bearded-ear Encodes a MADS Box Transcription Factor Critical for Maize Floral Development

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Although many genes that regulate floral development have been identified in Arabidopsis thaliana, relatively few are known in the grasses. In normal maize (Zea mays), each spikelet produces an upper and lower floral meristem, which initiate floral organs in a defined phyllotaxy before being consumed in the production of an ovule. The bearded-ear (bde) mutation affects floral development differently in the upper and lower meristem. The upper floral meristem initiates extra floral organs that are often mosaic or fused, while the lower floral meristem initiates additional floral meristems. We cloned bde by positional cloning and found that it encodes zea agamous3 (zag3), a MADS box transcription factor in the conserved AGAMOUS-LIKE clade. Mutants in the maize homolog of AGAMOUS, zag1, have a subset of bde floral defects. bde zag1 double mutants have a severe ear phenotype, not observed in either single mutant, in which floral meristems are converted to branch-like meristems, indicating that bde and zag1 redundantly promote floral meristem identity. In addition, BDE and ZAG1 physically interact. We propose a model in which BDE functions in at least three distinct complexes to regulate floral development in the maize ear.

INTRODUCTION

Organogenesis in plants requires the ongoing activity of meristems. Meristems are groups of totipotent cells that give rise to lateral organs, stems, and roots, while maintaining a population of stem cells. Meristsms can be indeterminate and give rise to an unlimited number of primordia, or they can be determinate and terminate in the production of primordia. Floral meristems (FMs) are determinate meristems: they produce a defined number of floral organs and terminate in the production of the ovule, which is contained in the carpel whorl. A typical eudicot flower contains four whorls of floral organs: sepals, petals, stamens, and carpels. Grass flowers contain stamens and carpels but also contain palea and lemma, organs unique to the grasses.

Floral development has been intensively studied in the model plant Arabidopsis thaliana. The molecular regulation of floral organ identity is described by the ABC model, which posits that floral organ identity is determined by the combinatorial action of the Class A, B, and C genes (Coen and Meyerowitz, 1991). Briefly, Class A genes alone specify whorl 1 organs (sepals), Class A and B genes together specify whorl 2 organs (petals), Class B and C genes together specify whorl 3 organs (stamens), and the Class C gene AGAMOUS (AG) specifies whorl 4 organs (carpels); AG also promotes FM determinacy. The ABC model has also been expanded to include Class D genes, which specify ovules (Colombo et al., 1995; Dreni et al., 2007), and Class E, or SEPALLATA genes, which function with the Class A, B, C, and D genes to specify organ identity (Pelaz et al., 2000; Honma and Goto, 2001; Ditta et al., 2004).

Much less is known about floral development in the grasses, although some aspects of the ABCDE model are applicable. For example, mutations in maize (Zea mays) and rice (Oryza sativa) homologs of the Arabidopsis Class B gene APETALA3 (AP3) result in homeotic conversions consistent with Class B function (Ambrose et al., 2000; Nagasawa et al., 2003). Furthermore, maize Class B homologs have similar biochemical activities as their Arabidopsis counterparts (Whipple et al., 2004). The Class C gene AG has been duplicated in the grasses and C function subfunctionalized. In rice, the AG homologs MADS3 and MADS58 regulate floral organ identity and FM determinacy, respectively (Yamaguchi et al., 2006). Similarly, the maize AG homolog zag1 is required for FM determinacy, suggesting that at least some Class C function is conserved (Mena et al., 1996). Mutants in maize Zea mays mads2, the other AG homolog, have not been identified. In rice, leafy hull sterile1 (hs1) mutants harbor mutations in MADS1, a member of the SEP clade (Jeon et al., 2000; Prasad et al., 2005). hs1 mutants make extra palea/ lemma-like organs and also make an aberrant number of stamens and carpels, indicating that LHS1 functions in meristem determinacy and organ fate. In maize, indeterminate floral apex1 (ifa1) is also required for FM determinacy, although the molecular identity of ifa1 is unknown (Laudencia-Chingcuano and Hake, 2000).
Thus, the ABCDE model provides a framework for the molecular regulation of floral development in grasses, but more genes need to be identified and characterized to understand how unique floral morphologies are specified in the grasses (Thompson and Hake, 2009).

All MADS box genes required for floral development encode MIKC-type MADS box transcription factors (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994; Jofuku et al., 1994). The MIKC class is named for its characteristic structure, which includes the MADS box (M), the intervening domain (I), the keratin-like domain (K), and C-terminal domain (C) (reviewed in Kaufmann et al., 2005). The MADS box binds DNA, the K-domain is involved in protein–protein interactions, the C terminus also contributes to protein–protein interactions, and some MADS box proteins harbor a transcriptional activation domain (Fan et al., 1997; Honma and Goto, 2001).

