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barren inflorescence2 regulates axillary meristem development in the maize inflorescence

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SUMMARY

Organogenesis in plants is controlled by meristems. Shoot apical meristems form at the apex of the plant and produce leaf primordia on their flanks. Axillary meristems, which form in the axils of leaf primordia, give rise to branches and flowers and therefore play a critical role in plant architecture and reproduction. To understand how axillary meristems are initiated and maintained, we characterized the *barren inflorescence2* mutant, which affects axillary meristems in the maize inflorescence. Scanning electron microscopy, histology and RNA in situ hybridization using *knotted1* as a marker for meristematic tissue show that *barren inflorescence2* mutants make fewer branches owing to a defect in branch meristem initiation. The construction of the double mutant between *barren inflorescence2* and *tasselsheath* reveals that the function of *barren inflorescence2* is specific to the formation of branch meristems rather than bract leaf primordia. Normal maize inflorescences sequentially produce three types of axillary meristem: branch meristem, spikelet meristem and floral meristem. Introgression of the *barren inflorescence2* mutant into genetic backgrounds in which the phenotype was weaker illustrates additional roles of *barren inflorescence2* in these axillary meristems. Branch, spikelet and floral meristems that form in these lines are defective, resulting in the production of fewer floral structures. Because the defects involve the number of organs produced at each stage of development, we conclude that *barren inflorescence2* is required for maintenance of all types of axillary meristem in the inflorescence. This defect allows us to infer the sequence of events that takes place during maize inflorescence development. Furthermore, the defect in branch meristem formation provides insight into the role of *knotted1* and *barren inflorescence2* in axillary meristem initiation.

Key words: Meristem, Axillary meristem, Inflorescence, Floret, Flower, *barren inflorescence2*, *ramosa1*, *tasselsheath1*, *knotted1*, Maize

INTRODUCTION

Organogenesis occurs throughout the lifetime of a plant through the action of meristems (Steeves and Sussex, 1989). Meristems achieve this continual production of organ primordia by maintaining a central population of undifferentiated cells to replenish the meristem as primordia are produced laterally. The shoot apical meristem forms at the apex of the plant and produces leaf primordia laterally. Axillary meristems, which arise in the axils of leaf primordia, produce branches and flowers and therefore play an important role in the architecture and reproduction of plants.

Two models for axillary meristem initiation have been proposed. The ‘detached meristem’ theory proposes that the shoot apical meristem gives rise to axillary meristems during the production of leaf primordia (Steeves and Sussex, 1989). Evidence for the detached meristem theory is provided by histological analysis, which shows that cells in the axils of leaf primordia do not undergo differentiation (Wardlaw, 1943; Garrison, 1955; Sussex, 1955; Cutter, 1964; Remphrey and Steeves, 1984). The alternative ‘de novo’ model proposes that axillary meristems are induced from previously differentiated cells by the subtending leaf (McConnell and Barton, 1998). Axillary meristems can form from apparently differentiated cells in some species (Majumdar, 1942). Additional support for the de novo model comes from evidence that the adaxial (adjacent to the meristem or upper) surface of leaf primordia has competence to form axillary meristems (Sinha et al., 1993; Chuck et al., 1996; McConnell and Barton, 1998; Lynn et al., 1999). A major difference between the models is that the detached meristem theory proposes that axillary meristem initials remain undifferentiated while the de novo model implies that axillary meristems can arise from previously differentiated cells.

During vegetative development, growth of the axillary meristem is often delayed relative to the subtending leaf primordium such that the axillary meristem is not visible until late in leaf development (Steeves and Sussex, 1989). Upon the onset of reproductive development, growth of the axillary meristem accelerates such that the axillary meristem becomes

prominent early in leaf development (Kaplan, 1967; Hempel and Feldman, 1994). Coincident with the acceleration of axillary meristem growth, the subtending leaf grows less, forming a small bract leaf in some species (for example, *Antirrhinum*; Bradley et al., 1996), or is suppressed completely in other species (such as *Arabidopsis* and maize; Bonnett, 1948; Long and Barton, 2000). Thus in many species, the switch from vegetative growth (making leaves) to reproductive growth (making flowers) is accompanied by a switch from pronounced leaf development to pronounced axillary meristem development.

In maize, the reproductive phase is complicated by the production of reproductive branches that bear the flowers (Bonnett, 1948; McSteen et al., 2000). The male inflorescence, the tassel, is highly branched with long lateral branches at the base of the main spike (Fig. 1A). Short branches, called spikelet pairs, are produced by the main axis and the long branches. Each spikelet is composed of two reduced leaf-like glumes enclosing two florets (Fig. 1B). Each floret consists of two reduced leaves called the lemma and palea, two lodicules (the remnants of the petals) (Ambrose et al., 2000), three stamens and a tricarpellate gynoecium. In the tassel, the gynoecium aborts resulting in the formation of male florets (Cheng et al., 1983; Irish, 1996). The female inflorescence (the ear shoot) forms from an axillary meristem located in the axil of a leaf five to six nodes below the tassel. The ear does not produce long lateral branches but does produce paired spikelets with paired florets like the tassel. Subsequently, the lower floret and the stamens abort resulting in the formation of single female florets (Cheng et al., 1983; Irish, 1996).

To generate this complex inflorescence, three types of axillary meristem are produced sequentially in maize (Bonnett, 1948; Irish, 1997; McSteen et al., 2000). The first axillary meristems produced by the inflorescence meristem are the branch meristems. Branch meristems at the base of the tassel produce the long lateral branches while later arising branch meristems (also called spikelet pair primordia) produce two spikelet meristems. Each spikelet meristem forms two glumes and two floral meristems. Subsequently, each floral meristem gives rise to the floral organs. Therefore, unlike model dicotyledons such as *Antirrhinum* and *Arabidopsis*, which produce floral meristems directly from the inflorescence meristem, the maize inflorescence meristem produces branch and spikelet meristems before producing floral meristems.

To identify genes required for axillary meristem development, we isolated maize mutants with fewer branches and spikelets in the tassel. Here, we characterize the *barren inflorescence2* (*bif2*) mutant, which makes fewer ear shoots, branches, spikelets, florets and floral organs owing to defects in the formation and maintenance of all reproductive axillary meristems.

