Maize *SBP-box* transcription factors *unbranched2* and *unbranched3* affect yield traits by regulating the rate of lateral primordia initiation

George S. Chuck[a,1], Patrick J. Brown[b], Robert Meeley[c], and Sarah Hake[a]

[a]Plant Gene Expression Center, Albany, CA 94710; [b]Department of Crop Sciences, University of Illinois, Urbana, IL 61801; and [c]Pioneer Hi-Bred International, Johnston, IA 50131

Edited by Enrico Sandro Coen, John Innes Centre, Norwich, United Kingdom, and approved November 20, 2014 (received for review April 22, 2014)

The separation of male and female flowers in maize provides the potential for independent regulation of traits that affect crop productivity. For example, tassel branch number controls pollen abundance and length of shedding time, whereas ear row number directly affects kernel yield. Mutations in duplicate *SBP-box* transcription factor genes *unbranched2* (*ub2*) and *ub3* affect both of these yield traits. Double mutants display a decrease in tassel branch number and an increase in ear row number, both of which are enhanced by loss of a related gene called *tasselsheath4* (*tsh4*). Furthermore, triple mutants have more tillers and leaves—phenotypes seen in *Corngrass1* mutants that result from widespread repression of *SBP-box* genes. Immunolocalization of UB2 and UB3 proteins revealed accumulation throughout the meristem but absence from the central domain of the meristem where cells regenerate. Thus, *ub2*, *ub3*, and *tsh4* function as redundant factors that limit the rate of cell differentiation to the lateral domains of meristems. When these genes are mutated, cells are allocated to lateral primordia at a higher rate, causing a net loss of cells from the central domain and premature termination of the inflorescence. The *ub3* locus is tightly linked to quantitative trait loci (QTL) for ear row number and tassel branch number in both the nested association mapping (NAM) and intermated B73 by Mo17 (IBM) populations of maize recombinant inbreds, indicating that this gene may be agronomically important. Analysis of ear and tassel QTL across biparental families suggests that multiple mutations in *ub3* independently regulate male and female inflorescence development.

maize | meristem | yield | inflorescence | QTL

Meristems are groups of totipotent cells responsible for forming all of the tissues and organs of plants throughout their lifecycle, and as such, they have a direct effect on crop yield. Unlike animal systems, where growth is determinate, plants display indeterminate growth and constantly regenerate cells to maintain their apical meristems. If the rate of lateral primordia initiation is unregulated, too many primordia initiate at the expense of the meristem. Hence, coordination is required between pathways that renew the apical meristem and deplete it through initiation of lateral primordia.

The rate of lateral organ initiation from the meristem is characterized by the plastochron index (1). A plastochron is defined as the amount of time between successive lateral organ initiation events. Several genes have been described that regulate plastochron in grasses. The first gene described in maize was *terminal ear1* (*te1*), which encodes an *MEI-2*–like RNA-binding protein (2) as well as its ortholog in rice *PLASTOCHRON2* (*PLA2*) (3). Both mutants initiate several extra leaves, even into the reproductive phase when leaf initiation is normally suppressed. The *pla1* mutant in rice displays a similar phenotype. *PLA1* encodes a cytochrome (P450) and is expressed in leaf bases and internodes but not meristems (4), similar to the expression of *te1* in maize (2). The fact that these plastochron regulators affect meristem function and yet, are not expressed in the meristem indicates that they may function noncell-autonomously. This idea is supported by work in *Arabidopsis*, where it was shown that the *SQUAMOSA PROMOTER BINDING* (*SBP*)-*box* transcription factors *SPL9* and *SPL15* function noncell-autonomously to regulate plastochron index independent of the *PLA1* pathway (5). This finding raises the intriguing possibility that SPB-box proteins or their downstream targets may act as mobile signals that travel to meristems to act as inhibitory factors and thereby, regulate the timing of leaf initiation.

Positional cloning of *Corngrass1*, a dominant phase-change mutant in maize, revealed that it overexpresses miR156, which represses *SBP-box* genes (6). In *Arabidopsis*, *SBP-box* genes have been shown to play a role in regulating the rate of leaf initiation (5), flowering time, and developmental timing (7). In maize, the *SBP-box* mutant *tasselsheath4* (*tsh4*) showed that these transcription factors also play an important role in suppression of leaf development during the floral phase (8). Interestingly, derepression of floral leaves was linked to reduction of tassel branches in *tsh4*, raising the possibility that *SBP-box* genes control partitioning of cells between lateral organs vs. meristems. This hypothesis was supported by studies showing that branch meristem (BM) markers, such as *ramosa2* (*ra2*) (9), are ectopically expressed in the derepressed leaves of *tsh4* (10). Such results are consistent with the phytomer concept for plant development, in which leaf, axillary meristem, and internode all form from a common group of progenitor cells that must be directed to specific compartments (11). Thus, if plastochron regulators, such as *SBP-box*, factors are missing, cells are inappropriately directed to the wrong compartment at the expense of other phytomer components.

A reverse genetic analysis of genes similar to *tsh4* revealed a pair of redundant genes [*unbranched2* (*ub2*) and *ub3*] that functions in the specification of lateral primordia, such as leaves, tassel branches, and kernels. Immunolocalization using anti-UB3 serum

Significance

Crop yields are dependent on the number of lateral primordia made by the inflorescence. In maize *unbranched* mutants, excess lateral primordia are made at the expense of the stem cells located in the center of the meristem. Ultimately, the *unbranched* mutant meristem lacks enough cells to regenerate and thus, terminates prematurely. This study shows that the duplicate transcription factors *unbranched2* and *unbranched3* function together to decrease the rate of lateral primordia initiation, thus giving the stem cells of the meristem enough time to regenerate. Variants of the *unbranched3* gene affect different aspects of lateral primordia initiation that control crop yield.

