (McSteen and Hake, 2001). ba1, LAX1, and MOC1 encode transcription factors, whereas bif2 encodes a Ser/Thr protein kinase protein involved in auxin signaling (Komatsu et al., 2003b; Li et al., 2003; Gallavotti et al., 2004; McSteen et al., 2007).

Another group of mutants in maize and rice increase inflorescence branching. The maize mutants ramosa1 (ra1), ra2, and ra3 change spikelet-pair meristems to branch meristems, resulting in more branches in the tassel and the development of branches in the ear (Vollbrecht et al., 2005; Bortiri et al., 2006; Satoh-Nagasawa et al., 2006). Likewise, mutants in the orthologous ethylene response factor (ERF) family of APETALA2 (AP2) transcription factor genes branched silkless1 (bd1) in maize and FRIZZY PANICLE (FZP) in rice result in highly ramified inflorescences due to conversion of spikelet meristems to branch meristems (Colombo et al., 1998; Chuck et al., 2002; Komatsu et al., 2003a; Zhu et al., 2003; Yi et al., 2005).

Although many inflorescence architecture genes have been identified in grass species that have branched inflorescences, much less is known about the factors regulating unbranched inflorescence development (Malcomber et al., 2006). In an unbranched inflorescence, known as a spike, axillary meristems produced by the inflorescence meristem develop directly into spikelets. As with wheat and barley, the inflorescence of Brachypodium (Brachypodium distachyon) is a spike (Draper et al., 2001; Vogel et al., 2006). To understand the regulation of spike development, we have carried out a study of the stages of Brachypodium spike development. Here, we present a detailed analysis of inflorescence ontogeny as it relates to meristem development in wild-type Brachypodium (accession no. Bd21). Furthermore, to begin studying the genetic regulation of Brachypodium inflorescence architecture, we established conditions for Brachypodium fast neutron and chemical mutagenesis. The fast neutron mutant populations had a high frequency of mutants, and from these populations, we identified a range of mutants. Three inflorescence mutants, awnless1 (awn1), no inflorescence1 (nif1), and tillerless1 (til1), had defective spikelet and/or floret development, and in one mutant, more spikelets1 (mos1), spikelets were converted into branches. We show that the inflorescence defect in mos1 results from changes in spikelet meristem fate during inflorescence development. Finally, we show that mos1 likely corresponds to a mutation in the Brachypodium gene most closely related to maize bd1 and rice FZP. Thus, bd1, FZP, and MOS1 have a conserved function to promote determinate spikelet meristem fate within the grasses. However, MOS1 in Brachypodium appears to be involved in initiation of the terminal spikelet and thus may control the number of spikelets formed in an unbranched determinate spike.

RESULTS

Brachypodium Inflorescence Development

To establish a foundation for descriptions of Brachypodium inflorescence development, we performed a detailed analysis of wild-type inflorescence ontogeny. Following germination, the first true leaf was visible 6 d after sowing, and six to seven additional leaves formed prior to emergence of an inflorescence meristem, approximately 22 d after sowing (Fig. 1A). Axillary meristems at the base of the plant elongated to form multiple basal tillers that were similar to the main stem (culm; Fig. 1A). The degree of tiller formation varied according to the growth conditions. More tillers formed under lower light intensity, and under these conditions, the transition to flowering was delayed.

The inflorescence of Brachypodium was composed of two or three lateral spikelets and a terminal spikelet (Fig. 2A). Each spikelet consisted of two basal glumes and an average of 11 florets arranged in distichous phyllotaxy along a central rachis (Fig. 2B, Table I). Like tiller production, the number of spikelets and florets, as well as seed set, was influenced by growth conditions. Under less than optimal growth conditions, there was low fertility, and in this case, the spikelet produced more florets. Florets were composed of an outer awned lemma, a translucent palea, two lodicules, three stamens, and a central pistil (Fig. 2, C and D). The two lateral stamens matured to produce pollen, whereas the abaxial stamen remained rudimentary and sterile.

To determine the timing of the vegetative to reproductive transition, we prepared longitudinal sections of plant apices and examined the morphology of the shoot apex from 6-, 10-, 14-, and 18-d-old plants grown under long-day conditions. In 6-d-old plants, the shoot apical meristem comprised a short dome of cells flanked by developing leaf primordia (Fig. 1B). In 10-d-old plants, the apical meristem was more elongated than 6-d-old plants, and the terminal spikelet had initiated (Fig. 1C). Therefore, the transition to a reproductive fate occurred in 7-d-old to 9-d-old plants. Floral meristems of the terminal spikelet were apparent in apices of 10-, 14-, and 18-d-old plants in longitudinal sections, whereas lateral spikelets with obvious floral meristems were evident only at a later stage in 18-d-old plants (Fig. 1, D and E). Development of the terminal spikelet therefore precedes that of the lateral spikelets.