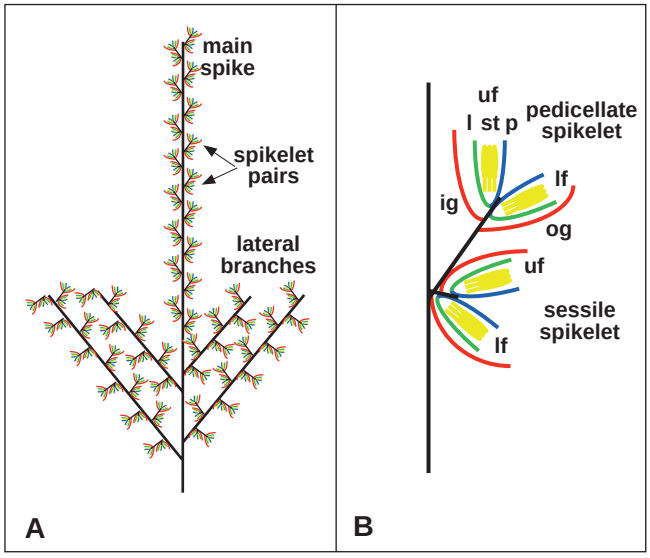

Fig. 1. Diagram of a normal tassel and spikelet pair. (A) Diagram of a normal tassel (male inflorescence). The tassel consists of a central main spike with long lateral branches at the base. Short branches called spikelet pairs cover the main spike and the lateral branches. (B) Diagram of a spikelet pair from a normal tassel. The pedicellate spikelet is borne on a pedicel while the sessile spikelet is attached at the base. Each spikelet contains two florets, the upper floret (uf) and the lower floret (lf) enclosed by two glumes, the inner glume (ig) and the outer glume (og). Each floret consists of lemma (l), palea (p), two lodicules (not shown) and three stamens (st).

MATERIALS AND METHODS

Origin of *bif2* alleles

The reference allele, *bif2-2354* was generated by EMS (ethylmethane sulfonate) mutagenesis by M. G. Neuffer (obtained from the Maize Coop Stock Center (www.ag.uiuc.edu/maize-coop) stock #301B; Neuffer and Briggs, 1994). Six additional alleles were identified from lines containing active *Mutator* (*Mu*) transposable elements: *bif2-1606* (P. Chomet, DeKalb, NJ), *bif2-47330* and *bif2-1512* (S. Briggs, Pioneer Hi-bred International, Johnston IA; Briggs and Johal, 1992), *bif2-70* and *bif2-77* (G. Johal, University of Missouri, Columbia, MO) and *bif2-1504* (R. Schneeberger and M. Freeling, University of California, Berkeley, CA). Each of the alleles failed to complement *bif2-2354* and/or *bif2-1606*. Introgression of the alleles into standard inbred genetic backgrounds did not show significant differences in phenotype between alleles. Therefore, the phenotypic and double mutant analysis was performed with *bif2-1606*.

bif2 maps to chromosome 1

bif2-2354 had previously been assigned to the long arm of chromosome 3 (Neuffer and Briggs, 1994) based on B-A translocation mapping (Beckett, 1993). We subsequently showed that *bif2* actually mapped to the long arm of chromosome 1 using both B-A mapping and RFLP analysis. *bif2-2354* and *bif2-1606* were crossed by the B-A translocation stocks, TB1La (Maize Coop Stock Center, stock#122A, which tests most of the long arm of chromosome 1) and TB3La (Maize Coop Stock Center, stock#327A, which tests the long arm of chromosome 3). *bif2-1606* and *bif2-2354* plants that were hypoploid for the long arm of chromosome 1 had a severe barren tassel phenotype, while *bif2-1606* and *bif2-2354* plants that were hypoploid for the long arm of chromosome 3 had a mild barren tassel phenotype, implying that *bif2* was either on the long arm of chromosome 1 or 3. RFLP mapping showed that *bif2* was unlinked to chromosome 3 and instead mapped near the centromere on the long arm of chromosome 1. *bif2-1606* maps within 3 cM of the RFLP marker *umc67* in bin 1.06 (0 recombinants out of 32 chromosomes).

Quantitative analysis

Quantitative analysis of the *bif2* mutant phenotype was performed with allele *bif2-1606* that had been backcrossed four times to the inbred lines B73 and A188, and three times to the inbred lines A619, W22 and W23. Branch and spikelet number were counted on plants

grown in the field in the summer (Brentwood, CA). Analysis of floral organ number was carried out with *bif2-1606* plants that had been backcrossed three times to A619, grown in the spring in the greenhouse (Albany, CA). The results presented were from all 103 spikelets of a single mutant plant, but similar results were observed in other mutant plants from the same genetic background. Similar trends, though with different severity, were obtained when *bif2* mutants were grown under different environmental conditions and when *bif2* mutants had been introgressed into other genetic backgrounds (B73, A188 and W22).

Double mutant analysis

tasselsheath-57333 (*tsh*) was obtained from S. Briggs (Pioneer Hi-Bred International, Johnston, IA) in the A632 genetic background (Briggs, 1992). *bif2*;*tsh* double mutants were identified as plants exhibiting characteristics of both parents segregating one sixteenth in the F_2 of a cross between *tsh-57333* and *bif2-1606*. Plants with *tsh* phenotypes were self pollinated in the F_2. Some of these families segregated one quarter *bif2*;*tsh* double mutants in the F_3 confirming the double mutant phenotype.

ramosa1-ref (*ra1)* was obtained from the Maize Coop Stock Center (stock#708A) and introgressed into the B73 genetic background. *bif2*;*ra1* double mutants were identified as plants with characteristics of both parents segregating one sixteenth in the F_2 of crosses between *ra1* and *bif2*. F_3 crosses were not performed because of sterility of the phenotype. However, the double mutant phenotype was observed in four separate families grown in the field over several seasons and was never observed in families segregating for either mutant alone.

tasselseed4-ref (*ts4*) was obtained from the Maize Coop Stock Center (stock#316A). *ts4*;*bif2* double mutants could not be identified in the F_2 owing to the presumed epistasis of *bif2*. Plants with the *ts4* phenotype were self-pollinated in the F_2. Some of these families segregated one quarter *bif2* mutant phenotype in the next generation confirming that *bif2* was epistatic to *ts4*.

SEM and histology

Families that were segregating *bif2* and normal siblings in the B73 genetic background were grown to 5-weeks old for tassels or 8-weeks old for ears. For scanning electron microscopy (SEM), inflorescences were dissected and molded with dental impression medium (Exaflex Type 3 viscosity, GCAmerica Inc, Chicago, IL). The molds were then filled with two ton epoxy resin (Ace Hardware, Oakbrook, IL), allowed to harden overnight and cured in a 60°C oven overnight. The casts were removed from the mold and allowed to outgas under vacuum for 3 days. Casts were sputter coated with gold palladium and viewed by SEM (ISI 30 model) at 10 kv accelerating voltage. For sectioning, inflorescences were dissected and fixed at 4°C overnight in 4% formaldehyde in phosphate-buffered saline for ears or FAA (3.7% formalin, 50% ethanol, 5% acetic acid) for tassels, dehydrated in an ethanol series and embedded in paraffin wax (Paraplast, Oxford Labware, St. Louis, MO). Sections 8 to 10 μm thick were cut with a Microm HM340 microtome and mounted on coated slides (Probe-On plus, Fisher Biotech). DIG-labeled antisense RNA probes of *kn1* were prepared and RNA in situ hybridization performed according to the method of Jackson et al. (Jackson et al., 1994). Immunolocalization with anti-KN1 antibody was performed according to the method of Smith et al. (Smith et al., 1992). For histological analysis, slides were dewaxed in histoclear (National Diagnostics, Atlanta, GA), hydrated in series, stained for 30 seconds in 0.05% Toluidine Blue O (TBO), rinsed, dehydrated and mounted with Merckoglas (Mikroskopic, Germany).