Author contributions: G.S.C. designed research; G.S.C. and P.J.B. performed research; G.S.C., P.J.B., R.M., and S.H. contributed new reagents/analytic tools; G.S.C., P.J.B., and S.H. analyzed data; and G.S.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. Email: georgechuck@berkeley.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1407401112/-/DCSupplemental.

PLANT BIOLOGY

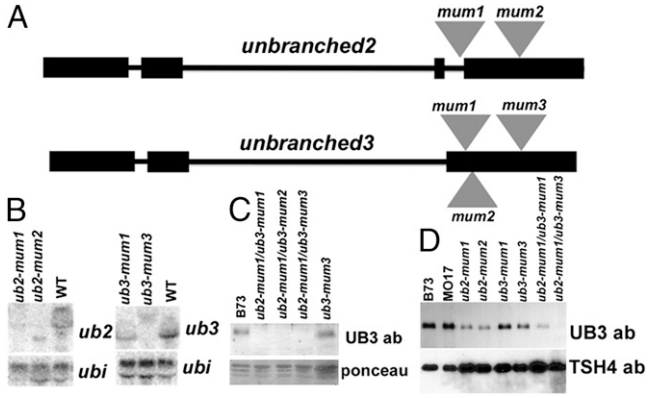

Fig. 1. Gene structure and expression of *ub2* and *ub3*. (*A*) Positions of *Mu* transposon insertions in *ub2* and *ub3* genes. (*B*) RNA gel blot analysis of *ub2* and *ub3* alleles from 3.5-wk-old dissected shoot apices. The *ubiquitin* (*ubi*) gene was used as a loading control. (*C*) Western blot analysis using 3.5-wk-old nuclear extracts from inbred B73, *ub2*/*ub3* double mutants, and *ub3-mum3* single mutants. Ponceau protein staining was done as a loading control. (*D*) Western blot of nuclear extracts from 0.5-cm ear primordia of B73, Mo17, and *ub2* and *ub3* single and double mutants. The TSH4 antibody was used as a protein loading control.

showed expression throughout lateral domains of meristems but not in the central domain, where meristem renewal occurs. These results indicate that *ub2* and *ub3* function together with *tsh4* to restrict initiation of lateral primordia, thus allowing cell renewal within the central domain of the meristem. The *ub2* and *ub3* mutant phenotypes affect several important agronomic properties, and genome-wide association study analysis implicates two independent natural variants at the *ub3* locus with increased tassel branch number (TBN) and increased ear row number (ERN), respectively.

Results

***ub2* And *ub3* Regulate Primordia Initiation.** A reverse genetic analysis was performed for duplicate *SBP-box* genes that group with *tsh4*, a gene responsible for repression of bract primordia in maize inflorescences (8). These genes, formerly known as *TC305612* and *TC282500* (6), were renamed *ub2* and *ub3* based on their mutant phenotypes. UB2 and UB3 share 79% overall amino acid identity and 86.1% identity within the DNA-binding domains (Fig. S1). Phylogenetic analysis showed that *ub2* and *ub3* are duplicated loci in maize, with single-copy orthologs present in sorghum, rice, and *Brachypodium* (Fig. S2). The closest maize homolog to *ub2* and *ub3* is *tsh4*. Two independent *Mutator* (*Mu*) transposon insertions (12) into *ub2* were identified: one in a 99-bp intron near the exon border and one into an exon. In addition, three *ub3* insertions into the last exon of *ub3* were also identified (Fig. 1*A*). From RNA gel blots, both *ub2* alleles completely disrupt transcription, whereas the *ub3-mum1* allele only partially reduces transcription (Fig. 1*B*).

To test whether these alleles make functional protein, an antibody was raised to full-length UB3 protein. Given the similarity of UB2 and UB3, the serum was predicted to detect both proteins. This result was confirmed by Western blots using nuclei from 3.5-wk-old shoots or 0.5-cm ear primordia, where accumulation was seen in WTs and single mutants but not several double-mutant combinations (Fig. 1 *C* and *D*). For example, a band was detected in *ub3-mum3* single-mutant shoots and ears, although it lacks functional transcript. This band is likely to correspond to UB2 protein, because *ub2-mum1*/*ub3-mum3* double mutants show no detectable protein (Fig. 1*C*). In ear tissue, however, the *ub2-mum1*/*ub3-mum1* double mutant had a small amount of protein, indicating that *ub3-mum1* is only a partial loss of function (Fig. 1*D*). Thus, the anti-UB3 antibody recognizes both UB2 and UB3 proteins, and the lack of any protein in *ub2-mum1*/*ub3-mum2* and *ub2-mum1*/*ub3-mum3* double-mutant combinations indicates that these alleles are likely loss-of-function null mutants.

The *ub2-mum1* and *ub3-mum1* alleles were introgressed into the WT W22 background and observed for phenotypic differences relative to W22. Both *ub2-mum1* and *ub3-mum1* single mutants showed a modest but significant reduction in TBN, with the *ub3-mum1* allele being slightly more severe (Fig. 2*A* and Fig. S3*A*). This decrease in TBN was greatly enhanced in three different double-mutant combinations (Fig. 2*A* and Fig. S3*A*). In addition, thickening appeared near the tip of the central spike of the tassel of the *ub2-mum1*/*ub3-mum3* double mutant (Fig. 2*A*), indicating the presence of excess spikelets. A similar reduction in TBN was observed in single mutants of *tsh4* (8). To determine whether *tsh4* may function redundantly with *ub2* and *ub3*, double- and triple-mutant combinations were made. This putative redundancy was confirmed by a reduction in TBN in two different

