The Relationship between Auxin Transport and Maize Branching[1][C][W][OA]

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Maize (*Zea mays*) plants make different types of vegetative or reproductive branches during development. Branches develop from axillary meristems produced on the flanks of the vegetative or inflorescence shoot apical meristem. Among these branches are the spikelets, short grass-specific structures, produced by determinate axillary spikelet-pair and spikelet meristems. We investigated the mechanism of branching in maize by making transgenic plants expressing a native expressed endogenous auxin efflux transporter (ZmPIN1a) fused to yellow fluorescent protein and a synthetic auxin-responsive promoter (DR5rev) driving red fluorescent protein. By imaging these plants, we found that all maize branching events during vegetative and reproductive development appear to be regulated by the creation of auxin response maxima through the activity of polar auxin transporters. We also found that the auxin transporter ZmPIN1a is functional, as it can rescue the polar auxin transport defects of the Arabidopsis (*Arabidopsis thaliana*) *pin1-3* mutant. Based on this and on the groundbreaking analysis in Arabidopsis and other species, we conclude that branching mechanisms are conserved and can, in addition, explain the formation of axillary meristems (spikelet-pair and spikelet meristems) that are unique to grasses. We also found that *BARREN STALK1* is required for the creation of auxin response maxima at the flanks of the inflorescence meristem, suggesting a role in the initiation of polar auxin transport for axillary meristem formation. Based on our results, we propose a general model for branching during maize inflorescence development.

Two major stem cell systems, the shoot and root apical meristems, form the apical-basal axis of plant growth. Secondary axes of growth are established by axillary meristems that are responsible for the formation of branches and flowers. The plant hormone auxin is essential for the formation of secondary axes of growth and of different organ primordia, from leaves to flower organs (Okada et al., 1991; Reinhardt et al., 2000, 2003; Benkova et al., 2003). Auxin accumulation at these sites is accomplished through the concerted action of influx and efflux transporters, whose activities create dynamic fluxes of the hormone (Benkova et al., 2003; Reinhardt et al., 2003). Auxin starts to accumulate from the stem epidermis at the site of primordium initiation. Once the primordium starts to grow out, auxin is depleted from the surrounding area and a new auxin sink forms at the site of the next primordium in a regularly arranged fashion (Reinhardt et al., 2003; Heisler et al., 2005). One of the most investigated auxin transporters is the PINFORMED1 (PIN1) protein of Arabidopsis (*Arabidopsis thaliana*), a member of the PIN family of auxin efflux transporters (Galweiler et al., 1998; Paponov et al., 2005). Localized on the plasma membrane, PIN1 actively transports auxin out of cells. Its polar localization determines the direction of auxin efflux (Petrasek et al., 2006; Wisniewska et al., 2006), and dynamic changes in polarity are thought to be responsible for the creation of auxin response maxima that precede new primordium formation (Heisler et al., 2005). PIN1 dynamics have also been used to model how polarized auxin transport can form regular phyllotactic patterns (de Reuille et al., 2006; Jonsson et al., 2006; Smith et al., 2006). *pin1* mutants are characterized by the lack of floral meristem (FM) initiation by the inflorescence meristem (IM) and give rise to a naked inflorescence stem (Okada et al., 1991; Bennett et al., 1995; Galweiler et al., 1998).

In maize (*Zea mays*), mutants similar to *pin1* are found in the barren class of mutants and include *barren stalk1* (*ba1*), *Barren inflorescence1* (*Bif1*), and *bif2* (Neuffer et al., 1997; McSteen and Hake, 2001; Gallavotti et al., 2004; McSteen et al., 2007; Barazesh and McSteen, 2008). These mutants are severely affected in the formation of vegetative branches (tillers) and of all axillary reproductive structures (McSteen and Hake, 2001; Ritter et al., 2002; McSteen et al., 2007). *BIF2* is

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a co-ortholog of the *PINOID* (*PID*) Ser/Thr kinase (McSteen et al., 2007), which in Arabidopsis is involved in the correct subcellular localization of PIN proteins (Friml et al., 2004; Michniewicz et al., 2007), and *BA1* encodes a basic helix-loop-helix transcription factor (Gallavotti et al., 2004). *ba1* mutants completely lack all axillary meristems, resulting in a complete sterile plant essentially composed of a stalk and several leaves (Ritter et al., 2002; Gallavotti et al., 2004). The analysis of *ba1* and other barren mutants suggests that a common pathway for branching operates in all maize axillary meristems.

Among the grasses, maize has the peculiar feature of having two morphologically and physically distinct unisexual inflorescences, the tassel and the ear. Despite these differences, both inflorescences share many similarities in early development (Cheng et al., 1983; Irish, 1997; McSteen et al., 2000; Bommert et al., 2005). In the tassel, the IM first produces a series of axillary meristems, named branch meristems (BMs), that will make the long basal branches of the mature tassel (Figs. 1, A and B, and 5H). The ear lacks long branches, and the first axillary meristems formed are determinate spikelet-pair meristems (SPMs; Fig. 1C) that are also made by the tassel after the initiation of the BMs (Fig. 1B). As the SPMs develop, they form spikelet meristems (SMs; Fig. 1, D and E), which in turn make FMs and eventually floral organs (Fig. 1, F and G). This progression of branching events culminates in the production of a mature spikelet, a grass-specific structure bearing flowers, in both tassel and ear. Spikelets are found in all grasses, but spikelet pairs are found only in certain species, such as maize and sorghum (*Sorghum* spp.), belonging to the Andropogoneae (Vollbrecht et al., 2005; McSteen, 2006; Kellogg, 2007).

Previously, it was reported that differences in PIN1 functions exist in maize compared to Arabidopsis (Carraro et al., 2006). These conclusions were based on immunolocalization of PIN1 proteins in maize and suggested that the role of polar auxin transport in organ initiation might differ among species (Carraro et al., 2006). Here, we present evidence that the patterns of PIN localization are conserved in maize and Arabidopsis, supporting a conserved branching mechanism, and we show that dynamic gradients of auxin, established by the activity of polar auxin transporters, are likely responsible for the formation of all vegetative and reproductive axillary meristems and organs during maize development. Furthermore, we show that a maize auxin transporter, ZmPIN1a, is capable of re-establishing the formation of FMs and lateral organs in the Arabidopsis *pin1* mutant. These results indicate that this pathway is evolutionarily conserved among very diverse plant species. We also characterize *BA1* and *BIF1* as regulators of the polar auxin transport

