Auxin signaling modules regulate maize inflorescence architecture

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Edited by Mark Estelle, University of California, San Diego, La Jolla, CA, and approved September 18, 2015 (received for review August 19, 2015)

In plants, small groups of pluripotent stem cells called axillary meristems are required for the formation of the branches and flowers that eventually establish shoot architecture and drive reproductive success. To ensure the proper formation of new axillary meristems, the specification of boundary regions is required for coordinating their development. We have identified two maize genes, *BARREN INFLORESCENCE1* and *BARREN INFLORESCENCE4* (*BIF1* and *BIF4*), that regulate the early steps required for inflorescence formation. *BIF1* and *BIF4* encode AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins, which are key components of the auxin hormone signaling pathway that is essential for organogenesis. Here we show that BIF1 and BIF4 are integral to auxin signaling modules that dynamically regulate the expression of BARREN STALK1 (BA1), a basic helix-loop-helix (bHLH) transcriptional regulator necessary for axillary meristem formation that shows a striking boundary expression pattern. These findings suggest that auxin signaling directly controls boundary domains during axillary meristem formation and define a fundamental mechanism that regulates inflorescence architecture in one of the most widely grown crop species.

auxin signaling | inflorescence development | axillary meristems | maize | boundary domains

Plant shoot architecture is primarily determined by small groups of pluripotent stem cells called meristems. Throughout their life cycle, plants generate different types of meristems whose main function is to drive postembryonic organ initiation. In particular, reproductive axillary meristems (AMs) form branches and flowers that contribute to naturally occurring variations in inflorescence architecture. Genes regulating AM function have been frequent targets during crop domestication (1), and several recent examples have demonstrated how modulation of meristem activity can directly affect yields (2, 3).

Mutations that affect the initial steps in reproductive AM formation often result in the formation of characteristic pin-like inflorescences. Several such mutants, first described in *Arabidopsis*, are predominantly affected in genes related to the hormone auxin, including *PIN-FORMED1* (*PIN1*) and *MONOPTEROS* (*MP*) (4–6). Analysis of these and other mutants has established that auxin is central to the generation of all primordia. Auxin is polarly transported to the site of primordia initiation, where it is perceived by the nuclear auxin receptor TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB), part of an E3 ligase that rapidly degrades AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) coreceptor proteins and disrupts their recruitment of TOPLESS (TPL) corepressors. The auxin-dependent degradation of Aux/IAAs frees interacting activating AUXIN RESPONSE FACTOR (ARF) transcription factors from TPL repression, allowing them to activate downstream genes (7). Although it is known that ARFs bind to auxin-responsive cis-regulatory elements (AuxREs) composed of the core TGTC sequence, few downstream developmental pathways have been characterized (8–13). All components of the auxin signaling machinery are encoded by multimember gene families, and the combinatorial complexity afforded by the various members may contribute to auxin's capacity to regulate multiple aspects of plant development (14–19). How auxin regulatory components work together to trigger specific developmental responses in reproductive tissues, including grain-bearing inflorescences, remains an unaddressed aspect with important implications for crop productivity and improvement.

Grasses such as maize and rice contain inflorescences with multiple types of specialized reproductive AMs. In maize, these AMs give rise to two types of inflorescences: kernel-laden ears and tassels optimized for pollen dispersal. Maize inflorescence mutants with a pin-like phenotype are classically called *barren* mutants, with the founding member, *barren stalk1* (*ba1*), originally described more than 85 years ago. *BA1* encodes a basic helix-loop-helix (bHLH) transcription factor (20), and loss-of-function *ba1* mutants produce earless plants with tassels devoid of reproductive AMs. Additional *barren* mutants led to the discovery of proteins involved in auxin transport and biosynthesis (21–23), indicating that auxin-related defects often underlie this family of mutants.

Here we provide insight into the molecular mechanisms of auxin signaling during reproductive AM initiation by characterizing two *barren* mutants of maize. We identify the Aux/IAA proteins BARREN INFLORESCENCE1 and BARREN INFLORESCENCE4 (BIF1 and BIF4) and show that they are essential for organogenesis in maize inflorescences. We demonstrate that BIF1 and BIF4 are integral parts of functionally redundant signaling modules that directly control the transcription of *BA1*, thereby establishing critical boundary domains that ensure the formation of new AMs.

Significance

Axillary meristems are groups of plant pluripotent stem cells responsible for the formation of secondary axes of growth, such as branches and flowers. A crucial step in the initiation of new axillary meristems is the establishment of boundary domains that allow organ separation and prevent fusion defects during development. This work provides clues on the molecular mechanism by which the plant hormone auxin is involved in the formation of axillary meristems in maize inflorescences. Auxin signaling modules containing the AUXIN/INDOLE-3-ACETIC ACID proteins BARREN INFLORESCENCE1 and BARREN INFLORESCENCE4 and AUXIN RESPONSE FACTOR (ARF) transcriptional regulators are involved in the regulation of the boundary basic helix-loop-helix transcription factor BARREN STALK1, suggesting auxin is directly responsible for establishing boundary regions.