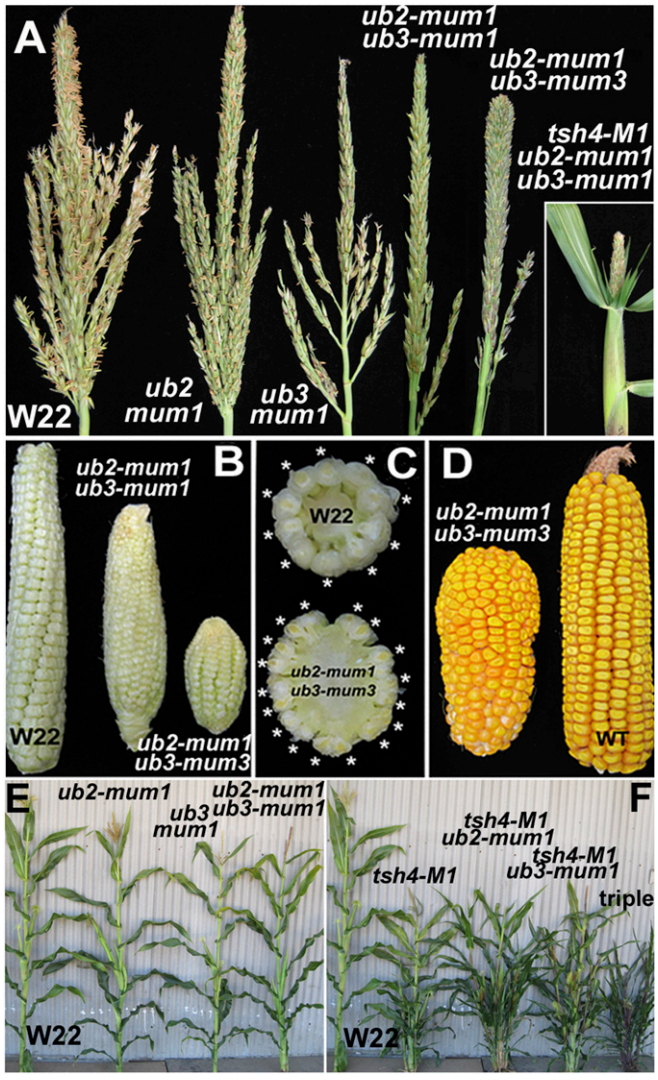

Fig. 2. Floral phenotypes of *ub2* and *ub3* mutants. (*A*) Tassel phenotypes of *ub2* and *ub3* mutant alleles; *ub2-mum1*, *ub3-mum1*, and the double mutant are in the W22 inbred background. Triple-mutant tassel in the W22 background is shown in *Inset*. (*B*) Ear phenotypes of W22 and double mutants. (*C*) Razor blade hand sections of the midpoint of the ears in *C*. Asterisks indicate kernel rows. (*D*) Fertilized ears of the double mutant compared with WT A619. (*E*) Mature field-grown plants of W22, *ub2*, *ub3*, and the double mutant. (*F*) Mature field-grown plants of *tsh4* and double- and triple-mutant combinations in W22 background showing an increase in tillering.

double-mutant combinations. In addition, two different triple-mutant combinations completely lacked tassel branches (Fig. 2*A* and Fig. S3*A*).

Defects in ear length and diameter were observed in single and double mutants (Fig. 2 *B*–*D*). The most severe length defect was observed in the triple mutant that was approximately one-half that of WT (Fig. S3*B*), leading to greatly reduced fertility. Significant increases in ear diameter were seen in *ub3* single and *ub2*/*ub3* double mutants (Fig. S3*C*) that also displayed fasciated tips (Fig. 2 *B* and *D*). Sections through the midpoint of the double-mutant ears showed nearly two times the number of kernel rows in the double mutants (Fig. 2*C*). When fertilized, the tips of these ears were disorganized, and the kernels crowded together (Fig. 2*D*). Taken together, these phenotypes indicate that meristem function during the floral phase of male and female inflorescences is altered by the simultaneous loss of *ub2* and *ub3*.

***ub2* And *ub3* Repress Tillering and Leaf Initiation Redundantly with *tsh4*.** Vegetative phenotypes of *ub2* and *ub3* single and double mutants were observed in the field. Although *ub2*/*ub3* double mutants do not affect tiller number, tillering was significantly increased in all double-mutant combinations with *tsh4* (Fig. 2 *E* and *F*), with the strongest increase observed in two different triple-mutant combinations (Fig. S4*A*).

Leaf number was also altered in the double and triple mutants. Because juvenile leaf number is difficult to assay in the field because of senescence and predation, leaf number above the upper most ear node was counted instead. Apart from *ub2-mum1*/*ub3-mum1*, which may not be a complete loss of function, double mutants made significantly more leaves, whereas triple mutants made up to two times as many upper leaves (Fig. S4*B*), mostly found at the base of the tassel (Fig. 2*A*). Thus, *ub2*/*ub3*/*tsh4* functions together redundantly to initiate tassel branches and repress excess tiller and leaf initiation. Despite the increase in leaf number, the double and triple mutants flowered at the same time as the WT, suggesting that plastochron is, in fact, shortened in *SBP-box* mutants.

Lateral Primordia Form at the Expense of the Apical Meristem in Double Mutants. SEM was performed on the double mutants to determine the origin of the extra primordia in the inflorescence. In normal maize tassels, the main inflorescence meristem (IM) first initiates a limited number of branch meristems (BMs) that form the long branches of the tassel (Fig. 3*A*). Later, the IM initiates several rows of spikelet pair meristems (SPMs), each of which forms two spikelet meristems that ultimately form the flowers and kernels (Fig. 3*B*). The *ub2*/*ub3* double mutants rarely initiate BMs and often have extra leaves at the base of the tassel instead (Fig. 3*C*). In addition, widening and fractionation of the IM are often seen at the tip (Fig. 3*C*). At later stages, the IM continues to widen, and many extra rows of SPMs initiate (Fig. 3*D*), often at the expense of the main IM that is either left as a sunken residual primordium (Fig. 3*E*) or completely consumed. Immunolocalization using antibodies to early BM markers, such as KNOTTED1 (KN1) (13–15) and RA2 (10), revealed no expression at the base of the tassel in the double mutant, supporting the observation that BMs do not initiate (Fig. S5 *A*–*C*).

