barren inflorescence2 Encodes a Co-Ortholog of the PINOID Serine/Threonine Kinase and Is Required for Organogenesis during Inflorescence and Vegetative Development in Maize1[C][W][OA]

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Organogenesis in plants is controlled by meristems. Axillary meristems, which give rise to branches and flowers, play a critical role in plant architecture and reproduction. Maize (Ze a m a y s) and rice (O r y z a s a t i v u s) have additional types of axillary meristems in the inflorescence compared to Arabidopsis (Arabidopsis thaliana) and thus provide an excellent model system to study axillary meristem initiation. Previously, we characterized the barren inflorescence2 (bif2) mutant in maize and showed that bif2 plays a key role in axillary meristem and lateral primordia initiation in the inflorescence. In this article, we cloned bif2 by transposon tagging. Isolation of bif2-like genes from seven other grasses, along with phylogenetic analysis, showed that bif2 is a co-ortholog of PINOID (PID), which regulates auxin transport in Arabidopsis. Expression analysis showed that bif2 is expressed in all axillary meristems and lateral primordia during inflorescence and vegetative development in maize and rice. Further phenotypic analysis of bif2 mutants in maize illustrates additional roles of bif2 during vegetative development. We propose that bif2/PID sequence and expression are conserved between grasses and Arabidopsis, attesting to the important role they play in development. We provide further support that bif2, and by analogy PID, is required for initiation of both axillary meristems and lateral primordia.

1 This work was supported by the National Science Foundation (grant no. DBI-0110189 to S.H. and E.K. and grant no. IBN-0416616 to P.M.).
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[OA] Open Access articles can be viewed online without a subscription.
www.plantphysiol.org/cgi/doi/10.1104/pp.107.098558

Organogenesis in plants is controlled by meristems (Steeves and Sussex, 1989). In the shoot, organ primordia are produced in the peripheral zone of the meristem, whereas the central zone supports further growth. Axillary meristems, which arise in the axils of leaves, play an important role in organogenesis by producing branches and flowers. During vegetative development, axillary meristems produce several leaf primordia and then arrest as dormant buds (Esau, 1967; Steeves and Sussex, 1989). During inflorescence development, axillary meristems develop into flowers or branches and the subtending leaf, called a bract, is often so reduced as to be cryptic or invisible (Bonnett, 1948; Long and Barton, 2000). Genetic and hormonal factors that regulate the initiation, arrest, and subsequent outgrowth of axillary meristems have been identified and, in particular, auxin transport has been shown to play a critical role (Gribic, 2005; McSteen and Leyser, 2005; Paponov et al., 2005; Reinhardt, 2005; Schmitz and Theres, 2005; Beveridge, 2006).

Treatment of plants with auxin transport inhibitors shows that auxin transport is required for leaf and floral meristem initiation (Okada et al., 1991; Reinhardt et al., 2000; Scanlon, 2003). In Arabidopsis (Arabidopsis thaliana), pinformed1 (pin1), pinoid (pid), yucca (yuc), and monopteros (mp) mutants have similar phenotypes to auxin transport-inhibited plants forming a pin-shaped inflorescence with very few flowers (Okada et al., 1991; Bennett et al., 1995; Przemeck et al., 1996; Cheng et al., 2006). PIN1 encodes a transmembrane protein that has been shown to transport auxin (Galweiler et al., 1998; Petrasek et al., 2006). PID encodes a Ser/Thr kinase that is proposed to positively regulate auxin transport, possibly through direct effects on PIN1 localization or trafficking (Christensen et al., 2000; Benjamins et al., 2001; Friml et al., 2004; Lee and Cho, 2006). The YULCA gene family of flavin monooxygenases regulates the rate-limiting step in auxin biosynthesis (Zhao et al., 2001; Cheng et al., 2006). MP is a member of the auxin...
response factor transcription factor family that activates transcription when auxin is present (Hardtke and Berleth, 1998; Tiwari et al., 2003). Auxin is thus seen to play a key role in floral meristem initiation in Arabidopsis.

Both maize (Zea mays) and rice (Oryza sativa) inflorescences are more branched than Arabidopsis due to the presence of additional axillary meristem types (Bonnett, 1948; Cheng et al., 1983; Clifford, 1987; Irish, 1997; McSteen et al., 2000; Shimamoto and Kyozuka, 2002). Maize is monocious with two types of inflorescence, male (tassel) and female (ear). The tassel forms at the apex of the plant and consists of several long lateral branches and a central main spike (Fig. 1A). On the spike and the lateral branches, short branches, called spikelet pairs, form. Two florets are produced in each spikelet. The ear forms from an axillary meristem in the axil of the leaf about five nodes below the tassel. Rice inflorescences have similarities with maize inflorescences (Shimamoto and Kyozuka, 2002; Bommert et al., 2005b). Long lateral branches form, but the main apex aborts early in development. The branches bear spikelets singly, instead of in pairs, and terminate in a spikelet. Each spikelet bears one fertile floret, although there is evidence of two additional sterile florets (Stapf, 1917; Malcomber et al., 2006). Branching in the rice inflorescence is controlled by three types of axillary meristem—branch, spikelet, and floral meristem—whereas branching in the maize inflorescence is controlled by four types of axillary meristem—branch, spikelet pair, spikelet, and floral meristem. Therefore, maize and rice are excellent model systems for studying axillary meristem initiation during inflorescence development.