Author contributions: M.G., B.L.M., S.M., J.L.N., and A.G. designed research; M.G., Q.L., B.L.M., S.M., W.L., C.G., S.F., J.R., and A.G. performed research; R.M. contributed new reagents/analytic tools; M.G., Q.L., B.L.M., S.M., J.L.N., and A.G. analyzed data; and M.G., B.L.M., S.M., and A.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1516473112/-/DCSupplemental.

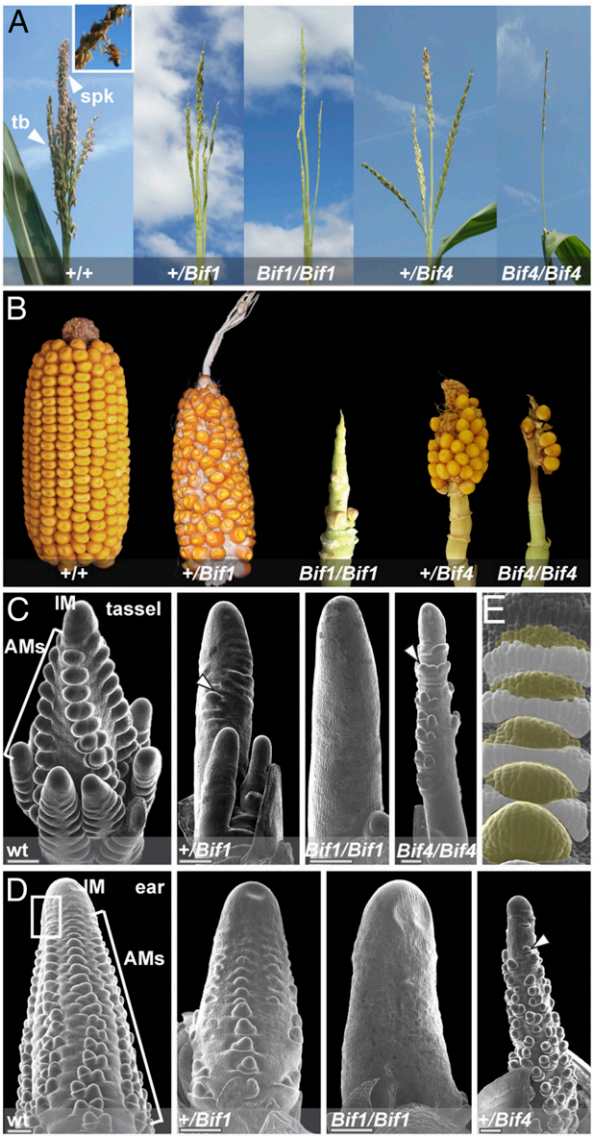

Fig. 1. *Bif1* and *Bif4* mutant phenotype. (*A*) Mature tassel phenotype. Normal tassels produce spikelets and flowers that are reduced in both mutants. (*Inset*) Spikelets with protruding anthers. tb, tassel branch. spk, spikelet. (*B*) Mature ear phenotype. (*C–E*) Scanning electron microscope image of early tassel and ear development in normal and mutant plants. Arrowheads point to a few axillary meristems forming in mutant plants. Primordia are absent in homozygous *Bif1* mutants. Boxed region in *D* marks the peripheral zone of the IM. (*E*) Close-up of the peripheral zone of the IM. White and yellow colors mark suppressed bract primordia and AMs, respectively. Note the acropetal development of primordia (from top, younger, to bottom, older).

Results

***Bif1* and *Bif4* Are Semidominant Mutants Affected in Reproductive Organogenesis.** The semidominant *barren* mutants *Bif1* (24, 25) and *Bif4* were originally isolated from ethyl methanesulfonate (EMS) mutagenesis screens and displayed similar inflorescence defects. After undergoing a normal vegetative-to-reproductive transition, *Bif1* and *Bif4* plants developed tassels with reduced numbers of branches and spikelets, the floral unit of grass inflorescences (Fig. 1*A* and Fig. S1). Ears appeared shortened and displayed disorganized rows of kernels, as well as areas completely devoid of kernels (Fig. 1*B*). These defects were more pronounced in homozygous *Bif1* and *Bif4* plants (Fig. 1 *A* and *B* and Fig. S1).

SEM analysis of young inflorescences revealed that the *Bif1* and *Bif4* phenotypes resulted from defects in primordium initiation at the peripheral zone of the apical inflorescence meristem (IM), where organogenesis occurs (Fig. 1 *C* and *D*). Normally in maize inflorescences, the first primordia to appear are suppressed bracts (SBs; Fig. 1*E*). These structures are followed shortly after by the formation of a series of reproductive AMs (branch, spikelet-pair, spikelet and floral meristems) that initiate at the bract axils and eventually give rise to spikelets and flowers (Fig. 1 *C* and *D*). In *Bif1* tassels and ears, a severe reduction in the initiation of AMs was observed (Fig. 1 *C* and *D*). Homozygous mutants produced smooth structures (albeit with a normal IM), indicating that both bract primordia and AM initiation were defective. Similar defects were also observed in *Bif4* mutant tassels and ears (Fig. 1 *C* and *D*). Double-mutant analysis of *Bif1* and *Bif4* showed a strong synergistic effect (Fig. 2*A*). In +/*Bif1*;+/*Bif4* tassels and ears, all primordia were missing, resulting in pin-like inflorescences in which organogenesis was often completely impaired (Fig. 2 *A* and *B*). Because of the missing floral organs, we were only able to generate +/*Bif1*;*Bif4*/*Bif4* plants that resembled +/*Bif1*;+/*Bif4* double heterozygotes. No significant vegetative defects were observed in either single or double mutants (Fig. S1). On the basis of this analysis, we conclude that *BIF1* and *BIF4* are essential for organogenesis during inflorescence development and function together in the initiation of lateral primordia (SBs and AMs).