We also used the KN1 antibody to follow the fate of the residual IM in the double mutant and determine whether it retained meristem identity. Recessive *kn1* mutants are shootless in some backgrounds (16) or have smaller inflorescences with reduced numbers of lateral primordia in others (17). KN1 is found in all meristems, and it is down-regulated in lateral organs (13–15). KN1 immunolocalization experiments showed that the residual meristem of the double mutant has no KN1 expression (Fig. 3 *F* and *G*). Moreover, the central cells of the residual meristem appear vacuolated rather than densely cytoplasmic, further indicating that they have lost meristem identity and terminally differentiated (Fig. 3*G*). These results indicate that meristem maintenance and renewal are compromised in the *ub2*/*ub3* double mutants.

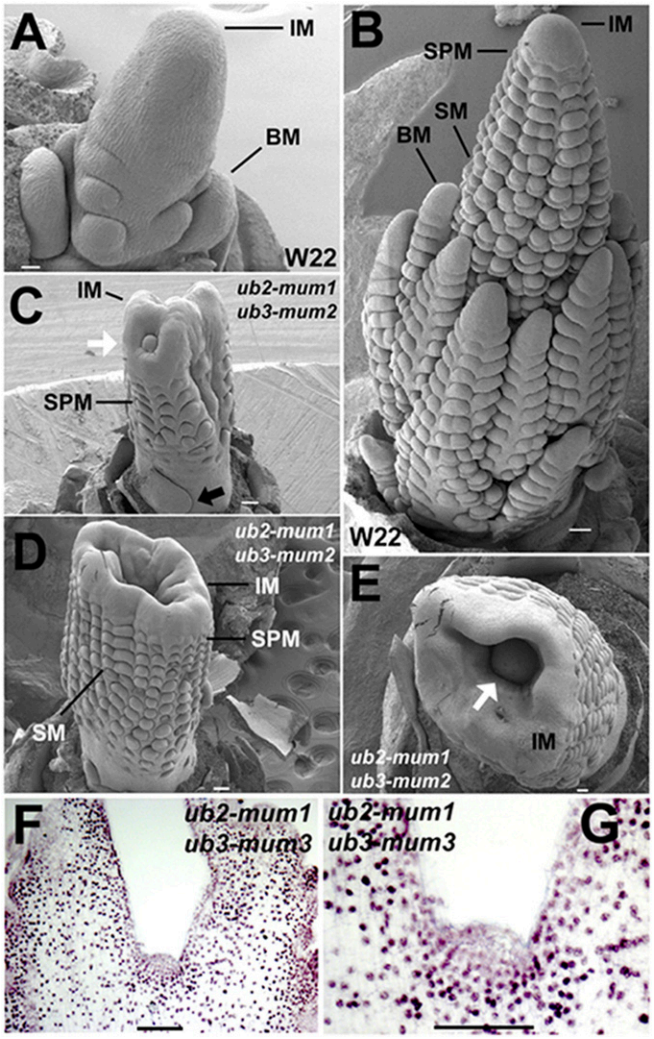

Fig. 3. SEMs and immunolocalization of KN1 in the *ub2*/*ub3* double mutant. (*A*) Early W22 tassel showing IMs and BMs. (*B*) Older W22 tassel showing rows of SPMs and spikelet meristems (SMs). (*C*) Early *ub2*/*ub3* double mutant tassel showing absence of BM and displaced residual IM (white arrow). Black arrow points to extra leaf primordium. (*D*) Older double mutant showing increased rows of SPM and widened IM. (*E*) Top view of same double mutant showing sunken residual IM in the center (arrow). (*F*) KN1 immunolocalization in residual IM of double-mutant tassel. (*G*) Close-up view of residual IM showing absence of KN1 and vacuolated cells. (Scale bars: *A*, 30 μm; *B*–*E*, 100 μm; *F* and *G*, 500 μm.)

Localization of UB2 and UB3 Proteins. To determine where *ub2* and *ub3* are expressed, anti-UB3 serum was used for immunolocalization experiments in shoot and floral apices. Because the serum recognizes both UB2 and UB3 proteins, immunolocalization experiments were performed on *ub3* and *ub2* single mutants to determine the tissue-specific localization of UB2 and UB3 proteins, respectively (Fig. 4 *A* and *B*). In the shoot apex, UB2 protein was found in initiating leaf primordia and the base of the shoot apical meristem (SAM) but not in the meristem tip (Fig. 4*A*). UB3 protein localized in a similar pattern in SAMs of the same age (Fig. 4*B*). In WT shoot apices, both proteins were found everywhere in the SAM, the stem, and young leaf primordia but absent from the center of the meristem tip, where cell renewal normally occurs (Fig. 4*C*).

To determine if this meristem expression pattern changes on flowering, florally determined transition-stage meristems were isolated and analyzed (Fig. 4*D*). Adjacent sections were probed with an anti-TSH4 antibody (8) for comparison (Fig. 4*E*). UB2

and UB3 proteins continue to be expressed throughout the transition-stage meristem, young leaves, and stem (Fig. 4*D*), although meristem tip expression is still restricted. In the adjacent section, TSH4 protein was found in a limited domain (Fig. 4*E*) in leaf primordia and older leaves but not in axillary buds or stems. The expression of TSH4 overlaps completely with UB2 and UB3, consistent with genetic redundancy between all three genes. In female inflorescence ears, UB2 and UB3 were excluded from the tip of the IM as well as the SPMs (Fig. 4*F*), although high expression was found in bracts subtending the SPM. In early-stage tassels, high UB2 and UB3 expression was found at the base of the tassel, where BMs form, but not in BMs themselves or the inflorescence tips (Fig. 4*G*). Double labeling with TSH4-specific antibodies showed localization in young bracts subtending the SPM primordia in the upper part of the tassel. At this stage, UB2 and UB3 were expressed in the portion of the tassel that defines the branch zone (BZ) but not the zone that makes SPMs. This complementary expression pattern was confirmed in double-labeling experiments with UB3 and TSH4 or RA2 in radial sections of tassels (Fig. S5 *D* and *E*). Thus, UB2 and UB3 are not expressed in the meristems that are affected in the double mutant, including IMs, SPMs, and BMs.