To characterize later stages of Brachypodium inflorescence development further, we dissected apices from 17- to 22-d-old plants and examined the progression of early inflorescence development by scanning electron microscopy (SEM). The inflorescence meristem generated lateral spikelets in a distichous
phyllotaxy, whereas the terminal spikelet was oriented 90° to the plane of the lateral spikelets (Fig. 2E). Consistent with results from longitudinal sections, SEM analysis showed that development of the terminal spikelet was more advanced than that of lateral spikelets (Fig. 2E–G). As such, progression of inflorescence development was most readily followed through examination of the terminal spikelet. At the earliest stage of inflorescence development examined, the terminal spikelets had initiated several floral meristems (Fig. 2E). Initiation of the outermost floral organ, the lemma, was evident as a ridge of cells at the base of floral meristems, whereas other floral organs were not apparent. The two glumes associated with the terminal spikelet had also initiated, with the upper glume more prominent and the lower glume present only as a small ridge of tissue. We called this the “naked” stage of inflorescence development, as the floral meristems of the terminal spikelet were fully exposed. The next stage of development occurred when several florets of the terminal spikelet had initiated and was characterized by growth of the glumes and the lemma of basal florets (Fig. 2F). The developing lemma elongated and differential growth of the distal midregion of the lemma resulted in formation of an awn. We classified this as the “awn initiation” stage. Subsequently, the upper glume and the most basal floret elongated to cover the terminal spikelet, and this occurred prior to elongation of lateral spikelet glumes (Fig. 2G; Supplemental Fig. S1). We referred to this as the “enclosed terminal spikelet” stage.

In both the terminal and lateral spikelets, the floral meristems developed acropetally (Fig. 2F). To confirm the progression of floral meristem development within the terminal spikelet and to establish an informative molecular marker for inflorescence meristem development, we used the whole genome sequence to identify Brachypodium genes encoding class I KNOX transcription factors. Class I knotted-like homeobox (KNOX) genes are required for shoot meristem function and are expressed in shoot meristems and down-regulated in lateral organs (Jackson et al., 1994; Long et al., 1996; Sentoku et al., 1999). Brachypodium has seven class I KNOX genes (Vogel et al., 2010). Maximum-likelihood phylogenetic analysis of Brachypodium, maize, and rice class I KNOX proteins showed Brachypodium encodes a single ortholog within each class I KNOX gene clade, with the exception of the knox3 clade, where there is no Brachypodium gene, and the knox10 clade, where there are two genes, Bradi1g12677 and Bradi1g12690, which are located adjacent to each other on chromosome 1 (Fig. 2I). Compared with other class I KNOX genes, Bradi1g12677 is truncated and may be a pseudogene.

The protein encoded by Bradi1g10047 was most closely related to KNOTTED1 (KN1) of maize and rice HOMEOBOX1 (OSH1; Fig. 2I). Bradi1g10047 was therefore designated BdKN1. We used in situ hybridization to examine the expression pattern of BdKN1.
within the developing terminal spikelet of wild-type Brachypodium and found it was highly expressed in spikelet and floral meristems. (Fig. 2H). Similar to kn1, BdKN1 expression was not detected in the outer L1 cell layer of all shoot meristems.

BdKN1 was down-regulated in a small region adjacent to the terminal spikelet meristem, which corresponded to the site of an initiating organ. From the apex toward the base of the spikelet, floral meristems became progressively larger, and BdKN1 was down-regulated in developing organs associated with these meristems. Thus, the BdKN1 expression pattern matches that of the closely related genes in other plants and marks active meristems in Brachypodium. The expression pattern also confirms acropetal maturation of spikelet florets.

**Mutagenesis of Brachypodium**

In order to identify genes regulating inflorescence development in Brachypodium, we generated mutagenized...
populations that were screened for inflorescence development mutants. We used two mutagenesis approaches, chemical ethyl methanesulfonate (EMS) mutagenesis and fast neutron radiation, which have been successful in generating mutants in other species. Mutants were obtained using both approaches (Supplemental Fig. S2). The fast neutron mutagenized populations produced a higher frequency of mutants and therefore became the focus of subsequent screening.