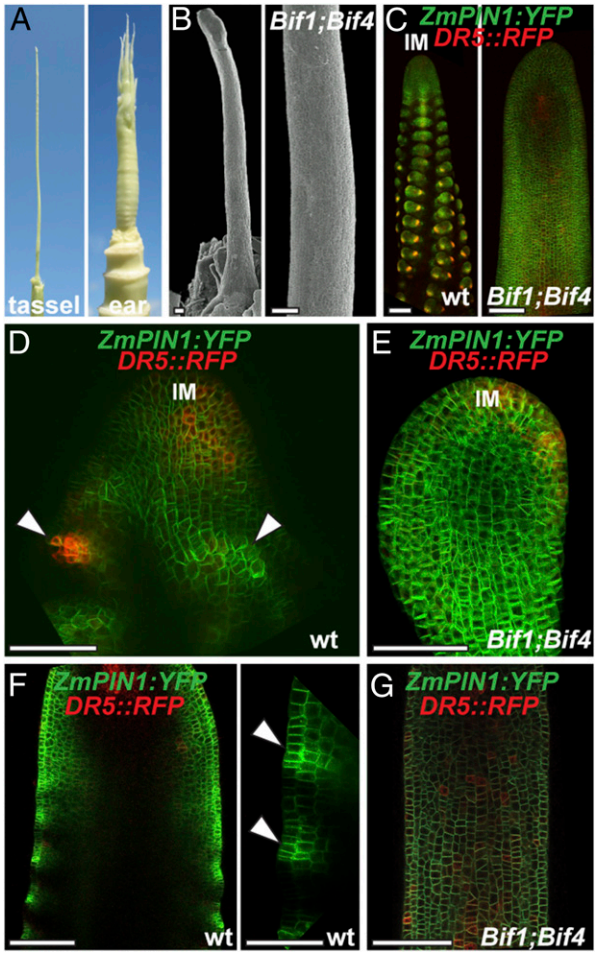

Fig. 2. *BIF1* and *BIF4* are required for patterning of primordium initiation. (*A*) Mature inflorescence phenotype of the double heterozygous *Bif1* and *Bif4* mutant. (*B*) Scanning electron microscope image of a young *Bif1*;*Bif4* tassel showing lack of primordium initiation. (Scale bars, 100 μm.) (*C*) Confocal images of normal and *Bif1*;*Bif4* tassels expressing ZmPIN1a-YFP fusion proteins and DR5::RFP. (*D* and *E*) Maximum projections of confocal images of wild-type and *Bif1*;*Bif4* mutant IMs. (*F* and *G*) Confocal images of the peripheral zone of immature tassels showing up-regulation of ZmPIN1a-YFP signals in normal tassels (*F*, arrowheads in close-up right panel) that is missing in double *Bif1*;*Bif4* mutants (*G*).

Because of the striking similarity between the inflorescences of the double +/*Bif1*;+/*Bif4* mutant and *Arabidopsis* auxin transport mutants, we used confocal microscopy to investigate how the expression of the membrane-localized auxin efflux transporter ZmPIN1a:YFP was affected in +/*Bif1*;+/*Bif4* inflorescences. We simultaneously monitored auxin signaling using the *DR5rev::RFP* reporter (26) (Fig. 2 and Fig. S2). Whereas in wild-type immature tassels the patterning of primordia at the periphery of the IM was marked by increased ZmPIN1a-YFP and RFP signals, in +/*Bif1*;+/*Bif4* plants, this patterning was completely absent ($n = 5$; Fig. 2 *C*–*G* and Fig. S2). This indicates that although ZmPIN1a is still expressed in +/*Bif1*;+/*Bif4* IMs, the normal auxin-driven patterning of primordia is completely disrupted and suggests that BIF1 and BIF4 are required for the upregulation of PIN-mediated auxin transport. In double-mutant tassels, RFP signal was observed in the IM, confirming that the IM is unaffected (Fig. 2*E*), and in occasional cells and inner tissue along the main axis (Fig. 2*G* and Fig. S2), suggesting that auxin signaling is not completely disrupted in these plants.