UB3 Maps to Quantitative Trait Loci for TBN and ERN. Quantitative trait loci (QTL) analysis of inflorescence traits was done using the nested association mapping (NAM) population. This population was made by crossing B73 with 25 different inbreds and then selfing to create recombinant inbred lines (18), and detected QTL for TBN, ERN, and BZ at the marker most closely linked to *ub3* (19). The *ub3*-linked ERN QTL has the largest effect of any inflorescence QTL in the NAM population and explains 12% of the variance in ERN across the entire NAM population (Figs. S6 and S7). A larger set of HapMap2 SNPs has now become available for the parents of the NAM population (20). From an ∼18-Mb region encompassing the 95% confidence intervals of the *ub3*-linked ERN and TBN QTL on chromosome 4, we extracted a set of 8,851 polymorphic HapMap2 SNPs that contained no missing data and no heterozygous genotypes among 27 NAM founder lines, including Mo17, and tested each SNP for association with ERN and TBN (*Materials and Methods* and Fig. 5*A*). For each trait, we found separate, highly significant nonsynonymous substitutions within the third exon of *ub3* downstream of the SBP domain. One variant (Val260Met; 199,457,430 bp) is found in four NAM founder lines (CML103, Mo17, Oh7B, and Tx303) and associated with increased TBN. Another variant (Ser220Asn; 199,457,549 bp) is found in two NAM founders (B73 and Hp301) and associated with increased ERN (Fig. 5*B*). For both TBN and ERN, genome-wide associations slightly more significant than the nonsynonymous substitution in *ub3* were found within the QTL confidence interval (Fig. 5*A*). These SNPs could represent synthetic associations (21) resulting from either additional alleles at *ub3* or closely linked QTL. No correlation was found between QTL effects for TBN and ERN in the *ub3* region (Table S1), suggesting that independent mutations, rather than a single pleiotropic mutation, are responsible for the TBN and ERN QTL colocalization.

Another high-resolution mapping resource, intermated B73 by Mo17 (IBM), was made by crossing B73 with Mo17 and intermating the F1 progeny four times before selfing (22). Using a cleaved amplified polymorphic sequence (CAPS) marker that distinguishes the two polymorphisms in *ub3*, 167 families derived from the IBM population were genotyped as possessing either the B73 Asn220 allele or the Mo17 Met260 allele. These data were correlated with BZ length, branch number, and ERN scores (19) from 2,839 progeny of 167 geno-typed families. Those families that received the Mo17 *ub3* allele were found to have, on average, a BZ that was 8.45 mm longer with 1.17 more tassel branches but 0.65 fewer ear rows (Fig. 5*C*). Thus, the two polymorphisms seem to affect the male and female inflorescences differently: the B73 allele correlates with robust ear growth, whereas the Mo17 allele correlates with robust tassel growth. These results are consistent with the NAM associations. Neither polymorphism is present in teosinte, the progenitor of maize (Fig. 5*B*). Furthermore, the *ub3* locus is not linked to any of the major QTL responsible for differences in inflorescence architecture between maize and teosinte (23). This finding suggests that natural variants in *ub3* arose during the process of crop improvement rather than crop domestication.

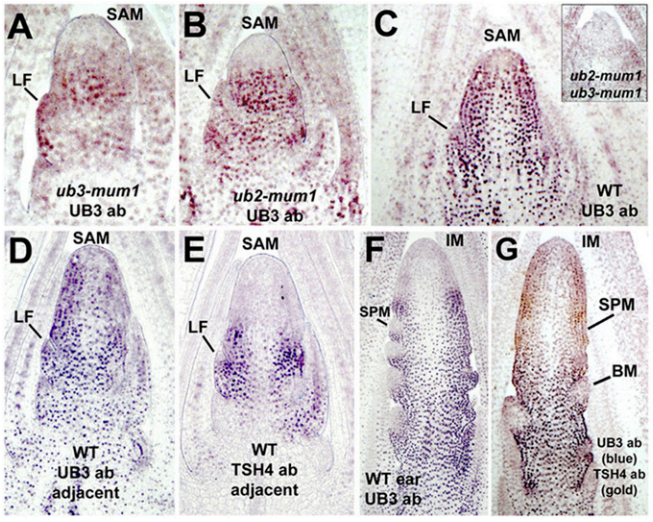

Fig. 4. Immunolocalization of UB2 and UB3 proteins. (*A*) UB2 expression in 3-wk-old *ub3-mum1* mutant shoot showing expression in leaves (LFs) and the stem. (*B*) UB3 expression in 3-wk-old *ub2-mum1* mutant shoot showing a similar expression pattern. (*C*) UB2 and UB3 localization in 3.5-wk-old WT shoots showing absence of expression near the tip. Double-mutant negative control showing no localization is shown in *Inset*. (*D*) Four-week-old WT transition-stage shoot probed with anti-UB3 serum. (*E*) Section adjacent to *D* probed with anti-TSH4 serum. (*F*) UB2 and UB3 in ear primordia with initiating SPM. (*G*) UB2 and UB3 (blue) in young tassel initiating BM. The same tassel was also labeled with TSH4 protein (gold) to show complementary expression in SPM above the BZ.