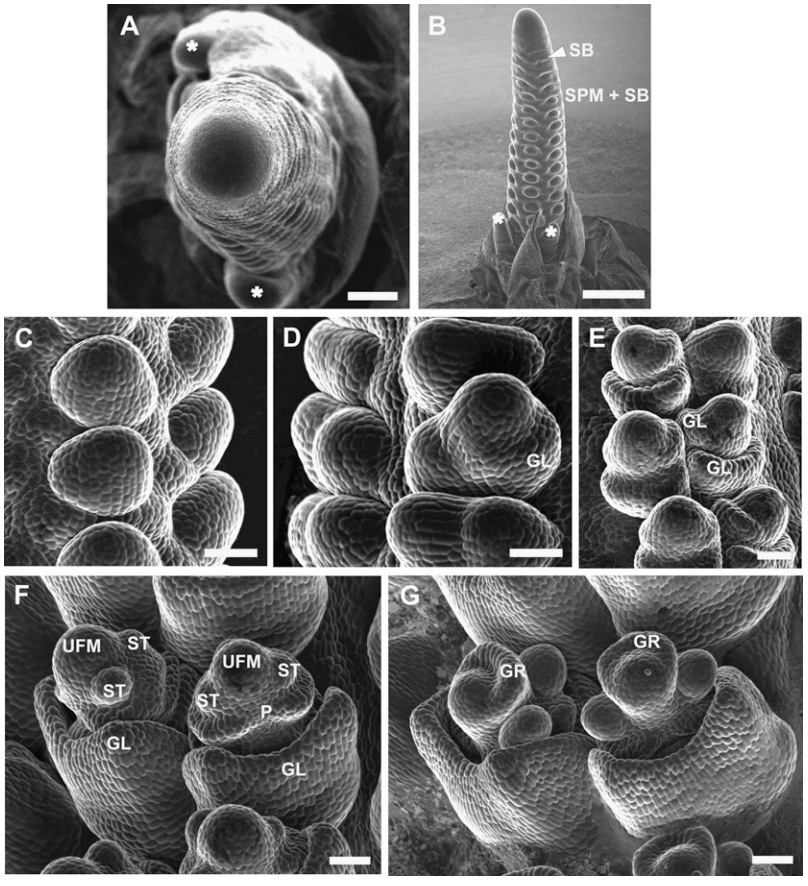

Figure 1. SEMs of axillary meristems during maize inflorescence development. A, Top view of a tassel IM forming suppressed bracts and SPMs at its flanks. Asterisks mark basal BMs. B, Immature tassel showing BMs at its base (asterisks) and regular rows of SPMs formed at the axils of suppressed bracts (SB). C, SPMs. D, SPMs giving rise to two SMs. The outer glume primordia (GL) is the first visible sign of an SM. E, SMs with two glume primordia before giving rise to two FMs, the upper (UFM) and lower FMs (not visible). F and G, Ear FMs forming floral organ primordia: stamens (ST), palea (P), gynoecial ridge (GR). Bars = 100 μm in A, 500 μm in B, 50 μm in C to G.

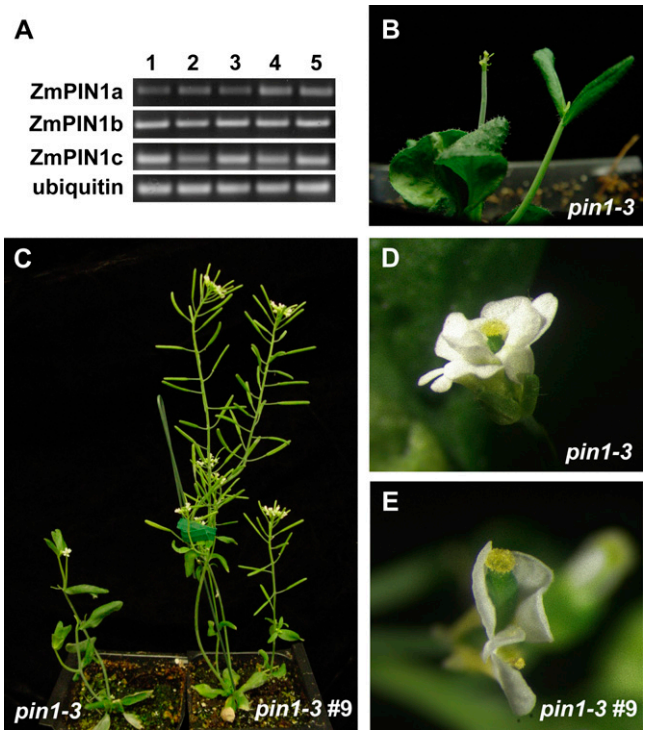

Figure 2. A, Three *ZmPIN1* genes are similarly expressed during vegetative and reproductive development, as assessed by RT-PCR (lane 1, seedling; lane 2, immature tassel 2 mm; lane 3, immature tassel 6 mm; lane 4, immature ear 2 mm; lane 5, immature ear 6 mm). *ZmPIN1a* is capable of rescuing the Arabidopsis *pin1-3* mutant (B–E). B, *pin1-3* homozygous mutant showing the characteristic pin-like inflorescence stem. C, Comparison of the *pin1-3* mutant with a *pin1-3* mutant line (no. 9) rescued by the *ZmPIN1a* gene. Some late floral defects of the *pin1-3* mutant, such as lack of stamens (D), are still present in some flowers of the rescued line (E). [See online article for color version of this figure.]

pathway, providing new insights into the development of maize inflorescences.

RESULTS

ZmPIN1a Is a Functional Auxin Efflux Transporter

There are at least three maize PIN1-like genes, *ZmPIN1a*, *ZmPIN1b* (Carraro et al., 2006), and *ZmPIN1c*, which map on chromosomes 9, 5, and 4, respectively. The amino acid sequences of ZmPIN1a and ZmPIN1c are the most diverse, with 77% identical residues, whereas ZmPIN1a and ZmPIN1b and ZmPIN1b and ZmPIN1c each share 87% identity (Carraro et al., 2006). The three genes were similarly expressed during vegetative and reproductive development (Fig. 2A). Given the overlapping expression patterns and the high amino acid identity among the ZmPIN1 family members, it is likely that they act redundantly (Moore and Purugganan, 2005), and we focused on *ZmPIN1a*. To assess its function as an auxin transporter, we transformed Arabidopsis plants carrying the *pin1-3* mutation (Fig. 2, B and D; Bennett et al., 1995) with a construct containing the *AtPIN1* promoter driving the *ZmPIN1a* complementary DNA. This construct was capable of fully rescuing the branching defects of Arabidopsis *pin1-3* mutants (Fig. 2, C and E). Although flower initiation was fully rescued, some defects were visible in later stages of flower development. We sometimes observed extra petals and a reduced number of stamens (Fig. 2E; Table I), defects normally observed in *pin1-3* mutants (Table I; Fig. 2D; Bennett et al., 1995). Nonetheless, the complete rescue of branching and floral initiation shows that ZmPIN1a is a functional auxin efflux transporter and is capable of restoring polar auxin transport in a heterologous system.