***Bif1* and *Bif4* Harbor Mutations in *Aux/IAAs* Expressed in the Early Stages of Inflorescence Development.** The underlying molecular cause of the *Bif1* mutant has remained unknown since its discovery in 1977 (24). On the basis of its auxin-related phenotype and semidominance, we reasoned that *BIF1* might encode one of the 38 maize *Aux/IAA* genes (27, 28), known negative regulators of auxin signaling that give rise to dominant mutants (7). We searched the maize genome for *Aux/IAAs* that were located in the region of chromosome 8, where the *BIF1* locus was previously mapped (25). Single-amino acid substitutions in the degron domain of GRMZM2G130953/IAA27 were observed in all *Bif1* alleles (Fig. 3 *C* and *D*). The degron domain is a highly conserved amino acid sequence found in Aux/IAA proteins that confers auxin-induced degradation and is consistently mutated in all known dominant *aux/iaa* mutants. Because *Bif4* showed an identical phenotype to *Bif1*, we used a similar approach, which revealed an amino acid substitution in the degron domain of GRMZM5G864847/IAA20 (Fig. 3*B*). These results show that *BIF1* encodes an Aux/IAA protein, and equally suggest it for *BIF4*.

Phylogenetic analysis of *BIF1/IAA27* and *BIF4/IAA20* revealed that the two genes belong to separate clades and share only 39% amino acid identity (Fig. S3*A*). To examine their expression pattern, we carried out RNA in situ hybridizations in developing inflorescences (Fig. 3 *C*–*J*). Both genes were broadly expressed in the IM, and in its peripheral zone in both tassels and ears (Fig. 3 *C*, *E*, *G*, and *I*). As the newly formed AMs developed, *BIF1* and *BIF4* showed strong expression in the central zone of all AMs (Fig. 3 *D*, *F*, *H*, and *J*). Similarly, maize transgenic lines expressing a VENUS-BIF4 fusion protein driven by the endogenous *BIF4* promoter showed VENUS-BIF4 protein in AMs (Fig. 3*K*). These expression patterns are consistent with the mutant phenotypes and support a role for *BIF1* and *BIF4* in initiating reproductive primordia.

BIF1 and BIF4 Show Distinct Auxin-Response Dynamics. Despite having similar phenotypes and localization patterns, *BIF1* and *BIF4* displayed different degrees of auxin inducibility when subjected to exogenous auxin treatments (Fig. 3*L*), as previously reported for other *Aux/IAA* genes. Because auxin signaling relies on the rapid degradation of Aux/IAA proteins, we monitored the stability of the two proteins in the presence of auxin, using a yeast synthetic assay (29, 30). We engineered yeast expressing BIF1 or BIF4 and monitored their degradation dynamics in combination with the *Arabidopsis* auxin receptor TIR1 (Fig. 3*M*). This analysis revealed that BIF1 displayed a slower rate of degradation than BIF4. We also tested the degradation rates of mutant alleles of BIF1 and BIF4, and all showed strong auxin insensitivity (Fig. 3*M*). These data, together with the observation that the same mutation in IAA20 and BIF1-N1440 stabilizes both proteins, provided additional confirmation that the *Bif4* phenotype is caused by a mutation in *IAA20*. The degradation rate of BIF1 was slower compared with the closest putative co-ortholog AtIAA15 and with other closely related Aux/IAAs (Fig. 3*N* and Fig. S4). In contrast, we observed similar degradation rates of BIF4 compared with its respective co-orthologs in *Arabidopsis*, suggesting there may be an evolutionarily conserved sequence-based bias for the stability of certain Aux/IAAs. Overall, this analysis indicates that *BIF1* and *BIF4* have unique auxin-response dynamics, suggesting the two genes may have subtle functional differences.