Discussion

The number of lateral primordia made by the male and female inflorescences of maize is a major determinant of yield. In tassels, such primordia include tassel branches, spikelet pairs, and spikelets, all of which control the length of shedding time and pollen quantity. In ears, the number of spikelet pairs affects kernel yield. Because all lateral primordia are products of meristems, increasing the number of these primordia to improve yield requires alteration of meristem activity. We hypothesize that *ub2* and *ub3* control the rate at which cells leave the undifferentiated central zone of the meristem and enter the peripheral zone where lateral primordia initiate (Fig. 5*D*). In *ub2* and *ub3* double mutants, extra lateral primordia are made too quickly at the expense of the apical meristem, which lacks enough cells to regenerate (Fig. 3 *D* and *E*), loses expression of meristem markers, such as *kn1* (Fig. 3 *F* and *G*), and terminates prematurely. The roles of these genes in regulating the rate of lateral primordia initiation are similar to the functions of *SPL9* and *SPL15* in vegetative shoots of *Arabidopsis* (24, 25), although *tsh4*, *ub2*, and *ub3* primarily affect the floral phase of development.

UB2 and UB3 accumulate throughout the base of the meristem but are excluded from the central tip of the SAM, where cell renewal occurs. Because these *SBP-box* transcription factors are targets of miR156 (6), the lack of expression in the central tip of the meristem may be a consequence of microRNA repression. In support of this idea, *tsh4*, which is also targeted by miR156, is expressed in a pattern complementary to miR156 in the IMs (8). Recent reports show repression of miR156 by sugar (26, 27), which itself is a noncell-autonomous mobile signal. Because

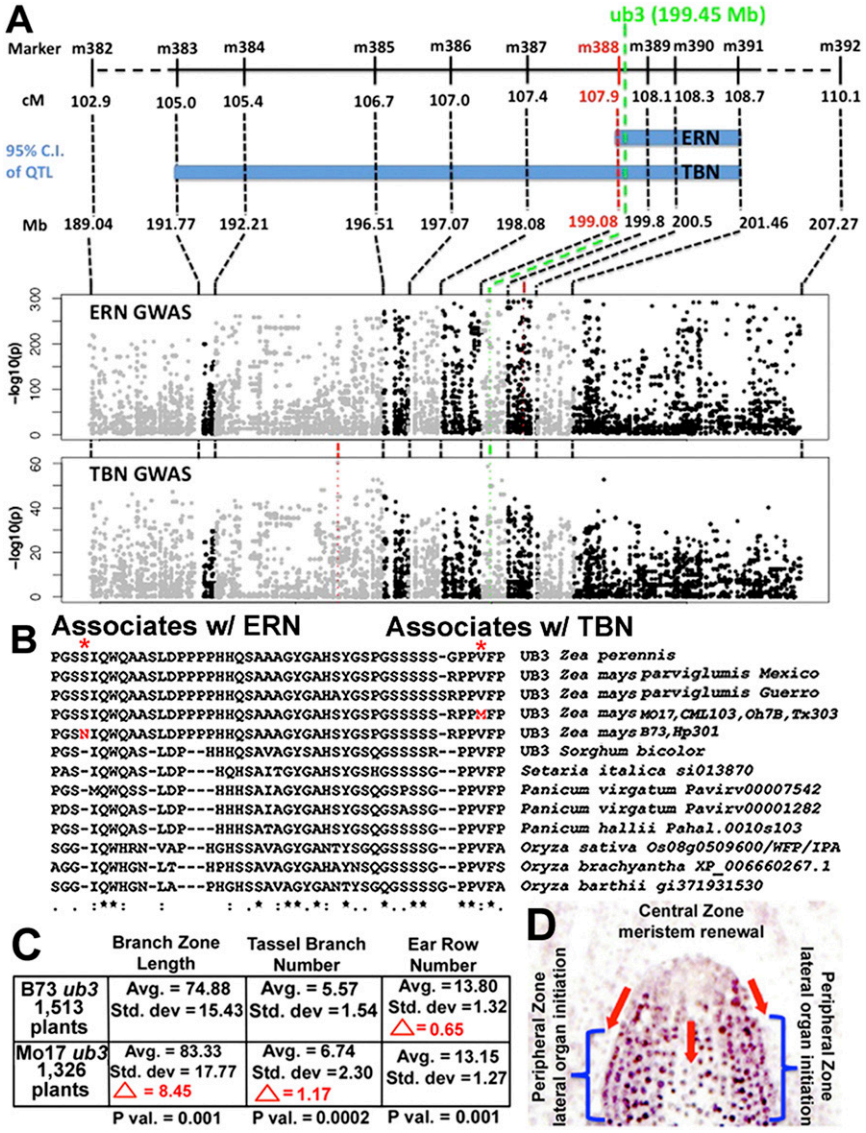

Fig. 5. Allelic diversity in *ub3* and its association with inflorescence variation with a model for function. (*A*) Physical and genetic positions of *ub3* relative to marker–trait associations for ERN and TBN in the NAM population. The 95% confidence intervals (95% CIs) for *ub3*-linked ERN and TBN joint linkage QTL are shown in blue. The positions of the most significant markers/SNPs for joint linkage and genome-wide association study (GWAS) analyses of ERN and TBN are shown as red dots. The position of *ub3* on each map is shown as green dots. The 95% CI for the *ub3*-linked ERN QTL extends 2.386 Mb and contains 44 predicted genes, whereas the 95% CI for the *ub3*-linked TBN QTL extends 9.695 Mb and contains 191 predicted genes. All physical positions refer to AGPv2.1. (*B*) Clustal alignments of UB3 from teosinte, maize, and other panicoid grasses. Polymorphisms are indicated by asterisks, and amino acid differences are in red. (*C*) Variation in average BZ length, branch number, and ERN in B73 vs. Mo17 populations of IBM lines. Differences between the two populations are highlighted in red. (*D*) Model for UB2/UB3 function. UB2/UB3 is not expressed in undifferentiated cells at the tip of the meristem, where renewal occurs. Red arrows signify UB2/UB3 control of cell movement out of the central zone into the peripheral zone, where cell differentiation and lateral organ initiation occur.

leaves are carbohydrate sources, perhaps the sugar that they produce travels to the peripheral zone of the meristem to repress microRNA expression and thus, allow *ub2* and *ub3* expression. It is possible that these sugars either do not travel far enough to the central tip of the SAM or are actively excluded from the central tip of the SAM, allowing miR156 to remain active and repress *ub2* and *ub3*.