In maize and rice, mutants that fail to initiate branches, spikelets, and florets during inflorescence development have been used to identify genes that regulate axillary meristem initiation (Coe et al., 1988; Doebley et al., 1995; Komatsu et al., 2001, 2003a; McSteen et al., 2000; Shimamoto and Kyozuka, 2002). brk1 is required for initiation of axillary meristems during both vegetative and inflorescence development (Komatsu et al., 2001, 2003a; Gallavotti et al., 2004). The maize and rice orthologs of PIN1 have been cloned, but no loss-of-function phenotypes in the inflorescence have been reported (Xu et al., 2005; Carraro et al., 2006). The term co-ortholog (Sonnhammer and Koonin, 2002) is used rather than ortholog because multiple rounds of genome duplication have occurred in both monocot and eudicot lineages (Sonnhammer and Koonin, 2002; Bowers et al., 2003; Paterson et al., 2004; Yu et al., 2005).

In this article, we describe the cloning of bif2 from maize and seven other grasses and show by phylogenetic analysis that bif2 is a co-ortholog of PID from Arabidopsis. Expression studies show that bif2 is expressed in all axillary meristems and lateral primordia in both maize and rice. Previous phenotypic analyses showed that bif2 is required for inflorescence development. Here we report that bif2 also plays a role during vegetative development.

RESULTS

Cloning bif2 by Transposon Tagging

To identify a transposon insertion in bif2, transposon-induced alleles were subjected to DNA gel-blot analyses using a number of different restriction enzymes and probes from the most common Mutator (Mu) elements (Chandler and Hardeman, 1992; Lisch, 2002). DNA gel-blot analysis of the bif2-47330 allele identified an 8.5-kb MuI hybridizing fragment present in mutants and absent from wild-type cousins (Fig. 1C). This fragment was cloned by constructing a subgenomic DNA λ library in this size range and screening with Mu1. After purification and excision, the 8.5-kb fragment was restriction mapped and subcloned. A 7.1-kb EcoRI-NotI unique restriction fragment flanking the Mu1 insertion hybridized to the same 8.5-kb fragment on the DNA gel blot and the expected 7.1-kb fragment lacking Mu1 (1.4 kb) in normal cousins, indicating that the correct fragment had been cloned (data not shown). The 8.5-kb restriction fragment was sequenced and found to contain the full-length bif2 gene, including 4 kb of promoter sequence (Fig. 1D). Four cDNA clones were identified from a cDNA library made from immature tassels using the same 0.7-kb EcoRI-NotI restriction fragment as a probe. Full-length cDNA was obtained using reverse transcription (RT)-PCR on B73 tassel RNA with gene-specific primers. The predicted bif2 gene encodes a Ser/Thr kinase similar to PID of Arabidopsis (Christensen et al., 2000).

To characterize the molecular defect in bif2 alleles, PCR was performed on mutant alleles using gene-specific primers and a Mu degenerate primer. Mu1 elements were identified near the beginning of the second exon in bif2-47330, 20 bp downstream of this insertion in bif2-1504 and in the intron of bif2-RM (Fig. 1D). Using gene-specific primers, bif2-77 was determined to have a 168-bp insertion at amino acid position 108 that produces a 9-bp host site duplication. This insertion is not similar to sequences in the current databases, indicating that it may be a novel transposon. The bif2-77 insertion introduces four in-frame stop codons into BIF2 likely causing truncation of the protein before the kinase domain and therefore should be nonfunctional. bif2-2354 contains a single-nucleotide insertion rather than ortholog because multiple rounds of genome duplication have occurred in both monocot and eudicot lineages (Sonnhammer and Koonin, 2002; Bowers et al., 2003; Paterson et al., 2004; Yu et al., 2005).
Figure 1. Cloning of \textit{bif2} by transposon tagging. A, Normal tassel with several long lateral branches at the base of the main spike. Spikelet pairs cover the branches and main spike. B, \textit{bif2} tassel with no long lateral branches. A few single spikelets are visible on the main spike. C, DNA gel-blot analysis of \textit{EcoRI}-digested genomic DNA probed with \textit{Mu1}. An 8.5-kb band (arrowed) is present in \textit{bif2} mutants (b) and absent in normal cousins (+). D, Schematic of the \textit{bif2} gene showing exons as large rectangles and UTRs as narrow rectangles. The 11 subdomains of the kinase catalytic domain are shaded. The positions of transposon insertions are indicated with triangles. Probes used in Southern and northern hybridization (0.7-kb \textit{EcoRI}-\textit{NotI} restriction fragment) and RNA in situ hybridization (0.6-kb \textit{PstI} restriction fragment) are indicated as lines beneath the map. E, Multiple sequence alignment of \textit{PID} (Arabidopsis), \textit{PsPK2} (pea), \textit{bif2} (maize), and \textit{Osbif2} (rice). The conserved kinase subdomains are indicated with a line over the alignment. The insertion domain required for localization of \textit{PID} is indicated with a dotted line over the alignment. The amino acids shown to be important for activation of \textit{PID} by PDK1 are indicated with asterisks.
change compared to its Mo17 progenitor, causing substitution of the conserved Pro 193 with Leu. Sequence changes in five independent alleles confirm that we had cloned the bif2 locus. All alleles have similar severity of phenotype and are predicted to be null alleles.