RESULTS

To identify genes required for axillary meristem development, we collected mutants that made few, if any, branches and spikelets in the tassel. Complementation tests showed that we had identified seven independent alleles of *bif2* (see Materials and Methods). Genetic analysis showed that *bif2* was a single, recessive nuclear mutation. *bif2* mapped close to the centromere on the long arm of chromosome one using genetic and molecular analysis (see Materials and Methods). As all seven alleles had the same severity of phenotype, we performed phenotypic and double mutant analyses with one allele, *bif2-1606* (hereafter referred to as *bif2*).

Fig. 2. *bif2* mutants make fewer branches and spikelets in a background-dependent manner. (A) Normal tassel after anthesis (B73 genetic background). The main spike and long lateral branches produce pairs of spikelets. (B) *bif2* mutant tassel in the B73 genetic background. In severe cases, *bif2* mutants produce no branches and almost no spikelets, resulting in a barren rachis (inflorescence stem). (C) *bif2* mutant tassel in the A188 genetic background. The tassel has a sparse appearance with few branches and few spikelets on the branches and main spike. (D) *bif2* mutant tassel in the A619 genetic background. Single spikelets form on the rachis. The tip of the rachis is split. (E) Normal ear (the female inflorescence). The outer protective husk leaves are removed to reveal rows of female florets with elongated silks covering the rachis. (F) *bif2* mutant ear. Inside the husk leaves is a bare rachis with no spikelets or florets. The tip of the rachis is split. Scale bar, 2.7 cm.

bif2 mutants produced fewer branches and spikelets

bif2 mutants had fewer lateral branches in the tassel. To quantify the defect, *bif2* mutants were backcrossed four times into standard inbred lines. The decrease in the number of branches produced by *bif2* mutants was dependent on genetic background (Fig. 2; Table 1). In the inbred line A188, which produced many lateral branches in the tassel and was early flowering (7 weeks to anthesis), the *bif2* phenotype was weak. After four backcrosses to A188, *bif2* mutants produced one or two branches whereas normal siblings had about 24 branches (Fig. 2C; Table 1). In B73, an inbred that produced relatively few tassel branches (Fig. 2A) and flowered late (9 weeks to

Table 1. *bif2* mutants make fewer branches, spikelets and ear shoots

		Tassel branch no.				Tassel spikelet no.				Ear shoot	
Inbred		Mean	s.d.	Range	*n*	Mean	s.d.	Range	*n*	%	*n*
A188	N	20	3.9	12-31	28	1162	165.6	1016-1346	3	100	28
	bif2	1	1.8	0-6	16	66.4	70.6	5-227	16	22.3	16
B73	N	10.1	1.8	7-15	25	573	n.d.	n.d.	1	100	25
	bif2	0	0	0	13	16.8	11.9	0-41	13	43.3	13

Branch and spikelet number were counted from tassels of *bif2* and normal plants grown under field conditions. Ear shoot refers to the percentage of plants that made at least one ear shoot.

N, normal siblings; s.d., standard deviation; *n*, no. of plants; n.d., not determined.

anthesis), the *bif2* phenotype was more severe. After four backcrosses to B73, *bif2* mutants produced no lateral branches in the tassel while normal siblings had approximately 10 branches (Fig. 2B; Table 1). *bif2* mutants also produced no lateral branches in the inbred line A619 (Fig. 2D), which normally has an intermediate number of tassel branches and time to anthesis.

Spikelet number was also drastically reduced in *bif2* mutants in a background-dependent manner (Table 1). Similar to the effect on branch number, the phenotype was weaker in A188, more severe in B73 and intermediate in A619. Depending on the inbred line and growing conditions, normal tassels produced 500 – 1000 spikelets in pairs (Table 1). In A188, *bif2* mutants produced an average of 66 spikelets compared to normal siblings that produced over a thousand (Table 1). In B73, *bif2* mutants produced an average of 17 spikelets compared to normal siblings, which produced about 500 spikelets (Table 1). As seen from the high standard deviations, the number of spikelets produced was still quite variable within a family.

Normal plants usually produced at least one ear shoot (the female inflorescence) in the axil of a leaf, five to six nodes below the tassel (Fig. 2E). In contrast to normal siblings, less than half of *bif2* mutants produced ear shoots (Table 1). When an ear shoot formed in *bif2* mutants, defects similar to those in the tassel were observed. A bare rachis (inflorescence stem) was seen inside the husk leaves (Fig. 2F). Sometimes a few spikelets were present, usually at the base of the rachis. The tip of the ear was sometimes fasciated and split into several growing points.

In contrast to the dramatic effect on inflorescence development, vegetative development of *bif2* mutants appeared normal. There were no obvious defects in leaf morphology or phyllotaxy and the number of leaves produced was not significantly different from wild type (data not shown).

bif2 mutants failed to initiate branch meristems

The absence of branches and spikelet pairs in *bif2* mutants was indicative of a very early defect in inflorescence development. Scanning electron microscopy (SEM) was used to determine when *bif2* inflorescence development differed from wild type. The inflorescence forms a convenient developmental series with branch meristems near the inflorescence apex and progressively older stages of development towards the base of the inflorescence stem. As early development of male and female inflorescences are similar and the *bif2* mutation affected both in the same way, we do not distinguish between them when referring to the inflorescence.

The first step in normal inflorescence development was the formation of branch meristems, visible as bumps, on the flanks of the inflorescence (Fig. 3A; Bonnett, 1948; Cheng et al., 1983). In contrast, *bif2* inflorescence meristems did not produce branch meristems (Fig. 3B). Undulations visible on the surface

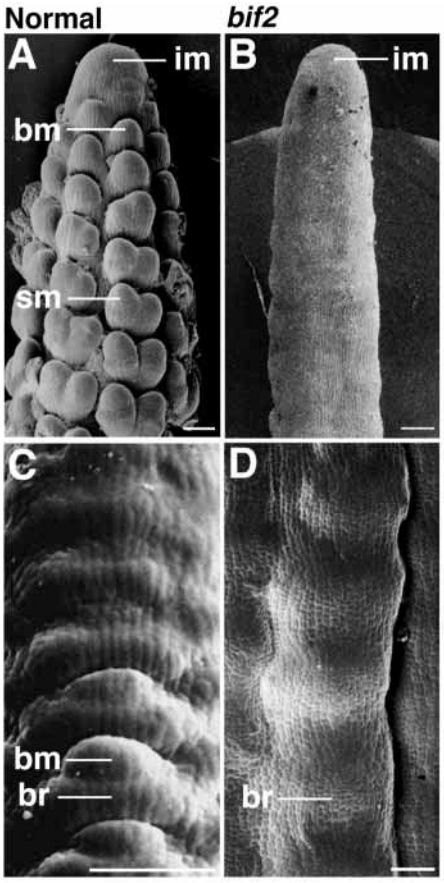

Fig. 3. *bif2* is required for branch meristem formation: scanning electron micrographs (SEM) of normal and *bif2* mutant inflorescences. (A) A normal male inflorescence at 5-weeks old. The inflorescence meristem (im) produces axillary meristems called branch meristems (bm) which then form two spikelet meristems (sm). (B) A *bif2* male inflorescence at 5-weeks old. The inflorescence meristem fails to produce branch meristems. Ripples are visible on the surface of the rachis. (C) A normal female inflorescence at 8 weeks of age. The higher magnification shows that branch meristems (bm) form in the axils of bract primordia (br), which are suppressed. (D) A *bif2* female inflorescence at 8 weeks. The ripples on the surface of the rachis resemble bract primordia. Bm, branch meristem; br, bract primordium; sm, spikelet meristem; im, inflorescence meristem. Scale bar, 200 μm.

