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RESEARCH ARTICLES

Differentiating Arabidopsis Shoots from Leaves by Combined YABBY Activities [W] [OA]

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In seed plants, leaves are born on radial shoots, but unlike shoots, they are determinate dorsiventral organs made of flat lamina. YABBY genes are found only in seed plants and in all cases studied are expressed primarily in lateral organs and in a polar manner. Despite their simple expression, *Arabidopsis thaliana* plants lacking all YABBY gene activities have a wide range of morphological defects in all lateral organs as well as the shoot apical meristem (SAM). Here, we show that leaves lacking all YABBY activities are initiated as dorsiventral appendages but fail to properly activate lamina programs. In particular, the activation of most CINCINNATA-class TCP genes does not commence, SAM-specific programs are reactivated, and a marginal leaf domain is not established. Altered distribution of auxin signaling and the auxin efflux carrier PIN1, highly reduced venation, initiation of multiple cotyledons, and gradual loss of the SAM accompany these defects. We suggest that YABBY functions were recruited to mold modified shoot systems into flat plant appendages by translating organ polarity into lamina-specific programs that include marginal auxin flow and activation of a maturation schedule directing determinate growth.

INTRODUCTION

Leaves are determinate laminar structures specialized for photosynthesis. Evidence in the fossil record suggests that seed plant leaves evolved from lateral branch systems (Galtier, 1981; Stewart and Rothwell, 1993; Beerling and Fleming, 2007; Sanders and Rothwell, 2009). Over time, the ancestral radial, three-dimensional lateral shoot systems evolved to become determinate, planar, dorsiventral, and eventually laminar. This would have required mechanisms to repress radial, axial growth and promote laminar growth. Lamina growth facilitated the development of specialized leaf surfaces, with leaves of many seed plant species having one surface specialized for light capture and the other for gas exchange.

Leaves of seed plants are derived from the periphery of the shoot apical meristem (SAM) and display a distinct adaxial–abaxial axis. This asymmetry is thought to reflect inherent positional differences in the developing organ relative to the SAM from which it is derived; the adaxial side is adjacent to the meristem, while the abaxial side faces away from the meristem. In classical experiments in which an incision was made between the SAM and an incipient leaf primordium, the isolated leaf primordium developed as a radial unifacial organ, indicating not only that adaxial-abaxial polarity establishment requires communication with the SAM but that adaxial–abaxial polarity is necessary for lamina growth (Sussex, 1954). Later observations of ectopic lamina development in *Antirrhinum phantastica* mutants led to the proposal that juxtaposition of adaxial and abaxial cell types results in lamina growth (Waites and Hudson, 1995).

Experiments in model angiosperm species have identified several families of transcription factors, some of which are regulated by small RNAs, that direct adaxial–abaxial polarity establishment, or its interpretation, and in so doing promote laminar growth (Canales et al., 2005; Chitwood et al., 2007). Adaxial fates in angiosperm leaves are regulated by the activities of the AS2/ARP (*PHANTASICA*-related) and class III HD-Zip transcription factors and *trans*-acting small interfering RNAs, which are generated through the miR390-TAS3-RDR6 pathway (Waites and Hudson, 1995; McConnell and Barton, 1998; Emery et al., 2003; Chitwood et al., 2007; Iwakawa et al., 2007). In *Arabidopsis thaliana*, abaxial fates are the result of the activities of the KANADI, YABBY, and AUXIN RESPONSE FACTOR (ARF3/4) transcription factor families and the miR165/166 small RNAs (Eshed et al., 1999, 2001; Sawa et al., 1999a; Siegfried et al., 1999; Kerstetter et al., 2001; McConnell et al., 2001; Juarez et al., 2004; Pekker et al., 2005). Homologs of nearly all of these genes

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can be found in the genomes of *Selaginella moellendorffii* (lycophyte) and *Physcomitrella patens* (moss), demonstrating that most of the gene families identified as important for leaf polarity and lamina growth existed long before seed plant leaves evolved (Harrison et al., 2005; Floyd and Bowman, 2006, 2007; Floyd et al., 2006; Prigge and Clark, 2006). This suggests that most of the genetic machinery for seed plant leaf polarity establishment was coopted from preexisting genetic programs operating in plants with indeterminate branching radial shoot systems; however, neofunctionalization following duplication may have been critical. The lone known exception is the YABBY gene family, which is seed plant–specific; thus, its evolutionary history coincides with the origin of leaves in seed plants (Floyd and Bowman, 2007; Figure 1). YABBY genes have unique expression patterns that include exclusion from apical meristems, activation in initiating lateral organ primordia, and, often, asymmetric restriction to the abaxial domain of eudicot primordia but to the adaxial domain of initiating maize (*Zea mays*) leaves (Sawa et al., 1999a; Siegfried et al., 1999; Golz et al., 2004; Juarez et al., 2004).

YABBY genes have been identified in all seed plants examined, with most eudicotyledonous angiosperms possessing five different classes (Bowman and Smyth, 1999; Yamada et al., 2004; Lee et al., 2005). The *Arabidopsis* genome contains six YABBY genes (*FILAMENTOUS FLOWER* [*FIL*], *CRABS CLAW* [*CRC*], *INNER NO OUTER* [*INO*], *YABBY2* [*YAB2*], *YAB3*, and *YAB5*), with *FIL* and *YAB3* the result of a recent duplication (Bowman and Smyth, 1999; Sawa et al., 1999b; Siegfried et al., 1999; Villanneva et al., 1999; Lee et al., 2005). The expression of two family members, *CRC* and *INO*, is restricted to floral organs (Bowman and Smyth, 1999; Villanneva et al., 1999). In contrast, *FIL*, *YAB3*, *YAB2*, and *YAB5* are referred to as the "vegetative YABBY genes" of *Arabidopsis*, with *FIL*, *YAB2*, and *YAB3* expressed in the abaxial domains of all leaf-derived organs, including cotyledons, leaves, and floral organs (Sawa et al., 1999a; Siegfried et al., 1999; Watanabe and Okada, 2003; Golz et al., 2004).

The molecular identification of YABBY genes, along with the discovery of their polar expression patterns, led to the proposal that YABBY factors promote abaxial cell fate (Eshed et al., 1999; Sawa et al., 1999b; Siegfried et al., 1999). A role in polar differentiation is supported by both gain- and loss-of-function data. For example, *Arabidopsis* loss-of-function alleles of *CRC* can result in adaxial carpel tissues developing in abaxial positions, while gain-of-function alleles of *FIL* and *YAB3* promote the differentiation of abaxial tissues in adaxial positions (Eshed et al., 1999; Sawa et al., 1999b; Siegfried et al., 1999). Consistent with classical observations, expression of *FIL* and *YAB3* is closely associated with lamina expansion in *Arabidopsis*, with boundaries of abaxial YABBY gene expression marking the abaxial–adaxial boundary (Siegfried et al., 1999; Kumaran et al., 2002; Eshed et al., 2004). Expression of *FIL* and *YAB3* in leaf primordia displays a longitudinal gradient, presumably following the cell division arrest front, which passes from leaf tip to base (Nath et al., 2003). Previous studies have demonstrated a partial loss of polar differentiation in leaves and other lateral organs of YABBY mutants, with both adaxial and abaxial tissues being affected, implying nonautonomous YABBY gene activity in the adaxial leaf domain (Siegfried

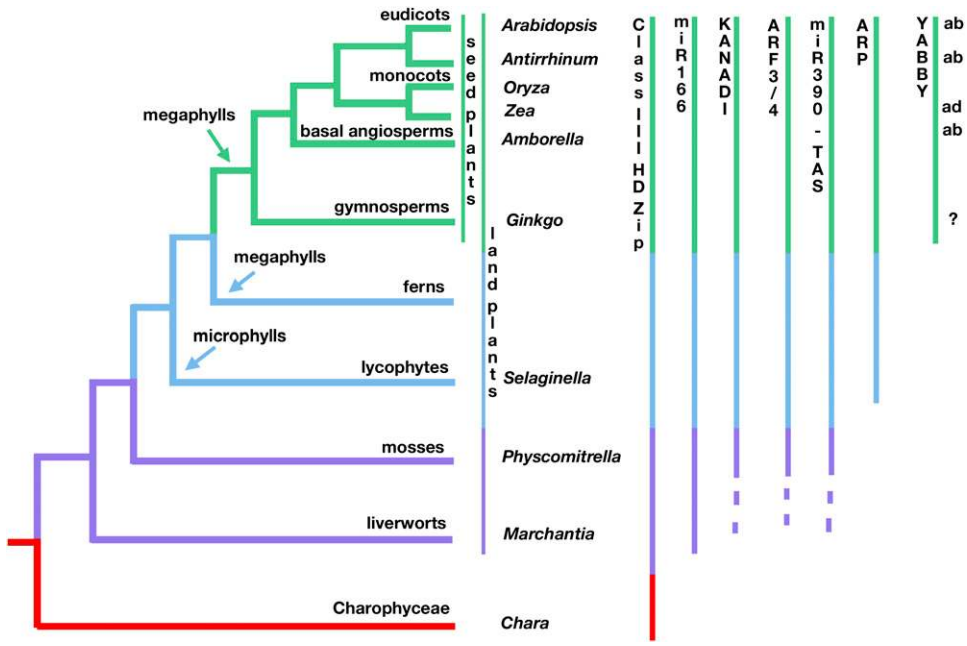

Figure 1. Phylogenetic Distribution of Angiosperm Leaf Polarity Genes.

Origins of leaves, megaphylls, and microphylls are indicated, as are expression patterns of YABBY genes in seed plants (ab, abaxial; ad, adaxial). Dashed lines indicate that the antiquity of gene families is not known. The phylogenetic distribution of selected genes involved in angiosperm leaf polarity was determined utilizing genome sequence data available at present for land plant lineages (Sawa et al., 1999a; Siegfried et al., 1999; Golz et al., 2004; Yamada et al., 2004; Harrison et al., 2005; Floyd et al., 2006; Prigge and Clark, 2006; Floyd and Bowman, 2007; Rensing et al., 2008).

et al., 1999; Kumaran et al., 2002; Golz et al., 2004; Stahle et al., 2009). Furthermore, loss of YABBY activity results in altered anatomy at the margin of *Antirrhinum* leaves (Golz et al., 2004). The implications for a role for YABBY genes in marginal patterning and growth are intriguing in light of recent evidence for the importance of marginal auxin transport and PIN1-mediated maxima in the patterning of leaves in *Cardamine* and *Solanum* (Scarpella et al., 2006; Barkoulas et al., 2008; Koenig et al., 2009). It is unknown how loss of YABBY function might affect important auxin-mediated processes in leaf margins.

Several lines of evidence suggest that the role of YABBY genes extends beyond promoting abaxial identity and laminar growth. Based on nonautonomous phenotypic defects in both loss- and gain-of-function alleles, YABBY gene activity has been implicated in communication between developing leaves and the SAM in both *Arabidopsis* and *Antirrhinum* (Eshed et al., 2004; Golz et al., 2004; Goldshmidt et al., 2008; Stahle et al., 2009) and in downregulating class I KNOX expression in leaves of *Arabidopsis* (Kumaran et al., 2002). Thus YABBY genes are implicated in both promoting polarity and laminar growth as well as repressing SAM-patterning genes.

Since at least some YABBY gene family members are abaxially expressed in *Amborella trichopoda*, the basal-most angiosperm taxon, abaxial expression may be the ancestral state in angiosperms (Yamada et al., 2004). However, abaxial expression is not universal within angiosperms, as some YABBY genes in *Zea* are expressed in a polar, but adaxial, pattern (Juarez et al., 2004). Furthermore, in restricted lineages of angiosperms, YABBY genes have been coopted for other developmental roles. For example, *CRC* orthologs are important for midrib development in grasses and for nectary development in core eudicots (Yamaguchi et al., 2004; Lee et al., 2005; Toriba et al., 2007). While no functional data are available for YABBY genes from basal angiosperms or gymnosperms, functional conservation in *Arabidopsis* and *Antirrhinum* and expression patterns in *Amborella* and *Zea* suggest an ancestral association of YABBY genes with leaf polarity, and by inference lamina growth within angiosperms.