ZmPIN1a Is Up-Regulated during Every Branching Event in Maize Development

To obtain evidence of a relationship between branching and the expression of the ZmPIN1a auxin efflux transporter, we produced maize transgenic plants expressing ZmPIN1a fused to yellow fluorescent protein (YFP) under the control of its endogenous promoter (*pZmPIN1a*::*ZmPIN1a*:*YFP*). Six independent events were recovered and five of them showed similar expression patterns. Confocal images of the fusion protein during maize vegetative and reproductive development showed a clear up-regulation of ZmPIN1a expression at the site of each axillary meristem or lateral organ primordium. During the first few days of seedling development, ZmPIN1a was detected in the epidermis of the leaf primordia adjacent to the axil of immature leaves where the axillary meristem will form (Fig. 3A). Following initiation, the axillary meristems themselves form leaf primordia, and these were also marked by an up-regulation of ZmPIN1a expression (Fig. 3, B and C). After the switch to the reproductive phase, the inflorescence shoot apical meristem makes axillary meristems in the axils of suppressed bracts. During the formation of BMs by the tassel and of SPMs by both tassel and ear (Fig. 1, A and B) on the flanks of the IM, there was strong expression of ZmPIN1a predicting the sites where the suppressed bracts and the axillary meristems will form (Fig. 3, D and E; Supplemental Movie S1). This expression was maintained during the early stages of their development (Fig. 3, D and E). Subsequently, two foci of ZmPIN1a expression were localized on opposite sides of an SPM, one marking the initiation of an SM, the other marking the formation of a glume primordium

Table I. *Floral organ count of Arabidopsis pin1-3 mutant in noncomplemented and complemented pAtPIN1a::ZmPIN1a lines*

The numbers reported are the average and the SD (in parentheses).

	n	Sepals	Petals	Stamens
Wild type	10	4	4	6
pin1-3/pin1-3	13	4.8 (0.8)	6.3 (1.18)	0
pin1-3/pin1-3 family no. 9	25	4.2 (0.9)	5 (1.4)	3.6 (1.9)

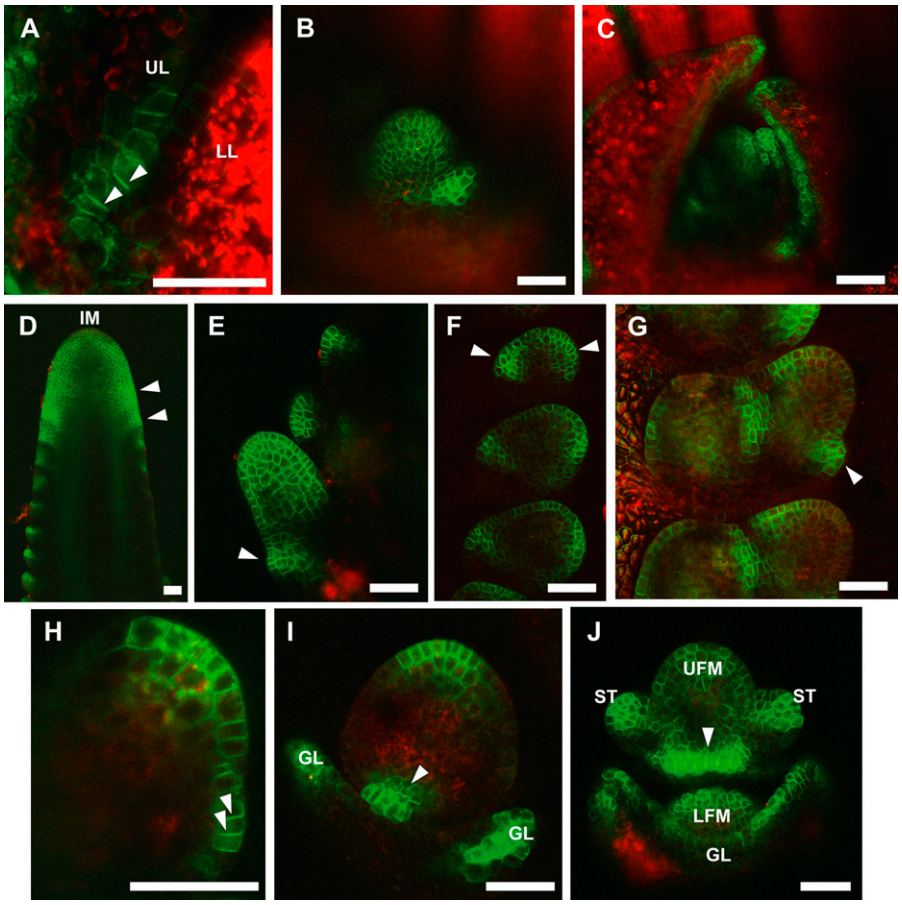

Figure 3. A to J, Confocal images of the *pZmPIN1a*::*ZmPIN1a*:*YFP* transgenic lines during vegetative (A–C) and reproductive (D–J) development. A, ZmPIN1a-YFP is localized in the epidermis of the upper leaf primordia (UL; arrowheads), suggesting an auxin flow directed toward the axils of the subtending lower leaf (LL). B and C, Vegetative axillary buds, forming leaf primordia. D, IM showing a periodic up-regulation of ZmPIN1a expression at its flanks (arrowheads). E, Tassel BM, forming its first SPM (arrowhead). F, A series of SPMs in the process of giving rise to two SMs. Note the strong signal (arrowheads) at the opposite sides of the developing SPM. G, Developing SMs, showing a strong signal from the newly formed glume primordia (arrowhead). H, Basal localization of ZmPIN1a (arrowheads) during SM initiation. I, SM forming the lower FM (arrowhead). J, FM forming floral organs: two lateral stamen primordia (ST) and one central palea primordium (arrowhead) are visible. GL, Glume primordia; UFM, upper FM; LFM, lower FM. Bars = 50 μm.

(Fig. 3, F and G, arrowed; Supplemental Movie S2). The outer glume primordium is the first morphologically distinguishable sign of an SM (Fig. 1D). Each SM is committed to form two FMs. The emerging lower FM showed an up-regulation of ZmPIN1a expression (Fig. 3I), as did the emergence of all floral organ primordia (Figs. 1, F and G, and 3J; Supplemental Movie S3). ZmPIN1a subcellular polar localization was usually directed toward the emerging primordium (Fig. 3H) and was strongly expressed in the L1 layer of every meristem and in the epidermis of all developing primordia (Fig. 3, A–J). In summary, throughout maize shoot development, ZmPIN1a was consistently up-regulated to predict the position of axillary meristems and lateral organs of both tassel and ear, suggesting that polar auxin transport is at the core of a general mechanism for maize primordium initiation and branching.