The quartet model has been proposed to explain the molecular underpinnings of the ABCDE model, which posits that four different combinations of A, B, C, and E class transcription factors determine floral organ identity in the four floral whorls (Thiessen, 2001). Indeed, biochemical and genetic evidence suggests that MADS box proteins form trimers and tetramers, and these higher-order complexes regulate transcriptional programs required for floral organ identity (Egea-Cortines et al., 1999; Honma and Goto, 2001). Thus, uncovering the protein interaction network is critical to understand MADS box function in floral development.

Here, we describe the cloning and characterization of the maize floral mutant bearded-ear (bde). bde is critical for multiple aspects of floral development, including FM determinacy, organ development, and sex determination. bde encodes a MADS box transcription factor belonging to the AGL6 clade. AGL6-like genes are conserved among diverse angiosperms; however, the role of AGL6-like genes in development has remained elusive due to a lack of phenotypes in single loss-of-function mutants. Thus, characterization of bde in maize provides key insights into the roles of AGL6-like genes.

RESULTS

bde Regulates Multiple Aspects of Floral Development

To understand the molecular regulation of floral development in maize, we characterized the bde mutant phenotype in detail. bde is defined by three alleles, bde-McClintock (bde-McC), bde-TR864, and bde-N868. bde-McC was obtained from Barbara McClintock’s collection via the Maize Genetics Cooperative; bde-TR864 was ethyl methanesulfonate (EMS) induced in the A619 inbred background; bde-n868 was EMS induced and found segregating in the et*N868A stock from the Maize Genetics Cooperative. All alleles are completely recessive. Both bde-TR864 and bde-n868 fail to complement bde-McC, indicating these three alleles define a single locus. Phenotypic characterization focused on bde-McC, backcrossed three times to A619. All mutant alleles exhibit qualitatively similar phenotypes with variations in severity depending on allele and inbred background.

Maize has two types of inflorescences, the tassel and the ear, which produce male and female florets, respectively. Spikelet pair meristems (SPMs) arise on the flanks of the inflorescence meristem (IM) and give rise to two spikelet meristems (SMs). Each SM produces two bract leaves, called glumes, and two FMs (Figure 1A). The SM initiates the lower floral meristem (LFM), and then the SM either initiates the upper floral meristem (UMF) or is itself converted to the UFM. The FM initiates a specific number of floral organs in a defined phyllotaxy, including two lodicules, three stamens, and three carpel primordia. Surrounding these floral organs are two bracts, called lemma and palea, but it is not clear if these bracts are the product of the SM or FM. Carpels abort in the tassel, and stamens arrest in the ear,
resulting in unisexual flowers (Cheng et al., 1983). In the ear, the LFM aborts, producing a single floret per spikelet (Figures 1B and 1C).

bde mutants exhibit multiple defects in floral development but not during earlier stages of inflorescence or vegetative development. The ear phenotype is striking and obvious in all alleles and inbred backgrounds examined, whereas the severity of the tassel phenotype is more variable. In the tassel, spikelets contain extra florets, which produce extra floral organs and often silks (Figures 2A to 2F). In the ear, bde mutants make extra silks and are mostly sterile (Figures 2G to 2L). We dissected individual spikelets and found they contain multiple florets. In addition, the florets produce an excess of palea/lemma-like organs, and multiple silks often emerge from a single ovule (Figures 2M to 2R). bde mutants often exhibit protruding nucelli (Figure 2R), suggesting a defect in ovule and/or integument development.

To confirm the defects we observed in the mature inflorescences, we examined earlier stages of development by scanning electron microscopy. Early inflorescence development was indistinguishable from normal development, and no defects were observed in branch meristems (BMs), SPMs, or SMs (Figures 3A to 3D; data not shown). In both ears and tassels, SM initiated LFM, although FMs were abnormal and misshapen compared with those in normal siblings (Figures 3E to 3H). The UFM initiated an excess of organ primordia in an aberrant phyllotaxy (Figures 3I to 3L). In rare instances (<1% of spikelets), the UFM bifurcated and formed two meristems (see Supplemental Figures 1A and 1B online). The LFM initiated what appear to be ectopic meristems in the axils of bract leaves (bracts were often removed during dissections), which are likely the source of the extra florets observed in the mature inflorescence (Figures 3N and 3P). To confirm these were meristems and not aberrant floral organs, we examined the expression of the meristem marker knotted1 (kn1) (Jackson et al., 1994) in bde mutants. Indeed, multiple loci of kn1 expression were apparent in bde mutants in the region corresponding to the LFM, indicating these primordia are meristems (see Supplemental Figure 1C online). Since FMs are terminal meristems and never initiate other meristems, this result suggests that FM identity has been lost in the LFM in bde mutants. Thus, bde mutants affect the UFM and LFM differentially.