Two fast neutron mutagenesis trials demonstrated that exposure to 20 to 30 Gray (Gy) was sufficient to obtain plants that set viable seed (Supplemental Fig. S2, A and B). Using these two independent fast neutron mutant populations, we screened the M2 progeny from a total of 872 M1 lines for visible shoot phenotypes. Mutants were classified into three general categories: albino, pale, and defective development (Supplemental Figs. S2, C and D, and S3). The two mutant populations had 5% of lines segregating albino mutants and 4% of lines segregating pale mutants (Supplemental Fig. S3A). The defective development class of mutants encompassed phenotypes disrupting plant shoot development, and these mutants were identified in 2% and 4% of lines in the two mutant populations. Developmental mutants included plants that were extremely dwarf mutants, a crinkled leaf mutant, and a downward-growing mutant (Supplemental Fig. S3, B–D). The frequency of mutant phenotypes, especially the high frequency of albino mutants, in the fast neutron lines indicates effectiveness of 20 to 30 Gy for Brachypodium mutagenesis.

Three mutants, awl1, nif1, and til1 disrupted inflorescence development. awl1 was similar to the wild type during vegetative development; however, in reproductive development, the floret lemma failed to form an awn, and pistil development was abnormal (Fig. 3, A–E). nif1 was also similar to the wild type during vegetative development; however, upon transition to inflorescence development, nif1 often failed to form any spikelets and instead produced a naked stem (Fig. 3, F and H). The til1 mutant was disrupted in both vegetative and inflorescence development. In the wild type, tillers arise in the axils of leaves at the base of the plant, whereas in the til1 mutant, tillers were absent (Fig. 3G). In the til1 inflorescence, terminal spikelet and lateral spikelets had defective and reduced numbers of florets (Fig. 3, G and I). A fourth mutant, mos1, also affected inflorescence development and produced more spikelets than the wild type. This mutant was selected for further analysis. mos1 Converts Spikelets to Branches

The mos1 mutant was fertile and segregated as a recessive mutant. During vegetative growth, mos1 did not differ significantly from the wild type and could only be distinguished from the wild type in the inflorescence (Fig. 4, A and B). However, during reproductive growth, the inflorescence produced approximately twice the number of lateral spikelets compared with the wild type (Fig. 4C; Table II). The lateral spikelets of mos1 were not subtended by a glume and therefore were considered to be branches. Each branch had an average number of florets equal to the sum of the lateral spikelet numbers, and there were approximately twice as many lateral spikelets compared with the wild type. In addition, the lateral spikes produced approximately twice as many spikelets compared with the wild type.

Table 1. Spikelet and floret numbers in the Brachypodium spike

| Genotype          | Spikelets a | Florets in TS | Florets in LS1 b | Florets in LS2 |
|-------------------|-------------|---------------|------------------|---------------|
| Wild type, n = 12 | 3 ± 0       | 11.44 ± 0.5   | 10.56 ± 1.1      | 11.11 ± 0.6   |

aSpikelets includes the terminal spikelet (TS) and lateral spikelets (LS). bLateral spikelets were numbered basipetally such that number 1 was the apical-most lateral spikelet and number 2 the next and more basal lateral spikelet.

Figure 3. Inflorescence mutants. A, awl1 flowering plant. B and C, Spike of the wild type (B) and awl1 (C). D and E, Reproductive organs of the wild type (D) and awl1 (E). Anthers are marked by arrows. F and G, nif1 (F) and til1 (G) flowering plant. H and I, Spikes in nif1 (H) and til1 (I). [See online article for color version of this figure.]
of 2.2 to 3.9 spikelets, including the terminal spikelet and primary lateral spikelets (Table II). Branches produced several orders of spikelets, such that a single branch produced primary lateral spikelets, which in turn produced secondary lateral spikelets (Fig. 4, D and E). The branch terminal spikelet also produced an average of three secondary spikelets, and these had an average of 7.6 florets, which is less than the number of florets in a wild-type spikelet (Tables I and II). Florets did not have organ defects. Spikelets at the base of branches tended to be small with rudimentary florets having reduced numbers of organs (Fig. 4C; Table II). Thus, mos1 results in ramification of the Brachypodium spike.

mos1 inflorescence development was further characterized using SEM. In the naked stage of development, lateral branches appeared similar to wild-type spikelets, and each branch had initiated axillary meristems subtended by a developing organ (Fig. 5, A and B). As branches transitioned to the awn initiation stage, the branch axillary meristems formed lateral spikelets (primary lateral spikelets; Fig. 5C). Each branch produced from two to four primary lateral spikelets (Table II). The terminal spikelet of a branch could be distinguished, as this was subtended by a glume and was developmentally more advanced than the lateral spikelets.