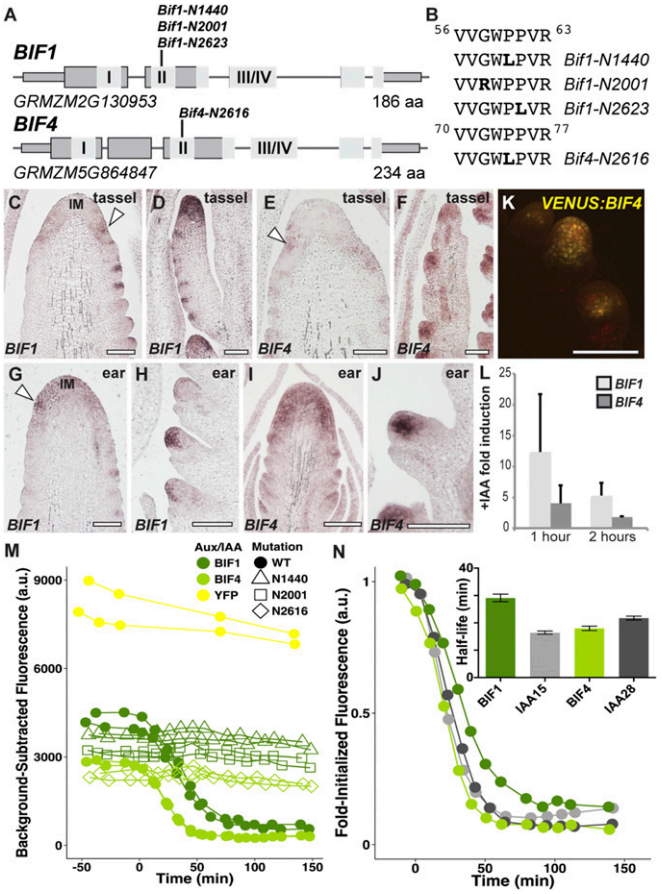

Fig. 3. *BIF1* and *BIF4* encode Aux/IAA proteins. (*A*) Schematic representation of *BIF1* and *BIF4* genes. Exons are depicted as gray rectangles. I and II represent the EAR repressor motif and the degron domain; III/IV corresponds to the dimerization domain. (*B*) The amino acid sequence of the degron domains of BIF1 and BIF4 and the mutations identified. (*C*–*J*) mRNA in situ hybridizations of immature inflorescences with *BIF1* and *BIF4* antisense probes. Arrowheads, localized signals at the peripheral zone of the IM. (*D* and *F*) Branch meristems are shown; (*H* and *J*) spikelet meristems. (Scale bars, 100 μm.) (*K*) Confocal image of VENUS-BIF4 in spikelet meristems. (*L*) Auxin inducibility of *BIF1* and *BIF4*. Error bars show SD. (*M* and *N*) Auxin-induced degradation profiles of normal and mutant BIF1 and BIF4 proteins.

Maize-Activating ARFs Are Expressed in Defined Domains of the Inflorescence Meristem. Aux/IAA proteins interact with and regulate the activity of ARF transcription factors. To determine which ARFs function with BIF1 and BIF4, we first took a reverse genetic approach. When grown to the adult stage, the *Arabidopsis mp* mutant shows pin-like inflorescences (5). Therefore, we hypothesized that the closest maize homologs of *MP*, *ZmARF4*, and *ZmARF29* (duplicated genes with 96% aa similarity; Fig. S3*B*) would be likely candidates to work with BIF1 and BIF4 in reproductive organogenesis. We identified exonic transposon insertions in both genes (Fig. S5*A*); however, double *arf4;arf29* mutants showed no phenotype in either shoot or reproductive development.

Suspecting *ARFs* may work redundantly, we mined public transcriptome databases and found 13 maize-activating *ARFs* expressed in inflorescences. To obtain an expression map of these *ARFs* and assess whether they were coexpressed with *BIF1* and *BIF4*, we performed in situ hybridizations on immature inflorescences (Fig. S5 *B*–*D*). All *ZmARFs* except *ARF16* were expressed in specific

domains of the IM: *ARF1* and *35* showed broad expression; *ARF4*, *ARF18*, *ARF20*, *ARF22*, *ARF29*, and *ARF34* showed strong expression at the peripheral zone of the IM; and *ARF3*, *ARF27*, and *ARF30* showed narrow expression in developing primordia. Expression patterns of the different *ARFs* also varied in developing AMs; the majority were predominantly restricted to the meristematic core of the different types of AMs (*ARF1*, *ARF4*, *ARF9*, *ARF16*, *ARF20*, *ARF22*, *ARF29*, *ARF34*, *ARF35*), and others such as *ARF3* and *ARF30* appeared localized in more restricted domains at the base of AMs and at their boundary, whereas *ARF18* and *ARF22* localized to the suppressed bracts and glume primordia (Fig. S5). Strong vasculature expression was also observed for *ARF4*, *ARF9*, *ARF20*, and *ARF29*. Overall, these domains largely overlapped with those of *BIF1* and *BIF4*.

To determine whether all activating ARFs were capable of physically interacting with BIF1 and BIF4 proteins, we performed yeast 2-hybrid (Y2H) assays and detected interaction of BIF1 and BIF4 with all activating ARFs (Fig. S6). We verified by Y2H, BiFC, and in vitro pull-down that BIF1 and BIF4 interacted with REL2, a functional homolog of the *Arabidopsis* TPL corepressor (31). Furthermore, we showed that BIF1 and BIF4 homo and heterodimerize (Fig. S6 *A–C*). Overall, our expression and protein interaction data suggest functional redundancy among BIF1/BIF4-ARFs transcriptional repression modules and that multiple ARFs work together with BIF1 and BIF4 during the initial stages of reproductive organogenesis.

***BARREN STALK1* Is an Early Target of the Auxin Signaling Pathway.** One of the earliest genes expressed at the peripheral zone of the IM is *BA1*, whose mRNA expression marks a boundary domain in newly forming AMs that is necessary for meristem formation (20). Severe *ba1* mutants lack all AMs but form enlarged suppressed bracts (Fig. S7*A*). Because of the phenotypic resemblance between *ba1* and *Bif1;Bif4* mutants, we hypothesized that *BA1* may be directly regulated by transcriptional repressor complexes containing BIF1 and BIF4. To investigate this possibility, we first checked the genetic interaction between *Bif1, Bif4*, and *ba1*, using a weak, fertile allele of *ba1* (*ba1-mum1*) (20). Analysis of double *+/Bif1; ba1-mum1/ba1-mum1* and *+/Bif4;ba1-mum1/ba1-mum1* mutants showed that *ba1* strongly enhanced the phenotype of heterozygous *Bif1* and *Bif4* mutants in both tassels and ears, impairing both branch and spikelet formation (Fig. 4*A* and Fig. S7 *B* and *C*). These data suggest that *BIF1*, *BIF4*, and *BA1* function either in the same or in parallel pathways contributing to AM formation.