Because of the unique association of the YABBY gene family with leaves in flowering plants and their phylogenetic restriction to the seed plant clade, understanding the role of YABBY genes in shoot development may provide insight into the fundamental nature of the angiosperm leaf and provide clues about the origin of leaves in the seed plant clade. Previous studies of YABBY gene function in leaves have been primarily limited to *FIL* and *YAB3*. Given similar expression patterns of all vegetative YABBY genes and the previously noted functional redundancy of *FIL* and *YAB3*, it is likely that *YAB2* and *YAB5* also play overlapping roles during leaf development (Siegfried et al., 1999). Therefore, we undertook a genetic approach to characterize *Arabidopsis* plants compromised in the function of all four vegetative YABBY genes (*FIL*, *YAB3*, *YAB2*, and *YAB5*) simultaneously. We demonstrate that YABBY genes govern embryo patterning and leaf lamina growth along the abaxial–adaxial boundary. In the absence of YABBY activities, leaf primordia maturation programs and restriction of SAM programs are partially lost; the leaves fail to activate early stages of lamina development marked by CINCINNATA-class TCP (CIN-TCP) activities and, instead, reactivate shoot programs marked by *WUSCHEL* (*WUS*) expression. The developmental aberrations in YABBY loss-of-function mutants are both autonomous and nonautonomous in nature and are also accompanied by changes in the distribution and flow of auxin. Based on the new types of auxin flow patterns and the novel molecular composition of mutant leaves, we postulate that YABBY genes act as integrators of an ancestral shoot system genetic program, molding it into a leaf-specific program, and thus have been integral to the evolution of laminar organs in seed plants.

RESULTS

Expression of *YAB2* and *YAB5*

To investigate *YAB5* expression patterns, we constructed a promoter:β-glucuronidase (GUS) reporter gene using 4.1 kb of upstream sequence (extending from the *YAB5* initiation codon to the next annotated gene). We constructed a similar reporter gene using 1.3 kb of the *YAB2* upstream region. Like *FIL* and *YAB3* (Figures 2A, 2D, 2G, and 2H), *pYAB5:GUS* and *pYAB2:GUS* are expressed in the abaxial domain of lateral organs (Figures 2I and 2J). Overall, the *pYAB5:GUS* construct was broadly expressed in young seedlings, with strongest expression in the petiole and midrib region (Figures 2B and 2E). Expression of the *pYAB2:GUS* construct was most pronounced in the petiole/midrib region (Figures 2C and 2F). It is notable that both *pYAB2:GUS* and *pYAB5:GUS* constructs continued to drive GUS expression in the midrib region of developed leaves (Figures 2E and 2F), while in leaves of equivalent age GUS expression in the *yab3-2* allele was no longer apparent (Figure 2D). In several independent *pYAB2:GUS* and *pYAB5:GUS* transgenic lines, reporter gene expression was also observed in stem vascular tissues, an expression that was confirmed by RT-PCR (data not shown). Thus, *FIL* and *YAB3* share overlapping expression domains with *YAB2* and *YAB5* in young leaf primordia.

Effects of Eliminating All YABBY Activity on Shoot Development

To eliminate all YABBY activity in young leaf primordia, mutations in *YAB2* and *YAB5* were identified and combined with strongest available alleles of *FIL* (*fil-8*), *YAB3* (*yab3-2*), and *CRC* (*crc-1*; Alvarez and Smyth, 1999; Kumaran et al., 2002). Loss-of-function alleles of *YAB2* and *YAB5* genes were identified by screening TILLING lines (McCallum et al., 2000; Till et al., 2003). The *yab5-1* allele is a Q>stop nonsense mutation in the highly conserved YABBY domain. The mutation in *yab2-1* results in the loss of a splice site acceptor site and leads to a frameshift within the YABBY domain in the mRNA (see Supplemental Figure 1 online). Both mutations disrupt the YABBY domain, and based on phenotypes of similar alleles of *FIL* and *CRC*, they should be strong, likely null, alleles (Bowman and Smyth, 1999; Siegfried et al., 1999).

Leaves of homozygous *yab2-1* and *yab5-1* mutant lines and the *yab2-1 yab5-1* double mutant exhibit a morphology similar to wild-type leaves (Stahle et al., 2009). However, the triple mutant *fil-8 yab3-2 yab5-1* (*yab135*) and quadruple mutant *fil-8 yab2-1 yab3-2 yab5-1* (*yab1235*) plants, hereafter referred to as

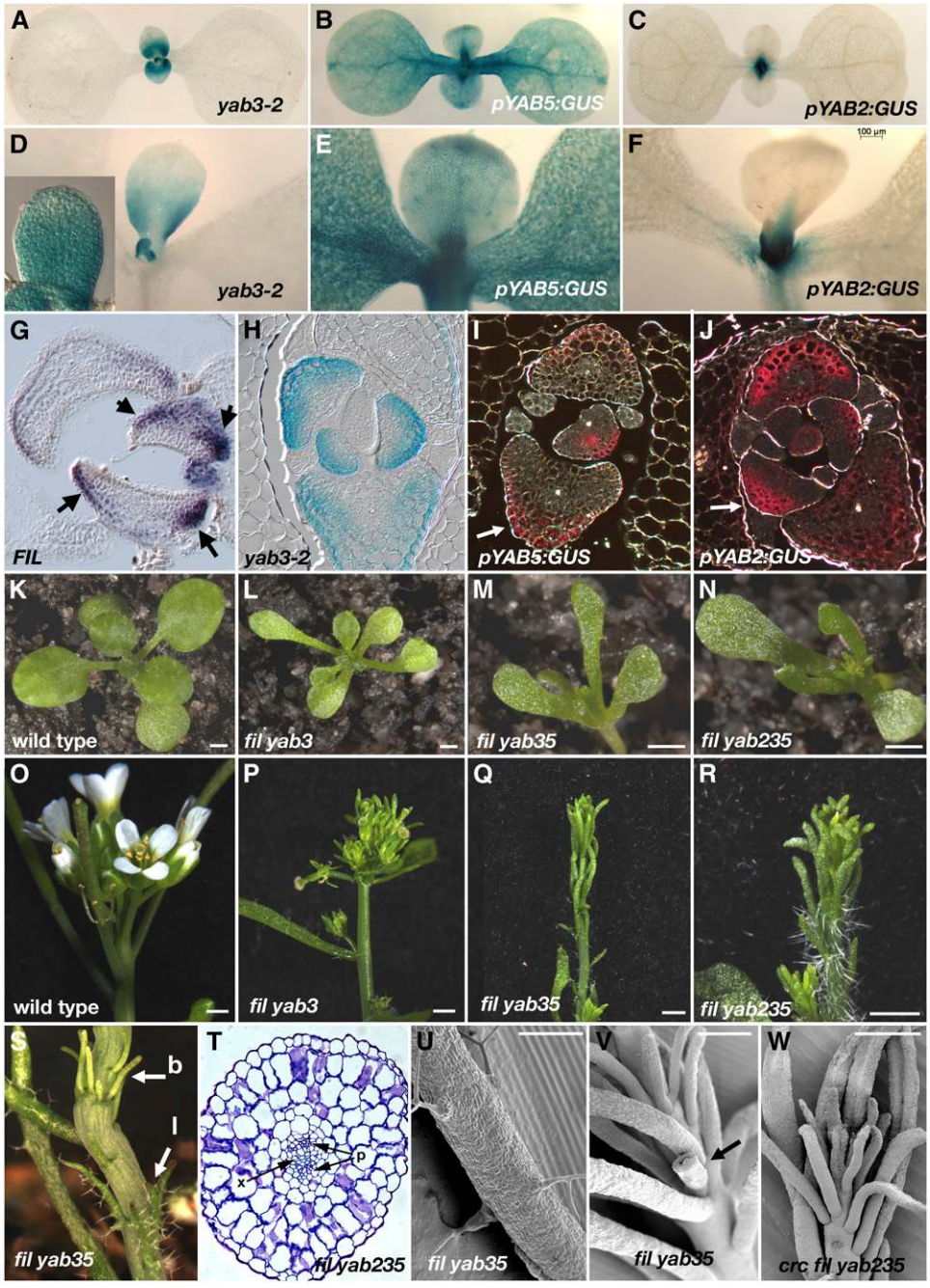

Figure 2. Redundancy of YABBY Gene Activity.

(A) to **(C)** *YAB3* (*yab3-2*), *YAB5* (*pYAB5:GUS*), and *YAB2* (*pYAB2:GUS*) display unique gene expression patterns (9-d-old seedlings, top view).
(D) *YAB3* is initially expressed throughout the leaf primordium (inset) but is switched off as the leaf differentiates. Note also the absence of expression in the petiole and midrib region.
(E) *YAB5* expression is more widespread, with highest levels in the petiole and midrib region.
(F) *YAB2* expression is restricted sharply to the petiole and midrib region.
(G) and **(H)** Cross sections reveal that at early stages, *FIL* and *YAB3* expression is throughout the abaxial regions of leaves (arrowheads), but at later stages, *FIL* expression becomes localized to the margins (arrows).
(I) *pYAB5:GUS* is initially detected near vascular bundles and is later primarily in the central abaxial region of leaf primordia (arrow).
(J) Expression of *pYAB2:GUS* is limited to the abaxial midrib (arrow).
(K) to **(N)** Whole plant images of wild-type and YABBY multiple mutant plants.
(O) to **(S)** Inflorescence structure of wild-type and YABBY mutant plants. The transition from leaf (l) production to trichomeless bract-like (b) structures is

YABBY triple and quadruple mutants, respectively, displayed a significant enhancement of the *fil-8 yab3-2* double mutant phenotype (Figures 2K–2N): diminutive and bushy plants that lack apical dominance, and with all lateral organs displaying a dramatic loss of lamina expansion and polarity defects.

The leaves of YABBY triple and quadruple mutants develop in various shapes, ranging from organs with some lamina to fully radialized organs (Figures 2M, 2N, and 2Q–2S). An age-dependent gradient exists, such that the first few leaves have the greatest amount of lamina, whereas later produced leaves, especially the stem (cauline) leaves, tend to be radialized (Figures 2Q–2S). The flowers of YABBY triple and quadruple mutant plants consist almost entirely of single filamentous organs (Figures 2Q–2V). Occasionally, a filamentous organ subtends a solitary gynoecium composed of two carpels (Figure 2V), suggesting that some filamentous organs observed in the YABBY mutants (Figure 2P) represent the normally suppressed bracts of *Arabidopsis* flowers (Long and Barton, 2000), which are also occasionally initiated in *fil-8* single mutants. In both triple and quadruple mutants, the inflorescence meristem terminates in either a cluster of filaments (Figures 2Q, 2R, and 2V) or in carpelloid tissue with morphologically and anatomically normal ovules (data not shown). The presence of carpel tissue in the YABBY quadruple mutant is due to residual YABBY activity supplied by a functional *CRC* gene, since no evidence of carpel tissue was detected in the *crc-1 fil-8 yab2-1 yab3-2 yab5-1* pentuple mutant (Figures 2V and 2W).

Polarity of YABBY Quadruple Mutant Leaves

Our analyses of *yabby* mutant leaves were primarily focused on the first formed leaves, since SAM activity, which may affect polarity establishment in leaf primordia, is disrupted in YABBY mutants. In wild-type *Arabidopsis* plants, trichomes are limited to the adaxial surfaces of the first four to six leaves (Figure 3A), with later produced leaves having trichomes on both surfaces. The first few rosette leaves of YABBY quadruple mutants display trichomes exclusively on their adaxial surface, indicating that some adaxial–abaxial polarity exists (Figure 3B).

In wild-type leaves, the adaxial and abaxial epidermal surfaces are distinct, with the adaxial epidermis consisting of cells of relatively uniform size and sparsely interspersed stomata, while the abaxial epidermal cells are variably sized with a higher density of stomata (Figures 3C and 3D). Epidermal cells of YABBY quadruple mutant leaves are uniformly large compared with the wild type and are neither identical to wild-type adaxial nor wild-type abaxial cells (Figures 3E and 3F). Internally, the lamina of wild-type *Arabidopsis* leaves comprises six cell layers: the adaxial epidermis, a single adaxial layer of palisade mesophyll, three abaxial layers of spongy mesophyll, and the abaxial epidermis (Figure 3G). The dark green leaves of YABBY quadruple mutants are noticeably thicker than the wild type, with a total of 7 to 12 cell layers, usually lacking a clear spongy/palisade differentiation (Figure 3H). Occasionally, the adaxial-most subepidermal cell layer retains anatomical aspects of the palisade mesophyll, being slightly elongated perpendicular to the laminar surface and without large air spaces between the cells (Figures 3I to 3J). In radialized leaves, there is little to distinguish the different mesophyll layers (Figure 3K). In wild-type *Arabidopsis* leaves, the xylem is located adaxially and phloem abaxially (Figure 3I), but in bundles of YABBY quadruple mutant leaves that exhibit lamina growth, this polarity is usually skewed in orientation such that the arrangement of phloem and xylem is not perpendicular to the leaf axis (Figure 3J). In contrast, radialized leaves of quadruple mutants tend to have an amphicribal arrangement of vascular bundles, where central xylem tissue is surrounded by clusters of phloem (Figures 2T and 3K), an arrangement typically found in abaxialized radial leaves (Waites and Hudson, 1995).

Expression of Polarity Markers in the YABBY Quadruple Mutant

The *yab3-2* gene trap allele faithfully expresses GUS in the *YAB3* expression domain and serves as a useful molecular marker of adaxial–abaxial polarity during lateral organ development (Kumaran et al., 2002). In YABBY triple and quadruple mutant backgrounds, GUS expression, although extremely weak compared with the wild type, is clearly abaxial (Figures 3L–3N). Even radialized organs retain this biochemical signature of polarity, although expression in older leaves may be expanded to the adaxial regions (Figure 3N; Stahle et al., 2009).

