Asymmetric leaf development and blade expansion in *Arabidopsis* are mediated by KANADI and YABBY activities

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Summary

Asymmetric development of plant lateral organs is initiated by a partitioning of organ primordia into distinct domains along their adaxial/abaxial axis. Two primary determinants of abaxial cell fate are members of the KANADI and YABBY gene families. Progressive loss of KANADI activity in loss-of-function mutants results in progressive transformation of abaxial cell types into adaxial ones and a correlated loss of lamina formation. Novel, localized planes of blade expansion occur in some *kanadi* loss-of-function genotypes and these ectopic lamina outgrowths are YABBY dependent. We propose that the initial asymmetric leaf development is regulated primarily by mutual antagonism between KANADI and PHB-like genes, which is translated into polar YABBY expression. Subsequently, polar YABBY expression contributes both to abaxial cell fate and to abaxial/adaxial juxtaposition-mediated lamina expansion.

Key words: *Arabidopsis*, Leaves, Abaxial-adaxial polarity, Pattern formation, *YABBY*, *KANADI*

Introduction

Most leaves are laminar structures with two distinct surfaces that are specialized for their distinct functions. Leaves are produced from the flanks of the apical meristem, and thus, there exists a fundamental positional relationship between leaves and the meristem from which they are produced. The adaxial side (ad – next to) of the leaf is derived from cells adjacent to the meristem, while the abaxial side (ab – away from) is derived from cells in the leaf primordium at a distance from the meristem. In many species, including *Arabidopsis*, the adaxial side differentiates to form the upper surface and is specialized for light capture, with the lower, or abaxial, specialized for respiration.

The formation of the leaf lamina requires the establishment of polarity and is proposed to be a result of interactions between juxtaposed abaxial and adaxial cells (Sussex, 1955; Waites and Hudson, 1995). The initial establishment of ad-abaxial polarity may result from a signal emanating from the apical meristem to induce or maintain adaxial identity, and in the absence of signal, abaxial identity may be the default. As communication between the abaxial and adaxial domains has been proposed to be responsible for lamina growth, factors promoting the identity of one or both domains are probably involved in directing the initial stages of lamina formation. In *Arabidopsis*, adaxial fates are promoted by the expression of members of the class III HD-ZIP family of genes, such as *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003). Abaxial fates are promoted by members of two gene families, the KANADIs and the YABBYs (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 1999; Kerstetter et al., 2001; Eshed et al., 2001). Gain-of-function alleles of some class III HD-ZIP genes (*PHB*, *PHV*) result in adaxialized radial leaves lacking lamina expansion (McConnell and Barton, 1998; McConnell et al., 2001), and loss-of-function alleles result in abaxialized radial cotyledons (Emery et al., 2003). Conversely, gain-of-function alleles of KANADI result in radial abaxialized organs (Eshed et al., 2001; Kerstetter et al., 2001). Thus, homogenization, either to all abaxial or all adaxial identities results in a loss of lamina development, consistent with the hypothesis of Waites and Hudson (Waites and Hudson, 1995) that juxtaposition of these two domains is critical for lamina expansion.

Previously, members of the YABBY gene family have been proposed to promote abaxial cell fates. This hypothesis was based on the observations that gain-of-function alleles of several members, *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*) and *CRABS CLAW* (*CRC*), result in abaxial tissues differentiating in adaxial positions, namely in the cotyledon, leaf and petal epidermises (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 1999). Conversely, loss-of-function alleles of *CRC*, when in combination with *kan1* mutations result in adaxial tissues developing in abaxial positions in the gynoecium (Eshed et al., 1999). During leaf development *FIL* and *YAB3* are expressed in the abaxial regions of *Arabidopsis* leaves and their expression patterns parallel that of the progress of leaf differentiation (Siegfried et al., 1999; Sawa et al., 1999; Kumaran et al., 2002). We present several lines of evidence that YABBY gene activity is associated with lamina expansion and propose that boundaries

of YABBY gene expression marking the abaxial-adaxial boundary are intimately linked with the proposed communication between the abaxial and adaxial domains during leaf development.