As in the wild-type inflorescence, development of the terminal spikelet of the mos1 inflorescence was more advanced than that of lateral branches (Fig. 5D). Furthermore, differences in maturation of branches along the inflorescence were pronounced. At the enclosed terminal spikelet stage of inflorescence development, four to six basal branches were at an awn initiation stage while two to three apical branches were at a naked stage of development. MOS1 therefore appears to be involved in lateral spikelet production by promoting the transition of the inflorescence meristem to a terminal spikelet and in promoting the transition of axillary meristems produced by the inflorescence to spikelet fate.

mos1 Carries a Chromosomal Rearrangement Upstream of an ERF Transcription Factor Gene Related to bd1

The orthologous ERF genes bd1 in maize and FZP in rice are required to maintain spikelet meristem identity (Chuck et al., 2002; Komatsu et al., 2003a; Zhu et al., 2003). Mutations in both genes result in conversion of determinate spikelet meristems to indeterminate branch meristems reminiscent of mos1. Given these similarities, we tested whether mos1 might be disrupted in a Brachypodium bd1-related gene. BD1 and FZP are ERF transcription factors and there are 142 AP2/ERF transcription factors encoded in the Brachypodium genome (Vogel et al., 2010). Using BD1 and FZP amino acid sequences to search Brachypodium annotated protein sequences, we found only one closely related protein, encoded by Bradi1g18580, which shared amino acid identity in the ERF domain and extending beyond this domain (Fig. 6A; Vogel et al., 2010). We also identified Sb02g042400 from

Table II. Numbers of branches, primary spikelets, and florets in mos1

| Genotype | Branches and TSa | Spikelets in TSb | 1’ Spikelets | Branch TS Florets |
|----------|------------------|-----------------|-------------|-----------------|
| mos1, n = 12 | 5.6 ± 0.5 | 3 ± 0.7 | 2.2 ± 0.8 | 2.9 ± 1.1 | 3.8 ± 0.7 | 3.9 ± 0.7 | 3.9 ± 0.4 | 7.6 ± 0.9 |
| Rudimentary spikeletsc | 0% | 23% | 20% | 24% | 34% | 37% |

aBranches and spikelets were counted on the main axis of the inflorescence and included the terminal spikelet (TS) and lateral branches. bBranches were numbered basipetally such that number 1 was the apical-most branch, number 2 was the next and more basal lateral spikelet, and so on. cPercentage of primary lateral spikelets on branches that were rudimentary spikelets.
sorghum (*Sorghum bicolor*) and a predicted protein in the barley genome sequence as most closely related to BD1 and FZP (Paterson et al., 2009; Mayer et al., 2012). The Bradi1g18580 protein shared 59%, 54%, 57%, and 58% identity with BD1, FZP, Sb02g042400, and the barley protein, respectively (Fig. 6A). Maximum-likelihood phylogenetic analysis of these BD1-related proteins was consistent with the relationship between these species and showed the Brachypodium and barley proteins were most closely related (Supplemental Fig. S4A; Kellogg, 2001; Vogel et al., 2006). To test whether this candidate was disrupted in *mos1*, Bradi1g18580 was amplified by PCR from the wild type and *mos1*. Sequencing of PCR products revealed no mutations in the coding region of Bradi1g18580. However, a PCR amplification of the 5′ region of Bradi1g18580 revealed a product size difference between the wild type and *mos1*. Sequencing these amplification products revealed a 10-bp deletion extending from –170 to –180 bp upstream of the ATG start codon of Bradi1g18580, as well as a unique 991-bp sequence insertion at –170 bp upstream of the ATG start codon in *mos1* relative to the wild type (Fig. 6, B and C). Interestingly, the insertion was identical to a sequence on chromosome 3, suggesting the mutation resulted from a chromosomal rearrangement where a segment of chromosome 3 inserted into chromosome 1. Sequence analysis indicated the chromosome 3 insertion did not have features of a transposable element and did not have any open reading frames indicative of protein coding sequence. To determine whether this insertion might be responsible for the *mos1* phenotype, we tested for cosegregation between the *mos1* phenotype and the insertion. In lines segregating *mos1*, all 108 mutants examined were homozygous for the insertion allele, and all 36 phenotypic wild-type plants examined were heterozygous for the wild-type and insertion allele. Based on published recombination frequencies in Brachypodium, the insertion upstream of Bradi1g18580 and *mos1* are estimated to be linked by less than 100 kb (Huo et al., 2011).

The insertion in Bradi1g18580 disrupts a highly conserved sequence in Brachypodium, barley, rice, maize, and sorghum, including a TATA box transcriptional start site (Fig. 6C; Supplemental Fig. S4B). To determine whether the insertion alters transcript levels of Bradi1g18580, we performed quantitative reverse transcription (RT)-PCR and found that the transcript levels of Bradi1g18580 were reduced in *mos1* compared with the wild type (Fig. 6D). The modest reduction in *MOS1* transcript levels in the mutant indicates *mos1* is a weak allele, which is consistent with the mild phenotype of *mos1*, compared with alleles of *bd1* and *fpz*. Taken together, genetic, phenotype, and transcript data suggest *MOS1* corresponds to Bradi1g18580.