We examined global polar gene expression patterns in *fil yab3* and *fil yab3 yab5* mutants. We identified sets of polarly expressed genes, defined as genes modified in the adaxial (*phb-1d*) and the abaxial (*pANT>>KAN2*) genotypes relative to the wild type (Figure 3O; see Supplemental Data Set 1 online). Using the set of identified genes as a proxy for polar gene expression, YABBY mutants are neither clearly abaxialized nor adaxialized; rather, overall polar gene expression appears reduced. YABBY triple mutants display reduced expression of both adaxial (e.g., *PHB*, *REV*, *AS2*) and abaxial (e.g., *ARF4*) polarity markers (see Supplemental Table 1 online). These data are in agreement with the mixed morphological attributes of the mutant leaves.

Lamina of the YABBY Mutants Have a Mixture of Leaf and Shoot Characters

When the activities of two of the principal regulators of organ polarity, *PHB*-like HD-Zip genes and *KAN1*-like GARP family genes, are expanded throughout leaf primordia, polarity is not

Figure 2. (continued).

noted in **(S)**.

(T) and **(U)** Radial leaf of a *fil yab235* plant **(T)** and a cross section of a radial leaf (**U**). p, phloem; x, xylem.

(V) Occasionally formed axillary flower (arrow) consisting of a solitary gynoecium.

(W) *fil yab235 crc* pentuple mutants lack carpelloid organs.

Bar in **(K)** to **(R)** = 1 mm; bar in **(U)** and **(V)** = 250 μm; bar in **(W)** = 500 μm.

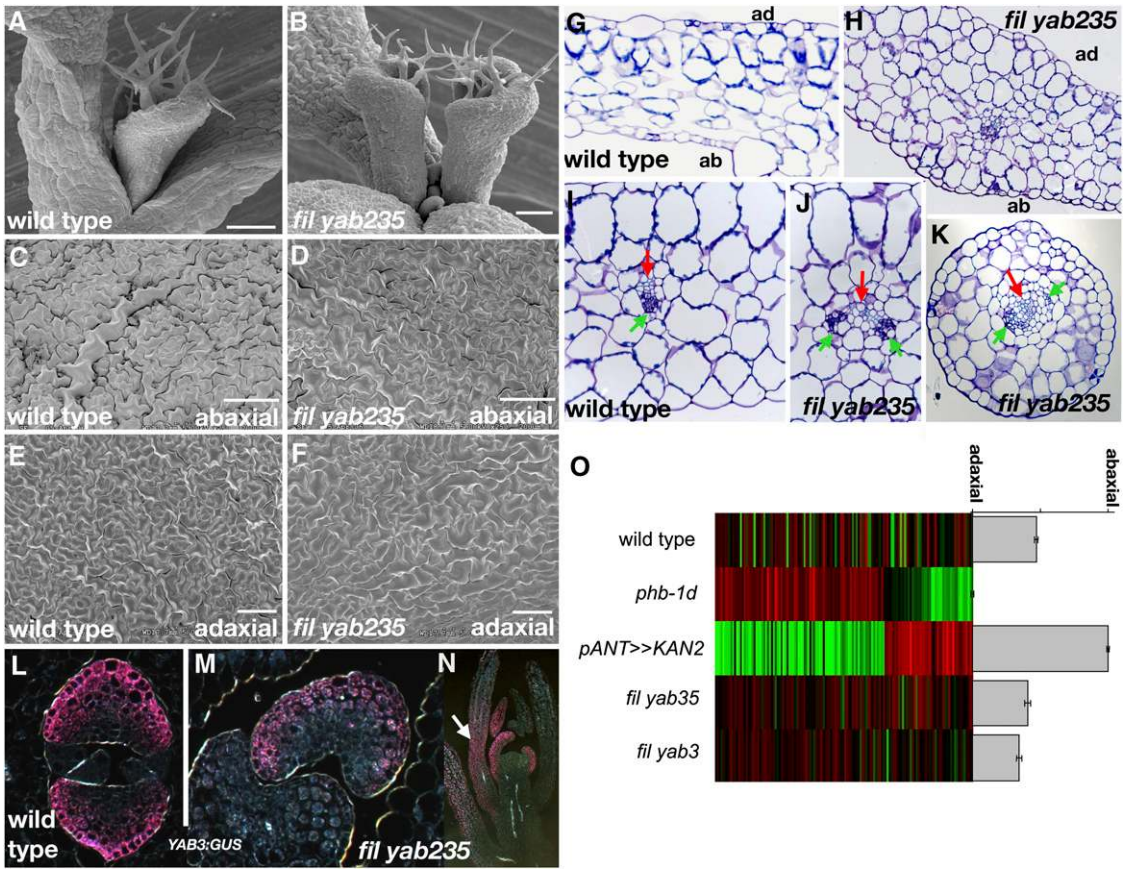

Figure 3. Morphological and Molecular Markers Show a Loss of Polar Differentiation in *yabby* Leaves.

(A) and **(B)** Scanning electron microscopy images of wild-type and *fil yab235* seedlings showing adaxial trichomes on the first two leaves.
(C) to **(F)** The adaxial and abaxial epidermises of wild-type leaves show distinct cell types, while this is lost in *fil yab235*leaves.
(G) and **(H)** Transverse leaf sections showing a loss of polar differentiation in *fil yab235* leaves and additional cell layers. ab, abaxial; ad, adaxial.
(I) to **(K)** Leaf vasculature: phloem (green arrows) and xylem (red arrows).
(L) to **(N)** GUS expression in *yab3-2*.
(O) The polarity index (right) and color-coded normalized expression of leaf polarity genes (left), derived from genes modified in the adaxial (*phb-1d*) and the abaxial (*pANT>>KAN2*) leaves. YABBY mutants show no overall bias in the expression of these genes. Bars = 100 μm.
Bar in **(A)** to **(F)** = 100 μm.

established and lamina are not initiated (Eshed et al., 2001; McConnell et al., 2001). YABBY activity is greatly reduced in the apolar *phb-1d* primordia (Siegfried et al., 1999), but in YABBY quadruple mutant leaves, polarity is established but not maintained. Thus, a common theme to the miniature filamentous leaves of YABBY quadruple mutants and the apolar leaves of *pANT>>KAN2* and *phb-1d* is a lack of lamina growth. To characterize the "YABBY-dependent" leaf programs, we examined global gene expression patterns in *fil yab3* and YABBY triple mutant shoots. Overall, expression of 1190 genes (fold change > 2, false discovery rate $P < 0.05$) was altered in theses apices, and attempts to find specific signatures in the form of enriched pathways among these genes failed. This failure may reflect the pleiotropic nature of the YABBY mutants that lack specific cell types such as stipules, are impaired in growth, and, as will be discussed below, have altered behavior of the SAM.

We took advantage of the other mutant backgrounds that, similar to the YABBY quadruple mutants, also lacked lamina. "Leaf lamina" genes were thus defined as genes downregulated in the three laminaless genotypes (*phb-1d*, *pANT>>KAN2*, and *fil yab3 yab5*; see Methods) relative to expression in the wild type (Figure 4A). These criteria were met by 587 lamina marker genes (see Supplemental Data Set 2 online). Looking for enriched gene families, we identified many cell cycle genes, including CYCB and CYCA families, histones, kinesins, and DNA replication genes, along with three members of the FAMA bHLH clade, previously reported to regulate stomata development (MacAlister et al., 2007). Notably, five CIN-TCP genes were found to be downregulated in all laminaless genotypes, and particular examination of this family showed that all eight CIN-TCP genes are downregulated to different degrees (Figure 4B). Indeed, many of the leaf lamina genes were also greatly downregulated in the *35S:*

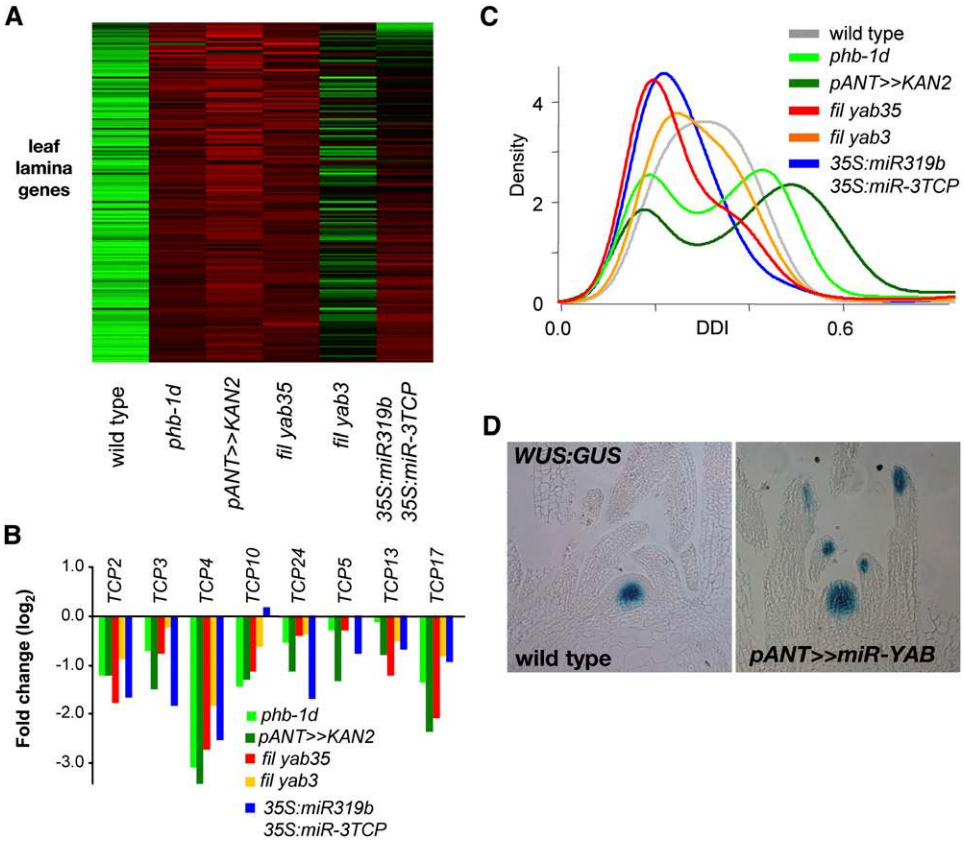

Figure 4. YABBY Mutants Are Defective in Lamina Production and Repression of Meristem Gene Expression.

(A) Expression of leaf lamina genes, defined as downregulated in three laminaless genotypes: *phb-1d*, *pANT>>KAN2*, and *fil yab3 yab5*. Note that most of these genes are downregulated in the loss of eight *TCP* genes (right).
(B) The *CIN-TCP* gene family is downregulated in polarity and YABBY mutants.
(C) DDI based on a set of genes with leaf expression modified with age reveals that YABBY triple mutants have a younger transcriptome, on par with that of loss of eight TCPs. Note that severe polarity mutants have a bimodal DDI distribution.
(D) *pWUS:GUS* marker is ectopically expressed in the *pANT>>miR-YAB13* leaves.

miR319b;35S:miR-3TCP genotype relative to the wild type (Figure 4A), suggesting that downstream factors of the CIN-TCP pathway may not be activated in laminaless genotypes. Transcriptome analysis has previously identified gene expression patterns that reflect maturation and differentiation during leaf development, and these have been used to formulate a digital differentiation index (DDI) reflecting the developmental state of leaves (Efroni et al., 2008). When applied to evaluate the differentiation status of YABBY mutant leaves, the majority of gene expression is at a low DDI, reflecting a young stage of leaf differentiation, similar to the *35S:miR319b;35S:miR-3TCP* genotype (Figure 4C). In contrast, adaxialized or abaxialized leaves exhibited a bimodal DDI, indicating that the young status of YABBY mutants is not simply due to a loss of lamina or a failure to maintain polarity.

Given that YABBY mutant leaves appear to be stalled at a young differentiated state, and that they do not appear to establish lamina-specific genetic programs, we examined whether SAM-specific genetic programs were properly downregulated. Since the quadruple YABBY mutant displays GUS activity (due to the presence of the *yab3-2* allele), we utilized a transactivation line in which YABBY function has been reduced using a synthetic microRNA designed to target *FIL* and *YAB3* (Moore et al., 1998; Alvarez et al., 2006). The transactivation line (*pANT>>miR-YAB13*) closely resembles the *fil yab3* double mutant (Goldshmidt et al., 2008). Remarkably, the *pWUS:GUS* marker, which is normally only expressed in the central zone of the SAM (Mayer et al., 1998; Williams et al., 2005), is expressed at the adaxial tips of YABBY mutant leaves (Figure 4D). Expression of *pWUS:GUS* appears to be a reactivation, since neither younger *pANT>>miR-YAB13* leaves nor primordia anlagen express the GUS marker. The domain of *pWUS:GUS* marker expression is also expanded in the *pANT>>miR-YAB13* shoot apex relative to its expression in the wild type, consistent with previous observations demonstrating expansion of WUS expression in both inflorescences and flower meristems of YABBY mutants (Goldshmidt et al., 2008).