If BIF1 and BIF4 formed repressor complexes targeting *BA1* transcription, *BA1* expression should be down-regulated in auxin-insensitive tassels. Quantitative RT-PCR on immature *+/Bif1;+/Bif4* tassels supported this prediction, as no significant expression of *BA1* was detected (Fig. 4*B* and Fig. S7 *D* and *F*). Conversely, in situ hybridizations showed that both *BIF1* and *BIF4* expression were unchanged in strong *ba1* mutant tassels, as were *SPI1,* an auxin biosynthetic gene, *ARF4,* and *ZYB15*, a marker for SBs (Fig. 4*C*) (22, 32), suggesting that auxin biosynthesis, signaling, and SB patterning are unaffected in *ba1* mutants. Furthermore, expression of *SPI1* was observed in the peripheral zone of the IM before the appearance of *BA1*, whereas *ARFs* showed expression patterns that preceded but subsequently partially overlapped with *BA1*, indicating that *BA1* functions downstream of auxin biosynthesis and signaling (Fig. S7 *G–I*). Finally, in situ hybridizations of *BA1* and *BIF1* on consecutive sections showed that *BIF1* was broadly expressed in the peripheral zone of the IM, whereas *BA1* was present only in a small number of cells (Fig. 4*D*). However, as the AM developed, the two genes showed a striking complementary expression, with *BIF1* being expressed in the center of the meristem and *BA1* in its characteristic boundary domain (Fig. 4*E*). This analysis shows that *BA1* and *BIF1* expression patterns, although initially overlapping, are subsequently partitioned in two distinct domains of the AM: the boundary domain and the meristem center. Overall, these results are consistent with the hypothesis that BIF1 and BIF4 directly repress *BA1* transcription.

To assess whether coexpressed BIF-ARF repression modules directly bind the *BA1* promoter, we expressed a subset of

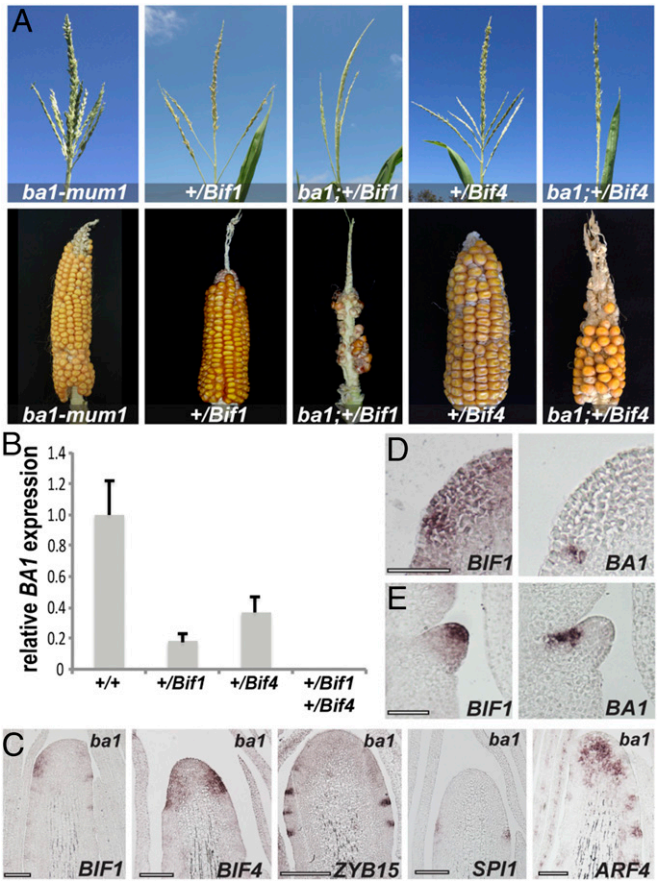

Fig. 4. Genetic and expression analysis of *ba1* mutants. (*A*) Double-mutant analysis of *Bif1* and *Bif4* with *ba1-mum1* in A619 background. (*B*) qRT-PCR of *BA1* in double *Bif1;Bif4* mutants. Error bars, SD. (*C*) In situ hybridization of immature *ba1-ref* tassels with specific markers. (Scale bars, 100 μm.) (*D* and *E*) mRNA in situ hybridizations on consecutive sections of immature inflorescences with *BIF1* and *BA1* antisense probes. (Scale bars, 50 μm.)

nonparalogous maize-activating ARFs and carried out electrophoretic mobility shift assays (EMSAs) with four regions of the ∼7-kb *BA1* promoter enriched for the core TGTC AuxRE element (Fig. 5*A*). ARF4, ARF16, ARF27, ARF29, and ARF34 strongly bound to all four probes, whereas ARF22 and ARF35 bound only a subset of these regions (Fig. 5*B* and Fig. S8). Competition with unlabeled probe or mutation of the core TGTC elements inhibited binding (Fig. 5*C* and Fig. S8). No detectable binding was observed for ARF1, ARF3, ARF9, or ARF30; however, all ARFs bound to the *DR5* promoter, albeit with varying intensities (Fig. S8*B*). No ARFs bound to regions containing only a single AuxRE (Fig. 5*D*, probes E and F). These results demonstrate that various activating ARFs directly bind to the *BA1* promoter and suggest that multiple BIF1,4-ARF modules regulate the expression of *BA1* (Fig. 5*E*).