**DISCUSSION**

**Fast Neutron Mutants in Brachypodium**

Brachypodium provides a useful model system for investigating the genetic regulation of spike development because it is a fast-growing and readily cultivated species with a relatively small genome that has recently been sequenced (Draper et al., 2001; Vogel et al., 2010; Brkljacic et al., 2011). We have established conditions for fast neutron and EMS mutagenesis. Screening fast neutron mutant populations identified a range of phenotypic mutants. Several of the developmental mutants had phenotypes similar to mutants reported in other plant species. For example, a mutant with a downward-growing shoot was similar to agravitropic mutants *lazy* in maize and rice and *serpentina* in barley (Van Overbeek, 1936; Jones and Adair, 1938; Sheridan, 1988; Türkkan and Suge, 1991).

A number of mutants with defects in inflorescence development were identified. The *awl1* mutant lacked awns and had defective pistil development. Awns are present in other grass species, such as rice, barley, and wheat, and likely serve a function in protection against animal foraging and to promote seed dispersal, although awns may also have a physiological role in seed development (Grundbacher, 1963). Although it remains to be determined whether a single gene controls awn and pistil development, the *awl1* mutant will provide insight into both processes in inflorescence development.

**Figure 5.** *mos1* inflorescence development. A and B, Naked stage. C, Branch at awn initiation stage. D, Inflorescence enclosed terminal spikelet stage showing the terminal spikelet (1) and five branches. a, Awn; am, axillary meristem; b, branch; gl, glume; l, lemma; pls, primary lateral spikelet; ts, terminal spikelet. Bars = 100 µm.
Figure 6. MOS1 corresponds to a mutation in the Brachypodium ortholog of bd1 and FZP. A, Amino acid sequence alignment of predicted proteins from Brachypodium MOS1 (Bradi1g18580-encoded protein), maize BD1, rice FZP, and the sorghum and barley BD1 orthologs. Color shading indicates percentage identity. The ERF domain is underlined. B, PCR amplification products of wild type and mos1 amplified with primers shown in C. Primers F5 and R5 reveal an insertion polymorphism in mos1. C, Diagrammatic representation of Bradi1g18580 in mos1 with single open-reading frame (green box) and chromosome 3 insertion (red line). Numbers are nucleotides, with number 1 being the start of the open reading frame. Arrows indicate the location of primers. Nucleotide sequence shows the region carrying the rearrangement. Nucleotides in red are the ends of the chromosome 3 insertion. Nucleotides in gray are deleted in mos1. Yellow boxes highlight nucleotides that are conserved in Brachypodium, barley, maize, rice, and sorghum. D, Quantitative RT-PCR showing transcript levels of MOS1 relative to ACTIN2 in the wild type and the mos1 mutant. Bars = se of mean.
The $nif1$ and $til1$ mutants shared similarity with other reported mutants. The $nif1$ mutant that lacked spikelets resembled the auxin transport mutants $pin1$, $pin2$, and $monopterous$ in Arabidopsis ($Arabidopsis_thaliana$), and $bif2$ in maize (Okada et al., 1991; Bennett et al., 1995; Przemeck et al., 1996; McSteen et al., 2007). $PINFORMED1$ encodes an auxin efflux carrier that is expressed at high levels in localized regions in the peripheral of the meristem and is required to form local auxin maxima associated with organ initiation (Reinhardt et al., 2003). Likewise, mutants in $bif2$, which encodes a Ser/Thr protein kinase protein orthologous to PINOID, have fewer inflorescence branches, spikelets, and floral organs, and fail to establish localized auxin maxima in peripheral regions of the meristem (McSteen et al., 2007; Skirpan et al., 2009). Potentially, $nif1$ is also defective in auxin transport. The $til1$ phenotype was similar to that of $ moc1$ in rice and to mutants in the orthologs $ba1$ in maize and $LAX1$ in rice. $MOC1$ encodes a GRAS family transcription factor gene, whereas $ba1$ and $LAX1$ encode orthologous basic helix-loop-helix transcription factors (Komatsu et al., 2003b; Li et al., 2003; Gallavotti et al., 2004). The Brachypodium orthologs of these genes therefore represent candidates for $TIL1$.