Leaf Margin Structure in YABBY Loss-of-Function Plants

While most leaves of YABBY triple and quadruple mutants lack lamina, the first formed leaves, although reduced in size, are

somewhat laminar, allowing comparisons with wild-type leaves. In the wild type, leaf margins are serrated with hydathodes differentiating at the tips of serrations. At the cellular level, conspicuous leaf margin cells mark the boundary between the adaxial and abaxial epidermises. These highly elongated cells are arranged in a continuous cell file along the perimeter of the leaf (Figures 5A and 5C). On the adaxial side, directly adjacent to the leaf margin cells, are two to three rows of small isodiametrically shaped epidermal cells (Figure 5E). YABBY mutant leaves lack the typical marginal elaborations that characterize wild-type leaves, such as serrations and hydathodes. Neither the specialized leaf margin cells nor the adjacent small adaxial cells are present in YABBY quadruple mutant leaves (Figures 5B, 5D, and 5F), irrespective of whether the mutant leaves exhibit laminar expansion or are fully radialized. Occasionally, isolated large cells are observed in the margins of quadruple leaves with some lamina expansion, but these resemble abaxial epidermal cells rather than typical marginal cells (Figure 5B). In addition, stipules, another marginal and lateral leaf structure (Nardmann et al., 2004), are also lacking in *fil yab3*, YABBY triple, and YABBY quadruple mutant leaves. Thus, all specialized marginal cell types do not form in YABBY mutants.

To investigate leaf margin establishment, we made use of an enhancer trap line (YJ158) that displays GUS activity in leaf margin cells (Eshed et al., 2004). In wild-type leaves, strong GUS expression is observed in a sharp and continuous marginal domain around the entire leaf margin (Figures 5G and 5I), while in *pANT>>miR-YAB13* leaves, GUS activity was weak, patchy, and mostly distal (Figures 5H and 5J). Thus, the leaf margin, a domain that is derived from the establishment of leaf polarity, is missing from YABBY mutant leaves, even from those that are partially bifacial.

Venation and Auxin Patterning in YABBY Mutants

Since the leaf margin plays an important role in organizing movement of auxin during leaf development, in particular during vascular patterning and leaf serration formation, we investigated auxin dynamics in YABBY mutants. As a proxy for auxin maxima formation along the leaf margin, we first examined patterns of leaf venation. The venation patterns of YABBY mutant leaves are highly simplified compared with the normal reticulate patterns observed in wild-type *Arabidopsis* leaves (Figures 6A–6C). Leaves of YABBY double and quadruple mutants fail to form continuous loops of secondary veins. In radialized leaves of quadruple YABBY mutants, only a single central vascular strand differentiates.

We next analyzed the auxin response markers *ATHB8* and DR5 in wild-type and YABBY mutant leaves. The preprocambial marker, *ATHB8*, is one of the earliest markers of vasculature (Baima et al., 1995). *ATHB8* expression in young leaf primordia was similar in both wild-type and *pANT>>miR-YAB13* backgrounds (Figures 6E–6H). However, continuous loops of vasculature fail to form in YABBY mutants, and venation patterning is severely distorted (Figures 6I and 6J). DR5 is a synthetic reporter composed of auxin-responsive elements transcriptionally fused with a reporter gene, GUS or green fluorescent protein (GFP; Ulmasov et al., 1999; Friml et al., 2003). In wild-type plants, an auxin response maximum is observed at the tips of young leaf

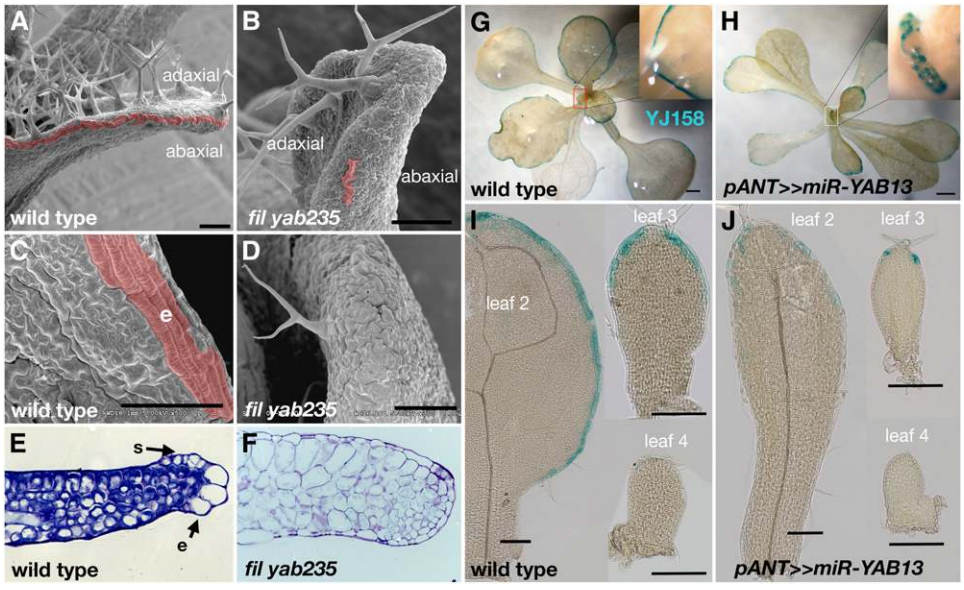

Figure 5. Loss of YABBY Function Is Associated with a Loss of Leaf Margin Cells.

(A), **(C)**, and **(E)** Wild-type margins are characterized by elongate marginal cells (e; red in **[A]** and **[C]**) and small isodiametric adaxial cells (s).
(B), **(D)**, and **(F)** *yab1235* leaves lack these cell types, with occasional large cells (red in **[B]**) resembling large abaxial cells (see Figures 3C and 3D).
(G) to **(J)** Marginal cell marker YJ158 is uniformly expressed in wild-type leaf marginal cells (**[G]** and **[I]**), and expression is patchy in *pANT>>miR-YAB13* leaves (**[H]** and **[J]**).
Bars = 100 μm.

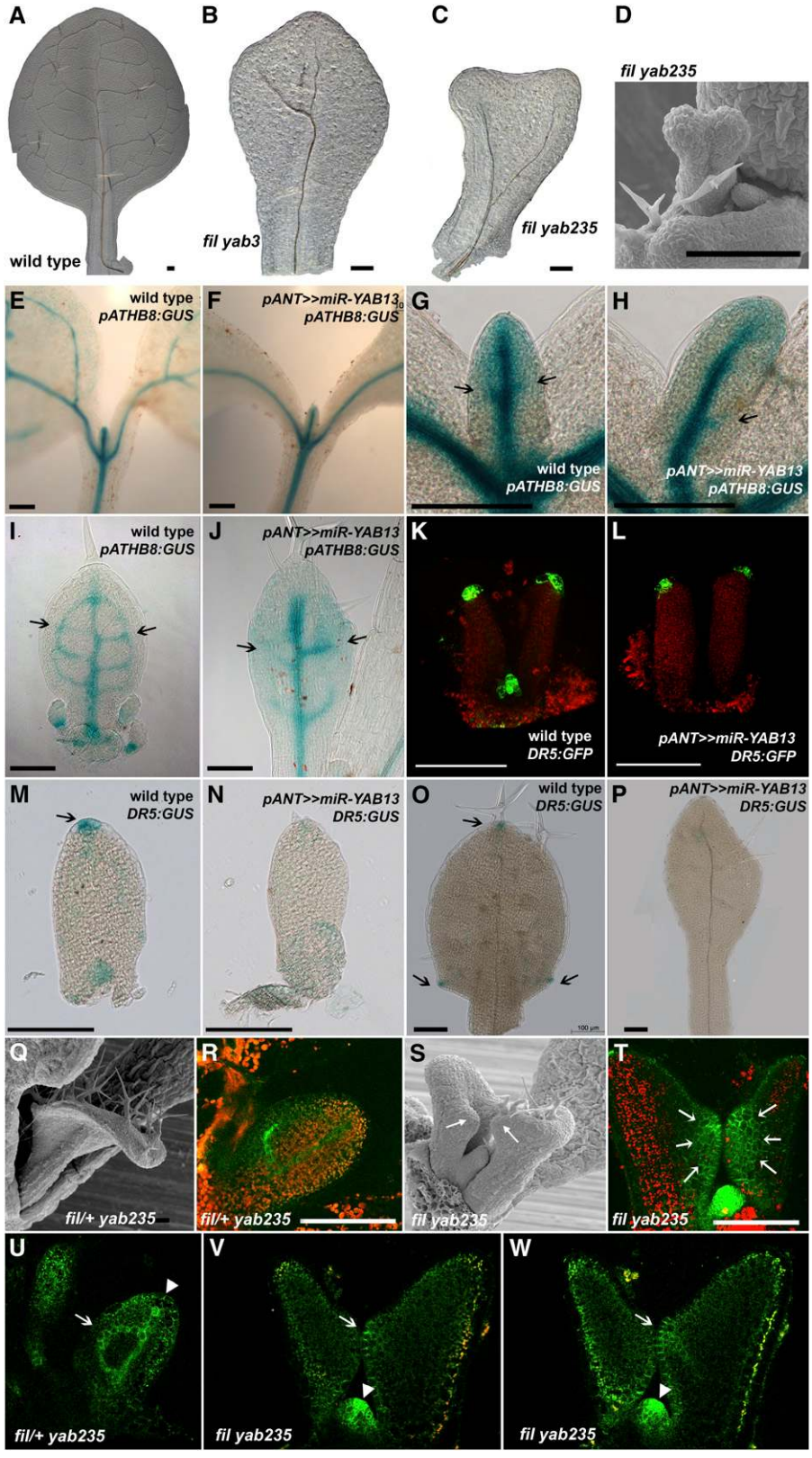

Figure 6. Loss of YABBY Function Is Associated with Defects in Auxin Patterning.

primordia (Figure 6M). As leaf development proceeds, a DR5 maximum is maintained at the leaf tip and additional foci of DR5 expression are observed at hydathodes (Figures 6M and 6O, arrows). In *pANT>>miR-YAB13* seedlings, DR5 expression is much weaker and fails to be strongly maintained (Figures 6L, 6N, and 6P). Hydathodes, with their corresponding points of DR5 expression, are not observed (Figure 6P). A weak DR5 response is consistent with overall lower levels of auxin and reduced PIN1-GFP expression (see below).

We examined *pPIN1:PIN1-GFP* expression during leaf development in YABBY quadruple mutants. *PIN-FORMED1* (*PIN1*) encodes an auxin efflux carrier, and its localization on the plasma membrane is thought to indicate directional auxin flow (Benkova et al., 2003; Reinhardt et al., 2003). We utilized a translational fusion of PIN1 to GFP (*pPIN1:PIN1-GFP*; Heisler et al., 2005) to visualize PIN1 expression in YABBY quadruple mutants. During wild-type leaf development, PIN1-GFP is initially expressed uniformly in the primordium epidermis, where its apical polarity facilitates auxin transport distally (Scarpella et al., 2006). At this stage, a single primary PIN1 convergence point is located at the primordium tip, where the incipient midvein is positioned (Scarpella et al., 2006). Since YABBY quadruple mutants form a midvein, as expected, we observe epidermal PIN1-GFP expression in both leaf primordia and the incipient midvein (Figures 6V and 6W, third formed leaf). However, the initially strong PIN1-GFP expression in the midvein is not maintained and quickly fades below detectable levels (Figures 6V and 6W, compare the third leaf primordium with the first and second formed leaves). In wild-type leaves, the continuous loops of secondary veins are patterned by transient "secondary" PIN1 convergence points occurring exclusively at leaf margins (Figures 6Q, 6R, and 6U; Scarpella et al., 2006). Continuous loops of PIN1-GFP expression are not observed in the YABBY quadruple mutant. Instead, we often observed additional PIN1-GFP convergence points at sites where ectopic bulges form on the primordium (Figures 6S, 6T, 6V, and 6W, arrows). These secondary PIN1 convergence points are broader than those formed in the wild type, with PIN1 expression not limited to the margins but extending in several cells of the epidermis on both "sides" of YABBY mutant leaves. This stable PIN1-GFP convergence point funnels auxin downward to meet the existing vasculature and resembles auxin patterning at the primordium tip rather than auxin patterning associated with secondary vein formation. Thus, secondary PIN1 convergence points in YABBY mutant leaves resemble those formed at leaf initiation rather than marginal secondary PIN1 convergence points of wild-type leaves.