Auxin Response Maxima Predicted by a DR5 Reporter Precede the Formation of All Axillary Meristems and Lateral Organs of Maize

PIN proteins act as auxin efflux transporters (Petrasek et al., 2006; Wisniewska et al., 2006), and their subcellular polar localization therefore predicts the direction of auxin flux and creates auxin response maxima where a new primordium will emerge. To correlate ZmPIN1a expression with the dynamics of auxin accumulation during maize development, we used the synthetic auxin-responsive promoter DR5 (Ulmasov et al., 1997). The DR5 promoter has been widely utilized as a reporter of auxin gradients in root and shoot development (Sabatini et al., 1999; Benkova et al., 2003; Friml et al., 2003). We used the DR5rev sequence (Benkova et al., 2003; Friml et al., 2003) to drive the expression of a monomeric red fluorescent protein (mRFP1; Campbell et al., 2002), targeted to the endoplasmic reticulum to act as a cell autonomous marker for auxin concentration (*DR5rev*::*mRFPer*). Maize transgenic plants carrying this construct showed RFP fluorescence in sites where auxin levels are predicted to be high. The spatial distribution of DR5-RFP-marked auxin response maxima was very similar to the expression pattern of ZmPIN1a (Figs. 3 and 4), suggesting that the auxin maxima arise following auxin transport by ZmPIN1a. In particular, auxin response maxima were detected on the flanks of the IM preceding the formation of the first suppressed bracts and SPMs (Fig. 4, A and C, arrowed; Supplemental Movie S4). In the BMs, this signal was stronger and localized to the L1 layer (Fig. 4B). The mRFP signal was also seen on opposite sides of SPMs. Also in this case, similarly to ZmPIN1a-YFP signal, the DR5-RFP signal was detected in the emerging lateral SM and in the glume primordia

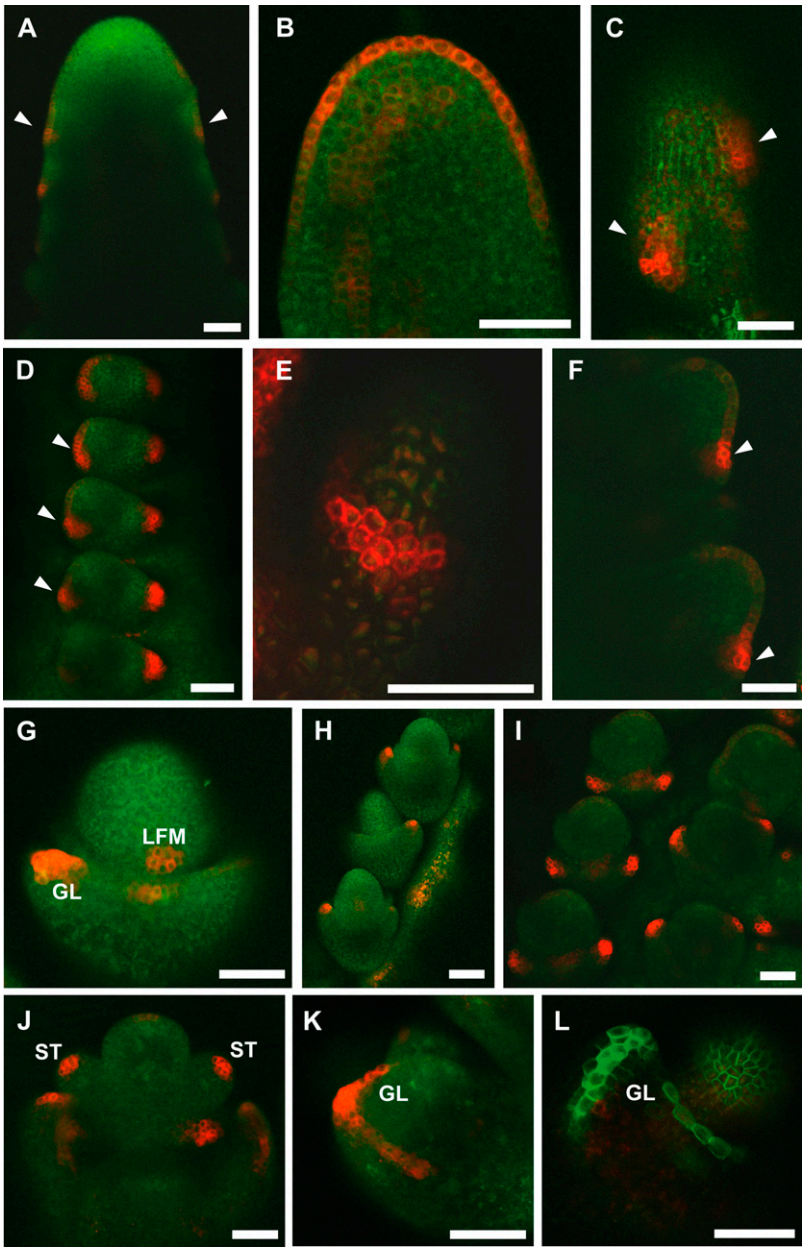

Figure 4. Confocal images of *DR5rev::mRFPer*, a marker for auxin response maxima, during reproductive development. A, IM forming SPMs (arrowheads) at its flanks. B, BM with strong expression in the L1 layer. C, Surface view of the newly formed SPM bulges (arrowheads). D, Developing SPMs giving rise to two SMs. Arrowheads point to developing glume primordia in one of the SM. E, Top view of a newly formed SM. F, Longitudinal view of SMs giving rise to glume primordia (arrowheads point to emerging glume primordia). G, Developing SM, showing signal in the growing tips of the glume primordium (GL) and at the site of the lower FM formation. H and I, Series of developing spikelets, with strong expression in the glume tips. J, FM forming floral organs; two stamen primordia (ST) are visible. K and L, Glumes; note a strong expression in the glume middle vein in both *DR5rev::mRFPer* and *pZmPIN1a::ZmPIN1a:YFP* lines. Bars = 50 μm.

of the main SM (Fig. 4, D–F). A similar pattern was observed in the SMs, prior to the formation of the two FMs, marking the site where the LFM starts to emerge (Fig. 4G; Supplemental Movie S5). Developing spikelets expressed DR5-RFP in the margins of glume primordia (Fig. 4, H and I). Subsequently, auxin response maxima were clearly associated with the initiation of floral organ primordia, including stamen primordia in both tassel and ear (Fig. 4J) and the gynoecial ridge in the ear (Supplemental Movie S6). DR5-RFP expression was also observed in the developing vasculature of the glume primordia (Fig. 4K). This expression colocalized with ZmPIN1a-YFP expression (compare Fig. 4, K and L).

The Inhibition of Polar Auxin Transport in Wild-Type Maize and *ba1* Mutants Abolishes the Formation of Axillary Meristems and Suppressed Bracts

With the previous analysis, we clearly established a connection between auxin transport and all branching events taking place during maize shoot development. We next asked how inhibiting auxin transport affects the formation of axillary meristems and of the suppressed bracts subtending them. Maize plants were irrigated with the auxin transport inhibitor *N*-1-naphtylphthalamic acid (NPA; 10, 30, or 50 μM). This treatment completely abolished the ability of the IM to form any axillary meristem if treated at the transition

to the reproductive phase (Fig. 5, A and B; see also Wu and McSteen [2007]). The IM retained its meristematic identity, as marked by *KNOTTED1* expression, but its ability to form axillary meristems was severely compromised in both tassel and ear (Fig. 5, C and D). Sometimes small ridges are present and these appear to have meristematic identity, as assessed by *KNOTTED1* expression (Fig. 5C). We interpret these structures to be arrested axillary meristems that initiated at or near the time of NPA exposure. NPA treatments also resulted in a decrease of ZmPIN1a-YFP and DR5-RFP expression (Fig. 5, E and F) and in a loss of ZmPIN1a up-regulation that is normally observed on the flanks of the IM (Fig. 3D).