of the *bif2* rachis (Fig. 3D) were similar to the bract primordia that normally subtend branch meristems (Fig. 3C). As in wild type, these bract primordia did not develop further. The SEM results suggest that *bif2* mutants do not produce branches and spikelet pairs because they do not produce branch meristems.

Histological analysis was performed to determine if there was any cellular evidence of branch meristem formation in *bif2* mutants. Meristematic cells stain more intensely with histological dyes than differentiated cells owing to their smaller vacuolar volume (Steeves and Sussex, 1989). In normal plants, the inflorescence meristem and its peripheral region stained intensely with Toluidine Blue O (TBO; Fig. 4A). Branch meristems with subtending bract primordia arose in this peripheral region. Branch meristems were first visible as densely stained groups of cells that extended many cell layers into the flanks of the inflorescence (Fig. 4B). Branch meristems remained densely stained later in development, as they grew out to form a bulge. Bract primordia that subtended branch meristems were not as densely stained. These bract primordia did not develop further and became less obvious as the branch meristems grew out (base of Fig. 4A). In *bif2* mutants, the inflorescence meristem and periphery were densely stained as in wild type (Fig. 4C). Primordia that arose from the flanks of the inflorescence were less densely stained than wild-type branch meristems and instead resembled bract primordia. These bract primordia had stronger staining on their adaxial side (side facing the inflorescence meristem) than abaxial side (side facing away from the meristem) (arrow in Fig. 4D). The staining of these primordia extended only a few cells thick (Fig. 4D), unlike the staining of branch meristems in wild type (Fig. 4B). Farther from the inflorescence tip, the dense staining disappeared, as the cells became vacuolated. There was no evidence of cell wall collapse indicative of cell death.

To test whether the densely stained cells on the adaxial side of bract primordia in *bif2* mutants were cells at an early stage of meristem formation or were indicative of the normal differences in cytoplasmic density that characterize the adaxial and abaxial sides of leaf primordia, we performed RNA in situ hybridization using *knotted1* (*kn1*) as a marker for meristematic tissue (Jackson et al., 1994). In normal inflorescences, *kn1* was highly expressed in the inflorescence meristem and was specifically down regulated on the flanks of the inflorescence meristem (Fig. 5A). The down regulation of *kn1* was the first indication of bract primordium initiation. *kn1* was also expressed in a small group of cells located between two successive bract primordia that we hypothesized were branch meristem initials (Fig. 5A). *kn1* was subsequently highly expressed in branch meristems as they grew out. Later in development, *kn1* was also expressed in spikelet and floral meristems as they formed (Fig. 5D). In *bif2* inflorescences, *kn1* was expressed in the inflorescence meristem and was down regulated in bract primordia as in wild type (Fig. 5B). Unlike wild-type inflorescences, however, *kn1* was not expressed anywhere along the flanks of the inflorescence meristem even later in development (Fig. 5E). There was no evidence of branch meristem formation or of branch meristem initials. Immunolocalization with the anti-KN1 antibody (Smith et al., 1992) revealed a similar pattern of KN1 protein localization in *bif2* mutants (Fig. 5C). Occasional fasciation of *bif2* inflorescence meristems was also observed (Fig. 5B,C,F). The absence of *kn1* expression in the primordia on the flanks of the inflorescence provides strong evidence that *bif2* mutants fail to initiate branch meristems.

We used a genetic test to determine if *bif2* was specifically required for axillary meristem function or whether it also played a role in the formation of the subtending bract leaf primordium by constructing double mutants with *tasselsheath* (*tsh*; Briggs, 1992). In *tsh* mutants, bract primordia that subtend branch meristems were no longer suppressed (Fig. 6A,C). Large bracts subtended the long branches at the base of the tassel (Fig. 6A) whereas smaller bracts subtended the spikelet pairs on the main spike (Fig. 6C). These bracts became smaller acropetally such that they were no longer visible on the upper portion of the main spike. If *bif2* was required for the formation of bract primordia as well as axillary branch meristems, then the *bif2*;*tsh* double mutant would have the same phenotype as *bif2* mutants. Instead, the *bif2*;*tsh* double mutant had an additive phenotype (Fig. 6B,D). At the base of the tassel, large bracts were produced but there were no branches in their axils (Fig. 6B). On the lower half of the main spike, smaller bracts were produced but no spikelet pairs formed in their axils (Fig. 6D). The double mutant with *tsh* clearly shows that *bif2* is not required for the formation of bract primordia but is specifically required for the formation of branch meristems in the axils of bract primordia.

If *bif2* was required for branch meristem formation then it should be epistatic to mutants affecting later stages of development. To test this hypothesis, double mutants with *tasselseed4* (*ts4*) (Hayes and Brewbaker, 1928; Phipps, 1928) were constructed. *ts4* is required for the transition from branch meristem to spikelet meristem identity (Irish, 1997). In *ts4* mutants, branch meristems continued to reiterate the formation of branch meristems resulting in tassels with increased indeterminacy (Fig. 6E). If *bif2* acted before *ts4*, then the *bif2*;*ts4* double mutant would have the same phenotype as *bif2*. In agreement with this hypothesis, *bif2* was epistatic to *ts4* (Fig. 6F) (see Materials and Methods for genetic evidence).

Branch meristems that formed in *bif2* mutants were defective

We next determined if *bif2* played a role in the function of branch meristems, once branch meristems had initiated. On the main spike of normal tassels, branch meristems produced short branches consisting of two spikelets, the pedicellate spikelet (with a pedicel) and the sessile spikelet (without a pedicel; Figs 1B, 7D). When spikelets formed in *bif2* mutants, most of them occurred singly instead of in pairs (50-75%; Fig. 7E). The spikelets that formed had pedicels, implying that the pedicellate spikelet had formed though the pedicels were longer than normal. Intermediates were sometimes seen in which the sessile spikelet was visible as a filament (6.8%) or as a single glume (9.7%) attached at the base of the pedicellate spikelet. Therefore, branch meristems that formed in *bif2* mutants were defective because they were unable to initiate the normal complement of spikelets.