Loss of YABBY Gene Function Affects Embryo Development

The abnormal DR5 and PIN1 distribution in YABBY mutant shoots promoted further analyses of these markers in mutant embryos, where the gradual and slow development of simple shoot elements permits easier scoring of patterning events. Wild-type *Arabidopsis* seedlings have two round-shaped cotyledons of equal size that symmetrically flank the SAM. The origin of this bilateral symmetry can be traced back to the transition stage of embryogenesis, when cotyledons are initiated (Figure 7A). In contrast, cotyledons of YABBY double (*fil yab3*), triple, and quadruple mutants are small and narrow, with only a single vascular strand (Figures 7D, 7E, 7I, and 7M). Strikingly, YABBY triple and quadruple mutant seedlings often have extra, or sometimes split, cotyledons (Figures 7B and 7C). The extent of polycotyly in YABBY mutants is variable, both in terms of cotyledon number and degree of cotyledon separation. For example, some quadruple YABBY mutant seedlings display three or four cotyledons, while others display only partial extra cotyledons, ranging from slight bulges to deep lobes (see Supplemental Figure 2 online). The observed polycotyly phenotype is partially penetrant, with ~28% of triple mutants and 50% of quadruple mutant seedlings having extra cotyledons (Table 1).

Because auxin maxima are thought to promote cotyledon establishment, we followed PIN1-GFP localization and *DR5* activity during YABBY mutant embryo development. *PIN1* is required for initial positioning of cotyledon primordia in the embryo by facilitating the formation of two auxin maxima at the late globular stage of embryogenesis (Benkova et al., 2003; Reinhardt et al., 2003). In wild-type embryos, expression of PIN1-GFP marks the sites of cotyledon initiation, with two focal points

Figure 6. (continued).

(A) to **(C)** Venation patterns of wild-type **(A)**, *fil yab3* **(B)**, and *fil yab235* **(C)** second formed leaves. Leaves of *fil yab235* seedlings arise in a variety of unusual shapes (see Supplemental Figure 4 online).

(D) Scanning electron microscopy image of a *fil yab235* leaf similar in shape to the one shown in **(C)**.

(E) to **(J)** *pATHB8:GUS* expression in wild-type and *pANT>>miR-YAB13* seedlings at 3 (**[E]** and **[F]**), 5 (**[G]** and **[H]**), and 14 (**[I]** and **[J]**) d after germination.

(K) to **(P)** DR5 expression in wild-type and *pANT>>miR-YAB13* seedlings.

(K) to **(L)** *DR5:GFP* signal in leaves 1 and 2 of 4-d-old seedlings. Red signal is chlorophyll autofluorescence.

(M) and **(N)** *DR5:GUS* expression in leaf 3 of 10-d-old seedlings.

(O) and **(P)** *DR5:GUS* expression in leaf 2 of 10-d-old seedlings.

(Q) and **(S)** Scanning electron microscopy images of *fil/+ yab235* **(Q)** and *fil yab235* **(S)** seedlings. Note the unusually shaped leaves of the YABBY quadruple mutant **(S)** with bulges of tissue at the sides (arrows).

(R), **(T)**, and **(U)** to **(W)** PIN1-GFP (green) expression in the first two to three leaves of *yab235* (**[R]** and **[U]**; top view) and *fil yab235* (**[T]**, **[V]**, and **[W]**; side view) seedlings. **(U)** to **(W)** show longitudinal optical sections showing the different pattern of PIN1-GFP expression in the YABBY quadruple mutant leaf (**[V]** and **[W]**) compared with a leaf from a seedling with a functional copy of the *FIL* gene **(U)**. Arrowheads point to original auxin maxima of leaf primordia, and arrows point to sites of secondary auxin maxima.

Bars = 100 μm.

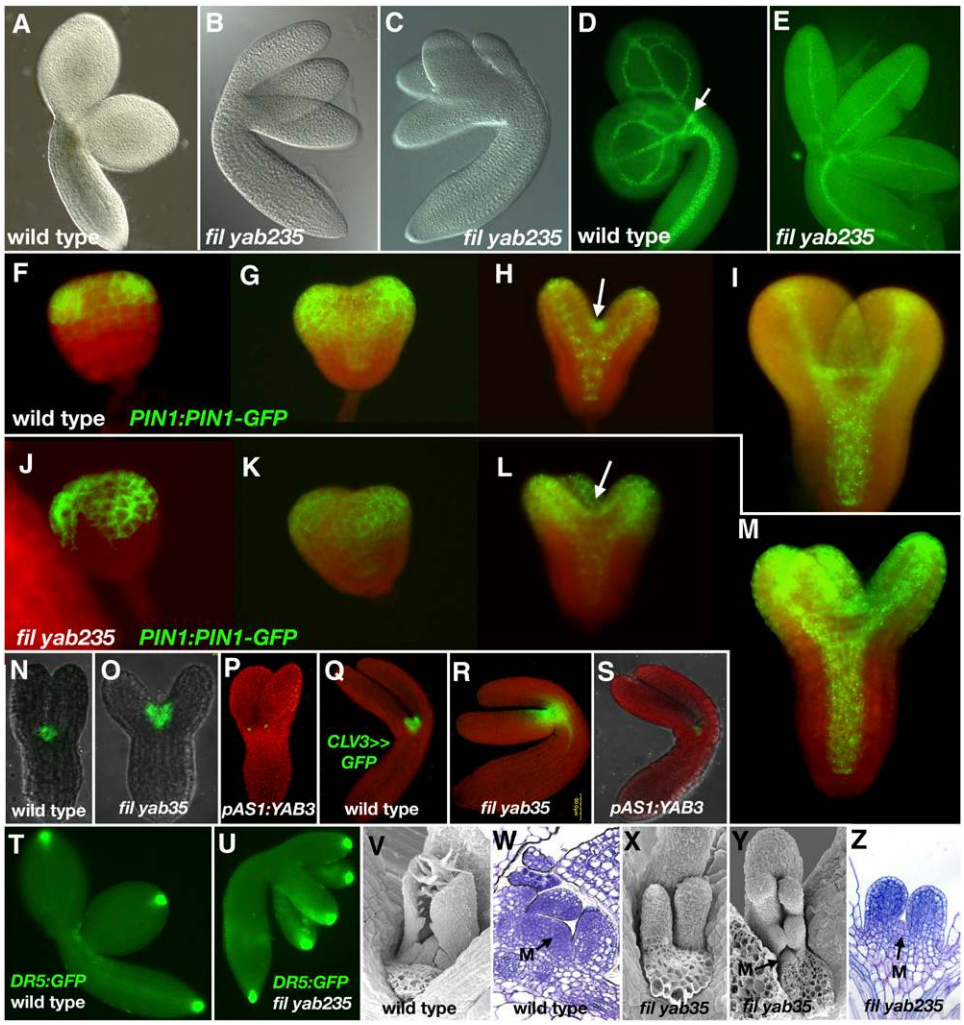

Figure 7. Embryogenesis in *yabby* Mutants.

(A) Wild-type embryo.
(B) and **(C)** *fil yab235* embryos with three distinct cotyledons **(B)** or multiple, sometimes fused cotyledons **(C)**.
(D) and **(E)** Reticulate vascular network of wild-type cotyledons as compared with solitary vascular traces of YABBY mutant cotyledons. Note conspicuous expression of PIN1-GFP in the SAM of wild-type plants (arrow).
(F) to **(M)** *PIN1:PIN1-GFP* in wild-type (**[F]**–**[I]**) and *fil yab235* (**[J]**–**[M]**) embryos. Red signal is chlorophyll autofluorescence. Arrows in **(H)** and **(L)** highlight prominent PIN1-GFP expression in the wild type that is lacking in YABBY quadruple mutants.
(N) to **(S)** *pCLV3>>GFP-ER* expression in wild-type, *fil-8 yab3-2*, and *pAS1:YAB3* embryos at the torpedo stage (**[N]**–**[P]**) and a later stage (**[Q]**–**[S]**) of embryogenesis.
(T) and **(U)** *DR5:GFP* in wild-type **(T)** and *fil yab235* **(U)** embryos.
(V) and **(W)** Wild-type seedling and apex.
(X) to **(Z)** *fil yab235* seedlings and apex. M, meristem.

evident from the late globular stage through the heart stage of embryogenesis (Figures 7F and 7G). In YABBY quadruple mutant embryos, during the early heart stage, PIN1-GFP expression does not appear to resolve cleanly into two focal points but rather is expressed more broadly across apical regions of the embryo (Figures 7J and 7K). At the late heart stage of embryogenesis, focal points of PIN1-GFP expression are often evident at more than two sites (Figure 7L) corresponding to developing cotyledons (Figure 7M). Each cotyledon is associated with a focal point of *DR5:GFP* expression at its tip, consistent with a local accumulation of auxin (Figures 7T and 7U).

To examine the effect of prolonged and widespread *PIN1* expression on cotyledon formation, we expressed *PIN1* under the control of the *FIL* promoter in the quadruple YABBY mutant background and scored for polycotyly. The frequency of polycotyledonous seedlings increased from 57 to 90% (Table 1). An increased frequency of polycotyly was observed in multiple independent lines. In contrast, expression of the bacterial

Table 1. Frequency of Polycotyly in YABBY Mutants

YABBY Mutant Genotype	No.	Percentage of Polycotyledonous Seedlings
*yab13 (*double)	371	15
yab135 (triple)	340	28
yab1235 (quadruple)	426	50
yab1235 (quadruple); *pFIL*::*PIN1*	213	96
yab1235 (quadruple); *pFIL*::*YUCCA4*	135	84
yab1235 (*quadruple*); *pFIL*::*iaaL*	200	32

auxin-metabolizing enzyme, iaaL, under the control of the *FIL* promoter in the quadruple YABBY mutant background, reduced the frequency of polycotyly to 32%. Thus, increasing PIN1-mediated auxin transport in the *FIL* expression domain results in a greater incidence of polycotyly in YABBY mutants, while increasing auxin conjugation has the opposite effect. No effect was observed in a wild-type background.

From the late heart stage, PIN1-GFP is conspicuous in the L1 of the wild-type SAM; however, this expression is reduced or not detected in quadruple YABBY mutant embryos (Figures 7H, 7I, 7L, and 7M). SAM gene expression was examined by following the expression of a *pCLV3>>GFP-ER* marker during embryogenesis and establishment of the vegetative SAM. In wild-type embryos, *pCLV3>>GFP-ER* is expressed in torpedo and later stage embryos demarcating the central zone of the SAM (Figures 7N and 7Q). In YABBY triple mutants, the expression domain of *pCLV3>>GFP-ER* is expanded relative to that seen in the wild type (Figures 7O and 7R). In contrast, embryos in which *YAB3* is ectopically expressed under the control of the *AS1* promoter (which drives expression throughout lateral organs) exhibit arrest of the SAM (see Supplemental Figure 3 online) and reduction in the expression of *pCLV3>>GFP-ER* (Figures 7P and 7S).

During seedling development of YABBY triple and quadruple mutants, the primary SAM (Figures 7V–7Z) is not maintained, and subsequent shoot growth is due to development from meristems at leaf axils or from ectopic meristems that develop on the adaxial side of the aberrant lamina (see Supplemental Figure 4 online). YABBY triple and quadruple mutant seedlings produce only two to four rosette leaves before the primary SAM terminates (Figures 7X–7Z). Leaves produced immediately prior to SAM termination are frequently radialized (Figure 7X). The wild-type SAM normally develops a tunica corpus structure characteristic of many angiosperms during the heart to torpedo stages of embryogenesis, and this is maintained throughout vegetative and reproductive development (Figure 7W; see Supplemental Figure 4 online). In histological sections of 10-d-old seedlings, the SAM of YABBY quadruple mutants is smaller than that of the wild type and with a less well-defined tunica corpus structure (Figure 7Z; see Supplemental Figure 4 online).

DISCUSSION

Since their discovery more than a decade ago, YABBY genes have been described as promoting abaxial leaf differentiation, lamina growth, floral organ identity, and as nonautonomously promoting SAM maintenance and adaxial leaf differentiation (Alvarez and Smyth, 1999; Chen et al., 1999; Eshed et al., 1999, 2004; Sawa et al., 1999a, 1999b; Siegfried et al., 1999; Golz et al., 2004; Juarez et al., 2004; Goldshmidt et al., 2008; Stahle et al., 2009). The combination of autonomous and nonautonomous defects results in a mutant phenotype that is enigmatic, with nearly every aboveground shoot–derived organ affected. This is surprising considering that YABBY mRNAs and proteins are largely only expressed in the abaxial regions of developing leaves (Sawa et al., 1999a; Siegfried et al., 1999; Watanabe and Okada, 2003; Golz et al., 2004; Navarro et al., 2004). Furthermore, the whole plant YABBY mutant phenotype is in many respects more severe than either KANADI loss-of-function or PHB gain-of-function mutant phenotypes, despite YABBY gene expression being greatly reduced in these genetic backgrounds (McConnell and Barton, 1998; Siegfried et al., 1999; Eshed et al., 2004).