**bif2 Encodes a Ser/Thr Protein Kinase Co-Orthologous to PID of Arabidopsis**

Sequence analysis indicated that bif2 encodes a Ser/Thr protein kinase with high sequence similarity to PID in Arabidopsis (Christensen et al., 2000). To determine the evolutionary history of the relationship between bif2 and PID, bif2-like genes were cloned from seven additional phylogenetically disparate grasses. PCR was performed with degenerate oligos on DNA extracted from common millet (Panicum miliaceum), foxtail millet (Setaria italica), green millet (Setaria viridis), herbaceous bamboo (Lithachne humilis), oats (Avena sativa), pearl millet (Pennisetum glaucum), and rice and the products sequenced. These sequences were aligned with 30 similar kinase sequences obtained from BLAST searches at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and analyzed using Bayesian phylogenetic software. Arabidopsis kinases were named according to Bogre et al. (2003) and Zegzouti et al. (2006b). The phylogeny was rooted using similar kinases from Physcomitrella (moss) and Adiantum (a fern). Phylogenetic analysis of 39 bif2-like protein kinases estimated a well-supported (100% clade credibility [CC]) grass bif2 clade that is sister to a well-supported (100% CC) eudicot PID clade, supporting the hypothesis that bif2 and PID are co-orthologs (Fig. 2). The presence of both grass and eudicot sequences within the bif2/PID clade indicates that the clade dates to at least the separation of the eudicot and monocot lineages approximately 125 million years ago. To date, no bif2/PID orthologs have been isolated from nonangiosperm species.

Sequence alignment of bif2/PID co-orthologs from maize (bif2), rice (Osbif2), Arabidopsis (PID), and pea (Pisum sativum; PsPK2) indicates that similarity is highest in the 11 subdomains that make up the catalytic core of Ser/Thr kinases (Fig. 1E). These proteins belong to subfamily VIIIAs of the AGC supergroup of kinases (Hanks et al., 1988; Christensen et al., 2000; Bogre et al., 2003; Bai et al., 2005). This subfamily of kinases is characterized by DFD instead of DFG in subdomain VII and an insertion domain of approximately 50 amino acids between subdomains VII and VIII (Fig. 1E). BIF2 and PID share 55% amino acid identity overall, with 67% amino acid identity in the kinase domain. BIF2 and OsBIF2 have an extended hydrophobic N terminus relative to PID and PsPK2. Outside the kinase domains, these proteins share little sequence conservation except they are Ser and Thr rich with many conserved Ser/Thr positions. The insertion domain between kinase subdomains VII and VIII, which has been shown to be important for subcellular localiza-

**bif2 Is Transiently Expressed in Axillary Meristems and Lateral Organs in Maize**

To determine the pattern of bif2 expression, RNA gel-blot and RNA in situ hybridization were performed.

**Figure 2.** Phylogenetic analysis of bif2 homologs from grasses and dicots. Bayesian consensus phylogram of 39 bif2-like protein kinases from diverse angiosperms rooted using Physcomitrella patens PHOTOTROPIN1 (PpPHOT1) and Adiantum capillus-veneris PHOTOTROPIN2 (Ac-vPHOT2). Thick branches are supported by 1.00 CC and medium thickness branches are supported by >0.95 CC. At = Arabidopsis (Brassicaceae); As = Avena sativa (Poaceae); Br = Brassica rapa (Brassicaceae); Cs = Cucumis sativus (Cucurbitaceae); Lh = Lithachne humilis (Poaceae); Os = Oryza sativa (Poaceae); Pg = Pennisetum glaucum (Poaceae); Pm = Panicum miliaceum (Poaceae); Ps = Pismum sativum (Fabaceae); Pw = Phaseolus vulgaris (Fabaceae); Si = Setaria viridis (Poaceae); Sv = Setaria italica (Poaceae); Zm = Zea mays (Poaceae).
RNA gel-blot analysis shows that bif2 is strongly expressed in young tassels and ears, with expression declining in older tassels and ears (Fig. 3A; data not shown). Using 10 μg of total RNA, bif2 expression was below the level of detection in roots, coleoptiles, leaves, and vegetative apices (Fig. 3A).

Expression of bif2 was monitored using a digoxigenin (DIG)-labeled antisense RNA probe from the 3’ end of the gene. Control hybridizations using the sense probe produced no detectable signal (Fig. 3D). bif2 is detected in the peripheral zone of the inflorescence meristem before axillary meristems arise (Fig. 3B). Subsequently, bif2 is expressed in branch meristems, spikelet pair meristems (Fig. 3, B and C), and each of the spikelet meristems and floral meristems (Fig. 3E). Expression is transient in floral meristems and becomes concentrated in floral organ primordia as they develop (Fig. 3, E and F). Expression is also transient in floral organs and fades as they grow out (see Fig. 3F, outer glume). During vegetative development, bif2 is expressed in axillary meristems that give rise to tillers (Fig. 3G) and is weakly expressed in leaf primordia (Fig. 3H). Expression is also detected in vasculature (Fig. 3, F–H). To conclude, bif2 is expressed in axillary meristems and lateral primordia during both vegetative and inflorescence development in maize.

Expression of Osbif2 in Rice

To determine whether the expression of Osbif2 was conserved in rice, RNA in situ hybridization was performed using a probe from the 3’ end of Osbif2. Expression of Osbif2 was detected in leaf primordia and vascular tissue of vegetative meristems in rice (Fig. 4A). Osbif2 expression was detected transiently in all stages of inflorescence development in branch meristems of young inflorescences (Fig. 4B), on the flanks for the spikelet meristem prior to floral organ initiation (Fig. 4C), in the proposed floral meristems of the two sterile, lower florets (Fig. 4D), in stamens and gynoecial tissue (Fig. 4E), and within the ovule (Fig. 4F). Expression was absent when sense probes were used (data not shown). Expression of Osbif2 in vegetative apical meristems and inflorescence branch meristems is supported by a recent report (Morita and Kyoizuka, 2007). Therefore, expression of Osbif2 in rice in axillary meristems and lateral primordia is similar to expression of bif2 in maize.

bif2 Plays a Role in Vegetative Meristems

Previous analysis had not identified a vegetative phenotype in bif2 plants (McSteen and Hake, 2001). However, in Pennsylvania growing conditions, we detected a statistically significant reduction in the number of leaves produced in bif2 mutants backcrossed to B73 (normal siblings = 20.08 ± 0.12 leaves, n = 36; bif2 = 18.29 ± 0.27 leaves, n = 14; P < 0.001). In addition, a small, but statistically significant, reduction in plant height was observed (normal siblings = 207.28 ± 2.08 cm; bif2 = 196.88 ± 2.16 cm; P = 0.009). These results contrast with Arabidopsis pid mutants, which have an increased number of leaves, although pin1 mutants have fewer leaves (Bennett et al., 1995).