To investigate the role of *bif2* in the branch meristem we constructed the double mutant between *bif2* and a mutant that made extra spikelets, *ramosa1* (*ra1*; Gernart, 1912). *ra1* mutants made more spikelets because long branches were produced in place of spikelet pairs (Fig. 7A). *bif2* was completely epistatic to *ra1* when the families had a severe *bif2* phenotype in which no branch meristems formed (data not

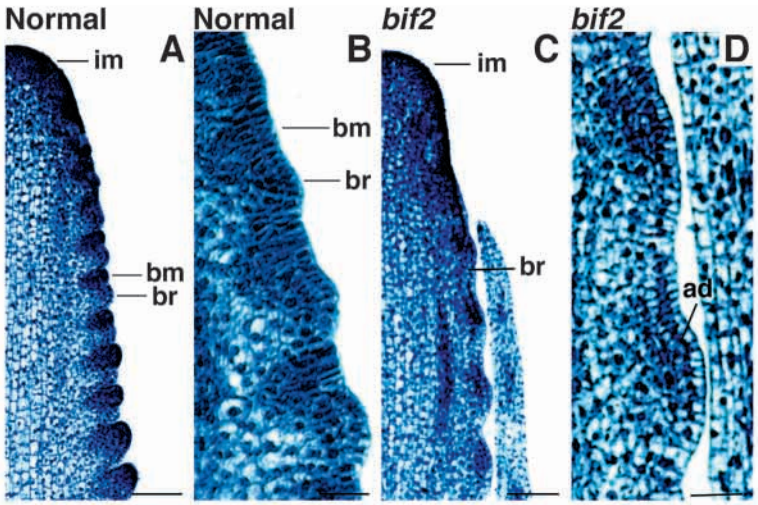

Fig. 4. *bif2* is required for branch meristem formation: histological analysis of normal and *bif2* mutant inflorescences. (A) Longitudinal section of a normal female inflorescence stained with TBO. The apex and the periphery of the inflorescence meristem (im) stain intensely. Branch meristems (bm) form densely staining bulges in the axils of less densely stained bract primordia (br). Scale bar, 80 μm. (B) Higher magnification of A. The branch meristem is visible as several densely staining cell layers. Scale bar, 250 μm. (C) Longitudinal section of a *bif2* female inflorescence stained with TBO. Like normal inflorescences, the inflorescence apex and periphery are densely stained, but branch meristems do not bud from the axils of bract primordia as in wild type. Scale bar, 80 μm. (D) Higher magnification of *bif2* bract primordia (from C) showing that several densely staining cells are visible on the adaxial side of the bract primordium (ad) though the staining does not extend through as many cell layers as normal. Note that *bif2* bract primordia are larger than normal bract primordia. Scale bar, 250 μm. ad, adaxial side of bract primordium; bm, branch meristem; br, bract primordium; im, inflorescence meristem.

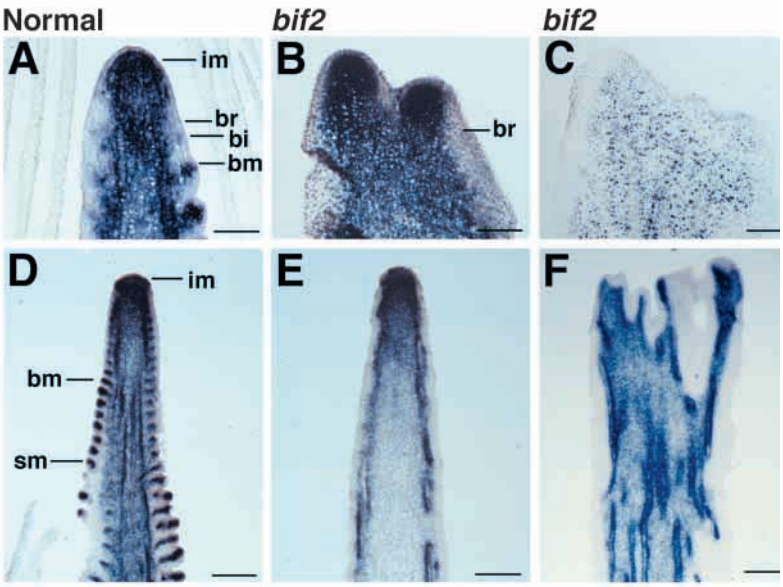

Fig. 5. *bif2* is required for branch meristem formation: expression analysis with *kn1*. (A) RNA in situ hybridization of *kn1* in a normal male inflorescence. *kn1* is strongly expressed in the inflorescence meristem (im) and is downregulated on the flanks of the inflorescence as bract primordia initiate (br). Branch meristem initials (bi) are visible as a small group of *kn1*-expressing cells separating successive bract primordia. *kn1* is highly expressed in branch meristems (bm) as they grow out. (B) *kn1* RNA in situ hybridization in a *bif2* male inflorescence. In this example, the inflorescence apex is fasciated and has split into two growing points. As in normal inflorescences, *kn1* is expressed in the inflorescence meristem and is downregulated as bract primordia (br) initiate. Unlike normal, *kn1* is not expressed on the flanks of the inflorescence and there is no evidence of branch meristem formation or branch meristem initials.
(C) Immunolocalization of KN1 protein in a *bif2* male inflorescence. KN1 protein is found in the inflorescence meristem but not on the flanks of the meristem. Note that KN1 protein extends into the epidermal layer of the inflorescence meristem (Smith et al., 1992; Jackson et al., 1994). (D) *kn1* RNA in situ hybridization in a normal female inflorescence. *kn1* is expressed in the inflorescence (im), branch (bm) and spikelet meristems (sm) as well as in the stem and vasculature. (E) *kn1* RNA in situ hybridization in a *bif2* female inflorescence. *kn1* RNA is not present on the flanks of the inflorescence owing to the absence of branch, spikelet and floral meristems. (F) *kn1* RNA in situ hybridization in a fasciated *bif2* female inflorescence. Down regulation of *kn1* within the inflorescence meristem occurs when the inflorescence apex has split into separate growing points. Bi, branch meristem initials, bm, branch meristem; br, bract primordium; im, inflorescence meristem;,sm, spikelet meristem. Scale bars, (A-C) 100 μm; (D-F) 300 μm.

shown). However, *bif2*;*ra1* double mutants (Fig. 7B) could be distinguished in families with less severe *bif2* phenotypes, in which branch meristems formed (Fig. 7C). The branches on *bif2*;*ra1* double mutants were elongated like *ra1* branches, but produced fewer spikelets than *ra1* single mutants. For example, at a mid point on the tassel main spike, a *ra1* branch produced 13 spikelets (Fig. 7G) while a *bif2*;*ra1* branch produced only three spikelets (Fig. 7F). As the number of spikelets produced by *bif2* branch meristems is affected even in a *ra1* mutant background we infer that *bif2* is required for branch meristem maintenance or for spikelet initiation.