YABBY Genes Help to Differentiate Leaves from Shoots

In this study, we analyzed plants in which all vegetative YABBY activity is compromised. Members of the *Arabidopsis* YABBY gene family are functionally redundant, and differences in expression in older leaves or specific organs are consistent with partial subfunctionalization or neofunctionalization following gene duplications (Figure 2). Using morphology, anatomy, global gene expression patterns, and distribution of auxin-related markers, several lines of evidence lead us to propose that YABBY gene expression helps to differentiate leaf and shoot identities.

First, YABBY gene activity is required for most aspects of leaf development; however, as YABBY gene expression is initially limited to the abaxial regions of the leaf anlagen in both the wild type (Watanabe and Okada, 2003) and in YABBY loss-of-function mutants, initial polarity establishment does not appear to require YABBY activity (Figure 3). Instead, establishment or maintenance of leaf developmental processes after leaf primordium initiation are lacking in YABBY mutants. The failure to maintain normal leaf development following initiation results, directly, or more likely indirectly, in a failure to maintain polar gene expression patterns established earlier. Thus, loss of YABBY function is associated with loss of leaf polarity in general rather than simply abaxialization or adaxialization.

Second, YABBY activity is required to establish a leaf marginal domain (Figure 5). In YABBY mutant leaves, auxin convergences subsequent to leaf initiation appear to be planar rather than linear (Figure 6). One interpretation is that YABBY mutant leaves fail to establish normal laminar growth and that subsequently formed auxin maxima recapitulate the pattern normally found at the leaf initiation stage. In this scenario, the bulges formed on the flanks of YABBY mutant leaves represent aberrant "leaf primordia" induced by secondary auxin maxima, which in the wild type would mark the sites of secondary leaf veins and the establishment of reticulate venation. As a consequence, the failure to properly organize marginal leaf tissues, which are required for the development of the reticulate venation in wild-type leaves, results in highly simplified venation patterns in YABBY mutant leaves. The lack of all other marginal elaborations, such as serrations and hydathodes, and other specialized marginal cells

may be a downstream consequence of the loss of proper marginal auxin flow. It is of note that marginal structures lacking in YABBY mutants include both abaxial and adaxial cell types, implying non-cell-autonomous YABBY activity, consistent with their loss being a downstream event.

Third, YABBY mutants fail to initiate lamina genetic programs and fully repress SAM genetic programs. YABBY mutants fail to establish a "lamina gene expression program," characterized by expression of a class of TCP transcription factors that act to sculpt later leaf development (Nath et al., 2003; Palatnik et al., 2003; Ori et al., 2007; Efroni et al., 2008; Figure 4). Based on indices of maturation of differentiation, YABBY mutants are neither abaxial nor adaxial but remain in a state of differentiation that characterizes young leaves. Furthermore, plants with reduced YABBY activity fail to appropriately repress genes that are normally expressed only in *Arabidopsis* shoots from developing leaves. *WUS* is expressed at the adaxial tips of YABBY mutant leaves in a shoot-like manner, and class I KNOX genes are ectopically expressed in *fil yab3* leaves (Kumaran et al., 2002). The increased thickness of YABBY mutant leaves relative to wild-type leaves (Figure 3) could be due to less focal auxin flow (see above) or, alternatively, ectopic expression of shoot meristem genes, or a combination of both processes.

YABBY proteins physically interact with LEUNIG, which is hypothesized to act as a transcriptional corepressor, raising the possibility that YABBY proteins may act to repress transcription of target genes (Navarro et al., 2004; Stahle et al., 2009). However, it is unlikely that YABBY proteins directly negatively regulate WUS, since it is adaxially expressed in YABBY mutant leaf tips and is temporally activated much later than the first phenotypic defects observed in YABBY mutant leaves (Figure 4). Thus, its ectopic expression appears to be a nonautonomous downstream consequence of loss of YABBY activity.

Fourth, expression of a SAM factor (*CLV3*) that does not overlap in its expression domain with any of the YABBY gene products is altered, consistent with nonautonomous patterning defects of the central SAM region in *fil-8* and *fil-8 yab3-2* mutants (Goldshmidt et al., 2008). In addition, SAM PIN1 expression is reduced in YABBY mutant embryos, another nonautonomous defect possibly related to changes in auxin flow in the newly formed leaves. While causation is not proven, it is plausible that gene expression changes during embryogenesis in YABBY mutants ultimately lead to their SAM arrest during seedling growth.

Finally, polycotyly is another surprising phenotype, considering that YABBY genes are initially expressed in two discrete domains in the heart-stage embryo representing cotyledon primordia (Figure 7). Polycotyly can be viewed as a failure to establish the lamina development program in the cotyledons, with a reiteration of "primordium" auxin maxima in developing cotyledons resulting in the development of lobed or additional cotyledons. Increasing auxin mobility or synthesis in the YABBY expression domain facilitates the formation of additional auxin maxima, while *iaaL* expression reduces their frequency, presumably by decreasing active auxin levels. Since initial YABBY expression follows the establishment of auxin maxima, marking cotyledon establishment, YABBY activity acts to stabilize the bilateral symmetry, perhaps by activating lamina-specific programs, including establishment of the linear marginal auxin flow characteristic of leaves.

On the basis of these observations, we present here a hypothesis explaining why the seed plant–specific YABBY genes have become indispensable for most aspects of shoot growth in

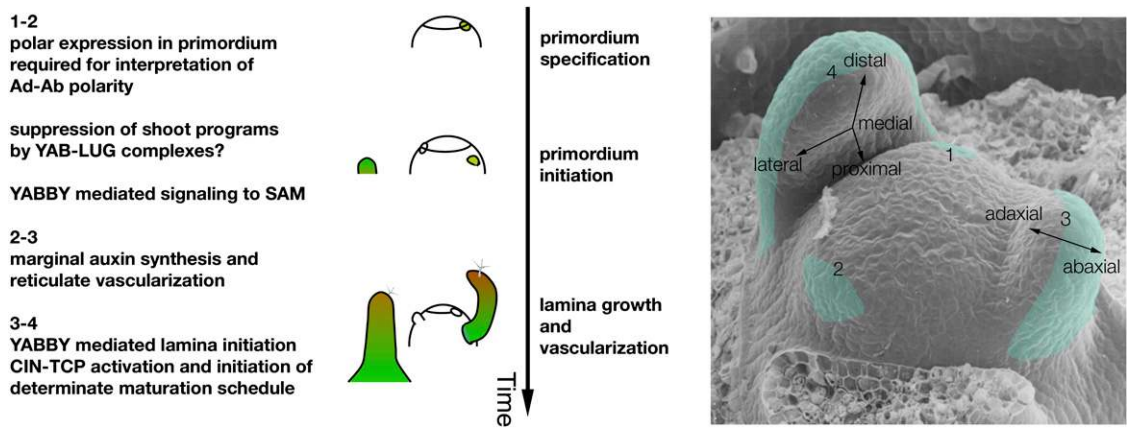

Figure 8. The Roles of YABBY Genes during Leaf Development.

YABBY genes perform different tasks at different stages of *Arabidopsis* leaf development. The numbers on the left correspond to the approximate leaf stages illustrated by the leaf primordium numbers on the petunia (*Petunia hybrida*) SAM, with green shading representing YABBY gene expression. During early stages (1 and 2), YABBY gene expression is activated abaxially in response to earlier acting polarity genes, and YABBY activity during these stages is required for proper signaling between leaf primordia and the SAM (Goldshmidt et al., 2008). YABBY-LEUNIG complexes likely act during these stages, since *leunig* mutations enhance SAM loss in a YABBY mutant background (Stahle et al., 2009). YABBY activity is required to limit auxin flows to lateral margins (stages 2 and 3), thus influencing leaf margin growth and differentiation and, consequently, reticulate vascularization of the leaf. YABBY activity is also required to initiate leaf-specific genetic programs, such as that defined by CIN-TCP genes, which lead to subsequent events in leaf differentiation (stages 3 and 4).

flowering plants. The complex YABBY mutant phenotype can be interpreted as a failure to establish a laminar growth pattern (e.g., TCP maturation/determination program), with leaves developing with a mixture of shoot-like characteristics (shoot-like *PIN1* expression and ectopic *WUS* expression) and YABBY-independent leaf characteristics. The further observation that loss of YABBY function leads to altered SAM gene expression indicates that YABBY genes alter meristem behavior from a distance, perhaps serving to modify the shoot to accommodate the development of leaves by signaling to the SAM that a group of cells has been recruited into leaf formation. Without YABBY activity, neither the leaf nor the SAM continues to develop normally, making YABBY genes important regulators integrating growth and development of the entire shoot in *Arabidopsis* (Figure 8).

YABBY Genes and the Evolution of the Seed Plant Leaf

While most developmental genetic studies of angiosperm shoots categorized genes as either leaf genes or meristem genes, it is becoming clear that most angiosperm leaves and SAMs share similar developmental genetic programs (Brand et al., 2007; Efroni et al., 2010; Floyd and Bowman, 2010). For example, PIN-mediated auxin transport is required for serration and leaflet development in leaves as well as for the initiation of leaves in the SAM peripheral zone (Benkova et al., 2003; Reinhardt et al., 2003; Hay and Tsiantis, 2006; Barkoulas et al., 2008; Koenig et al., 2009; Kawamura et al., 2010). CUP-SHAPED COTYLEDON (CUC) gene expression marks boundaries at the periphery of the SAM and boundaries between elaborations at the margins of leaves (Aida et al., 1999; Nikovics et al., 2006; Blein et al., 2008; Berger et al., 2009), and class I KNOX expression is required for SAM maintenance and promotes leaflet growth in species with complex leaves (Barton and Poethig, 1993; Long et al., 1996; Hay and Tsiantis, 2006; Barkoulas et al., 2008). Both CUC and class I KNOX genes have expression patterns that correlate with auxin flow and presumed auxin maxima, with both marking sites of auxin minima in the SAM and leaf (Furutani et al., 2004; Heisler et al., 2005; Hay et al., 2006; Barkoulas et al., 2008; Blein et al., 2008). Likewise, WOX-related gene expression is required for maintenance of growth of the SAM (*WUS*) and leaf margins (*PRESSED FLOWER*, *WOX1*; Laux et al., 1996; Mayer et al., 1998; Vandenbussche et al., 2009). However, there are exceptions to the shared genetic programs, with ARP (in many angiosperms), JAGGED, and YABBY gene expression restricted to leaves (Waites et al., 1998; Sawa et al., 1999a; Siegfried et al., 1999; Timmermans et al., 1999; Tsiantis et al., 1999; Watanabe and Okada, 2003; Dinneny et al., 2004; Ohno et al., 2004).

One hypothesis is that the common genetic programs in the SAM and leaf are due to homology (Floyd and Bowman, 2010). Seed plant leaves evolved from ancestral lateral branch systems. Thus, the leaf was evolutionarily transformed from an indeterminate radial structure to one that is determinate, dorsiventral, and laminar (Zimmerman, 1952; Stewart and Rothwell, 1993; Beerling and Fleming, 2007; Sanders and Rothwell, 2009). In this scenario, the genetic programs in the leaf would be derived from genetic programs present in the ancestral lateral branches (Floyd and Bowman, 2010). Because the origin of the YABBY gene family maps to the last common ancestor of extant seed plants (Figure 1) and YABBY genes are required for laminar growth, it has been proposed that YABBY genes may have been important in the origin and evolution of seed plant leaves (Floyd and Bowman, 2006, 2010). The failure to establish marginal leaf domains, to initiate lamina programs, and to maintain leaf polarity, the failure of suppression of SAM genetic programs, and the patterns of auxin flow in YABBY mutants provide further compelling support for this hypothesis.

The earliest seed plants bore leaves that were still in many respects stem-like and were only laminar at ultimate branches of highly ramified structures (Serbet and Rothwell, 1992; Stewart and Rothwell, 1993; Beerling and Fleming, 2007; Sanders and Rothwell, 2009), very unlike the leaves of *Arabidopsis* (with simple laminar development). In fact, the leaves of all extant seed plants are clearly distinct as lateral organs from their inception on the SAM. Although there are no data available on gene expression patterns of gymnosperm YABBY genes, this suggests that YABBY genes may function in gymnosperms as they do in *Arabidopsis*. Evolutionary developmental changes to produce the leaf of extant seed plants occurred not all at once but gradually through time, with a trend toward an earlier manifestation of dorsiventrality and laminar development (Floyd and Bowman, 2010). As the YABBY genes appear to map to the origin of seed plants, the function of YABBY genes may also have expanded gradually, integrating and differentiating leaf and stem development. Data for YABBY genes in gymnosperms and additional angiosperm species are needed to more accurately assess the ancestral role of YABBY genes for angiosperms and to confirm or reject the hypothesis that YABBY genes integrate leaf and stem development in all extant seed plants. The YABBY gene family is an intriguing and likely candidate for a gene family whose origin and evolution were partially responsible for seed plant leaves.