To characterize the bde tassel defects in more detail, we counted floral organs in the upper floret (Figure 4A). In normal florets, lemma and palea are easily distinguishable based on position and vasculature. However, in bde florets, lemma and palea were indistinguishable and therefore were scored together. bde mutants made excess palea/lemma and often an atypical number of stamens and lodicules. Normal tassel florets

Figure 2. bde Mutant Phenotype

(A) to (C) Mature tassels. (D) to (F) Dissected tassel florets; glumes have been removed. Arrows indicate stamens. Arrowheads indicate silks. (G) to (I) Mature ears. bde-McC (H) and bde-TR864 (I) ears make extra silks compared with normal (G). (J) to (L) Cross section of ears. Normal spikelets (J) have a single silk, while bde-McC (K) and bde-TR864 spikelets (L) produce multiple silks. (M) to (O) Dissected spikelets. A normal spikelet contains a single floret (M), while bde-McC (N) and bde-TR864 (O) spikelets contain multiple florets. (P) to (R) A normal ovule is contained within a single silk (P), while bde-McC (Q) and bde-TR864 (R) ovules are surrounded by multiple silks. bde-TR864 also exhibits a protruding nucellus. Bars = 2 mm.
do not produce silks due to carpel abortion. **bde** upper florets did not produce silks most of the time, in contrast with **bde** lower florets, which often produced silks. In addition, floral organs in the upper floret exhibited developmental defects, and organs were often mosaic or fused (Figures 4B to 4D). Thus, **bde** is required to specify proper floral organ number and development in the upper floret.

The scanning electron microscopy and *kn1* expression data indicate that the extra florets were the product of axillary meristems initiated from the lower floret. When we dissected spikelets, the orientation of the extra florets also suggested they arose from the lower floret. We counted florets that appeared to be derived from the UFM and LFM. The UFM produced a single floret, while the LFM usually produced multiple florets (Figure 4E). Florets derived from the LFM did not produce a normal complement of floral organs and often contained only a couple of lemma/palea-like organs (Figure 4F).

**bde Encodes a MADS Box Transcription Factor**

To identify the gene responsible for the **bde** mutant phenotype, we pursued a map-based cloning approach. **bde** was crossed to the B73 and Mo17 inbred lines and self-pollinated to generate F2 mapping populations. We established linkage to *bnlg118* on chromosome 5, bin 7 by bulked segregant analysis. We further defined the interval between the markers *umc1752* (34/2066...
chromosomes; 1.65 centimorgans) and umc1524 (7/2066; 0.34 centimorgans) (Figure 5A). We identified a syntenous region in rice on the long arm of chromosome 2 and designed single nucleotide polymorphism markers using maize ESTs corresponding to rice genes in the candidate region. Eventually, we narrowed the interval to a region that contained six genes in rice, four of which could be amplified from maize BACs present in the region (see Supplemental Figure 2A online).

Of these four genes, the MADS box gene zag3 was a particularly strong candidate to underlie the bde mutant phenotype given the role of MADS box genes in floral development and patterning. Furthermore, zag3 is specifically expressed in the inflorescence (Mena et al., 1995). We analyzed the zag3 genomic sequence in the three bde alleles and identified lesions in each allele (Figure 5B). bde-McC contains a single nucleotide insertion in the fourth exon of zag3, resulting in a frame shift that introduces 36 novel amino acids and a premature stop codon. bde-TR864 contains a large deletion that removes all zag3 coding sequences after the first exon. DNA gel blot analysis indicated a polymorphism between bde-TR864 and the A619 progenitor inbred (see Supplemental Figure 2B online), consistent with a deletion. To ask if other genes were also removed by the deletion, we identified syntenous genes in Sorghum bicolor, whose genome is fully sequenced (www.phytozome.net). We were able to amplify zag3-flanking genes from bde-TR864 homozygotes, indicating that only zag3 is likely to be disrupted in the bde-TR864 allele. The exact breakpoints of the deletion are unknown. bde-N868 contains an insertion/deletion in the 5' region, 825 bp upstream of the start codon, that deletes 39 bp and inserts ~150 nucleotides. A BLAST search with the inserted nucleotides yields no significant hits. bde-N868 also contains a missense mutation at position 180, a nonconserved residue in the C-terminal region of zag3. Together, these data indicate that the bde mutant phenotype is caused by mutations in the MADS box gene zag3.

Per convention, we refer to the zag3 gene as bde. To determine the effect of these mutations on bde gene expression, we compared bde mRNA levels in mutant alleles and the A619 inbred using quantitative RT-PCR (Figure 5C). bde mRNA levels are slightly reduced in bde-McC, consistent with decreased RNA stability due to the introduction of a premature stop codon and nonsense-mediated decay. As expected, bde mRNA is not detectable in bde-TR864; the primers flank the sequence deleted in this allele. Finally, the amount of bde mRNA is greatly reduced in bde-N868, suggesting that the insertion/deletion in the 5' flanking sequence affects transcriptional efficiency.

bde Expression

Previous work showed that bde was expressed in the tassel and ear but was not detectable in vegetative tissues (Mena et al., 1995), consistent with where we observe the bde mutant’s defects. To more carefully investigate bde expression in normal plants, we performed RNA in situ hybridization analysis. bde was first detected in the UFM and LFM in normal and bde tassels (left). The LFM produces a single floret in normal and bde tassels but multiple florets in bde (right).