If the NPA-treated plants were allowed to grow, a few of them (two out of 10) produced an apical inflorescence devoid of spikelets and with a few barren branches (Fig. 5G). Such defects are common to the barren class of mutants. Among these, *ba1* is the most severely affected, as no axillary meristems are formed during both vegetative and reproductive development (Ritter et al., 2002; Gallavotti et al., 2004). *ba1* tassels are able to form suppressed bracts (Ritter et al., 2002; Fig. 5, I and J), but when treated with 30 μM NPA, these suppressed bracts were abolished, resulting in a smoother inflorescence rachis compared to untreated plants (Fig. 5, M and N). These results suggest that polar auxin transport is required for the formation of the suppressed bracts in *ba1* plants.

Polar Auxin Transport in *ba1* and *Bif1* Mutants

We asked if ZmPIN1a-YFP expression was altered in *ba1* or in the semidominant *Bif1* mutants (Fig. 5, I–L). Tassels of *ba1* mutant plants showed periodic up-regulation of ZmPIN1a-YFP signal on the flanks of the

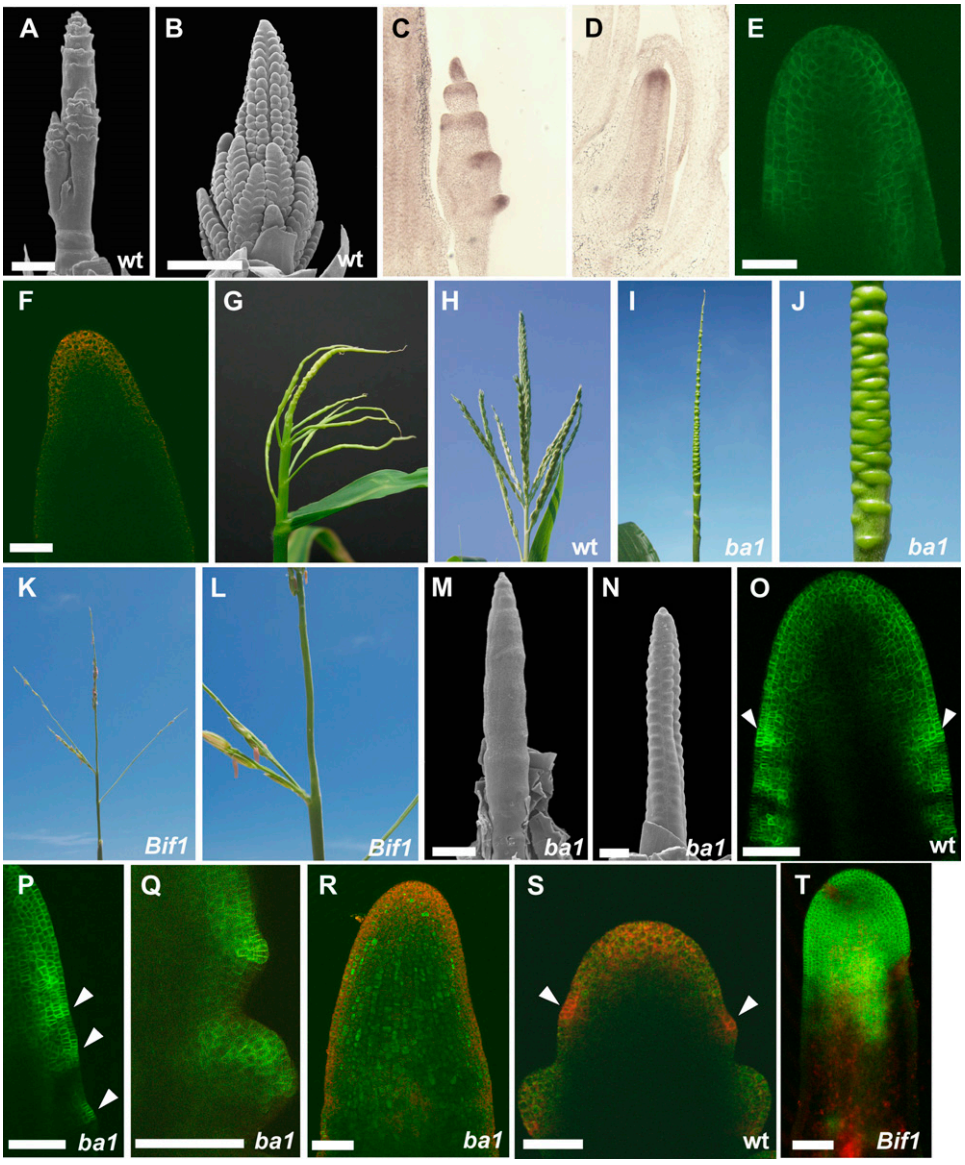

Figure 5. NPA treatment of wild-type maize inhibits the formation of axillary meristems at the flanks of the IM (A, 50 μM NPA; B, control). C and D, *KN1* in situ hybridizations of NPA-treated tassel (C) and ear (D). E and F, Confocal image of *pZmPIN1a*::*ZmPIN1a*:*YFP* (E) and *DR5rev*::*mRFPer* (F) tassels treated with NPA. G, After NPA treatment, few plants survive and give rise to naked tassels bearing only a few branches. H, Wild-type tassel. I and J, *ba1* mutant tassel showing enlarged suppressed bracts. K and L, *Bif1* mutant tassel, with few branches and spikelets, and smooth main rachis. M and N, NPA treatment of *ba1* mutant plants. *ba1*-treated tassel (30 μM NPA; M) lacks the suppressed bracts, compared to the control (N). Confocal images of *pZmPIN1a*::*ZmPIN1a*:*YFP* and *DR5rev*::*mRFPer* in *ba1/ba1* (O–S). *ba1* mutant tassels show a regular arrangement of ZmPIN1a up-regulation at the flanks of the IM (arrowheads), as observed in wild-type tassels (O and P), marking the enlarged suppressed bracts (Q, longitudinal view). DR5 expression is detected without the localized signal (arrowheads) observed in wild-type tassels (R and S). ZmPIN1a signal in +/*Bif1* tassels shows no periodic expression at the flanks of the main IM (T). Bar = 1 mm (A, B, L, and M) and 50 μm (E, M–S).

IM, similarly to wild-type plants (Fig. 5, O and P; Supplemental Fig. S1). This expression corresponded to the position of the suppressed bracts (Fig. 5Q). However, ZmPIN1a expression was absent in older primordia (Supplemental Fig. S1). This result, together with the results of NPA treatments, indicates that polar auxin transport by ZmPIN1a is responsible for the formation of the suppressed bracts and the failure to maintain ZmPIN1a expression or to express it in axillary meristem anlagens may be responsible for the absence of further development of the *ba1* inflorescence. We also examined the expression of the DR5 auxin-responsive promoter in *ba1* mutant tassels. In the IM and the area where the suppressed bracts are initiated no differences in DR5 expression were observed between wild type and *ba1*. However, unlike in wild type, no periodic DR5 expression was observed in *ba1* tassels (Fig. 5, R and S), indicating that the auxin response maxima of newly initiated axillary meristems are not created on the flanks of *ba1* IMs.