Materials and methods

Plant growth and genetics

All mutants were in the Landsberg *erecta* (L*er*) background except *kan3-1* and *yab3-1* (both in Wassilewskija) which were backcrossed into L*er*. Plants were grown under 18 hours cool white fluorescent light at 20°C. All mutant lines have been described previously: *kan1-2* (Eshed et al., 1999); *kan2-1*, *AS1>>KAN2* (Eshed et al., 2001); *kan3-1* (Emery et al., 2003); *fil-5*, *yab3-1* (Siegfried et al., 1999); *fil-8*, *yab3-2* (Kumaran et al., 2002); *phb-1d* (McConnell and Barton, 1998); *syd-2* (Wagner and Meyerowitz, 2002). YJ158 was identified in an enhancer trap screen as previously described (Eshed et al., 1999).

Plants mutant for *kan1*, *kan2*, *fil* and *yab3* were selected as follows: 25 phenotypically wild-type plants from the F_2 of *kan1 kan2* × *fil yab3* were self-fertilized and tested in the F_3. Families in which approximately 3/16 plants looked like either *kan1 kan2* or *fil yab3* (so *kan2* and *yab3* are homozygous) were subjected to further analysis. The rare 1/16 phenotype was considered to be the quadruple mutant, while the 3/16 plant that had trichomes on both sides of their first leaf were considered as *fil yab3 kan2 kan1*/+.

Isolation of *Solanum tuberosum* YABBY gene

Total mRNA was extracted from vegetative shoot tips using the RNeasy kit (Qiagen). First strand cDNA was generated using reverse transcriptase and an oligo(dT) primer. Two degenerate primers targeting the zinc finger domain (5′ GTIACIGTIMGITGYGG-ICAYTG 3′) and the YABBY domain (5′ GCCCARTTYTTIGCIGC 3′) were used to amplify *Solanum tuberosum* partial cDNA sequences. Products were cut from the gel and TA cloned using the TOPO TA cloning kit (Invitrogen). Phylogenetic analysis including a translation of the sequence obtained from *S. tuberosum* and the *A. thaliana* YABBY gene amino acid sequences indicate *S. tuberosum YABBY1* (GenBank accession no. AY495968) is orthologous to *FIL*/*YAB3*.

Microscopy

SEM, tissue clearing, GUS staining and in situ hybridization were carried out according to the methods of Eshed et al. (Eshed et al., 1999) and Emery et al. (Emery et al., 2003). *KAN1*, *KAN2*, *KAN3*, *FIL*, *PHV* and *PHB* probes were generated by linearizing cDNA plasmids and synthesizing DIG-labeled antisense RNA using T7 RNA polymerase. Histological analyses were carried out as described by Eshed et al. (Eshed et al., 1999) for *kan1 kan2* and Emery et al. (Emery et al., 2003) for *kan1 kan2 kan3*.

Results

Extensive redundancy relationships among *Arabidopsis* KANADI genes

Four closely related members of the GARP gene family, which we refer to as the KANADI genes (*KAN1-4*), are present in the *Arabidopsis* genome, all of which are capable of inducing abaxial cell fate upon uniform expression (Eshed et al., 2001; Kerstetter et al., 2001) (Y.E. and J.L.B., unpublished observations). *KAN1* is expressed in the abaxial regions of all lateral organs (Kerstetter et al., 2001). We examined whether other KANADI genes are expressed in a similar manner as *KAN1*. At the heart stage of embryogenesis, *KAN1*, *KAN2* and *KAN3* displayed a similar expression pattern in the abaxial basal portion of emerging cotyledon primordia (Fig. 1A-C). Based on reporter gene constructs, *KAN4* appears to have a more limited expression pattern in the ovules (data not shown). The use of promoter-reporter gene fusions suggested that *KAN1-3* genes also share phloem-associated vascular bundle expression (Emery et al., 2003).

Since none of the *kan1*, *kan2* or *kan3* single mutants exhibited a dramatic loss of polarity, we constructed the multiple mutant combinations *kan1 kan2* and *kan1 kan2 kan3*. All lateral organs had gross morphological defects in *kan1 kan2* plants as described previously (Eshed et al., 2001); leaves were narrow, dark green and developed ectopic outgrowths on their abaxial side (Fig. 1D,G,H), a feature never found in wild-type leaves (Fig. 1E-F). In contrast, the local blade outgrowths characteristic of the abaxial side of *kan1 kan2* leaves were greatly reduced in the triple mutant, and when outgrowths occurred in the triple mutant they developed much later temporally (Fig. 1I,L). While *kan1 kan2 kan3* leaves were nearly radial at initiation, these leaves still displayed some polarity, as trichomes were not present on the abaxial sides of the first leaves (Fig. 1L). In *kan1 kan2 kan3* leaves the mature blade expanded in various planes giving rise to long narrow leaves with a fan-like blade at their distal end (Fig. 1M). Ectopic meristems were commonly formed at the base of the abaxial side of the *phb-1d* leaves, a feature that has not been observed in *kan1 kan2* plants. However, ectopic meristems could occasionally be found at the abaxial base of *kan1 kan2 kan3* leaves (Fig. 1J,K). The flowers of *kan1 kan2 kan3* plants were similar to *kan1 kan2* plants (not shown) (Eshed et al., 2001).