![Figure 4. Quantification of Tassel Defects in bde Mutants.](image_url)

(A) Quantification of floral organs in the upper floret of normal and bde-McC tassels. Palea and lemma were scored together because they are indistinguishable in bde.

(B) to (D) Examples of floral organ defects in the upper floret of bde tassels.

(B) Floret containing a partially transformed lodicule (arrowhead) and four stamens.

(C) A partially transformed stamen (arrowhead).

(D) A stamen fused to a palea-like organ (arrowhead).

(E) Quantification of florets originating from the UFM and LFM in normal and bde tassels. The UFM produces a single floret in both normal and bde tassels (left). The LFM produces a single floret in normal tassels but multiple florets in bde (right).

(F) Three florets (white arrowheads) produced from the lower floret in a bde tassel. Bar = 2 mm.
carpel primordia but was downregulated later in carpel development (Figures 6F and 6G). In addition, *bde* was strongly expressed in the inner integument and the region underlying the ovule primordium (Figure 6G). The integument expression suggests a role for *bde* in integument development and might explain the protruding nucellar phenotype. *bde* was not detected in earlier stages of inflorescence development in the IM, SPM, or SM or with a sense probe.

**LFM Identity in ** _bde_ **Mutants**

The identity of the LFM is unclear in _bde_ mutants given that FMs normally do not initiate meristems. One possibility is that the LFM is converted to a SM, which normally initiates FM. To determine if the LFM has SM identity in _bde_ mutants, we examined expression of the SM marker _branched silkless1_ (*bd1*). *bd1* is transiently expressed in a semicircular pattern at the base of the SM (Chuck et al., 2002). In _bde-McC_, *bd1* was not detectable in the LFM at any stage of floral development, suggesting that the LFM does not have SM identity (see Supplemental Figure 3 online). To ask if the LFM retains FM identity, we examined expression of _bde_, which is only expressed in developing florets (Figure 6). We used the _bde-McC_ allele, in which _bde_ mRNA is reduced but still present (Figure 5C). *bde* was expressed in the UFM and LFM of _bde-McC_, and in some spikelets, the LFM contained additional foci of _bde_ expression (see Supplemental Figure 3 online). These foci likely correspond to the axillary meristems observed by SEM. Taken together, these results indicate that the LFM initiates additional FM yet retains at least some FM identity in _bde_ mutants.

**bde and zag1 Function Together to Regulate FM Determinacy**

*bde* likely functions in a pathway with additional genes to regulate floral development. One candidate gene is _zag1_, mutants in which make extra carpels in the ear, similar to the upper floret of _bde_, and is expressed in an overlapping domain (Schmidt et al., 1993; Mena et al., 1996; this work). We constructed _bde zag1_ double mutants to investigate their genetic interaction. _bde zag1_ double mutants had a novel ear phenotype (Figures 7A to 7E), not observed in either single mutant (Figures 7F and 7G), while _bde zag1_ tassels resembled _bde_ tassels. In place of normal ear florets were branch-like structures that initiated ectopic meristems in the axils of bracts. Occasionally, radialized organs that resembled silks were observed in the mature ear (Figure 7C); however, no other obvious floral organs were observed. In most plants, initiation of ectopic meristems was random. However, in one plant, the branch structure appeared to be a complete conversion to an inflorescence

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**Figure 5.** _bde_ Encodes the MADS Box Transcription Factor ZAG3.

(A) Schematic of genetic location of _bde_. _bde_ showed no recombination with the MADS box gene _zag3_. cM, centimorgans

(B) Schematic of _zag3_ genomic region and _bde_ lesions.

(C) Quantitative RT-PCR showing expression of _bde_ mRNA in _bde_ mutant alleles compared with the A619 inbred. _bde_ levels are normalized against _gapdh_ levels. Error bars represent 1 SD.
structure, as branches initiated spikelet pairs in a distichous phyllotaxy (Figures 7D and 7E).

To investigate the origin of these branch structures, we examined early development of bde zag1 double mutants by scanning electron microscopy. Early stages of inflorescence development appeared normal in double mutants, and the IM, SPM, and SM were indistinguishable from those of normal plants (Figure 7H; data not shown). bde zag1 double mutants initiated a meristem in the proper position for the LFM (Figure 7I); however, neither the UFM or the LFM initiated floral organs and instead continued to initiate ectopic meristems (Figures 7J to 7M). Sometimes structures that resembled a carpel ridge (Figure 7L, white arrowhead) were present, suggesting that the radial organs observed in the mature ear were silks.