Bif1 mutant tassels lack suppressed bracts along the main axis, and the branches are thinner and bear very few spikelets (Fig. 5, K and L; Barazesh and McSteen, 2008). By analyzing ZmPIN1a-YFP expression in +/*Bif1* tassel primordia, we observed a very different pattern. Mutant tassels lacked the periodic up-regulation of ZmPIN1a on the flanks of the IM (Fig. 5T; Supplemental Fig. S2), and expression was uniform throughout the inflorescence apex. This result indicates that BIF1 is required for the proper up-regulation of ZmPIN1a expression on the flanks of the IM.

In summary, we found that PIN expression and auxin response maximum patterns appear to be conserved in maize compared to Arabidopsis. This is also true for those branching events not found in dicot species. We also found that *BA1* and *BIF1* differentially regulate PIN expression, clarifying their roles in inflorescence architecture establishment.

DISCUSSION

ZmPIN1a and DR5 Expression Suggest That Spatial Organization of the Polar Auxin Transport Pathway Is Conserved between Maize and Arabidopsis

Polar auxin transport by the PIN proteins has been implicated in a variety of developmental processes, including the regular arrangement of leaf primordia at the flanks of the shoot apical meristem (phyllotaxy), the establishment of apical-basal polarity in embryo development, tropic responses, and the formation of pro-vascular strands during leaf development (Friml et al., 2002, 2003; Reinhardt et al., 2003; Scarpella et al., 2006). A recent study reported differences in ZmPIN1 localization between maize and Arabidopsis, implying differences in the mechanism of primordium initiation (Carraro et al., 2006). These conclusions were based on the observation that ZmPIN1 proteins did not accumulate in the L1 layer of apical and axillary meristems. However, our analysis shows a clear accumulation of ZmPIN1a-YFP in the maize L1 layer of axillary meristems and IMs, suggesting that the formation of all axillary meristems and lateral organs in maize conforms to the accepted model based on observations from Arabidopsis. The contrasting results were obtained using different methodologies and might reflect a different sensitivity of immunolocalization compared to our fluorescent protein approach. For our transgenic approach, we followed the guidelines reported for the analogous construct in Arabidopsis (Benkova et al., 2003), inserting the YFP at the same position in the ZmPIN1a protein and using an equivalent promoter fragment. Several lines of evidence support the functionality of our ZmPIN1a-YFP construct and that its reported expression is correct: (1) ZmPIN1a-YFP is polarly localized at the plasma membrane (see also Supplemental Fig. S2), consistent with its role in the creation of auxin response maxima, as reported by DR5-RFP expression; (2) ZmPIN1a-YFP expression is up-regulated at the sites where auxin response maxima form and where primordia will emerge; (3) ZmPIN1a-YFP expression is clearly detectable in the L1 layers of all meristems analyzed and in the epidermis of organ primordia, where it is proposed that auxin is transported (Benkova et al., 2003; Reinhardt et al., 2003); (4) ZmPIN1a-YFP expression is also observed in the inner layers of developing organs, consistent with the role of auxin transport in the differentiation of provascular strands (Benkova et al., 2003; Reinhardt et al., 2003; Scarpella et al., 2006; Smith et al., 2006); and (5) NPA disrupts ZmPIN1a-YFP expression, as previously described in Arabidopsis (Heisler et al., 2005). Furthermore, when driven by the Arabidopsis *PIN1* promoter, ZmPIN1a fully rescues all branching defects of *pin1-3* mutants. All these observations indicate that the creation of dynamic gradients of auxin during primordium development is a conserved mechanism between maize and Arabidopsis, despite their different shoot and inflorescence architectures. The conservation in polar auxin transport mechanisms between maize and Arabidopsis is also supported by a recent report that *BIF2*, a gene required for the initiation of axillary meristems and lateral organs, encodes a co-ortholog of the Arabidopsis PID protein (McSteen et al., 2007). PID is a Ser/Thr kinase that regulates the subcellular localization of PIN proteins (Friml et al., 2004; Michniewicz et al., 2007). Expression and sequence analysis across several members of the grass family showed a similar role of *BIF2/PID* genes in axillary meristems and primordia initiation (McSteen et al., 2007). These findings suggest that the conservation of the auxin transport pathway may extend to multiple components between the two species.

The PIN family of auxin transporters is present throughout the plant kingdom, and in Angiosperms it has undergone a substantial radiation (Paponov et al., 2005). There have also been extensive duplications in monocots, and three rice (*Oryza sativa*) *PIN1* genes

have been reported (Paponov et al., 2005; Xu et al., 2005) and at least three maize *PIN1* genes are present in its genome. The maize *PIN1* genes are all broadly expressed, suggesting the existence of functional redundancy among the different members. We found incomplete rescue of the flower organ defects of the Arabidopsis *pin1-3* by *ZmPIN1a*. This could be due to a limited sampling of transformed lines, or might suggest that some functional differences exist between maize and Arabidopsis PIN1 proteins. For example, subfunctionalization of the different members could have occurred (Moore and Purugganan, 2005), preventing a full rescue of *pin1-3* defects during floral development. The incomplete floral rescue could also be due to a lack of intron sequences that may be needed for proper spatiotemporal gene expression or by down-regulation of expression resulting from cosuppression.

The Role of Polar Auxin Transport in the Formation of Suppressed Bracts

The auxin transport inhibitor NPA has been used in several species to investigate the role of auxin transport during vegetative and reproductive development (Okada et al., 1991; Tsiantis et al., 1999; Reinhardt et al., 2000, 2003; Scanlon, 2003; Xu et al., 2005; Wu and McSteen, 2007). Here, we used it to assess the effects of impaired auxin transport during maize inflorescence development in wild-type and *ba1* mutant tassels. As expected, the formation of axillary meristems was abolished in wild-type tassels treated with NPA (Wu and McSteen, 2007). The partially suppressed bracts that typically subtend axillary meristems were also abolished. In *ba1* mutant tassels, the suppressed bracts are more pronounced and remain visible at maturity (Ritter et al., 2002; Fig. 5, I and J). NPA treatment also suppressed formation of these bracts. Because ZmPIN1a-YFP is expressed normally in the emerging bracts in *ba1* tassels, we propose that polar auxin transport is required for the formation of these suppressed bracts. It is interesting that the bracts are enlarged in *ba1* mutants, and this suggests that axillary meristems act in an inhibitory manner to limit bract growth in wild-type tassels.