Comparison of Inflorescence Development in Brachypodium with the Small-Grain Cereals Wheat and Barley

Brachypodium is a member of the subfamily Pooideae in the family Poaceae and is closely related to the two major small-grain cereal species wheat and barley (Hsiao et al., 1994; Vogel et al., 2006). Based on our analysis of wild-type Brachypodium, it is informative to compare this with inflorescence development in wheat and barley, as genetic regulation of inflorescence development is likely to be highly conserved in these related species. The inflorescence of all three species is a spike. The barley spike is characterized by an indeterminate inflorescence and determine spikelets (Bonnett, 1935; Kirby and Appleyard, 1981; Babb and Muehlbauer, 2003). Axillary meristems along the main axis of the inflorescence give rise to three spikelets, and each of these spikelets produces a single floret. In wheat, the inflorescence is determinate, and the inflorescence meristem continues to produce axillary meristems until a terminal spikelet is formed (Bonnett, 1936; Kirby and Appleyard, 1981). Axillary meristems on the inflorescence of wheat each develop into a single lateral spikelet. Both the terminal and lateral spikelets produce multiple florets. The inflorescence spike of Brachypodium is similar to that of wheat, whereby the inflorescence is determinate and the spikelets are indeterminate. Furthermore, florets within a spikelet mature acropetally and basipetally from the midregion of the inflorescence, and the terminal spikelet is the last to mature (Bonnett, 1935, 1936; Kirby and Appleyard, 1981). By contrast, development of the terminal spikelet in Brachypodium is advanced compared with the lateral spikelets. It is more difficult to draw conclusions on the relative maturation schedule of the few lateral spikelets in wild-type Brachypodium. However, in $mos1$, development of basal lateral branches was more advanced than that of apical lateral branches, suggesting that development along the main inflorescence axis in Brachypodium is acropetal. Thus there are striking similarities between inflorescence development in Brachypodium and wheat, but the subtle differences may reflect different regulation of meristem maturation rather than initiation.

$mos1$ Converts an Unbranched Spike to a Branched Spike

We have established a description of Brachypodium wild-type spike development and used this to define the role of $MOS1$ in spike architecture. Mutation in $MOS1$ results in an increase in the number of axillary meristems on the main axis of the inflorescence, and these develop as branches rather than spikelets. The increased number of axillary meristems may result from a delay in establishing the terminal spikelet. $MOS1$ may therefore either promote production of a terminal spikelet and determine meristem fate or repress indeterminate inflorescence meristem fate. The lateral axillary meristems in the $mos1$ inflorescence are not subtended by glumes and can therefore be considered branches rather than spikelets. Each branch resembles the main inflorescence of the wild type, forming several lateral spikelets and a terminal spikelet. Thus, the identity of spikelet meristems is converted to that of a branch meristem and is similar to phenotypes of $bd1$ and $fzp$ mutants of maize and rice.

Mutant alleles of $bd1$ and $fzp$ vary in phenotype severity. In severe $bd1$ mutants, which have mutations predicted to truncate the BD1 protein, tassels spikelets are indeterminate and produce a series of lateral spikelets, and ear spikelets are converted to indeterminate branches (Chuck et al., 2002). A weak allele, with reduced transcript levels due to a transposon insertion in the 5’ leader sequence of $bd1$, results in production of fewer branches in the ear compared with severe alleles and is partially fertile (Chuck et al., 2002). In severe $fzp$ mutants, all spikelets on the primary branches of the panicle are converted to secondary branches, whereas in plants with a weak $fzp$ allele, spikelets and occasionally fertile florets are produced at the tips of primary branches (Komatsu et al., 2001, 2003a; Zhu et al., 2003; Yi et al., 2005). The weak $fzp$ allele has an amino acid change in the ERF domain of FZP. Severe $fzp$ alleles have mutations that alter amino acids in the ERF domain or are predicted to produce C-terminal truncated proteins. Furthermore, two severe $fzp$ mutants carry transposon insertions more than 1 kb upstream of the coding region, which likely disrupts expression of FZP via a distant cis-regulatory region (Komatsu et al., 2003a; Zhu et al., 2003). Thus, in addition to protein
function, transcriptional regulation of \( bd1 \) and \( FZP \) is important in spikelet production and in determining degree of inflorescence branching in maize and rice. As with weak alleles of \( bd1 \) and \( fzp \), \( mos1 \) mutants are fertile. \( mos1 \) has a chromosomal rearrangement in the promoter region of the gene, and transcript levels are reduced compared with the wild type. We therefore propose that \( mos1 \) may be a weak allele, where the phenotype results from lower levels of transcript accumulation or from a delay in the onset of expression.