Discussion

A major outstanding question in auxin signaling is the degree of specificity existing among the various components, and whether combinatorial complexity plays a role in the multitude of processes controlled by auxin. The *Bif1* and *Bif4* mutants represent a striking case of stabilized Aux/IAAs that specifically confer phenotypes resembling the pin-like inflorescences of *Arabidopsis pin1* and *mp* mutants, indicating a specific and predominant role for both genes in reproductive organogenesis. However, previous analysis suggested a synergistic interaction of *Bif1* with *bif2*, an auxin transport mutant, during vegetative development (25), raising the possibility that other Aux/IAAs may function redundantly with BIF1 and BIF4 during shoot development.

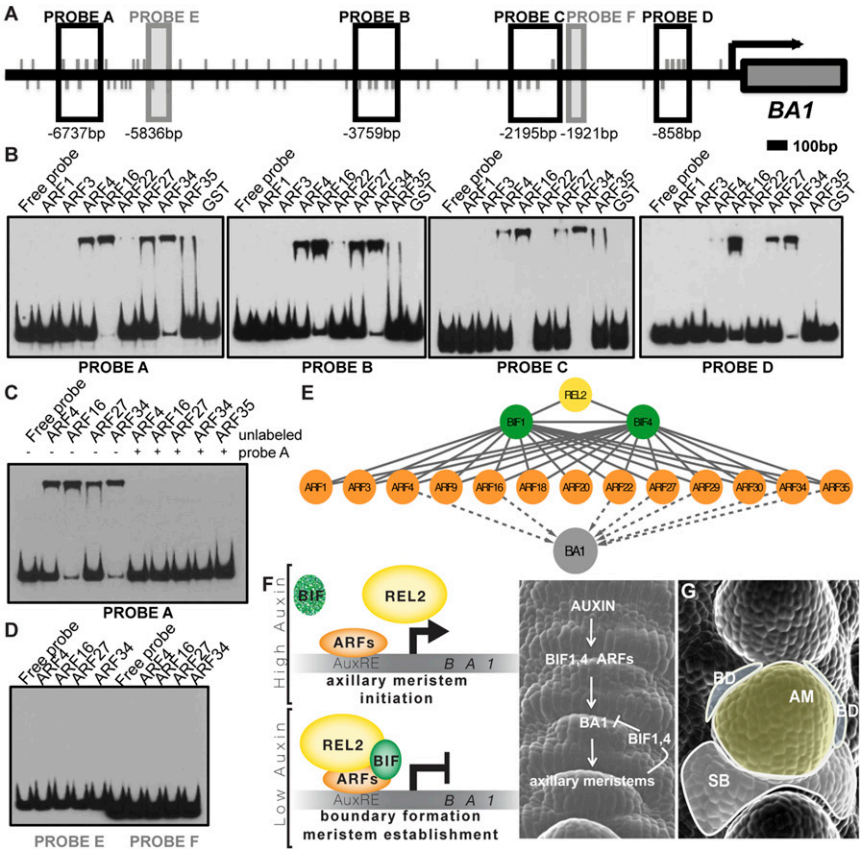

Fig. 5. *BA1* is a target of BIF/ARF transcriptional regulatory modules. (*A*) Schematic showing *BA1* genomic locus including 7 kb of putative promoter. Promoter fragments used as probes in EMSAs are shown as boxed regions. Values below boxes indicate position relative to BA1 start codon (+1). Gray lines indicate TGTC core AuxRE elements. (*B*) EMSAs show that various activating ARFs bind to BA1 promoter fragments; GST alone does not. (*C*) EMSA showing specificity of ARF binding to probe A. Addition of unlabeled probe A outcompetes binding to labeled probe A. (*D*) EMSA showing ARFs do not bind non-TGTC-enriched promoter fragments E and F. (*E*) Summary of protein–protein (solid lines) and protein–DNA (dashed lines) interactions identified in this study. (*F*) Molecular model of organogenesis in the peripheral zone of maize IMs. (*G*) Diagram of the resulting functional domains (false-colored). SB, suppressed bract; BD, boundary domain; AM, axillary meristem.