Spikelet and floral meristems were also defective in *bif2* mutants

The few spikelets that formed on a *bif2* mutant tassel produced fewer florets with fewer floral organs. We quantified the defect by dissecting spikelets and counting organ number from *bif2* mutants that had been backcrossed into the inbred line A619 in which *bif2* mutants had an intermediate phenotype. Normal spikelets had two glumes and two florets. Each floret consisted of a lemma, palea, two lodicules and three stamens (Fig. 8A). Spikelets on *bif2* mutants displayed a range of phenotypes (Fig. 8B-D). In the most severe cases,

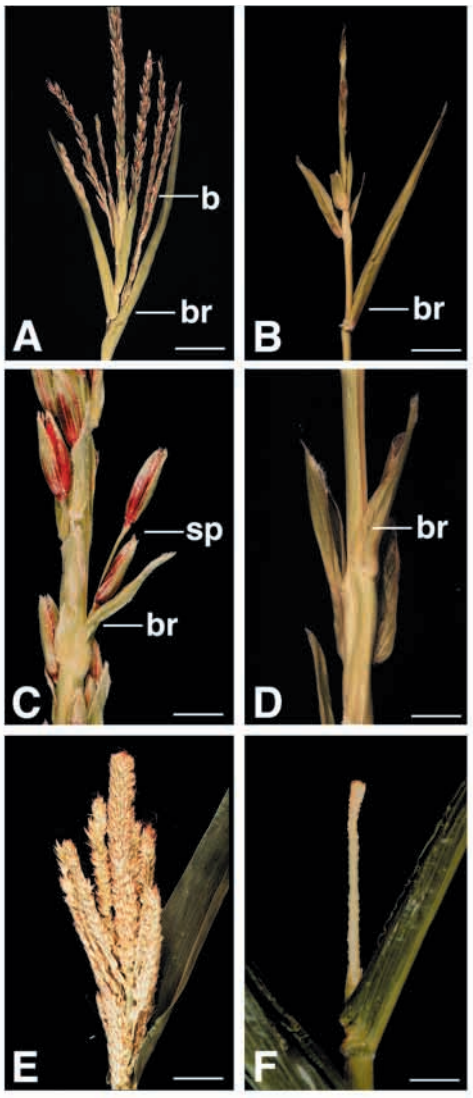

Fig. 6. *bif2* is required for branch meristem formation: double mutant analysis with *tsh* and *ts4*. (A) At the base of the *tsh* mutant tassel, large bracts (br) subtend the long branches (b). Scale bar, 3.33 cm. (B) At the base of the *bif2*;*tsh* double mutant tassel, large bracts (br) form but no branches are produced in the axils of the bracts. Scale bar, 3.33 cm. (C) On the main spike of the *tsh* mutant tassel, small bracts (br) subtend the spikelet pairs (sp). Scale bar, 0.7cm. (D) On the main spike of the *bif2*;*tsh* double mutant tassel, small bracts are produced as in *tsh* mutants, however, no spikelet pairs form in the axils of the bracts as in *bif2* mutants. Scale bar, 0.6 cm. (E) *ts4* mutant tassels are highly branched because of a delay in the transition from branch to spikelet meristem identity (Irish, 1997). Scale bar, 1.8cm. (F) The *bif;ts4* double mutant tassel has the same phenotype as a *bif2* mutant tassel. Note that in this case, the *bif2* mutant phenotype is severe and the tip of the rachis is fasciated. Scale bar,1.8 cm. B, branch; br, derepressed bract leaf; sp, spikelet pair.

spikelets consisted of one or two glumes with no florets (9.7%) (Fig. 8D). When florets formed, floral organs were missing from both florets. The upper floret was more severely affected than the lower floret (Fig. 8E). Only a quarter of *bif2* upper florets had the normal complement of organs (lodicules were not counted because of their small size). Phenotypes of the remaining upper florets ranged from florets missing one organ to florets consisting of only one organ (Fig. 8E). In extreme cases, the upper floret was absent or replaced by a filamentous structure (Fig. 8C). The majority of *bif2* lower florets were normal (Fig. 8B), while the remainder had two stamens instead of three (Fig. 8C). The floral defects mostly involved the absence of inner whorl organs though sometimes there were one or two extra organs resembling the lemma or palea (10.7%) and occasionally there were three florets instead of two (8.7%). Other defects seen were deformed stamens, missing lodicules and splitting of the lemma and palea. The defect in the production of glumes and florets indicates that *bif2* plays a role in the spikelet meristem while the defect in the production of floral organs indicates that *bif2* plays a role in the floral meristem. As most of the defects involve a reduction in the numbers of organs produced we infer that *bif2* plays a role in spikelet and floral meristem maintenance.

DISCUSSION

We have characterized the *bif2* mutant of maize, which makes fewer branches in the inflorescence. Genetic and histological analyses suggest that *bif2* is required for initiation of branch meristems and leads to a model for the role of *kn1* and *bif2* in axillary meristem initiation. Characterization of *bif2* mutants after introgression into lines in which the phenotype was less

Fig. 7. *bif2* is required for branch meristem maintenance: double mutant analysis with *ra1*. (A) In *ra1* mutant tassels, spikelet pairs are converted to branches resulting in a highly branched tassel (Gernart, 1912). Scale bar, 3.33 cm. (B) *bif2*;*ra1* double mutant tassels have a similar phenotype to *bif2* mutants, except that when branches form they have more spikelets than *bif2* mutants. (C) *bif2* mutant tassel. In this genetic background, the *bif2* mutant phenotype is weak and single spikelets form on the main spike. D to G are branches dissected from midway along the main spike of a family segregating normal (D), *bif2* (E), *bif2*;*ra1* (F) and *ra1* (G) mutant plants. Scale bar, 600 μm. (D) Normal spikelet pair with pedicellate and sessile spikelet. (E) *bif2* mutant spikelet. Note that *bif2* mutants produce spikelets singly instead of in pairs and that the pedicel is elongated. (F) *bif2*;*ra1* double mutant branch with several spikelets. G) *ra1* mutant branch with many spikelets.

severe reveals additional roles of *bif2* later in development. The spikelet and floral defects suggest that *bif2* plays a role in meristem maintenance and allows us to infer, for the first time, the sequence of events that occur during maize inflorescence development.

Role of *bif2* in branch meristem initiation

We show, using SEM and histology, that *bif2* mutants are unable to produce branches owing to an inability to form branch meristems. The branch meristem normally forms in the axil of a bract leaf which is suppressed in maize (Bonnett, 1948). In order to determine if the *bif2* defect also affects the subtending bract leaf primordia, we constructed the double mutant between *bif2* and *tsh*. In *tsh* mutants, bract leaves grow out, suggesting that the wild-type function of *tsh* is to repress bract outgrowth. Bract leaves also grow out in the *bif2;tsh* double mutant showing that *bif2* is not required for bract formation. This result provides convincing evidence that *bif2* is specifically required for the formation of branch meristems in the axils of bract leaf primordia.