The unbranched Brachypodium spike differs from the branched inflorescence of maize and rice, and one gene that may contribute to this difference is \( bd1/FZP/MOS1 \). We show \( MOS1 \) likely corresponds to a mutation that leads to a reduction in transcript levels in the Brachypodium ortholog of \( bd1 \) and \( FZP \). Further reduction of expression of \( MOS1 \) through isolation of additional mutant alleles or through the use of gene silencing will confirm this conclusion and will also reveal the extent to which \( MOS1 \) is required for meristem determinacy. In particular, the phenotype of a \( mos1 \) null mutant will establish whether specification of a terminal spikelet depends on expression of \( MOS1 \). Changes in the level or pattern of expression of this gene may determine whether a grass inflorescence is branched or not branched. Although \( bd1 \), \( FZP \), and \( MOS1 \) appear to have a conserved role in spikelet meristem fate, we show that \( MOS1 \) in Brachypodium also has a role in determinacy of the inflorescence meristem. One possibility is that \( MOS1 \) regulates the timing of initiation of the terminal spikelet. In wheat, differentiation of the terminal spikelet determines the number of spikelets produced (Bonnett, 1935, 1936). \( MOS1 \) is therefore a good target gene for genetic manipulation to increase grain number and yield in wheat.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Brachypodium (\( Brachypodium distachyon \)) Bd21, used as a wild type, was tenth generation of single-seed descent and was grown either in a growth chamber or in a glasshouse at 22°C with 18 h light. Seed was sown on damp filter paper, placed at 4°C in the dark for 7 d, and germinated either at room temperature or in a growth chamber for 4 d prior to transplanting to soil composed of 50% compost and 50% peat and grit mix supplemented with slow-release fertilizer. Detailed phenotypic studies of the wild type and mutants were carried out on plants grown in a growth chamber.

Fast neutron mutagenesis of Brachypodium seed was carried out at the Atomic Energy Research Institute in Budapest, Hungary. To determine the dose required for fast neutron mutagenesis, Brachypodium seed was initially exposed to 20, 30, 40, 50, and 60 Gy. M1 seed were screened for seedling viability. Seed exposed to a dose of 50 and 60 Gy all germinated but failed to produce any postembryonic organs. Exposure to 40 Gy resulted in 25% M1 seed germination and seedling growth, and 22% of plants were fertile. Exposure to 30 Gy produced 65% M1 seed germination, and 60% of plants were fertile. Based on this initial test, a second mutagenesis was carried out and seed were exposed to 20, 25, 30, and 35 Gy. Seed exposed to 35 Gy had poor seedling viability, and all plants that matured were sterile. Exposure to 20, 25, and 30 Gy resulted in 30% to 90% seedling viability. Only at 20 Gy were most viable seedlings fertile. Seed from individual fertile M1 plants was collected. We screened the M2 progeny from 203 M1 lines exposed to 30 Gy from the first mutagenesis and 669 M1 lines exposed to 20 and 30 Gy from the second mutagenesis for visible shoot phenotypes. To screen for mutant phenotypes, approximately 40 M2 seed from each M1 plant was germinated and planted to soil for visual examination.

EMS mutagenesis was carried out by initially imbibing dehusked seed then treating with EMS. Dehusked seed were imbibed by placing on damp filter paper for 48 h at 4°C. Seed was collected and treated with 0.2%, 0.4%, or 0.6% (v/v) EMS in 40 mL water for 8 h at room temperature. As a control, some seed was treated without EMS. Following chemical treatment, seed was washed with water six times for 30 min. Treated seed was placed on damp filter paper and placed at 4°C for 48 h, and then transferred to room temperature and allowed to germinate prior to transplanting to soil. In the control, 68% of seed germinated and produced fertile plants compared with 99% germination of untreated seed, indicating immersion of seed for a period of hours reduces viability. Screening for mutants was carried out as for fast neutron mutagenesis.

**Molecular Biology**

Isolation of genomic DNA was carried out using a cetly-trimethyl-ammonium bromide method as described previously (Long and Coupland, 1996). Sequencing of candidate genes was carried out on mutant plants and on wild-type parental plants used for the mutagenesis. \( MOS1 \) primers for genomic DNA amplification, sequencing, and genotyping were as follows: forward, 5'-CCCTTGTAGTACCTCCCTC-3'; reverse, 5'-GCCTGAGACATGCTCTG-3'; forward, 5'-GACCGGACCAACAGACG-3'; reverse, 5'-GGCTGCAACACGGAG-3'. Sequences were aligned using ClustalW, and the phylogenetic tree was constructed using MEGA version 9.0.0 (Kumar et al., 2018). For analysis of in situ hybridizations, \( FZP \) primers used for quantitative RT-PCR were 5'-CCTCAAGGATTAGCGATGAT-3' and 5'-CAGTGGGAGAGGAAGCTGAA-3'. Cosegregation of the insertion polymorphism in Bradi1g18580 and phenotypic mutant \( mos1 \) plants was determined by analysis of wild-type and mutant progeny from seven independent M2 lines. Quantitative RT-PCR analysis was carried out using nine inflorescence meristems dissected from 18-d-old plants. Total RNA was extracted from inflorescence tissue using TRI Reagent (Sigma-Aldrich) and DNase treated and purified with a column (Qiagen) prior to complementary DNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was carried out using SYBR Green Jump-Start quantitative RT-PCR kit (Sigma-Aldrich). Quantitative RT-PCR reactions were carried out in triplicate technical replicates. \( BdACTIN2 \) (Bradi1g10630) was used to normalize gene expression.