The Different Roles of *BA1* and *BIF1* in Polar Auxin Transport

As in wild type, we found that ZmPIN1a was expressed in *ba1* tassels on the flanks of the IM and in the developing suppressed bracts. This fits the hypothesis that *BA1* functions downstream of polar auxin transport (see also Wu and McSteen [2007]). However, although it is evident that ZmPIN1a expression does not require *BA1*, it is not clear if ZmPIN1a can be upregulated at the site of axillary meristem initiation in a *ba1* mutant. It is impossible to distinguish the suppressed bract primordia in the IM dome from the emerging axillary meristems, because no markers are available in maize to unequivocally identify suppressed bract initiation. However, if polar auxin transport were unaffected in *ba1*, we would expect an auxin response maximum to be formed in a pattern similar to that observed in wild type. The absence of periodic DR5-RFP expression on the flanks of the IM in *ba1* tassels (Fig. 5, R and S) suggests that axillary meristem anlagens are not defined by the polar auxin transport pathway, and therefore *BA1* may function upstream of polar auxin transport or auxin signaling for axillary meristem initiation. We hypothesize that the creation of an auxin response maximum at the site of new axillary meristem initiation (as detected by the DR5 promoter) is a process that requires *BA1* function. We noticed that ZmPIN1a-YFP expression is consistently stronger on the adaxial side of a suppressed bract in both wild type and *ba1* mutants, suggesting that polar auxin transport is mainly happening in this region (Fig. 5, O–Q). *BA1* may be required to integrate a signal from the suppressed bract with the polar auxin transport pathway to initiate a new meristem (Fig. 6A). In Arabidopsis, the interplay between leaf primordia and axillary meristem formation is well documented, and perturbation in the polarity of leaf primordia, for example, can result in ectopic meristem formation

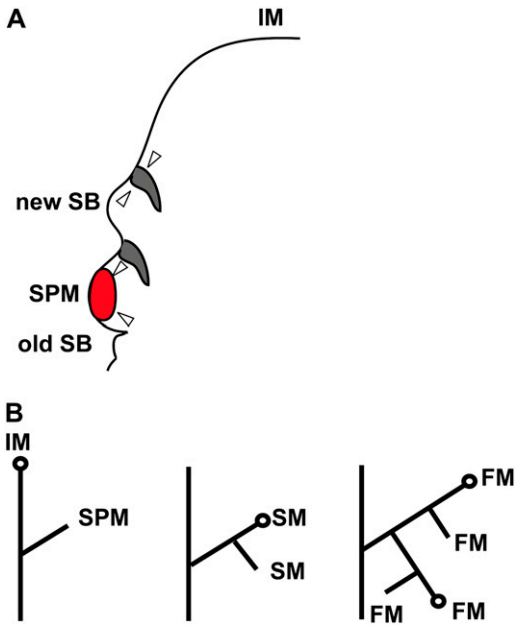

Figure 6. A, A model for *BA1* action in axillary meristem formation. Polar auxin transport (arrowheads) in the suppressed bract leads a signal to *BA1* (in gray, expression domain), which in turn activates polar auxin transport for axillary meristem initiation leading to an auxin response maximum (red area). B, A general model for branching during maize inflorescence development, based on ZmPIN1a expression pattern. Auxin response maxima are created at the flanks of an apical meristem to promote a branching event. Every axillary meristem in maize can be viewed as a result of a branching event by an apical meristem (either IM, SPM, or SM; circles). Subsequently, the apical meristems convert their identity to match that of the axillary meristem, according to the conversion model.

(McConnell and Barton, 1998). The absence of *BA1* expression in NPA-treated tassels, reported by Wu and McSteen (2007), could then be interpreted as an indirect consequence of the effects of NPA on the formation of the suppressed bracts (Fig. 5M).

The regular expression of ZmPIN1a is also altered in the semidominant *Bif1* mutant. In contrast to *ba1*, *Bif1* tassels do not form enlarged suppressed bracts. In *Bif1* mutant tassels, ZmPIN1a is expressed in the dome of the IM but lacks the periodic up-regulation on the flanks observed in wild type and *ba1* mutants. Therefore, *BIF1* is required for patterning of ZmPIN1a expression in the IM dome, suggesting it acts upstream of polar auxin transport during axillary meristem initiation. As mentioned above, another barren mutant, *bif2*, was recently isolated (McSteen et al., 2007). As in *Bif1*, *bif2* tassels have fewer branches and spikelets (McSteen and Hake, 2001). These similarities suggest that *BIF1* might also be involved in the regulation of auxin transport, as also suggested by measurement of polar auxin transport (Barazesh and McSteen, 2008).

A General Mechanism Explains All Branching Events during Maize Inflorescence Development

Grass inflorescences are defined by the spikelet, a specialized flower-bearing structure formed by the SM. In some grasses such as maize and sorghum, the spikelets are in pairs as a result of the activity of another type of axillary meristem, the SPM (Fig. 1C). We found that the specialized spikelet-pair and SMs are formed by a mechanism involving conserved patterns of ZmPIN1a expression. The colocalization of the DR5-RFP and ZmPIN1-YFP expression in SPMs indicates that the emerging SMs are the result of a branching event at the flank of the SPM, which then acquires the identity of an SM itself. This is also valid for the upper and lower FMs, where the latter is formed by a localized auxin response maxima on the flanks of an SM (compare Figs. 3I and 4G), which subsequently acquires an FM identity.

Two models have been proposed to explain the formation of SMs from SPMs and of FMs from SMs. In the conversion model, SMs or FMs are formed from lateral branching of the SPM or SM, respectively, which subsequently convert into an SM or an FM themselves (Irish, 1997; Kaplinsky and Freeling, 2003). In contrast, the lateral branching model proposes that SMs give rise to the two FMs by two successive lateral branching events, and the remnant SM remains in between the two FMs (Chuck et al., 1998). Recently, Wu and McSteen (2007) reported that NPA treatments result in formation of single spikelets. This suggests that the spikelet-pair is the result of a single branching event, affected by the inhibition of polar auxin transport, and strongly supports the conversion model for axillary meristem development in maize. Our data presented here indicate that a common pathway of PIN1-driven auxin transport regulates the initiation of all axillary meristems in maize, and the patterns of ZmPIN1a localization are also consistent with the conversion model for axillary meristem initiation. We therefore propose that the formation of all axillary meristems in maize is regulated by polar auxin transport in a common fashion. In this model, the apical meristem (either IM, or SPM or SM) creates auxin response maxima on its flanks to promote the formation of one or multiple new axillary meristems (either SPM or SM or FM; Fig. 6B). In the case of the SPM or SM, the apical meristem then acquires the same identity as the newly formed axillary meristem, consistent with the conversion model (Irish, 1997).

CONCLUSION

Conserved patterns of PIN1 and DR5 expression suggest a conserved polar auxin transport mechanism is necessary for the formation of all axillary meristems and lateral primordia in maize, as in Arabidopsis. This includes additional meristems such as SPMs and SMs that are not found in Arabidopsis. These meristems behave like determinate IMs, because they initiate only a single axillary meristem. This determinate growth pattern is required to produce the specialized maize inflorescence architecture and may be accomplished by blocking the transport or accumulation of auxin at additional axillary meristem sites.