METHODS

yab2-1 and *yab5-1*

Cleaved-amplified polymorphic sequence PCR markers were designed to identify *Arabidopsis thaliana yab2-1* and *yab5-1* alleles. For *yab2-1*, the ethyl methanesulfonate–induced mutation creates a *Pac*I site. Primers flanking the *Pac*I site amplify a 410-bp product. Digestion yields 190- and 220-bp products. For *yab5-1*, a partial *Eco*RI site (lacking the 3′ C) was designed at the 3′ end of a 40-bp primer used to amplify a 112-bp product. Upon *Eco*RI digestion, the wild-type allele (C) yields two fragments of 40- and 72-bp product, whereas the mutant allele (T) does not contain an *Eco*RI restriction site. Primers for genotyping *yab2-1* are Yab2-F (5′-TGCCTCCTATTCGCCGTATGT-3′) and Yab2-R (5′-TATAATTCTGACATCGTCGAT-3′). Primers for genotyping *yab5-1* are Yab5-F (5′-ATTTGTGTGTTTATATTAAAACCTTTGAAGAGAGGGAATT-3′) and Yab5-R (5′-CTTACATTCTTGGCAGCAGTGCTGAATGC-3′). Primers for the *YAB2* promoter are YAB2-PstI-F (5′-ATAACTGCAGACTTATTCACACGATCC-3′) and YAB2-BamHI-R (5′-CGCGGATCCTAGTTATCCCAATGAGATCA-3′).

Multiple Mutants

yab2-1 yab5-1 plants were generated by crossing homozygous *yab2-1* and *yab5-1* lines; double mutants were selected in the F2 by PCR

screening. *fil-8 yab3-2* plants were crossed to *yab5-1* plants to generate *fil-8 yab3-2 yab5-1* triple mutants. F2 plants exhibiting novel phenotypes were genotyped by PCR to confirm homozygosity for all three mutant alleles. *fil-8*/+ *yab3-2 yab5-1* plants were crossed to *yab2-1* plants to generate quadruple mutants and genotyped in a similar manner. *crc-1* plants were crossed to *fil-8*/+ *yab3-2 yab2-1 yab5-1* plants. In the F2 generation, *crc-1* plants were tested for GUS (the *yab3-2* allele is a Ds insertion allele exhibiting GUS staining). GUS-positive lines were further genotyped by PCR for *yab2-1*, *yab5-1*, and *fil-8*. A plant of genotype *fil-8*/+ *yab3-2 yab2-1 yab5-1 crc-1* was obtained and maintained to produce the pentuple mutant. Plants were grown in long days, with 18 h of light at 22°C. Since triple and quadruple YABBY mutants are sterile, the *FIL:IaaL*, *FIL:PIN1*, and *FIL:YUC4* transgenes were introduced into a *fil*/+ *yab235* background. *DR5:GFP*, *ATHB8:GUS*, and *PIN1:PIN1-GFP* were crossed into the *pANT>>miR-YAB13* background, and individuals harboring all three transgenes were selected in the F1.

Transactivation of Ectopic and Reporter Gene Expression

Ectopic and reporter gene expression was accomplished using the transactivation system of Moore et al. (1998). In this system, a driver transgene, such as *pAS1:LhG4*, produces a chimeric transcription factor in a defined expression pattern that can activate responder transgenes, such as *Op:YAB3* (Goldshmidt et al., 2008). Transactivation is denoted *pAS1>>YAB3*. ER-GFPx2 is an endoplasmic reticulum–localized GFP with two tandem GFP sequences such that it is cell autonomous.

Histology and Microscopy

Scanning electron microscopy was performed as described previously (Alvarez et al., 1992; Siegfried et al., 1999). GUS staining was performed according to the methods described by McConnell and Barton (1998). General histology was performed as described by Emery et al. (2003). For whole-mount analyses of embryos, developing seeds were excised from developing gynoecia with 27-gauge needles, cleared overnight in Hoyer's solution (Liu and Meinke, 1998), and observed as described by Izhaki and Bowman (2007).

To image fluorescent signals, young seedlings (3–4 d old) were carefully removed from Murashige and Skoog plates, dissected, and mounted in water between a glass slide and a cover slip as described previously (Marcos and Berleth, 2009). Imaging was performed using a Leica TCS SP5 inverted confocal laser-scanning microscope. Excitation was at 488 nm (25% of laser output). The collection wavelengths were 505 to 525 nm for GFP and 600 to 670 nm for plastid autofluorescence. Scanning speed was set at 400 Hz and 512 × 512 pixel frames. The pinhole was set at 1.7 airy units. The objective was a 63.0×, 1.20-numerical aperture Leica HCX PL APO CS water-immersion lens. Image analysis was performed using Imaris 5.7 software (Bitplane). The GFP signal was false colored green, and the chlorophyll signal was false colored red.

Tissue Collection, RNA Preparation, and Microarray Hybridization

Plants were grown for 14 d under short-day conditions. Tissue collection, using microscissors, of the different samples always took place at the same daily time interval (1–3 h after the beginning of the light period). In all experiments, two independent biological replicates were sampled. Total RNA (7 to 10 μg) was extracted with the RNeasy RNA isolation kit (Qiagen). Labeled complementary RNA was prepared and hybridized to Affymetrix ATH1 GeneChips according to the manufacturer's guidelines (Affymetrix).

Bioinformatic Analysis

To identify differentially expressed genes in *yabby* mutants, RNA was extracted from apices (leaves 1 and 2 and cotyledons removed) of short-day-grown, 14-d after sowing seedlings of the wild type (four repeats) and mutants (two repeats), using the Qiagen RNEasy kit and hybridized to ATH1 Affymetrix expression arrays according to the manufacturer's recommendation. Signal values were obtained and normalized using the GeneChip-Robust Multi-array Analysis protocol. Genes with expression values lower than $\log_2(10)$ were removed. All analysis was done with R 2.7.2 (www.r-project.org) and Bioconductor 2.2 (www.bioconductor.org/). False discovery rate correction was done using the multtest package of R.

To compare the expression of polarity-related genes in *yabby* mutants with polarity mutants, previously published data of 14-DAS apices (ALP, AYL [Efroni et al., 2008]; *phb-1d*, *pANT>>KAN2* [Malitsky et al., 2008]), along with ATH1 Affymetrix expression data of two repeats of similar-age apices of *fil-8 yab3-2*, *fil-8 yab3-2 yab5-1*, and *kan1 kan2* plants, were processed using MAS5, median normalized to 50, and their average was used for comparison. Genes with expression values below 30 in all samples were discarded from further analysis.

To define polarity genes, a 1.5-fold change cutoff was used, and a gene list was defined by the following criterion: *phb-1d* greater than wild type and wild type greater than *pANT>>KAN2* or *pANT>>KAN2* greater than wild type and wild type greater than *phb-1d*. Dividing by the maximal expression value detected among mutant apices was used to normalize each gene's expression level. A polarity index was calculated as average expression of abaxial genes (*pANT>>KAN2* is greater than wild type) minus the average expression of adaxial genes (*phb-1d* is greater than wild type). Lamina genes were defined as reduced in all *phb-1d*, *pANT>>KAN2*, and *fil yab35* compared with the wild type. Data for the TCP octuple mutant were published previously together with the DDI scripts (Efroni et al., 2008).

Accession Numbers

The Arabidopsis Genome Initiative locus identifiers for *CRC*, *FIL*, *YAB2*, *YAB3*, *YAB5*, and *INO* correspond to AtNg1g69180, AtNg2g45190, AtNg1G08465, AtNg4g00180, AtNg2G26580, and AtNg1G23420, respectively. Microarray data have been deposited in the Gene Expression Omnibus database, series number GSE21705.

Supplemental Data

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

Supplemental Figure 1. The YABBY Gene Family.

Supplemental Figure 2. Polycotyly in YABBY Quadruple Mutants.

Supplemental Figure 3. Effects of YABBY Activity on Vegetative Shoots.

Supplemental Figure 4. YABBY Mutants Fail to Maintain a Functional SAM, and Continued Growth Arises from Axillary and de Novo–Initiated Meristems.

Supplemental Table 1. Expression of Organ Polarity Markers in Selected Apices.

Supplemental Data Set 1. Genes Modified in Their Expression in *yabby* Triple Mutant Apices.

Supplemental Data Set 2. Genes Modified in Their Expression in Mutants Lacking Lamina Expansion.

Supplemental Data Set 3. Text File of Alignment Corresponding to the Phylogeny in Supplemental Figure 1 Online.

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REFERENCES

Aida, M., Ishida, T., and Tasaka, M. (1999). Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: Interaction among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. Development **126:** 1563–1570.

Alvarez, J., Guli, C.L., Yu, X.H., and Smyth, D.R. (1992). Terminal Flower—A gene affecting inflorescence development in Arabidopsis thaliana. Plant J. **2:** 103–116.

Alvarez, J., and Smyth, D.R. (1999). CRABS CLAW and SPATULA, two Arabidopsis genes that control carpel development in parallel with AGAMOUS. Development **126:** 2377–2386.

Alvarez, J.P., Pekker, I., Goldshmidt, A., Blum, E., Amsellem, Z., and Eshed, Y. (2006). Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. Plant Cell **18:** 1134–1151.

Baima, S., Nobili, F., Sessa, G., Lucchetti, S., Ruberti, I., and Morelli, G. (1995). The expression of the Athb-8 homeobox gene is restricted to provascular cells in Arabidopsis thaliana. Development **121:** 4171–4182.

Barkoulas, M., Hay, A., Kougioumoutzi, E., and Tsiantis, M. (2008). A developmental framework for dissected leaf formation in the Arabidopsis relative Cardamine hirsuta. Nat. Genet. **40:** 1136–1141.

Barton, M.K., and Poethig, R.S. (1993). Formation of the shoot apical meristem in Arabidopsis thaliana—An analysis of development in the wild-type and in the shoot meristemless mutant. Development **119:** 823–831.

Beerling, D.J., and Fleming, A.J. (2007). Zimmermann's telome theory of megaphyll leaf evolution: A molecular and cellular critique. Curr. Opin. Plant Biol. **10:** 4–12.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115:** 591–602.

Berger, Y., Harpaz-Saad, S., Brand, A., Melnik, H., Sirding, N., Alvarez, J.P., Zinder, M., Samach, A., Eshed, Y., and Ori, N. (2009). The NAC-domain transcription factor GOBLET specifies leaflet boundaries in compound tomato leaves. Development **136:** 823–832.

Blein, T., Pulido, A., Vialette-Guiraud, A., Nikovics, K., Morin, H., Hay, A., Johansen, I.E., Tsiantis, M., and Laufs, P. (2008). A conserved molecular framework for compound leaf development. Science **322:** 1835–1839.

Bowman, J.L., and Smyth, D.R. (1999). CRABS CLAW, a gene that regulates carpel and nectary development in Arabidopsis, encodes a novel protein with zinc finger and helix-loop-helix domains. Development **126:** 2387–2396.

Brand, A., Shirding, N., Shleizer, S., and Ori, N. (2007). Meristem maintenance and compound-leaf patterning utilize common genetic mechanisms in tomato. Planta **226:** 941–951.

Canales, C., Grigg, S., and Tsiantis, M. (2005). The formation and patterning of leaves: Recent advances. Planta **221:** 752–756.

Chen, Q.Y., Atkinson, A., Otsuga, D., Christensen, T., Reynolds, L., and Drews, G.N. (1999). The Arabidopsis FILAMENTOUS FLOWER gene is required for flower formation. Development **126:** 2715–2726.

Chitwood, D.H., Guo, M.J., Nogueira, F.T.S., and Timmermans, M.C.P. (2007). Establishing leaf polarity: The role of small RNAs and positional signals in the shoot apex. Development **134:** 813–823.

Dinneny, J.R., Yadegari, R., Fischer, R.L., Yanofsky, M.F., and Weigel, D. (2004). The role of JAGGED in shaping lateral organs. Development **131:** 1101–1110.

Efroni, I., Blum, E., Goldshmidt, A., and Eshed, Y. (2008). A protracted and dynamic maturation schedule underlies *Arabidopsis* leaf development. Plant Cell **20:** 2293–2306.

Efroni, I., Eshed, Y., and Lifschitz, E. (2010). Morphogenesis of simple and compound leaves: A critical review. Plant Cell **22:** 1019–1032.

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F., and Bowman, J.L. (2003). Radial patterning of Arabidopsis shoots by class IIIHD-ZIP and KANADI genes. Curr. Biol. **13:** 1768–1774.

Eshed, Y., Baum, S.F., and Bowman, J.L. (1999). Distinct mechanisms promote polarity establishment in carpels of Arabidopsis. Cell **99:** 199–209.

Eshed, Y., Baum, S.F., Perea, J.V., and Bowman, J.L. (2001). Establishment of polarity in lateral organs of plants. Curr. Biol. **11:** 1251–1260.