We tested whether bif2 functioned in vegetative axillary meristems by constructing double mutants with tb1-ref (Doebley et al., 1997). In normal maize, axillary meristems arise in the axil of every leaf, but arrest, except for the one or two that will grow out to form ear shoots (Fig. 5A, arrow). In tb1 mutants, however, all axillary buds grow out to form vegetative branches, called tillers, giving the plant a bushy appearance (Fig. 5A; Doebley et al., 1997; Hubbard et al., 2002). In addition, the branches at the ear node are masculinized, forming short tillers (Fig. 5A, arrowhead).

bif2;tb1 double mutants produced a reduced number of axillary branches compared to tb1 mutants (Fig. 5). To quantify the defects, the total number of primary tillers and ear shoots were counted in two F2 families segregating for both bif2 and tb1 (Fig. 5B). bif2;tb1 double mutants had significant reduction in the total number of axillary shoots compared to tb1 mutants (Fig. 5B; P < 0.001). This difference was mainly due to a difference in the number of tillers, which were significantly reduced in bif2;tb1 double mutants compared to tb1 mutants (Fig. 5C; P < 0.0001). bif2 mutants have a reduced number of ear shoots compared to normal plants (Fig. 5B; McSteen and Hake, 2001). When occasional ear shoots formed in bif2;tb1 double mutants, they were masculinized like tb1 mutants. Because the number of secondary tillers is dependent on the number of primary tillers, these numbers were not included in the graphs shown. However, the number of secondary tillers was also reduced (tb1 = 5.13 ± 0.92; bif2;tb1 = zero), showing that bif2 functions in the formation of both primary and secondary tillers. Therefore, bif2 plays a role in production of vegetative axillary meristems as well as axillary meristems in tassel and ear inflorescences.

bif2 Mutants Have Defects in Auxin Transport and Vasculature

Because PID plays a role in regulating auxin transport in Arabidopsis, we tested whether bif2 mutants had auxin transport defects in maize (Bennett et al., 1995; Benjamins et al., 2001; Friml et al., 2004; Lee and Cho, 2006). To compare our data to that of pid and pin mutants in Arabidopsis, we used the same protocol (Okada et al., 1991; Bennett et al., 1995), except we used [3H]indole acetic acid (IAA) instead of [14C]IAA, because it can be labeled to a higher specific activity. Inflorescence stem pieces from normal and bif2 tassels were placed in upward or downward orientation in tubes containing 1.5 μM [3H]IAA and incubated overnight in the dark. The top 0.5 cm was then counted in a liquid scintillation counter (Fig. 6A). At this stage of development, neither normal siblings nor bif2 had
an appreciable level of acropetal transport (Fig. 6A, lanes 1 and 2) and this level was not further reduced by coincubation with N-1-naphthylphthalamic acid (NPA), an auxin transport inhibitor (lanes 5 and 6). However, normal plants displayed a very high level of basipetal transport (lane 3), which was totally abolished by NPA (lane 7).

*bif2* mutants, on the other hand, had significant reduction in the amount of basipetal transport of [H\(^{3}\)]IAA compared to wild type (lane 4; *P*, 0.001) and the level was further reduced by NPA (lane 8). Therefore, *bif2* mutants display a comparable magnitude of reduction of auxin transport in inflorescence stems in maize (21% of wild type) compared to the defects in auxin transport that occur in *pid* and *pin1* (7%–40% of wild type) inflorescence stems in Arabidopsis (Okada et al., 1991; Bennett et al., 1995; Oka et al., 1998).

However, a possible explanation for the reduction in auxin transport could be differences in stem anatomy. Inflorescence stems from *bif2* mutants were significantly narrower than normal siblings (Fig. 6, B and C; diameter across widest point: normal siblings 5.08 + 0.15 mm, *n* = 18; *bif2* = 4.15 ± 0.17 mm, *n* = 16; *P* < 0.001) and had significantly fewer vascular bundles (normal siblings = 113.25 ± 4.03, *n* = 10; *bif2* = 47.33 ± 1.57, *n* = 9; *P* < 0.001). Therefore, some of the reduction in transport (4.57-fold) could be due to a reduction in the number of vascular bundles (2.39-fold).
DISCUSSION

We cloned \textit{bif2} from maize and seven evolutionarily disparate grasses and determined that \textit{bif2} is a co-ortholog of \textit{PID} from Arabidopsis. Sequence and expression patterns of \textit{bif2/PID} genes are conserved between grasses and eudicots, attesting to the important role of \textit{bif2/PID} in development. However, analysis of the \textit{bif2} loss-of-function phenotype in maize uncovers additional roles of \textit{bif2/PID}.