MADS box proteins form dimers and higher-order complexes to regulate the expression of downstream target genes (Fan et al., 1997; Honma and Goto, 2001). Both bde and zag1 mutants make extra carpels, raising the possibility that BDE and ZAG1 function in the same complex to regulate floral organ number. Furthermore, ZAG1 and BDE homologs interact in Arabidopsis and petunia (Petunia hybrida) (Fan et al., 1997; de Folter et al., 2005; Rijpkema et al., 2009). To establish if BDE and ZAG1 also physically interact, we first used the yeast two-hybrid system (Figure 8A). We tested if full-length BDE fused to the GAL4 DNA binding domain (BD-BDE) activated lacZ reporter gene expression and found that BD-BDE possesses transcriptional activity in the absence of the GAL4 activation domain. In other MADS box proteins, the activation domain resides in the C terminus (Honma and Goto, 2001). Therefore, we deleted the C terminus of BDE (amino acids 164 to 256) and found that the truncated protein (BD-BDE C) no longer autoactivated in the yeast two-hybrid system. We also examined if BD-BDE autoactivated in a more stringent assay, which assays growth in the absence of histidine and adenine, indicating activation of reporter genes. BD-BDE did not autoactivate in this assay; however, yeast grew on restrictive media when both BD-BDE and AD-ZAG1 were present, indicating that BDE and ZAG1 interact. To eliminate the possibility that observed growth was due to some transcriptional activation by BDE alone, we also tested if BD-BDE C, which did not autoactive in the β-gal assay, interacted with AD-ZAG1. Indeed BD-BDE ΔC interacted with AD-ZAG1. The interaction was also observed with ZAG1 fused to the DNA binding domain (BD-ZAG1) and BDE fused to the activation domain (AD-BDE). BDE does not interact with itself, indicating that the BDE-ZAG1 interaction is specific.

To confirm the interaction we observed in the yeast-two hybrid system, we used bimolecular fluorescent complementation (BiFC) (Citovsky et al., 2006). We fused full-length ZAG1 to the N-terminal portion of yellow fluorescent protein (ZAG1-nYFP) and full-length BDE to the C-terminal portion of YFP (ZAG3-cYFP) and cobombarded these constructs into epidermal onion cells, along with a plastid red fluorescent protein (RFP) marker as an internal control. We observed nuclear YFP expression in 50% of RFP-expressing cells (n = 290 cells), indicating that BDE and ZAG1 also interact in planta and are localized to the nucleus, consistent with their function as transcription factors (Figure 8B). We observed no YFP expression when ZAG1-nYFP or BDE-cYFP were cobombarded with the cYFP or nYFP, respectively.
(n = 193 cells for ZAG1-nYFP + cYFP and n = 175 cells for BDE-cYFP + nYFP). ZAG3-nYFP co-bombarded with cYFP resulted in nonspecific nuclear YFP expression; thus, we were unable to conduct BiFC experiments in the opposite orientation or ask if BDE interacts with itself in this assay. Based on the BiFC, yeast two-hybrid analysis, bde zag1 genetic interaction data, and the observation that the BDE and ZAG1 homologs interact in other species (Fan et al., 1997; de Folter et al., 2005; Rijpkema et al., 2009), we conclude that BDE and ZAG1 are likely to physically interact in vivo.

**DISCUSSION**

Here, we describe the characterization and cloning of the maize floral mutant bde. **bde** encodes a member of the AGL6 clade of the MADS box family. bde is expressed in FMs and a subset of floral organs. The phenotype reveals a role for bde in multiple stages of floral determinacy and floral organ fate. Given that AGL6-like genes are conserved among diverse angiosperms and that no other single loss-of-function mutants have yet been described, our analysis provides key insights into the roles of this group of MADS box regulators.

**bde Mutants Affect the Upper and Lower FM Differently**

Florets in the grasses are organized in spikelets, and the number of florets in a spikelet depends on the species. Maize and other members of the Andropogoneae contain two florets, which although seemingly identical, develop at different rates (Clifford, 1987). Interestingly, **bde** mutants affect the UFM and LFM differently. In the upper floret, bde is required to specify floral organ number and fate, and in the lower floret, bde is required to specify FM identity. One explanation for the difference between UFM and LFM could be differential gene expression. For example, a gene that functions redundantly with bde to promote FM fate might be expressed in the UFM, but not the LFM. Indeed, previous work suggests that many genes are differentially expressed in the upper and lower floret. Two MADS box genes, ZMM8 and ZMM14, are expressed in the UFM and their floral organs but not in the lower floret (Cacharrón et al., 1999). Microarray analysis indicates that ~9% of anther-expressed genes are differentially expressed in anthers from the upper and lower floret (Skibbe et al., 2008). If MADS box genes are differentially expressed in the UFM and LFM, BDE might participate in distinct complexes in the two florets. A second possibility is that the upper and lower florets have different developmental potentials based on the different history of these two meristems. Although the UFM and LFM are identical morphologically, their origins differ. For example, one model suggests the SM first initiates the LFM and then converts to the UFM (Irish, 1998). It is possible that the chromatin state may be different in the UFM and LFM and that some genes are unavailable for transcriptional meristems (black arrowheads) and form branch-like structures (I) to (M). In some spikelets, a carpel ridge is formed (L); white arrowhead. Bars = 2 mm in (B) to (G) and 100 µM in (H) to (M).
activation by BDE. Differentiating these two possibilities requires

determining which mRNAs are expressed in the upper and lower

tassel and determining BDE target genes.