Eshed, Y., Izhaki, A., Baum, S.F., Floyd, S.K., and Bowman, J.L. (2004). Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. Development **131:** 2997–3006.

Floyd, S.K., and Bowman, J.L. (2006). Distinct developmental mechanisms reflect the independent origins of leaves in vascular plants. Curr. Biol. **16:** 1911–1917.

Floyd, S.K., and Bowman, J.L. (2007). The ancestral developmental tool kit of land plants. Int. J. Plant Sci. **168:** 1–35.

Floyd, S.K., and Bowman, J.L. (2010). Gene expression patterns in seed plant shoot meristems and leaves: Homoplasy or homology? J. Plant Res. **123:** 43–55.

Floyd, S.K., Zalewski, C.S., and Bowman, J.L. (2006). Evolution of class III homeodomain-leucine zipper genes in streptophytes. Genetics **173:** 373–388.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature **426:** 147–153.

Furutani, M., Vernoux, T., Traas, J., Kato, T., Tasaka, M., and Aida, M. (2004). PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in Arabidopsis embryogenesis. Development **131:** 5021–5030.

Galtier, J. (1981). Structures foliaires de fougeres er pteridospermales du carbonifere inferieur et leur signification evolutive. Palaeontographica B **180:** 1–38.

Goldshmidt, A., Alvarez, J.P., Bowman, J.L., and Eshed, Y. (2008). Autonomous primordial YABBY-derived signals regulate phyllotaxis. Plant Cell **20:** 1217–1230.

Golz, J.F., Roccaro, M., Kuzoff, R., and Hudson, A. (2004). GRAMINIFOLIA promotes growth and polarity of Antirrhinum leaves. Development **131:** 3661–3670.

Harrison, C.J., Corley, S.B., Moylan, E.C., Alexander, D.L., Scotland, R.W., and Langdale, J.A. (2005). Independent recruitment of a

conserved developmental mechanism during leaf evolution. Nature **434:** 509–514.

Hay, A., Barkoulas, M., and Tsiantis, M. (2006). ASYMMETRIC LEAVES1 and auxin activities converge to repress BREVIPEDICELLUS expression and promote leaf development in Arabidopsis. Development **133:** 3955–3961.

Hay, A., and Tsiantis, M. (2006). The genetic basis for differences in leaf form between Arabidopsis thaliana and its wild relative Cardamine hirsuta. Nat. Genet. **38:** 942–947.

Heisler, M.G., Ohno, C., Das, P., Sieber, P., Reddy, G.V., Long, J.A., and Meyerowitz, E.M. (2005). Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. Curr. Biol. **15:** 1899–1911.

Iwakawa, H., Iwasaki, M., Kojima, S., Ueno, Y., Soma, T., Tanaka, H., Semiarti, E., Machida, Y., and Machida, C. (2007). Expression of the ASYMMETRIC LEAVES2 gene in the adaxial domain of Arabidopsis leaves represses cell proliferation in this domain and is critical for the development of properly expanded leaves. Plant J. **51:** 173–184.

Izhaki, A., and Bowman, J.L. (2007). KANADI and class IIIHD-zip gene families regulate embryo patterning and modulate auxin flow during embryogenesis in *Arabidopsis*. Plant Cell **19:** 495–508.

Juarez, M.T., Twigg, R.W., and Timmermans, M.C.P. (2004). Specification of adaxial cell fate during maize leaf development. Development **131:** 4533–4544.

Kawamura, E., Horiguchi, G., and Tsukaya, H. (2010). Mechanisms of leaf tooth formation in *Arabidopsis*. Plant J. **62:** 429–441.

Kerstetter, R.A., Bollman, K., Taylor, R.A., Bomblies, K., and Poethig, R.S. (2001). KANADI regulates organ polarity in Arabidopsis. Nature **411:** 706–709.

Koenig, D., Bayer, E., Kang, J., Kuhlemeier, C., and Sinha, N. (2009). Auxin patterns *Solanum lycopersicum* leaf morphogenesis. Development **136:** 2997–3006.

Kumaran, M.K., Bowman, J.L., and Sundaresan, V. (2002). YABBY polarity genes mediate the repression of KNOX homeobox genes in *Arabidopsis*. Plant Cell **14:** 2761–2770.

Laux, T., Mayer, K.F.X., Berger, J., and Jurgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development **122:** 87–96.

Lee, J.Y., Baum, S.F., Oh, S.H., Jiang, C.Z., Chen, J.C., and Bowman, J.L. (2005). Recruitment of CRABS CLAW to promote nectary development within the eudicot clade. Development **132:** 5021–5032.

Liu, C.M., and Meinke, D.W. (1998). The titan mutants of Arabidopsis are disrupted in mitosis and cell cycle control during seed development. Plant J. **16:** 21–31.

Long, J., and Barton, M.K. (2000). Initiation of axillary and floral meristems in Arabidopsis. Dev. Biol. **218:** 341–353.

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature **379:** 66–69.

MacAlister, C.A., Ohashi-Ito, K., and Bergmann, D.C. (2007). Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. Nature **445:** 537–540.

Malitsky, S., Blum, E., Less, H., Venger, I., Elbaz, M., Morin, S., Eshed, Y., and Aharoni, A. (2008). The transcript and metabolite networks affected by the two clades of Arabidopsis glucosinolate biosynthesis regulators. Plant Physiol. **148:** 2021–2049.

Marcos, D., and Berleth, T. (2009). Visualising auxin transport routes in Arabidopsis leaf primordia. In Plant Hormones: Methods and Protocols, B.A. Cutler, ed (New York: Springer), pp. 11–20.

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G., and Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell **95:** 805–815.

McCallum, C.M., Comai, L., Greene, E.A., and Henikoff, S. (2000). Targeting induced local lesions in genomes (TILLING) for plant functional genomics. Plant Physiol. **123:** 439–442.

McConnell, J.R., and Barton, M.K. (1998). Leaf polarity and meristem formation in Arabidopsis. Development **125:** 2935–2942.

McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. (2001). Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature **411:** 709–713.

Moore, I., Galweiler, L., Grosskopf, D., Schell, J., and Palme, K. (1998). A transcription activation system for regulated gene expression in transgenic plants. Proc. Natl. Acad. Sci. USA **95:** 376–381.

Nardmann, J., Ji, J.B., Werr, W., and Scanlon, M.J. (2004). The maize duplicate genes narrow sheath1 and narrow sheath2 encode a conserved homeobox gene function in a lateral domain of shoot apical meristems. Development **131:** 2827–2839.

Nath, U., Crawford, B.C.W., Carpenter, R., and Coen, E. (2003). Genetic control of surface curvature. Science **299:** 1404–1407.

Navarro, C., Efremova, N., Golz, J.F., Rubiera, R., Kuckenberg, M., Castillo, R., Tietz, O., Saedler, H., and Schwarz-Sommer, Z. (2004). Molecular and genetic interactions between STYLOSA and GRAMINIFOLIA in the control of Antirrhinum vegetative and reproductive development. Development **131:** 3649–3659.

Nikovics, K., Blein, T., Peaucelle, A., Ishida, T., Morin, H., Aida, M., and Laufs, P. (2006). The balance between the MIR164A and CUC2 genes controls leaf margin serration in *Arabidopsis*. Plant Cell **18:** 2929–2945.

Ohno, C.K., Reddy, G.V., Heisler, M.G.B., and Meyerowitz, E.M. (2004). The Arabidopsis JAGGED gene encodes a zinc finger protein that promotes leaf tissue development. Development **131:** 1111–1122.

Ori, N., Cohen, A.R., Etzioni, A., Brand, A., Yanai, O., Shleizer, S., Menda, N., Amsellem, Z., Efroni, I., Pekker, I., Alvarez, J.P., Blum, E., et al. (2007). Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato. Nat. Genet. **39:** 787–791.

Palatnik, J.F., Allen, E., Wu, X.L., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature **425:** 257–263.

Pekker, I., Alvarez, J.P., and Eshed, Y. (2005). Auxin response factors mediate *Arabidopsis* organ asymmetry via modulation of KANADI activity. Plant Cell **17:** 2899–2910.

Prigge, M.J., and Clark, S.E. (2006). Evolution of the class IIIHD-Zip gene family in land plants. Evol. Dev. **8:** 350–361.

Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. Nature **426:** 255–260.

Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P.F., Lindquist, E.A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., et al. (2008). The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. Science **319:** 64–69.

Sanders, H.L., and Rothwell, G.W. (2009). Key morphological alterations in the evolution of leaves. Int. J. Plant Sci. **170:** 860–868.

Sawa, S., Ito, T., Shimura, Y., and Okada, K. (1999a). FILAMENTOUS FLOWER controls the formation and development of *Arabidopsis* inflorescences and floral meristems. Plant Cell **11:** 69–86.

Sawa, S., Watanabe, K., Goto, K., Kanaya, E., Morita, E.H., and Okada, K. (1999b). FILAMENTOUS FLOWER, a meristem and organ identity gene of Arabidopsis, encodes a protein with a zinc finger and HMG-related domains. Genes Dev. **13:** 1079–1088.

Scarpella, E., Marcos, D., Friml, J., and Berleth, T. (2006). Control of leaf vascular patterning by polar auxin transport. Genes Dev. **20:** 1015–1027.

Serbet, R., and Rothwell, G.W. (1992). Characterizing the most primitive

seed ferns. I. A reconstruction of Elkinsia polymorpha. Int. J. Plant Sci. **153:** 602–621.

Siegfried, K.R., Eshed, Y., Baum, S.F., Otsuga, D., Drews, G.N., and Bowman, J.L. (1999). Members of the YABBY gene family specify abaxial cell fate in Arabidopsis. Development **126:** 4117–4128.

Stahle, M.I., Kuehlich, J., Staron, L., von Arnim, A.G., and Golz, J.F. (2009). YABBYs and the transcriptional corepressors LEUNIG and LEUNIG_HOMOLOG maintain leaf polarity and meristem activity in *Arabidopsis*. Plant Cell **21:** 3105–3118.

Stewart, W.N., and Rothwell, G.W. (1993). Paleobotany and the Evolution of Plants. (New York: Cambridge University Press).

Sussex, I.M. (1954). Experiments on the cause of dorsiventrality in leaves. Nature **174:** 352–353.

Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J.E., Burtner, C., Odden, A.R., Young, K., Taylor, N.E., Henikoff, J.G., Comai, L., et al. (2003). Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res. **13:** 524–530.

Timmermans, M.C.P., Hudson, A., Becraft, P.W., and Nelson, T. (1999). ROUGH SHEATH2: A Myb protein that represses knox homeobox genes in maize lateral organ primordia. Science **284:** 151–153.

Toriba, T., Harada, K., Takamura, A., Nakamura, H., Ichikawa, H., Suzaki, T., and Hirano, H.Y. (2007). Molecular characterization of the YABBY gene family in Oryza sativa and expression analysis of OsYABBY1. Mol. Genet. Genomics **277:** 457–468.

Tsiantis, M., Schneeberger, R., Golz, J.F., Freeling, M., and Langdale, J.A. (1999). The maize rough sheath2 gene and leaf development programs in monocot and dicot plants. Science **284:** 154–156.

Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (1999). Activation and repression of transcription by auxin-response factors. Proc. Natl. Acad. Sci. USA **96:** 5844–5849.

Vandenbussche, M., Horstman, A., Zethof, J., Koes, R., Rijpkema, A.S., and Gerats, T. (2009). Differential recruitment of WOX transcription factors for lateral development and organ fusion in petunia and *Arabidopsis*. Plant Cell **21:** 2269–2283.

Villanneva, J.M., Broadhvest, J., Hauser, B.A., Meister, R.J., Schneitz, K., and Gasser, C.S. (1999). INNER NOOUTER regulates abaxial-adaxial patterning in Arabidopsis ovules. Genes Dev. **13:** 3160–3169.

Waites, R., and Hudson, A. (1995). Phantastica—A gene required for dorsoventrality of leaves in Antirrhinum majus. Development **121:** 2143–2154.

Waites, R., Selvadurai, H.R.N., Oliver, I.R., and Hudson, A. (1998). The PHANTASTICA gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in Antirrhinum. Cell **93:** 779–789.

Watanabe, K., and Okada, K. (2003). Two discrete cis elements control the abaxial side-specific expression of the FILAMENTOUS FLOWER gene in *Arabidopsis*. Plant Cell **15:** 2592–2602.

Williams, L., Grigg, S.P., Xie, M.T., Christensen, S., and Fletcher, J.C. (2005). Regulation of Arabidopsis shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes. Development **132:** 3657–3668.

Yamada, T., Ito, M., and Kato, M. (2004). YABBY2-homologue expression in lateral organs of Amborella trichopoda (Amborellaceae). Int. J. Plant Sci. **165:** 917–924.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y., and Hirano, H.Y. (2004). The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in *Oryza sativa*. Plant Cell **16:** 500–509.

Zimmerman, W. (1952). Main results of the telome theory. The Paleobotanist **1:** 456–470.