Polar anatomical and expression pattern features of *kan1 kan2* and *kan1 kan2 kan3* seedlings

Anatomical differences along the abaxial/adaxial (ab/ad) axis of *Arabidopsis* leaves are evident shortly after leaf initiation. Wild-type leaves have distinct dense columnar palisade mesophyll cells on the adaxial side (Fig. 2A-C). The abaxial spongy mesophyll cells appear more cubic and intercelluar air spaces gradually became more prevalent. The distinct layers of mesophyll are maintained by predominantly anticlinal cell divisions (Fig. 2C). In contrast, leaf primordia of *kan1 kan2* and *kan1 kan2 kan3* seedlings were nearly radial (Fig. 2E,H), and only later displayed signs of polar anatomical features (Fig. 2F,I). Cells on the abaxial side of *kan1 kan2* leaf primordia maintained a densely cytoplasmic appearance for a prolonged time, and periclinal divisions were common (Fig. 2F). As a consequence, *kan1 kan2* leaves were 10-20 cells across, compared to only six cell layers in wild type. The excess cell divisions were not distributed equally, resulting in localized outgrowths on the abaxial side (Fig. 2F). Unlike those of the double mutant, leaves of the triple mutant remained radial until much later in development and did not exhibit the ectopic abaxial periclinal divisions characteristic of *kan1 kan2* leaves (Fig. 2I).

To further characterize the polar nature of *kan1 kan2 kan3* leaves, adaxial- and abaxial-specific gene expression in the mutant background was compared to that of the wild type. To assess abaxial gene expression, we assayed *YAB3* expression in *kan1 kan2 kan3* leaves using the *yab3-2* enhancer trap line, which faithfully reproduces *YAB3* expression in leaves (Kumaran et al., 2002). β-glucuronidase (GUS) activity was undetectable in the *kan1-2 kan2-1 kan3-1 yab3-2* background