Having demonstrated a role for *bif2* in the formation of branch meristems, we tested whether *bif2* was required for the initiation of branch meristems using *kn1* as a marker for meristems. *kn1* is a homeobox gene which is down-regulated within the meristem as lateral organ primordia are initiated (Smith et al., 1992; Jackson et al., 1994). The first indication of bract leaf initiation is the down regulation of *kn1* on the flanks of the inflorescence meristem. Similar down-regulation of *SHOOTMERISTEMLESS*, an *Arabidopsis kn1* homologue, occurs during bract formation in *Arabidopsis* (Long and Barton, 2000). Bract primordia are flanked by small groups of *kn1*-expressing cells that are in continuity with *kn1*-expressing cells in the inflorescence meristem and stem. We propose that these groups of cells are branch meristem initials. In *bif2* mutants, *kn1* is down regulated in bract primordia as in normal plants, supporting our genetic studies with *tsh* and suggesting that at least this aspect of bract formation occurs normally in *bif2* mutants. However, unlike wild-type inflorescences, *kn1* is not expressed in the axils of these bract primordia. It is possible that the densely cytoplasmic cells visible on the adaxial side of the bract primordia are competent to respond to signals to form an axillary meristem. However, as these cells do not express *kn1*, it is more likely that they result from the normal differences in cytoplasmic density that occur between the adaxial and abaxial sides of leaf primordia (Hagemann, 1970). Similarly, *kn1* homologues are not expressed on the flanks of the inflorescence meristem in other plants that fail to initiate axillary meristems (Reinhardt et al., 2000; Vernoux et al., 2000). As no evidence of branch meristem initiation is found in *bif2* mutants using *kn1* as an in situ probe, we conclude that *bif2* is required for branch meristem initiation.

We considered the role of *bif2* and *kn1* in axillary meristem initiation in light of existing theories on the origin of axillary meristems. One theory suggests that axillary meristems arise de novo from the adaxial side of leaf primordia (McConnell and Barton, 1998; Lynn et al., 1999). All of the indicators suggest that bract leaf primordia are normal in *bif2* mutants; they display down regulation of *kn1*, adaxial/abaxial distinctions, and elongate in a *tsh* mutant background. Thus, if axillary meristems arise directly from leaf primordia, then in *bif2* mutants, cells that will give rise to the axillary meristem are specifically defective in receiving that signal from the leaf. The other theory, referred to as the detached meristem theory, suggests that axillary meristem initials remain in a meristematic state in the axils of leaf primordia as leaf primordia separate from the inflorescence meristem (Steeves and Sussex, 1989). Thus, axillary meristem initials never differentiate. Our analysis of the expression of *kn1* in normal inflorescences, supports this theory as *kn1*, which is known to maintain cells in an undifferentiated state (Sinha et al., 1993; Kerstetter et al., 1997), is expressed in branch meristem initials. We propose that axillary meristems do not form in *bif2* mutants because branch meristem initials fail to maintain *kn1* expression and hence differentiate. Thus, BIF2 responds to the signal for axillary meristem formation, then, directly or indirectly, maintains *kn1* expression in the branch meristem.

bif2 mutants share similarities with mutants in *Arabidopsis*, tomato and rice that fail to make axillary or floral meristems (Okada et al., 1991; Szymkowiak and Sussex, 1993; Bennett et al., 1995; McConnell and Barton, 1995; Talbert et al., 1995; Przemeck et al., 1996; Bohmert et al., 1998; Chen et al., 1999; Lynn et al., 1999; Sawa et al., 1999; Komatsu et al., 2001; Otsuga et al., 2001). However, unlike many of these mutants, *bif2* mutants do not appear to affect leaf formation (Okada et al., 1991; Bennett et al., 1995; Talbert et al., 1995; Przemeck et al., 1996; Bohmert et al., 1998) or apical meristem formation (McConnell and Barton, 1995; Talbert et al., 1995; Chen et al., 1999; Lynn et al., 1999). In *Arabidopsis*, *PINOID*, *PINFORMED* and *MONOPTEROS* are implicated in auxin transport or perception (Okada et al., 1991; Bennett et al., 1995; Przemeck et al., 1996; Galweiler et al., 1998; Hardtke and Berleth, 1998; Christensen et al., 2000) while in tomato, *lateral suppressor* is implicated in gibberellic acid signaling (Schumacher et al., 1999) raising the possibility that BIF2 responds to a hormonal signal for axillary meristem formation.

In addition to the failure to initiate branch meristems, *bif2* mutants have a fasciated inflorescence meristem. Fasciation is also seen in other mutants that fail to make floral meristems (Bennett et al., 1995), in mutants that fail to make organs (Laufs et al., 1998) as well as in mutants that make extra organs (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). It is unlikely that the fasciation of the inflorescence meristem is directly responsible for the branch meristem defect in *bif2* mutants as fasciation occurred infrequently and not until relatively late in inflorescence development. Furthermore, increasing the size of the inflorescence meristem using the *Fascicled1* mutation (Orr et al., 1997) did not correct the ability of *bif2* mutants to initiate branch meristems (our unpublished results). Rather, fasciation may be a secondary effect of the failure to initiate branch meristems. We suggest that the inflorescence meristem fasciates because *kn1*-expressing cells do not detach from the inflorescence meristem to form branch meristem initials.

Role of *bif2* in meristem maintenance

The *bif2* mutant was introgressed into inbred lines in which the phenotype was less severe, allowing us to identify additional roles of the wild-type gene later in development. When branch meristems form in *bif2* mutants, they often make single spikelets instead of paired spikelets. In some cases, the spikelet pair consists of a normal pedicellate spikelet and a partial sessile spikelet consisting of one or two glumes. This result

could be explained if branch meristems in *bif2* mutants make a pedicellate spikelet but have insufficient cells remaining to form a complete sessile spikelet. This defect suggests that the wild-type function of *bif2* is to maintain branch meristems. In support of this conclusion, *bif2* mutants are defective at making multiple spikelets even in a *ra1* mutant background. Once spikelet meristems initiate in *bif2* mutants, they usually produce defective florets. The upper floret is consistently more affected than the lower floret and is sometimes replaced by a filamentous structure. This result suggests that, in *bif2* mutants, the spikelet meristem sets aside cells to form the lower floret but then has insufficient cells left for the formation of a complete upper floret. This defect suggests that *bif2* plays a role in spikelet meristem maintenance during wild-type development. The presence of fewer floral organs in *bif2* mutants provides evidence that the *bif2* gene is also required for floral meristem maintenance. Organs are most often missing from the center of the floret implying that, in *bif2* mutants, the floral meristem is either consumed during the production of the outermost floral organs or is smaller from inception. Other mutants, such as *shootmeristemless* (*stm*) and *wuschel* (*wus*) in *Arabidopsis*, that are defective in floral meristem maintenance also have fewer floral organs in inner whorls (Endrizzi et al., 1996; Laux et al., 1996). However, unlike *stm* and *wus*, *bif2* mutants specifically affect maintenance of axillary meristems without affecting maintenance of the shoot apical meristem. We propose that *bif2* is required for maintenance of all axillary meristems in the inflorescence, the branch, spikelet and floral meristem.