Stabilizing degron mutations in *Arabidopsis Aux/IAA* genes that are closely related to *BIF1* (*AXR2/IAA7*, *AXR3/IAA17*, *SLR/IAA14*, and *IAA16*) and *BIF4* (*IAA28*) (Fig. S3) were reported to show decreased shoot branching, dwarfism, and partial infertility (33–38). Some of these phenotypes may point to functional homology, as mutations in both species affect reproductive branching. Alternatively, the lack of severe pin-like inflorescence phenotypes in *Arabidopsis* may indicate that BIF1 and BIF4 were specifically co-opted for patterning maize reproductive AMs. Although *Arabidopsis mp* mutants display strong pleiotropic defects, no phenotype was observed in the orthologous maize *arf4;arf29* double mutants. Overall, our findings from maize suggest specificity among Aux/IAA function, as well as redundancy among activating ARFs. However, *ARF* expression patterns suggest that although several *ARFs* are expressed in the peripheral zone of the IM early in inflorescence development, they subsequently acquire more specific domains of expression (AMs vs. suppressed bracts and glumes).

The reproductive defects observed in both mutants suggest that the function of BIF1 and BIF4 is to negatively regulate organogenesis in the peripheral zone of the IM, and that their auxin-induced degradation is necessary for new primordia to initiate. Analysis of the *ZmPIN1a-YFP* reporter line in +/*Bif1*;+/*Bif4* tassels also indicates that BIF1 and BIF4 are part of a core signaling mechanism that regulates the patterning of maize inflorescences and is required for the local up-regulation of the polar auxin transport components necessary for organogenesis. Previous reports suggested that auxin negatively regulates boundary domain genes during embryo and leaf development (39, 40). Together with a general role in organogenesis, our data support a model in which multiple auxin signaling modules involving BIF1 and BIF4 directly regulate the formation of boundary regions during AM initiation (Fig. 5*F*). In this model, auxin, first synthesized and transported in the peripheral zone of the IM (22, 26), triggers the transcription of the early-response genes *BIF1* and *BIF4* (Fig. 3*L*). Both BIF1 and BIF4 proteins are, in turn, rapidly degraded in the presence of auxin (Fig. 3*M*), and activating *ARFs* expressed in this region can promote transcription of their targets to initiate organogenesis. Among these targets, *BA1* is specifically required for initiating AMs (20). As meristems develop, auxin is transported to the inner tissue for vasculature formation and to nearby areas to promote new primordia initiation (41, 42). Therefore, in the central zone of developing AMs, BIF1 and BIF4 are no longer efficiently degraded and can form stable repressor complexes on the *BA1* promoter. This repression restricts *BA1* expression and establishes boundary domains essential for AM formation (Fig. 5 *F* and *G*).

Recent reports in tomato and *Arabidopsis* have established that low auxin at the adaxial boundary of leaf primordia is necessary for vegetative AM formation (43–45). Whether a similar mechanism is established during reproductive development is not known. In maize inflorescences, SB (modified leaves) and AM primordia, although initially overlapping, subsequently resolve and acquire distinct identities (46), making it inherently difficult to test whether auxin minima exist at the axils of SBs. Nonetheless, the auxin-dependent regulation of *BA1* transcription, a key regulator of maize inflorescence architecture, ensures that axillary meristems are established throughout reproductive development. Our results pave the way for future biotechnological strategies aimed at modifying reproductive structures. For example, by modulating the auxin-dependent stability of BIF1 and BIF4 proteins, using engineered variants of their degron motifs (47), it may be possible to alter the position and number of primordia initiated by the IM. Similar strategies could be used in other species as well, allowing optimization of inflorescence architecture in crops.

Methods

All *Bif1* and *Bif4* alleles were generated by EMS mutagenesis by Gerry Neuffer. The Mutator transposon insertion lines were obtained from the UniformMu collection (mu1021266; *ARF4*) (48), and the Pioneer TUSC population (BT94 27C-05 and BT94 27E-08; *ARF29*) (49). Experimentally verified full-length cDNAs of *BIF1* and *BIF4* genes correspond to *GRMZM2G130953_T02* and *GRMZM5G864847_T01*, respectively (GenBank KT819172 and KT819173).

Full-length *ZmARFs* ORFs were cloned from B73 mixed-stage inflorescence cDNA. EMSAs were performed using recombinant ARFs and the Lightshift Chemiluminescent kit. Auxin-induced degradation assays were carried out as in Havens et al. (29). In situ hybridizations, qRT-PCRs, analysis of transgenic lines, and detailed description of all methods are provided in *SI Experimental Procedures*.

ACKNOWLEDGMENTS. We thank Gerry Neuffer and the Maize Inflorescence Project for generating mutants; the Maize Genetics Cooperation Stock Center for providing seeds; UniformMu for insertion lines; Marc Probasco for field and greenhouse care; David Jackson for SEM use; Sharon Stanfield for preliminary experiments; and Robert Schmidt, Brian Crawford, Rhiannon Macrae, and Mithu Chatterjee for critical reading of the manuscript. We acknowledge funding from National Science Foundation (IOS-08207291/1114484 to A.G. and S.M.; MCB-1411949 to J.L.N.), NIH (R01-GM107084 to J.L.N.; F32CA180514 to B.L.M.), the China Scholarship Council (Q.L.), and the Waksman Institute (A.G.). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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