**Histology and in Situ Hybridization**

For analysis of inflorescence development stages, plant apices were fixed in 4% (v/v) paraformaldehyde, dehydrated in an ethanol series, and embedded in wax. Embedded material was sectioned, cleared, and stained with 0.1% (v/v) toluidine blue. RNA in situ hybridizations were performed as previously described (Long et al., 1996). For \( BdKN1 \), a gene-specific fragment of 747 bp was amplified from complementary DNA with primers 5'-CATCTCCACCCCATATTACCTC-3' and 5'-TAGACGCCACCATCTGGTGA-3', cloned into the vector pCR-Blunt II-TOPO (Invitrogen). An antisense probe was prepared from this clone by in vitro transcription of the \( BdKN1 \) fragment in the presence of digoxigenin-UTP and subsequent hydrolysis into smaller fragments approximately 130 bp in length prior to use in hybridizations.

**SEM**

To prepare tissue for SEM, apices were dissected from plants, and surrounding leaf tissue was removed to expose meristems. The apices were mounted in a horizontal position on aluminum stubs and plunge-frozen in liquid nitrogen. After freezing, the samples were immediately loaded into the cryo-chamber of an ALTO 2500 cryotransfer system (Gatan) attached to a Zeiss Supra 55 VP FEG scanning electron microscope. Samples were sputter coated with platinum and imaged at 3 kV.

**Sequence Alignment and Phylogenetic Analysis**

KNOX and BD1 amino acid sequences were analyzed using MEGA version 5. Sequences were aligned using ClustalW, and the phylogenetic tree was constructed using the maximum-likelihood algorithm, with the JTT+G model.
of protein evolution and 1,000 bootstrap replicates. This was identified as the best-fitting model using Proton 3 (Darriba et al., 2011). Plant class I KNOX genes have been shown to be monophyletic (Kerstetter et al., 1994; Malcomber et al., 2006), therefore the tree was rooted with the Arabidopsis (Arabidopsis thaliana) class II KNOX protein Arabidopsis knotted-like7 (KNAT7) as an outgroup. The BD1 tree was rooted with a related protein, PUCHI (AT5G18560), from Arabidopsis and included BD1 and the closely related maize (Zea mays) gene ZMD2G458437 (Chuck et al., 2002). FZP, Sb02g024200, a predicted protein in the barley (Hordeum vulgare) morex-contig 1585332; and Bradi1g13850 (MO51). GenBank accession numbers for Arabidopsis, maize, and rice (Oryza sativa) KNOX protein sequences were as follows: BREVIPE-DICELLUS, AEE82597.1; GNARLEY1, AAPI76320.1; KN1, AAPI21661.1; KNAT2, AE38075.1; KNAT6, AEE0380.1; KNAT7 AF308451; KNOX3, AC507867.1; KNOX8, AF318686.1; KNOX10, AF35627.1; LUCILESS4a, AAP3149.1; LC25, AAD13611.1; OSCH, BAA63999.1; OSCH, BAA79223.1; OSHE, BAA79224.1; OSHE1, AAI87192.1; OSH15, BAA31688.1; OSH43, BAA79225.1; OSHE1, BAA79226.1; ROUGH SEATHE1, AAI66287.1; and SHOOT MERISTEMLESS, AEE33958.1. GenBank accession numbers for maize and rice BD1/FZP protein sequences were as follows: BD1, ACG14675.1; ZMCD458457, DAA41703.1; FZP, BAA79225.1; OSH15, BAA79226.1; OSH43, BAA79226.1; ROUGH SEATHE1, AAI66287.1; and SHOOT MERISTEMLESS, AEE33958.1. GenBank accession numbers for maize and rice BD1/FZP protein sequences were as follows: BD1, ACG14675.1; ZMCD458457, DAA41703.1; and FZP, BAC97264.1. Brachypodium sequences, and rice BD1/FZP protein sequences were as follows: BD1, ACG14675.1; ZMCD458457, DAA41703.1; FZP, BAC97264.1. 

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