Links between Sex Determination and
Meristem Determinacy

In addition to FM determinacy, bde is required for sex determina-
tion. Interestingly, this aspect of the phenotype is also expressed
differently in the upper and lower floret, since almost all ectopic
silks are produced from the lower floret in bde tassels. Charac-
terization of bde and other mutants suggest that meristem deter-
minacy and sex determination are linked. The recessive mutant
tasselseed4 (ts4) and the dominant mutant Ts6 fail to abort carpels
in the tassel and have indeterminate SPM and SM (Irish et al.,
1994; Chuck et al., 2007). ts4 encodes a miRNA that targets the
SM determinacy gene indeterminate spikelet1 (ids1). Ts6 harbors a
mutation in the miRNA binding site of ids1 mRNA, rendering it
immune to miRNA regulation (Chuck et al., 2007). One possible link
between sex determination and meristem determinacy is the plant
hormone, gibberellin (GA). GA has a well-established role in maize
sex determination, as application of exogenous GA results in
failure of carpel abortion in the tassel (Nickerson, 1959), and
mutants in the GA biosynthetic pathway do not abort stamens in
the ear (Bensen et al., 1995). GA is also required to promote FM
identity in Arabidopsis (Okamuro et al., 1996, 1997). Furthermore,
the MADS box genes AP3, PI, and AG are targets of GA signaling
(Yu et al., 2004), providing another possible link between MADS
box proteins, sex determination, and meristem identity.

bde Provides Insight into the Function of the AGL6 Clade

MADS box genes have well-studied roles in plant development,
particularly in floral development. In this work, we show that bde
encodes the MADS box gene previously described as zag3. bde is
a member of the AGL6 family of MIKC-type transcription
factors, which is sister to the SEP clade (Becker and Theissen,
2003; Parenicova et al., 2003). Although AGL6-like genes are
conserved among diverse angiosperms, their role in develop-
ment has remained elusive. Multiple lines of evidence, however,
suggest that AGL6-like genes function specifically in floral
development. In petunia, AGL6 functions redundantly with the sep
genes FBP2 and FBP6, although single AGL6 mutants have no
phenotype (Rijpkema et al., 2009). Overexpression of orchid

Figure 8. BDE and ZAG1 Protein Interactions

(A) BDE and ZAG1 interact in yeast two-hybrid assays. Left, BD-BDE autoactivates in a B-gal assay but not in a growth assay; BD-BDEΔC and BD-
ZAG1 do not autoactivate in either assay. Right, BD-BDE+AD-ZAG1, BD-ZAG1+AD-BDE, and BD-BDEΔC+AD-BDE are not. The ability to grow in the absence of histidine and adenine indicate activation of
reporter genes and a positive protein–protein interaction. Media lacking Trp and Leu select for bait and prey plasmids, respectively.

(B) BifC indicates that ZAG1 and BDE interact in planta. Top, yellow fluorescence indicates that ZAG1-nYFP and BDE-cYFP interact in the nucleus of
epidermal onion cells. Middle, ZAG1-nYFP does not interact with cYFP. Bottom, nYFP does not interact with BDE-cYFP.

(C) Model for ZAG1 and ZAG3 function. BDE and ZAG1 interact in Complex A to control floral organ number. BDE is present in a complex that does not
contain ZAG1 (Complex B) to control floral organ development. BDE and ZAG1 redundantly promote FM identity. BDE and ZAG1 might be able to
compensate for each other in the same complex (Complex C) or might be present in two different complexes that both promote FM identity.
Mutants in zag1, the maize homolog of the C-class gene AG, make extra carpels in ear florets, similar to the upper floret of bde mutants (Mena et al., 1996), suggesting they may function in a common genetic pathway. In addition, bde and zag1 are expressed in overlapping domains (Figure 6; Schmidt et al., 1993), and double mutants exhibit a novel ear phenotype, in which FM are converted to branch-like or inflorescence-like meristems. In contrast with the ear, bde zag1 double mutant tassels resemble bde tassels. Although zag1 is expressed in the tassel, zag1 mutant tassel florets are normal, suggesting other genes function redundantly with zag1 in the tassel (Mena et al., 1996).

MADS box proteins physically interact and form dimers and higher-order complexes to regulate the expression of downstream target genes (Fan et al., 1997; Honma and Goto, 2001; de Folter et al., 2005). In Arabidopsis and petunia, the BDE and AGL6 homologs physically interact, although the functional significance of this interaction is unknown (Fan et al., 1997; Rijkema et al., 2009). We also found that BDE and ZAG1 interact both in yeast and in planta.