\textit{bif2/Osbif2} Are Co-Orthologs of \textit{PID}

Given that there have been at least three rounds of whole-genome duplication since Arabidopsis and grasses last shared a common ancestor (Bowers et al., 2003; Paterson et al., 2004; Yu et al., 2005), orthologous relationships between maize/rice and Arabidopsis genes are not possible with a strict definition of orthology (Sonnhammer and Koonin, 2002). Frequently, especially with transcription factor families, a clade of grass genes is found to be sister to a clade of Arabidopsis genes and these clades are referred to as being co-orthologous (Sonnhammer and Koonin, 2002; Malcomber et al., 2006). Therefore, we refer to \textit{bif2/Osbif2} as co-orthologs of \textit{PID}. Because the maize genome has not been fully sequenced, it is not known how many \textit{PID} co-orthologs are in the maize genome. However, the fact that grass genes are co-orthologous to a single Arabidopsis gene suggests that there has been extensive loss of other paralogs. Another example where there is correspondence in a signaling pathway between maize and Arabidopsis is \textit{thick tassel dwarf1} (\textit{td1}) and \textit{fasciated ear2} (\textit{fea2}) from maize, which are co-orthologs of \textit{CLAVATA1 (CLV1)} and \textit{CLAVATA2} of Arabidopsis, respectively (Taguchi-Shiobara et al., 2001; Bommert et al., 2005a). However, despite the phylogenetic relationship, the \textit{td1/fea2/CLV} pathway has diverged between maize and Arabidopsis (Taguchi-Shiobara et al., 2001; Bommert et al., 2005a).

\textit{bif2} and \textit{Osbif2} share the highest similarity with \textit{PID} in the 11 subdomains that make up the catalytic domain of Ser/Thr kinases, implying that they also function as kinases (Hanks et al., 1988; Christensen et al., 2000). In addition, amino acids identified as being important for activation of \textit{PID} by \textit{PDK1} in Arabidopsis are also conserved, implying that \textit{bif2/Osbif2} may be activated by a \textit{PDK1} co-ortholog in maize and rice as in Arabidopsis (Zegzouti et al., 2006a). Outside the kinase domain, there is less sequence conservation, but, interestingly, the position of many Ser/Thr sites is conserved, implying that these positions may be important for autophosphorylation of \textit{bif2/PID}. On the other hand, both \textit{bif2} and \textit{Osbif2} have an extended hydrophobic domain at the N terminus that is not present in eudicots. In addition, the insertion domain shown to

\textbf{Figure 4.} \textit{Osbif2} is expressed in axillary meristems and lateral primordia in rice. RNA in situ hybridization expression patterns for \textit{Osbif2}. A, Vegetative meristem showing \textit{Osbif2} expression in leaf primordia (lp) including the next arising leaf primordia at plastochron 0 (P0) and vascular (v) tissue. B, Young inflorescence showing \textit{Osbif2} expression in branch meristems (bm). C, Young spikelet meristem (sm) showing \textit{Osbif2} expression on the flanks of the meristem where organs of the fertile upper floret will arise. D, Older spikelet showing \textit{Osbif2} expression in floral meristems (fm) of two sterile lower florets. E, In florets, \textit{Osbif2} expression is visible in the tips of the stamens (st) in the gynoecium (g). F, In older florets, \textit{Osbif2} expression is visible in the ovule (o). Scale bars in A to E = 100 μm; F = 50 μm.
be important for subcellular localization of PID in Arabidopsis is not conserved (Zegzouti et al., 2006b). Whether these differences in amino sequence cause a difference in the biochemical function of BIF2/OsBIF2 remains to be determined.

Role of bif2/PID in Development

*bif2* is expressed in branch, spikelet pair, spikelet, and floral meristems and all floral organs in both maize and rice, and *bif2* mutants have fewer branches, spikelets, florets, and floral organs (McSteen and Hake, 2001). In Arabidopsis, *PID* and *PIN* are both expressed in floral meristems and floral organs, and *pid* and *pin* mutants make fewer florets and floral organs (Okada et al., 1991; Bennett et al., 1995; Christensen et al., 2000; Benjamins et al., 2001; Reinhardt et al., 2003). Because floral meristems are axillary meristems produced by the inflorescence meristem in the axils of cryptic bracts (Long and Barton, 2000), the defects in *pid* and *pin* floral meristem initiation are analogous to the defects caused by *bif2* in axillary meristem initiation in maize. Therefore, the function of *bif2* and *PID* appears to be conserved in floral meristem and floral organ initiation during inflorescence development. However, because maize has additional types of axillary meristems in the inflorescence compared to Arabidopsis, the phenotype of *bif2* in maize extends the role of *bif2*/PID to all axillary meristems in the inflorescence.

*bif2* is also expressed in axillary meristems during vegetative development in maize and rice. Introduction of the *bif2* mutant into the *tb1* mutant background allowed its effects on vegetative branches (tillers) to be evaluated in maize. The *bif2* mutation causes a reduction in tiller number in the *tb1* mutant background, showing that *bif2* plays a role in vegetative axillary meristems. This is similar to the genetic interaction between *ba1* and *tb1*, except that no tillers form in *ba1;tb1* double mutants (Ritter et al., 2002). Mutants that cause a reduction in tiller number, as well as branch and spikelet number, have been identified in rice and other cereals (Babb and Muehlbauer, 2003; Komatsu et al., 2003a; Li et al., 2003). This contrasts with Arabidopsis, in which defects in the production of vegetative branches in *pid* or *pin* mutants have not been reported. The additional defects in *bif2* mutants reported here show that, similar to the role of *bif2* in inflorescence development, *bif2* also plays a role in the formation of lateral organs and axillary meristems during vegetative development.

Finally, *bif2*/Osbif2 and *PID* (Christensen et al., 2000; Benjamins et al., 2001) are all expressed in vasculature. Loss of *bif2*, however, has different effects on vasculature than in Arabidopsis. *pid* mutants have defects in the vasculature of flowers (Christensen et al., 2000), whereas *bif2* mutants have no defects in the vasculature of the spikelets (P. McSteen, unpublished data). *bif2* mutants have reduced numbers of vascular bundles in the inflorescence stem, whereas *pid* mutants have no apparent defects in the vasculature of the inflorescence stem (Christensen et al., 2000). This may be due to differences in the vascular pattern of monocots and eudicots (Esau, 1967). In maize, vascular bundles are scattered in the inflorescence stem and a reduction in density is easily detected compared to Arabidopsis, in which the vascular bundles coalesce into a ring. Alternatively, there may be differences in the function of *bif2* and *PID* in vasculature.