MATERIALS AND METHODS

Molecular Cloning and Transformation

The predicted coding sequence of the *ZmPIN1c* gene was determined by editing the gene prediction models FGENESHMAGI_48818.1 and FGENESHMAGI_48819.1 (http://magi.plantgenomics.iastate.edu) on the AZM4_22603 contig (http://maize.tigr.org/release4.0/assembly_summary.shtml). *ZmPIN1a*, *ZmPIN1b*, and *ZmPIN1c* were mapped on three different bacterial artificial chromosomes (b0080H22, c0463C23, and b0546F20, respectively) by database searches (www.maizesequence.org). The *pAtPIN1*::*ZmPIN1a* construct was made by PCR amplifying the AtPIN1 promoter from the clone *pPIN1*::*PIN1:GFP* (Benkova et al., 2003), introducing *Kpn*I and *Eco*RI sites and cloning into the same sites of pBluescript SK+ (pBS-pPIN1). A *ZmPIN1a* complementary DNA clone was amplified from the start codon to the 3′ untranslated region, introducing an *Eco*RI site upstream of the ATG and a *Sma*I site downstream of the 3′ untranslated region. The resulting fragment was ligated into the *Eco*RI/*Sma*I sites of pBS-pPIN1. The *Kpn*I/*Sma*I fragment containing the *pPIN1*::*ZmPIN1a* cassette was ligated in pMX202 vector cut with *Kpn*I and blunted from a *Bam*HI restriction site.

For the ZmPIN1a-YFP construct, a total of 6,568 bp of *ZmPIN1a* genomic DNA sequence was used. This sequence includes 2,766-bp upstream sequence and 1,152-bp downstream sequence of the *ZmPIN1a* open reading frame along with the *ZmPIN1a* open reading frame itself. Using the fluorescent tagging of full-length proteins method (Tian et al., 2004), YFP was internally inserted between the 218th and 219th amino acids. For this, the following primers were used: *ZmPIN1a*-P1, GCTCGATCCACCTAGGCTtttggaaaatcaggccaagc; *ZmPIN1a*-P2, CACAGCTCCACCTCCACCTCCAGGCCGGCCgtagatgtccgagcgcgac; *ZmPIN1a*-P3, TGCTGGTGCTGCTGCGGCCGCTGGGGCCtcccggcggtccatgg; *ZmPIN1a*-P4, CGTAGCGAGACCACAGGAcaaaacggcattatcaaacagg. Note that gene-specific sequences are shown in lowercase and the linker sequences are in uppercase.

The *DR5rev*::*mRFPer* construct was obtained by fusing the DR5rev promoter (Benkova et al., 2003) with a monomeric RFP (Campbell et al., 2002) targeted to the endoplasmic reticulum, as described above (primers: P1, GCTCGATCCACCTAGGCTgtcgacggtatcgcagcccag; P2, CACAGCTCCACCTCCACCTCCAGGCCGGCCgaattcggccgaggataatg; P3, TGCTGGTGCTGCTGCGGCCGCTGGGGCCcatgatgagctttaagag; P4, CGTAGCGAGACCACAGGA-

gcatgcctgcaggtcac). The fusion product was then cloned using a Gateway system (Invitrogen) in the pAM1006 vector for maize (*Zea mays*) transformation. pAM1006 is derived from the base vector pTF101.1 (Paz et al., 2004) by adding a Gateway cassette (A. Mohanty and D. Jackson, unpublished data). Fifteen independent events were obtained and analyzed for expression of the *DR5rev*::*mRFPer* construct and six events for the *ZmPIN1a*::*ZmPIN1a*:*YFP* construct.

For Arabidopsis (*Arabidopsis thaliana*) transformation, plants that were either heterozygous for *pin1-3* (*pin1-3* mutants are sterile) or wild type were infiltrated with Agrobacterium (Clough and Bent, 1998). After selection on Murashige and Skoog plates containing kanamycin, the transformants were genotyped for the *pin1-3* mutation by using a CAPS marker developed on the site of the mutation. After PCR amplification (primers: forward, TGGTTTGGAGGAAACTTATTCG; reverse, CATGAACAACCCAAGACTGAAC), the product was digested with *Sca*I (only the wild-type allele is cut). Homozygous and heterozygous *pin1-3* mutants were allowed to self-fertilize and were analyzed in the following generation for the rescue of the *pin1-3* phenotypes. Of 39 families carrying the *pin1-3* mutation, five showed complementation of the *pin1-3* phenotype (Supplemental Table S1). Family number 9 showed the strongest phenotypic rescue and was used for further analysis.

Confocal Microscopy

After dissection, seedlings, immature tassels, and ears (0.2–1 cm) were hand-sliced, laid on a microscope slide with water, and covered with a coverslip.

Confocal images were taken using a Zeiss LSM510 and a Leica TCS SP2. Images were acquired using the LSM software or the Leica confocal software and some images were analyzed with ImageJ 1.38X (http://rsb.info.nih.gov/ij). For preparing Supplemental Movies S1 to S6, we used the OsiriX medical imaging software (http://www.osirix-viewer.com).

Polar Auxin Transport Inhibition

Maize wild-type and transgenic plants were watered with NPA (ChemService) at different concentrations (10, 30, or 50 μM dissolved in dimethyl sulfoxide and diluted in water) for approximately 2 weeks. Control plants were watered with the same concentration of dimethyl sulfoxide. For the NPA treatment of *ba1* mutants, homozygous plants were identified by the absence of vegetative axillary buds at the axils of leaves, which form prior to the treatment stage (Gallavotti et al., 2004).

Scanning Electron Microscopy

For scanning electron microscopy, immature tassel and ear primordia were dissected and imaged without fixation using an S-3500 N SEM (Hitachi) as previously described (Taguchi-Shiobara et al., 2001). Samples were also fixed in formaldehyde-acetic acid (50% [v/v] ethanol, 3.7% [v/v] formaldehyde, 5% [v/v] acetic acid) for 12 h, dehydrated in a graded ethanol series, critically point dried, and coated with a gold/palladium mixture and viewed on a Quanta 600 scanning electron microscope at 20 kV.

Expression Analysis

In situ hybridizations were performed with a *KNOTTED1* probe (Jackson et al., 1994) as previously described (Gallavotti et al., 2004).

For reverse transcription (RT)-PCR, total RNA was isolated from pools of fiv immature tassels or ears (0.2 or 0.6 cm) or five seedling shoot apices (4 d after germination) after removal of the roots and most of the leaves. All samples were from the maize inbred line B73. After extraction with Trizol (Invitrogen), the RNA was further purified using the RNeasy plant mini-kit (QIAGEN), and 1 μg was treated with 2 μL of DNase following the manufacturer's instructions (Promega). Approximately 12 ng of total RNA was used in one-step RT-PCR reactions (one-step RT-PCR kit; Invitrogen). The list of the primers used is in Supplemental Table S2. *ZmPIN1a* and *UBIQUITIN* were amplified for 30 cycles, *ZmPIN1b* and *ZmPIN1c* for 38 cycles.

Supplemental Data

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

Supplemental Figure S1. Additional confocal sections of both *ba1* and *Bif1* mutants.

Supplemental Figure S2. ZmPIN1a-YFP polar localization.

Supplemental Figure S3. Confocal sections taken from the movies. It is meant to help the vision of the movies, together with the Supplemental Movie Legends S1.

Supplemental Table S1. List of *pin1-3* rescued families.

Supplemental Table S2. List of RT-PCR primers.

Supplemental Movies S1 to S6. Series of confocal images of ZmPIN1a-YFP and DR5-RFP lines at different stages of development.

Supplemental Movie Legends S1. Descriptions of Supplemental Movies S1 to S6.

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