The protein interaction data and double mutant analysis suggest that BDE functions in at least three distinct complexes in the ear (Figure 8C). BDE is likely to function in many more complexes, based on the numerous protein interactions of AGL6 in Arabidopsis (de Folter et al., 2005) and the pleiotropy of the bde mutant phenotype. The observations that bde and zag1 mutants both produce extra carpels in female florets and that BDE and ZAG1 physically interact suggest that BDE and ZAG1 interact in a complex that regulates floral organ number (Complex A). In addition, bde mutants have phenotypes that are not shared with zag1, including floral organ development and phyllotaxy defects. Thus, BDE is also likely to function in a second complex that does not contain ZAG1 but that is required to promote floral organ fate (Complex B). Finally, the bde zag1 double mutant phenotype suggests that BDE and ZAG1 redundantly promote FM identity and function in a complex required to promote FM fate (Complex C). BDE and ZAG1 may be able to compensate for the lack of the other in a single complex that promotes FM identity, or alternatively, BDE and ZAG1 may function in two separate complexes that promote FM fate. The different phenotypes of bde and zag1 in the ear and tassel also indicate that different MADS box complexes are present in the two inflorescences.

The Branch Meristem Is a Default Meristem Fate

The bde zag1 double mutants are reminiscent of ifa zag1 double mutants, which also produce branch-like meristems from ear florets (Laudencia-Chingcuanco and Hake, 2002). In ifa zag1 double mutants, however, branches are initiated from the carpel whorl of the floret, and other floral organs are specified normally. Analysis of both double mutants suggests that a branch-like meristem might be a default state in the ear if the floral development program cannot be executed. In bde zag1 double mutants, the FM cannot adopt FM fate and reverts to a default branch-like meristem. A similar situation occurs in ifa zag1 double mutants, although in this case, FM identity is maintained until the carpel whorl develops, at which point, the FM reverts to a BM.

Analysis of other mutants also supports the idea that a branch meristem is a default meristem in the ear. ids1 is required for SM meristem determinacy, and in the absence of ids1, spikelets produce extra florets (Chuck et al., 1998). In ifa ids1 double mutant ears, SM are converted to BM, indicating that ifa and ids1 are redundantly required for SM identity (Laudencia-Chingcuanco and Hake, 2002). Similarly, bd1 is also required for SM identity, and in the absence of bd1, SMs are converted to BMs (Chuck et al., 2002).

In contrast with the ear, a branch meristem is not likely to be a default meristem in the tassel. In the tassel of bde zag1, ifa zag1, and ifa ids1 double mutants, FMs and SMs do not convert to BM. In ifa zag1 double mutants, FMs are converted to SMs, while in ifa ids1 double mutants, SMs are converted to SPMs (Laudencia-Chingcuanco and Hake, 2002). In bd1 mutant tassels, SMs are converted to meristems with some branch-like characteristics, although the conversion is not as complete as in the ear (Chuck et al., 2002). Thus, a common theme between the tassel and ear is that determinate meristems are converted to less determinate meristems, but a complete conversion to a BM does not occur in the tassel.

ABCDE Model in Grass Floral Development

The simple yet elegant ABCDE model provides a mechanism to help understand how floral organ identity is determined in Arabidopsis and other dicot species. B and C class function is conserved in the grasses, although it is unclear if A and E class functions are also conserved or if other genes function in floral development specifically in the grasses. For example, in rice, the YABBY gene DROOPING-LEAF1 is a carpel identity gene, a function not conserved in Arabidopsis (Yamaguchi et al., 2004).

In this work, we described bde functions in maize floral development. bde is most closely related to AGL6 in Arabidopsis, which has no known biological function. In several respects, bde resembles Arabidopsis Class E or SEP genes. First, like the SEP genes, bde affects organ development in all four whorls. Second, BDE likely functions in multiple complexes (Figure 8C). BDE interacts with the C class protein ZAG1 to promote FM determinacy and likely interacts with B and C class proteins to regulate floral organ development. We also propose that BDE functions in a third complex with unknown members to promote FM identity. Similarly, SEP proteins act in multiple complexes with class A, B, and C proteins (Honma and Goto, 2001) as well as complexes important for ovule development (Favaro et al., 2003). Third, Arabidopsis SEP genes, particularly SEP4, have a
role in promoting FM identity, similar to bde (Pelaz et al., 2000; Ditta et al., 2004). The conclusion that bde resembles a Class E gene is consistent with the finding that agl6 functions redundantly with sep genes in petunia (Rijpkema et al., 2009).

Although aspects of the ABCDE model clearly apply to the grasses, there are multiple differences in the regulatory networks governing floral development in Arabidopsis and maize. A more complete understanding of maize floral development will require further understanding of MADs box protein interactions and if and how these interactions map onto the ABCD model, as well as identifying new genes that function specifically in maize.

**METHODS**

**Genetics**

To map the bde mutation, we followed the basic strategy for position based cloning in maize (Zea mays) outlined by Bortiri et al. (2006). Mapping populations for bde-McC were developed by crossing bde to B73 and Mo17 inbred lines and self-pollinating. Using bulked segregate analysis (Michelmore et al., 1991), we localized bde on chromosome 5, bin 7. We defined flanking markers and further refined the interval using markers from the IBM neighbors map. We designed additional single markers from the B73 and Mo17 inbred lines and self-pollinating. Using bulked segregate mapping populations for bde, we created a truncated version of BDE that lacked the C-terminal domain by digesting pBDGAL4-ZAG3 with PstI (removes amino acids 184 to 257) and religating the vector.