*bif2* Encodes a Serine/Threonine Protein Kinase

*bif2* is expressed in branch, spikelet pair, spikelet, and floral meristems and all floral organs in both maize and rice, and *bif2* mutants have fewer branches, spikelets, florets, and floral organs (McSteen and Hake, 2001). In Arabidopsis, *PID* and *PIN* are both expressed in floral meristems and floral organs, and *pid* and *pin* mutants make fewer florets and floral organs (Okada et al., 1991; Bennett et al., 1995; Christensen et al., 2000; Benjamins et al., 2001; Reinhardt et al., 2003). Because floral meristems are axillary meristems produced by the inflorescence meristem in the axils of cryptic bracts (Long and Barton, 2000), the defects in *pid* and *pin* floral meristem initiation are analogous to the defects caused by *bif2* in axillary meristem initiation in maize. Therefore, the function of *bif2* and *PID* appears to be conserved in floral meristem and floral organ initiation during inflorescence development. However, because maize has additional types of axillary meristems in the inflorescence compared to Arabidopsis, the phenotype of *bif2* in maize extends the role of *bif2*/PID to all axillary meristems in the inflorescence.

*bif2* is also expressed in axillary meristems during vegetative development in maize and rice. Introduction of the *bif2* mutant into the *tb1* mutant background allowed its effects on vegetative branches (tillers) to be evaluated in maize. The *bif2* mutation causes a reduction in tiller number in the *tb1* mutant background, showing that *bif2* plays a role in vegetative axillary meristems. This is similar to the genetic interaction between *ba1* and *tb1*, except that no tillers form in *ba1;tb1* double mutants (Ritter et al., 2002). Mutants that cause a reduction in tiller number, as well as branch and spikelet number, have been identified in rice and other cereals (Babb and Muehlbauer, 2003; Komatsu et al., 2003a; Li et al., 2003). This contrasts with Arabidopsis, in which defects in the production of vegetative branches in *pid* or *pin* mutants have not been reported. The additional defects in *bif2* mutants reported here show that, similar to the role of *bif2* in inflorescence development, *bif2* also plays a role in the formation of lateral organs and axillary meristems during vegetative development.

Finally, *bif2*/Osbif2 and *PID* (Christensen et al., 2000; Benjamins et al., 2001) are all expressed in vasculature. Loss of *bif2*, however, has different effects on vasculature than in Arabidopsis. *pid* mutants have defects in the vasculature of flowers (Christensen et al., 2000), whereas *bif2* mutants have no defects in the vasculature of the spikelets (P. McSteen, unpublished data). *bif2* mutants have reduced numbers of vascular bundles in the inflorescence stem, whereas *pid* mutants have no apparent defects in the vasculature of the inflorescence stem (Christensen et al., 2000). This may be due to differences in the vascular pattern of monocots and eudicots (Esau, 1967). In maize, vascular bundles are scattered in the inflorescence stem and a reduction in density is easily detected compared to Arabidopsis, in which the vascular bundles coalesce into a ring. Alternatively, there may be differences in the function of *bif2* and *PID* in vasculature.

*Rice is considered to have either a single- or a three-flowered spikelet, depending on whether the two structures beneath the fertile lemma are interpreted as being glumes or sterile lemmas, the sole remaining
organs of two sterile lower florets. The three-flowered spikelet hypothesis was first proposed by Stapf (1917) and this interpretation has recently been supported by gene expression patterns (Prasad et al., 2001; Komatsu et al., 2003b; Malcomber and Kellogg, 2004). Our expression analyses show that, within the spikelet, Osbif2 is expressed in the fertile upper floret. In addition, the distinct expression of Osbif2 on either side of the region subtending the fertile lemma and palea coincides with where the two lower floral meristems are predicted to initiate. This result adds support to the hypothesis that rice has a three-flowered, basipetally maturing, spikelet rather than a single-flowered spikelet.

Role of bif2 in Auxin Transport

Using the standard auxin transport assay on inflorescence stems, pid, pin, and bif2 mutants all have comparable reduction in the level of auxin transport relative to wild-type levels (Okada et al., 1991; Bennett et al., 1995; Oka et al., 1998). bif2 mutants, however, have a significant reduction in the number of vascular bundles and inflorescence stem girth, unlike pid, which does not have apparent defects in vasculature in the inflorescence stem. It is possible that the reduction in auxin transport could be indirectly caused by the reduction in vasculature; however, a recent report of the overexpression of a rice co-ortholog of PID (Morita and Kyozuka, 2007) supports a role for grass co-orthologs in auxin transport.

Expression of bif2 and PID overlaps with expression of the auxin efflux carrier PIN1/ZmPIN1 in vasculature as well as during lateral primordia and axillary meristem initiation (Christensen et al., 2000; Reinhardt et al., 2003; Carraro et al., 2006). PID is proposed to affect auxin transport by regulating PIN1 (Friml et al., 2004; Lee and Cho, 2006). In the pid inflorescence in Arabidopsis, the polarity of PIN1 localization is switched from the apical to the basal end of the cell (Friml et al., 2004). In maize, it has been reported that ZmPIN1 protein is not detected in strong bif2 mutant tassels (Carraro et al., 2006). However, this is likely to be due to the absence of spikelets in strong bif2 mutants because ZmPIN1 is detected when spikelets form in tassels (P. McSteen, unpublished data) and ears (Carraro et al., 2006). Occasional reversals of ZmPIN1 polarity in bif2 mutant tassels support the contention that bif2 and PID have conserved roles in regulation of PIN1 (Carraro et al., 2006).