The rare occurrence in *bif2* mutants of spikelets with three florets or the rare florets with one or two extra outer whorl organs could be a secondary effect of the formation of single spikelets or single florets. For example, if a branch meristem makes a single spikelet perhaps the spikelet meristem is slightly larger than normal and hence can give rise to three instead of two florets. Similarly, if a spikelet meristem allocates all its cells into a single floret then perhaps this floret has the capacity to make more organs. Alternative models for the role of *bif2*, including a role in primordia initiation, are also possible. In fact, similar mutant phenotypes in *Arabidopsis* have been interpreted as being due to a failure in primordia development (Christensen et al., 2000; Vernoux et al., 2000). The distinction between meristem maintenance and primordia outgrowth may be a matter of definition. As organs form from meristems, the failure to make organs can be considered a failure in the meristem itself.

Implications for maize inflorescence development

In normal maize inflorescence development, the branch meristem makes two spikelet meristems and the spikelet meristem makes two floral meristems (Bonnett, 1948; McSteen et al., 2000). SEM studies do not fully clarify which of the two spikelet meristems or which of the two floral meristems forms first (Cheng et al., 1983). Analysis of the *bif2* mutant phenotype, however, sheds light on this process. When *bif2* mutants make spikelet pairs, the pedicellate spikelet preferentially forms. This suggests that during normal development, the cells that will give rise to the pedicellate spikelet are allocated before cells that will give rise to the sessile spikelet. The fact that the upper floret is missing or more severely affected than the lower floret in *bif2* mutants, implies that the lower floret normally forms first as suggested by the lateral branching model for floret development (Chuck et al., 1998). Although, it is formally possible that the sequence of events is altered by the *bif2* mutation, we infer that during normal inflorescence development, the branch meristem forms

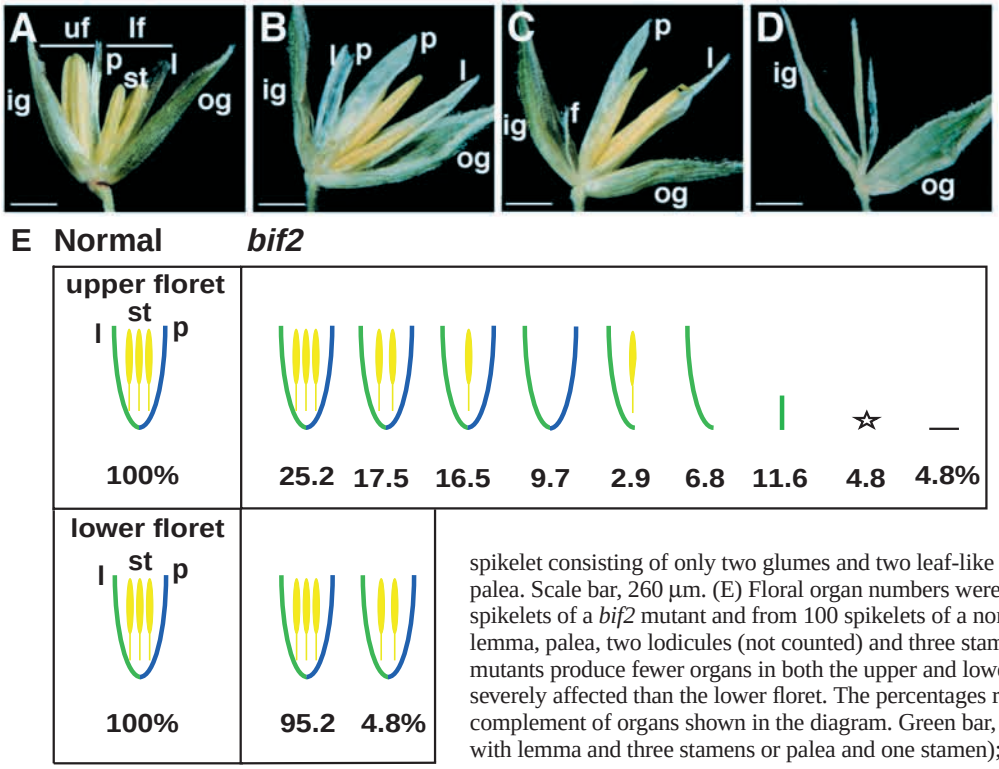

Fig. 8. *bif2* is required for spikelet and floral meristem maintenance. (A) Normal spikelet dissected open to reveal the two florets. The lower floret (lf) is flanked by the outer glume (og) and the upper floret (uf) is flanked by the inner glume (ig). Each floret consists of a lemma (l; flanked by the glume), palea (p; separating the flowers), lodicules (not visible) and three stamens (st). Scale bar, 230 μm. (B) *bif2* spikelet. The upper floret consists of lemma and palea only while the lower floret is normal. Scale bar, 230 μm. (C) *bif2* spikelet. The upper floret is replaced by a filamentous structure (f) and the lower floret has two stamens instead of three. Note that the stamens are deformed. Scale bar, 210 μm. (D) *bif2* spikelet consisting of only two glumes and two leaf-like structures that resemble the lemma or palea. Scale bar, 260 μm. (E) Floral organ numbers were counted from florets dissected from 103 spikelets of a *bif2* mutant and from 100 spikelets of a normal sibling. Normal florets have a lemma, palea, two lodicules (not counted) and three stamens in both upper and lower florets. *bif2* mutants produce fewer organs in both the upper and lower floret, though the upper floret is more severely affected than the lower floret. The percentages refer to the number of florets with the complement of organs shown in the diagram. Green bar, filamentous structure; star, other (florets with lemma and three stamens or palea and one stamen); dash, no floret.

the pedicellate spikelet meristem followed by the sessile spikelet meristem, then each spikelet meristem forms the lower floral meristem followed by the upper floral meristem.

Loss of *bif2* function does not completely abolish the ability of the maize inflorescence to make branches, spikelets and florets. The variable expressivity and background dependence of the phenotype provides evidence that additional factors are involved in branch, spikelet and floret development in maize. Differences in meristem size between inbreds could be partly responsible for the background dependence (Vollbrecht et al., 2000). Partial redundancy with other genes required for meristem function may also be involved. In fact, several other mutations in maize condition the phenotype of a reduction in branch, spikelet and floret number. For example, loss-of-function mutations in *kn1* result in fewer branches and spikelet pairs owing to defects in inflorescence meristem maintenance (Kerstetter et al., 1997). Mutants such as *barren stalk1* (*ba1*), *Suppressor of sessile spikelet1* (*Sos1*) and *Barren inflorescence1* (*Bif1*) have fewer branches and spikelets owing to defects similar to those in *bif2* mutants (Coe et al., 1988; Doebley et al., 1995). Double mutant analysis shows that there are multiple genetic pathways for branch meristem formation in the maize inflorescence (our unpublished results). Cloning of *bif2* and the other barren inflorescence mutants will provide further insight into the mechanisms of axillary meristem development.

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