For phenotypic characterization of single bde mutants, we used bde-McC introgressed three times in the A619 inbred background. To construct the bde zag1 double mutant, we used stocks of bde and zag1 introgressed in the B73 inbred background four or more times. bde-McC zag1-mum1 double mutants were genotyped using primers BT235 (5′-CAGCGACATGATCAATAGAT-3′) and BT237 (5′-TGCGATGGAG-AGAGAAACTCC-3′) for bde-McC and the simple sequence repeat marker umc1187 for zag1-mum1.

**Scanning Electron Microscopy**

Inflorescence primordia for scanning electron microscopy were fixed in FAA (50% ethanol, 5% glacial acetic acid, and 3.7% formaldehyde) overnight at 4°C and dehydrated in an ethanol series to 100% ethanol. Samples were critical point dried, and glumes were manually dissected to reveal developing florets. Samples were sputter coated with palladium for 60 s and viewed on a Hitachi S-4700 at an accelerating voltage of 2 kV. Images were processed using Adobe Photoshop CS.

**Expression Analysis**

RNA for quantitative PCR was isolated from approximately five ear primordia of bde-McC, bde-TR864, bde-N868, and A619, using Trizol (Invitrogen) according to the manufacturer’s instructions. Approximately 1 μg of total RNA was used as a template for cDNA synthesis, using an oligo(dt) primer according to the manufacturer’s recommendations (Superscript III first-strand synthesis kit for RT-PCR; Invitrogen). Quantitative PCR was performed on a MyIQ single-color real-time detection system (Bio-Rad), and data were processed in MyIQ and Excel. Data are from at least three RT-PCR and quantitative PCR replicates. Data were normalized against gapdh levels (primers used were ZmGAPDH-F, 5′-CGTGGCTTCATGAGATGTT-3′, and ZmGAPDH-R, 5′-TGAAGG- CAGGAAGGGAAC-3′).

In situ hybridization was done as described previously (Bortiri et al., 2006), except tissue was fixed overnight in FAA. The template for the bde probe was generated by cloning a fragment of bde cDNA lacking the MADS box into the pGEM-T Easy vector (Promega) (primers used zag3-5F, 5′-CAGGCAACTCACAACAGAG-3′, and zag3-3R, 5′-ATTCTATGACACCGGCTATG-3′). This fragment was amplified using M13 forward and reverse primers and used as a template for in vitro transcription (T7 RNA polymerase; Promega) using DIG RNA labeling mix (Roche Diagnostics).

**Protein Interaction Analysis**

BDE and ZAG1 interactions were tested by cloning bde and zag1 cDNAs into pBDGAL4 and pADGAL4 vectors (Stratagene) and transforming bait and prey plasmids into Y187 and AH109 yeast strains, respectively. To create double transformants, Y187 and AH109 yeast were mated overnight and plated on selective media. To avoid BDE autoactivation in pBDGAL4, we created a truncated version of BDE that lacked the C-terminal domain by digesting pBDGAL4-ZAG3 with PstI (removes amino acids 184 to 257) and religating the vector.

For BiFC assays, full-length zag1 and bde were cloned into pSAT4-neYFP-N1 and pSAT4-eYFP-N1 vectors (Citovsky et al., 2006), respectively, in frame with YFP. For microbombardment, 2 μg of each test plasmid and 250 ng pRecA-RFP (a kind gift from Ian Small), as an internal control, was absorbed onto S1000d gold beads according to the manufacturer’s instructions (Seashell Technology) and bombarded onto onion epidermal peels using a Helios gene gun system (model PDS-1000/He; Bio-Rad) at a pressure of 1100 p.s.i. After 26 to 30 h, samples were mounted and examined for RFP and YFP expression. A total of four replicates were performed for each plasmid combination. For ZAG1- and BDE-expressing cells, 60 to 100 RFP-expressing cells were examined for each replicate. For negative controls, 30 to 70 RFP-expressing cells were examined for each replicate. Images were processed using Adobe Photoshop CS.

**Accession Number**

Sequence data from this article can be found in GenBank/EMBL data libraries under accession number NM_001111862 (bde [zag3]).

**Supplemental Data**

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

**Supplemental Figure 1.** Ectopic Meristems in bde Mutants.

**Supplemental Figure 2.** Additional bde Mapping Data.

**Supplemental Figure 3.** The LFM Retains FM Identity in bde Mutants.

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bearded-ear Encodes a MADS Box Transcription Factor Critical for Maize Floral Development
Beth E. Thompson, Linnea Bartling, Clint Whipple, Darren H. Hall, Hajime Sakai, Robert Schmidt and Sarah Hake

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