CONCLUSION

To conclude, the sequence and expression of bif2 appears to be conserved between Arabidopsis, maize, and rice. Phylogenetic analysis was aided by identification of homologs from seven grass species, which demonstrated a well-supported co-orthologous relationship between bif2/Osbif2 and PID. Conservation of sequence and expression among grasses and Arabidopsis, which last shared a common ancestor 125 million years ago, attests to the important role of bif2/PID in development. Analysis of the bif2 phenotype in maize uncovers additional roles of bif2 in axillary meristem and lateral primordia initiation during both inflorescence and vegetative development.

MATERIALS AND METHODS

Maize Material and Growth

bif2 alleles were isolated as described (McSteen and Hake, 2001). An additional allele, bif2-KM, was identified in maize (Zea mays) Ms active lines generated by the Maize Gene Discovery Project (http://www.maizegdb.org/rescuemu-phenotype.php). For leaf number, plant height, auxin transport assays, and vasculature analysis, bif2-77 was backcrossed four or more times into the B73 genetic background. For bif2/D1606 double-mutant analysis, b1/D1606 was backcrossed four times into the A188 genetic background and b1-ref (obtained from the Maize Coop) was backcrossed three times into the A188 genetic background. A188 was used for these experiments because bif2 has a strong effect on ear number in this genetic background (McSteen and Hake, 2001).
2001) and bif2 does not exhibit a heterozygous effect in this background (Hubbard et al., 2002). For RNA in situ hybridization, meristems were dissected from B73 plants grown in the greenhouse for 5 weeks (tassels) or in the field for 8 weeks (ears). For phenotype and double-mutant analyses, plants were grown during the summer in the field at Penn State Agronomy Research Farm (Rock Springs, PA). Quantitative analysis of single- and double-mutant phenotypes was reported as mean ± se and statistical significance was determined using Student’s two-tailed t test.

bif2 Cloning

Genomic DNA of mutants and normal cousins (normal plants from sibling families that did not segregate the mutant phenotype) was extracted (Chen and DellaPorta, 1994), digested with EcoRI, separated on a 0.8% agarose gel, blotted, and probed with Mu1. An 8.5-kb Mu1 hybridizing band was found to cosegregate with the bif2 mutant phenotype in allele bif2-47330 (0 recombinants/59 chromosomes). DNA from the 8.5-kb size range was cut from a preparative gel, purified, and ligated into EcoRI cut pZapExpress vector (Stratagene). The subgenomic DNA library was screened with Mu1 and the 8.5-kb restriction fragment in vector pBK-CMV (pSFbis2) was purified and restriction mapped. The 8.5-kb insert was sequenced in its entirety by subcloning multiple restriction fragments and primer walking using the Big Dye sequencing kit on an ABI sequencer (Applied Biosystems). This clone was preparative gel, purified, and ligated into EcoRI cut pZapExpress vector (Stratagene). The molecular defect in alleles bif2-47330, bif2-1504, and bif2-RM was determined by PCR with a Mu1 primer (Ambion) and 5′ specific outer primer (5′-CAGGTTGATCCCCGAGCGAAG153-3′) with 1× betaine and 10× dimethyl sulfoxide (DMSO) in the reaction. Nested PCR was performed with RNA adaptor outer primer (Ambion) and 5′ specific outer primer (5′-CAGGTTGATCCCCGAGCGAAG153-3′) specific inner primer (5′-CAGGCTGCACGACGACGCTC-3′). To obtain a cDNA clone, 106 plaques of a cDNA library from immature tassels (R. Schmidt, University of California, San Diego) were screened with the 0.7-kb EcoRI-NolI restriction fragment. To obtain the 5′ end of the gene, 5′-RACE was performed using the First-Choice RLM-RACE kit (Ambion) on 10 µg total RNA from normal tassels, according to the manufacturer’s recommendations, except with the following modifications because bif2 is GC rich: annealing of oligo(dT) was performed at 65°C and SuperScript II enzyme (Invitrogen) was used at 50°C. PCR was performed with RNA adaptor outer primer (Ambion) and 5′ specific outer primer (5′-CAGGTTGATCCCCGAGCGAAG153-3′) 1× betaine and 10× dimethyl sulfoxide (DMSO) in the reaction. Nested PCR was performed with RNA adaptor inner (Ambion) and 5′ specific inner primer (5′-CAGGCTGCACGACGACGCTC-3′). To obtain a full-length cDNA clone from B73, RT-PCR was performed on 1 µg total RNA according to the SuperScript III (Invitrogen) protocol with the following modifications: annealing of oligo(dT) primer was at 70°C and SuperScript III reverse transcriptase was used at 50°C.

bif2 was amplified by PCR using bif2 5′ untranslated region (UTR) forward primers bif2-566F (5′-TGGTCCGGGAAGCAGGACG-3′) and bif2-3064—UTR reverse primers bif2-3064-C and SuperScript III reverse transcriptase was used at 50°C.

DNA Extraction, PCR Amplification, and Sequencing from Other Grasses

Total DNA was isolated from common millet (Panicum miliaceum), foxtail millet (Setaria italica), green millet (Setaria viridis), Lithochale humilis, maize, oat (Avena sativa), pearl millet (Pennisetum glaucum), and rice (Oryza sativa) using an SDS extraction protocol (DellaPorta, 1997). PCR products were amplified using the primers bif2-566F (5′-CACAAGCGCCGCTCCGCTCCGTCCCTC-3′) and bif2-1227R (5′-CGAGCGGCGCGCGSCAGAMCGGACG-3′), including 1× betaine and 5× DMSO in the PCR reaction. PCR fragments were purified, subcloned, sequenced, and assembled as described (Malcomber and Kellogg, 2004). All sequences were submitted to GenBank (accession nos. EF525201–EF525206).