It is unclear how *SBP-box* transcription factors affect the rate of organ initiation without being expressed in the central zone. Classic experiments performed in potato showed that a diffusible substance travels between leaves to control the rate of leaf growth and initiation (28). More recently, it has been shown that leaf-specific promoters driving *SPL9* expression in *Arabidopsis* were able to affect the rate of leaf initiation in the SAM (5). Such results are consistent with the hypothesis that SBP-box transcription factors or their target genes may function as non-autonomous signals that move through the shoot apex to regulate plastochron. Because we observed no sign of UB2 or UB3 protein throughout the IM, SPM, and spikelet meristem or in the apical tips of the vegetative meristem, it is more likely that a downstream factor in the *SBP-box* gene pathway is the noncell-autonomous signal rather than the SBP-box proteins themselves.

Loss-of-function mutations in *ub2*/*ub3* doubles display seemingly contradictory phenotypes in male vs. female inflorescences with regard to lateral meristems. For example, double mutants initiate several extra rows of SPMs in ears and tassels. In tassels, however, a different type of lateral meristem, the BM, is not made in excess, and the double mutant is nearly unbranched. This difference can be explained by the fact that double mutants make several extra leaves (Fig. S4*B*). These extra leaves, however, do not simply form in place of tassel branches, because the number of extra leaves does not equal the number of missing tassel branches in the mutants. It is more likely that BM can only form when vegetative growth is suppressed, allowing the tassel to initiate floral structures exclusively. Because both *ub2* and *ub3* are expressed exclusively in the basal zone of tassels, where BMs are made (Fig. 4*G*), it is possible that they function to suppress vegetative leaf initiation in that region. These observations suggest that highly branched inflorescences characteristic of panicoid grasses can only be obtained if vegetative leaf initiation is suppressed during the floral phase by *SBP-box* genes.

Because *ub2* and *ub3* affect TBN and ERN, two traits that directly affect yield, polymorphisms in these genes were tested for association with agronomically important inflorescence traits. A previous study using the NAM population showed the presence of *ub3*-linked QTL for TBN and ERN (19). In addition, a previous QTL study in a B73-Mo17 biparental family identified a TBN QTL that mapped close to *ub3* (29). A more detailed analysis

revealed the existence of two derived polymorphisms in the 3′-end of UB3 that are not present in teosinte, the progenitor of maize, which possesses two-rowed ears or several other panicoid grass orthologs (Fig. 5*B*). These two polymorphisms in the *ub3* gene are associated with distinct effects on male and female inflorescences: the Val260Met mutation associates with higher TBN in inbred lines, such as Mo17, whereas the Ser220Asn mutation associates with higher ERN in inbreds, such as B73 (Fig. 5*C*). Because both TBN and ERN are yield-related traits in maize, the physical proximity of the candidate SNPs for these traits in *ub3* (119 bp apart) could give rise to pseudooverdominance in hybrids that combine two advantageous alleles (e.g., B73 and Mo17). It is unclear at this time how these B73 and Mo17 polymorphisms might affect UB3 function. Western blots comparing B73 and Mo17 ear nuclear extracts appeared similar (Fig. 1*D*), indicating that the polymorphisms may affect protein function as opposed to protein amount or stability.

The potential for *ub3* to be used as a tool to improve the agronomic properties of crop plants is supported by the function of its ortholog in rice, *WEALTHY FARMER'S PANICLE (WFP)*/*IDEAL PLANT ARCHITECTURE* (*IPA*) (Fig. S2). Both *WFP* and *IPA* are dominant gain-of-function alleles that improve grain yield in rice by reducing tillering and increasing panicle branching (30, 31), phenotypes that are the opposite of the *ub2*/*ub3*/*tsh4* loss-of-function phenotype. *IPA* causes overexpression of *OsSPL14* through a mutation in the miR156-binding site that releases the gene from negative regulation by the microRNA (30). Extrapolating from the loss-of-function phenotypes of *ub2* and *ub3*, it is possible that overexpression of these *SBP-box* genes may delay lateral primordia initiation and differentiation, leaving more time for the meristem to renew and regenerate. After the floral transition, more meristem cells would then be available to produce a more complex inflorescence, thus leading to higher yield. Consequently, these *SBP-box* genes could be important tools with which to alter plant architecture and theoretically, increase yields in a variety of crop plants.

Materials and Methods

Isolation of *ub2* and *ub3* and Genetic Analysis. All *ub2* and *ub3* alleles were isolated from pools of *Mu* transposon-mutagenized populations and screened by PCR with *Mu*-end oligos and either *ub2* or *ub3* oligos. The *ub2-mum1* and *ub3-mum1* alleles were introgressed into the W22 background by backcrossing at least two times and then selfing; *ub2* and *ub3* were followed by PCR using the *Mu*-end primer *Mu9242*, the 5F1 primer (TAAAGAAGAGCTGCCGCAAACGCCT) for *ub2*, and the 7F1 primer (TGCTGGATTTCTCATACCCAAGG) for *ub3*. Triple mutants were made by crossing the single mutants to *tsh4-M1* in the W22 background, selfing, and scoring by PCR. All tassel, leaf, and tillering phenotypes were scored in the field.

Generation of a UB3 Antibody and Immunolocalization. Full-length UB3 protein coupled to an histidine tag was made into the pET21d expression vector (Novagen). Recombinant protein was isolated under denaturing conditions, dialyzed, and injected into two guinea pigs (Cocalico). The serum was affinity-purified using full-length UB3 protein coupled to a GST tag (Novagen). Immunolocalization was performed as previously described (8).