Phylogenetic Analyses

A total of 39 BIF2-like protein kinase sequences were examined from diverse green leaf plants. Seven sequences were cloned from disparate grass species (see above) and the remaining 22 genes were identified by BLAST (Altschul et al., 1997) searches at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov.). Nucleotide sequences were aligned based on the conceptual amino acid translation, using MacClade 4 (Maddison and Maddison, 2003) and ClustalX (Jeanmougin et al., 1998). Bayesian phylogenetic analyses were conducted using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) on the Beowulf parallel-processing cluster at the University of Missouri, using two separate searches of 5 million Monte Carlo Markov Chain generations using default flat priors and the GTR + I + Γ model of sequence evolution (as estimated by MODELLTEST [Posada and Crandall, 1998]). Trees were sampled every 500 generations and burn in was determined empirically by plotting the likelihood score against generation number. After burn-in trees had been removed, CC values and the 95% credible set of trees were estimated using MrBayes (Huelsenbeck and Ronquist, 2001).

Expression Analysis in Maize

For RNA gel-blot analysis, total RNA was extracted with TRizol (Invitrogen). Ten micrograms of total RNA were separated on a 1% agarose gel according to the northernMax-Gly protocol (Ambion), blotted, and hybridized using the 0.7-kb EcoRI-NolI clone at 68°C in 10× dextran sulfate, 1× NaCl, and 1% SDS.

For RNA in situ hybridization analysis, a 0.6-kb Psll fragment (which overlaps with the 0.7-kb EcoRI-NolI clone) was subcloned into SK+ Bluescript (Stratagene). This plasmid was linearized with XhoI and transcribed with T3 RNA polymerase to produce a DIG-labeled antisense probe. For sense probe, this plasmid was linearized with BamHI and transcribed with T7 RNA polymerase. Although this probe includes part of the conserved kinase domain, high-stringency DNA gel-blot hybridization shows that it detects a single major band in the maize genome and that there is no difference in the hybridization pattern compared to a probe that does not contain the kinase domain (Supplemental Fig. S1). RNA in situ hybridization was performed on developing maize inflorescences as described (Jackson et al., 1994).

Expression Analysis in Rice

Total RNA was extracted from developing rice inflorescences using RNAwiz solution (Ambion) according to the manufacturer’s instructions. The template for RNA in situ hybridization was generated using the SuperScript one-step RT-PCR kit (Invitrogen) with the primers Osbif2-566F and Osbif2-1705R (5′-AACACAAGGTTATTACGACG-3′) with a 65°C annealing temperature following the manufacturer’s instructions, except 4× DMSO and 1× betaine were included. The amplified fragment included 500 bp from the C-terminal region of the gene and 161 bp of the 3′-UTR. This probe is proposed to be specific for Osbif2 because the next closest homolog in the rice genome has 34% nucleotide difference in the coding region and is unalignable in the 3′–UTR. RNA in situ hybridization was performed on developing rice inflorescences, as described (Malcomber and Kellogg, 2004).

Analysis of bif2/tb1 Double Mutants

bif2/tb1 double mutants were identified by phenotype analysis for the bif2 locus and DNA analysis for the tb1 locus. Genomic DNA was extracted (Chen and DellaPorta, 1994), digested with HindIII, separated on a 0.8% agarose gel, blotted, and probed with the 3.4-kb HindIII genomic fragment from the bif2 locus (Doebley et al., 1997). Two F2 families totaling 122 individuals were grown in the field and tiller and ear number counted.

Auxin Transport Assays

Tassels were excised from normal and mutant bif2 plants (segregating 1:1 in the B73 genetic background) before they had fully extended from the leaf whorl. The inflorescence stem between the lowest tassel branch and the point...
of insertion of the flag leaf were used. Auxin transport assays were performed using the method of Okada et al. (1991) with the following modifications: 2-cm pieces of inflorescence stem were placed in normal or inverted orientation into tubes containing 100 μL 0.5% Murashige and Skoog medium, 1.5 μM 3-[3H]-indolyl acetic acid (specific activity 25 Ci/mmol; GE Healthcare), plus or minus 15 μM NPA (Chemservice) overnight in the dark. The following day, the pieces were blotted and 0.5 cm from the end that was not immersed in the solution was placed in scintillation fluid (Ready safe; Beckman-Coulter) and counted in a liquid scintillation counter. Statistical significance was determined using Student’s two-tailed t test.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EF525201 to EF525206, EF532402, EF532403, and EF532824.

Supplemental Data

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

Supplemental Figure S1. DNA gel-blot hybridization with four probes from the bzt locus.

ACKNOWLEDGMENTS

We thank Steve Briggs, Guri Johal, Richard Schneeberger, Paul Chomet, Gerry Nueffer, The Maize Gene Discovery Project, and the Maize Coop for genetic stocks. For plant care, we thank David Hantz at the Plant Gene Expression Center greenhouse, Tony Omeis at the Biology Department greenhouse, Penn State, and Bob Oberheim, Ron Shuey, and W. Scott Harkom at the Expression Center greenhouse. For plant care, we thank David Hantz at the Plant Gene Expression Center greenhouse, Tony Omeis at the Biology Department greenhouse, Penn State, and Bob Oberheim, Ron Shuey, and W. Scott Harkom. We thank Chuck and Bob Schmidt for the tassel cDNA library and Jeffrey Buterbaugh at Penn State Agronomy Research Farm, Rock Springs, PA. We thank George McSteen et al. for the tb1 locus.

Received February 26, 2007; accepted April 11, 2007; published April 20, 2007.

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