SEM. Samples were fixed in formaldehyde acetic acid overnight, critical point-dried, sputter-coated, and examined as previously described (8).

Association of *ub3* SNPs with ERN and TBN in the NAM and IBM Populations. Association analysis using NAM and IBM populations is described in detail in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Katsutoshi Tsuda, Michael Lewis, and China Lunde for critical reading of the manuscript. This work was supported by National Science Foundation Grants PGRP- 1339332 (to G.S.C.) and PGR-1238030 (to P.J.B.) and Binational Agricultural Research and Development Grant Bard IS-4536-12C (to S.H.).

1. Steeves TA, Sussex IM (1989) *Patterns in Plant Development* (Cambridge Univ Press, Cambridge, United Kingdom), 2nd Ed.
2. Veit B, Briggs SP, Schmidt RJ, Yanofsky MF, Hake S (1998) Regulation of leaf initiation by the *terminal ear 1* gene of maize. *Nature* 393(6681):166–168.
3. Kawakatsu T, et al. (2006) PLASTOCHRON2 regulates leaf initiation and maturation in rice. *Plant Cell* 18(3):612–625.
4. Miyoshi K, et al. (2004) PLASTOCHRON1, a timekeeper of leaf initiation in rice, encodes cytochrome P450. *Proc Natl Acad Sci USA* 101(3):875–880.
5. Wang JW, Schwab R, Czech B, Mica E, Weigel D (2008) Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in Arabidopsis thaliana. *Plant Cell* 20(5):1231–1243.
6. Chuck G, Cigan AM, Saeteurn K, Hake S (2007) The heterochronic maize mutant Corngrass1 results from overexpression of a tandem microRNA. *Nat Genet* 39(4):544–549.
7. Wu G, Poethig RS (2006) Temporal regulation of shoot development in Arabidopsis thaliana by miR156 and its target SPL3. *Development* 133(18):3539–3547.
8. Chuck G, Whipple C, Jackson D, Hake S (2010) The maize SBP-box transcription factor encoded by tasselsheath4 regulates bract development and the establishment of meristem boundaries. *Development* 137(8):1243–1250.
9. Bortiri E, et al. (2006) *ramosa2* encodes a LATERAL ORGAN BOUNDARY domain protein that determines the fate of stem cells in branch meristems of maize. *Plant Cell* 18(3):574–585.
10. Chuck G, Bortiri E (2010) The unique relationship between tsh4 and ra2 in patterning floral phytomers. *Plant Signal Behav* 5(8):979–981.
11. Bossinger G, Rohde W, Lundquist U, Salamini F (1992) Genetics of barley development: Mutant phenotypes and molecular aspects. *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology*, ed Shewry PR (Alden Press Ltd, Oxford), pp 231–264.
12. Bensen RJ, et al. (1995) Cloning and characterization of the maize *An1* gene. *Plant Cell* 7(1):75–84.
13. Smith LG, Jackson D, Hake S (1995) The expression of Knotted1 marks shoot meristem formation during maize embryogenesis. *Dev Genet* 16:344–348.
14. Smith LG, Greene B, Veit B, Hake S (1992) A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. *Development* 116(1):21–30.
15. Jackson D, Veit B, Hake S (1994) Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. *Development* 120(2):405–413.
16. Vollbrecht E, Reiser L, Hake S (2000) Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, *knotted1*. *Development* 127(14):3161–3172.
17. Kerstetter RA, Laudencia-Chingcuanco D, Smith LG, Hake S (1997) Loss-of-function mutations in the maize homeobox gene, *knotted1*, are defective in shoot meristem maintenance. *Development* 124(16):3045–3054.
18. McMullen MD, et al. (2009) Genetic properties of the maize nested association mapping population. *Science* 325(5941):737–740.
19. Brown PJ, et al. (2011) Distinct genetic architectures for male and female inflorescence traits of maize. *PLoS Genet* 7(11):e1002383.
20. Chia JM, et al. (2012) Maize HapMap2 identifies extant variation from a genome in flux. *Nat Genet* 44(7):803–807.
21. Brachi B, Morris GP, Borevitz JO (2011) Genome-wide association studies in plants: The missing heritability is in the field. *Genome Biol* 12(10):232.
22. Lee M, et al. (2002) Expanding the genetic map of maize with the intermated B73 x Mo17 (IBM) population. *Plant Mol Biol* 48(5-6):453–461.
23. Wills DM, et al. (2013) From many, one: Genetic control of prolificacy during maize domestication. *PLoS Genet* 9(6):e1003604.
24. Wang JW, Czech B, Weigel D (2009) miR156-regulated SPL transcription factors define an endogenous flowering pathway in Arabidopsis thaliana. *Cell* 138(4):738–749.
25. Schwarz S, Grande AV, Bujdoso N, Saedler H, Huijser P (2008) The microRNA regulated SBP-box genes SPL9 and SPL15 control shoot maturation in Arabidopsis. *Plant Mol Biol* 67(1-2):183–195.
26. Yang L, Xu M, Koo Y, He J, Poethig RS (2013) Sugar promotes vegetative phase change in Arabidopsis thaliana by repressing the expression of MIR156A and MIR156C. *eLife* 2:e00260.
27. Yu S, et al. (2013) Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. *eLife* 2:e00269.
28. Snow R (1929) The young leaf as the inhibiting organ. *New Phytol* 28:345–348.
29. Mickelson S, Stuber CW, Senior L, Kaeppler SM (2002) Quantitative trait loci controlling leaf and tassel traits in a B73 x MO17 population of maize. *Crop Sci* 42:1902–1909.
30. Jiao Y, et al. (2010) Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. *Nat Genet* 42(6):541–544.
31. Miura K, et al. (2010) OsSPL14 promotes panicle branching and higher grain productivity in rice. *Nat Genet* 42(6):545–549.