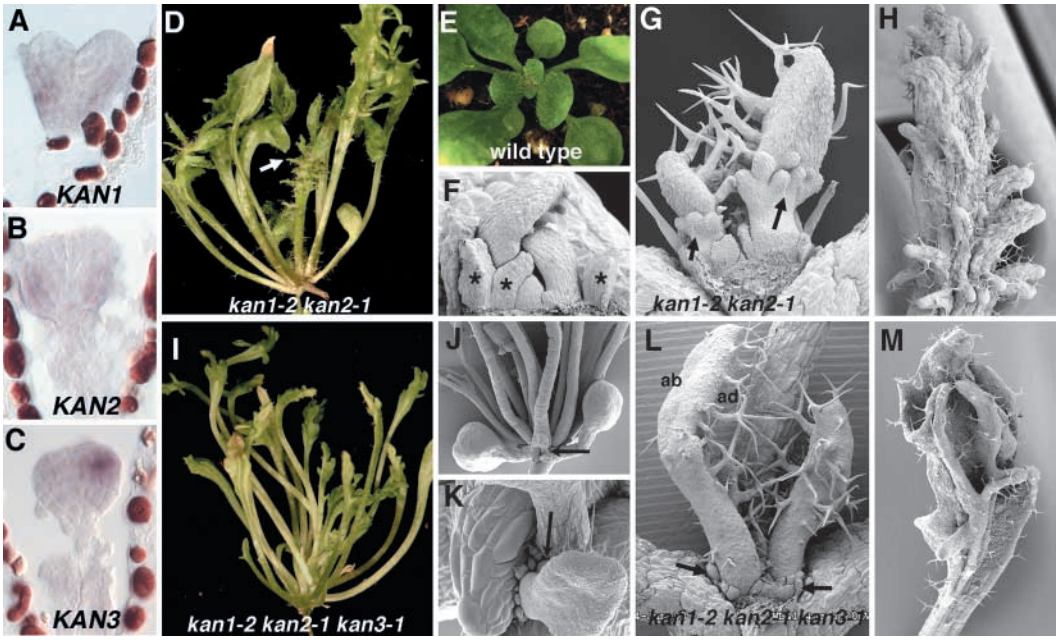

Fig. 1. KANADI loss-of-function morphological phenotypes. (A-C) All three KANADI genes, *KAN1* (A), *KAN2* (B), and *KAN3* (C) are expressed in the abaxial regions of developing embryos. Only one presumptive cotyledon is visible in the section showing *KAN3* expression. Unlike the mild or lack of phenotypic alterations of the single mutants *kan1* and *kan2*, respectively, *kan1-2 kan2-1* plants exhibit gross morphological aberrations in all lateral organs. (D) A *kan1-2 kan2-1* plant with narrow leaves which have outgrowths formed on their abaxial side (D). (G,H) The abaxial outgrowths (arrows) are visible shortly after leaf primordia have expanded, appearing first as a row along the bottom third of the leaf, and later in a less organized pattern as the leaf elongates. (E) A wild-type plant. (F) In wild-type leaves, two stipules (*) are formed on the flanks of each leaf, obscuring the apical meristem from view. (I) Leaves of *kan1-2 kan2-1 kan3-1* plants exhibit much less lamina expansion than do those of the double mutant, being radial except at their distal tips. (L) Leaves of the triple mutant are radial at inception, but they still retain some polar characteristics, such as a lack of abaxial trichomes on the first two leaves. (J-L) As in *phb-1d* homozygotes, axillary meristems (arrows) may form on the abaxial sides of *kan1 kan2 kan3* leaves (J,K) and similar to *kan1-2 kan2-1* and *phb-1d/+* leaves, stipules (arrows) develop all around the base of the leaves (L). (M) Disorganized blade tissue often forms at the distal tips of *kan1 kan2 kan3* leaves. ab, abaxial; ad, adaxial.

(Fig. 3B), while in an otherwise wild-type background, GUS activity was detected in the abaxial regions of developing *yab3-2* leaves (Fig. 3A). Thus, loss of lamina expansion is correlated with loss of YABBY gene expression in the triple mutant.

Various members of the class III HD-zip transcription factors are expressed in regions of the apical meristem and in the adaxial regions of lateral organs (McConnell et al., 2001; Otsuga et al., 2001; Eshed et al., 2001; Emery et al., 2003). For example, in wild type, *PHB* mRNA was localized to the SAM and was restricted to the adaxial domain as developing primordia separated from the meristem (Fig. 3F) (McConnell et al., 2001). Later expression was confined to the provascular and vascular tissues of leaves and stems. In contrast, *PHB* was expressed throughout *kan1 kan2 kan3* leaf primordia, although there was still a gradient with expression highest towards the adaxial side (Fig. 3C,D). In summary, loss of KANADI activity results in an adaxialization of leaves at the morphological, anatomical and molecular levels.

Effects of uniform KANADI expression

When KANADI was expressed uniformly throughout leaf primordia, such as in *AS1>>KAN2* plants, the leaves that developed were radialized and abaxialized (Fig. 3E) (Eshed et al., 2001). To further test the relationships between KANADI and YABBY or PHB, we examined their expression patterns in these plants. While *PHB* was localized to the adaxial regions of wild-type leaves (Fig. 3D), no *PHB* expression was detected in *AS1>>KAN2* leaves, and a low level of *PHB* expression was still present in the apical meristem (Fig. 3G). In contrast, *FIL* expression was detected throughout young *AS1>>KAN2* leaf primordia (Fig. 3I). However, unlike wild-type leaves in which *FIL* expression is maintained in the marginal abaxial regions (Fig. 3H), *FIL* expression in *AS1>>KAN2* was transient, only being detected in one leaf per apex (Fig. 3I). Thus, even though *AS1>>KAN2* leaves were abaxialized, *FIL* expression was ephemeral. We conclude that KANADI can induce uniform *FIL* expression in organ primordia, but that lack of maintenance of *FIL* expression correlates with the limited extent of lamina formation.

The abaxial leaf outgrowths have symmetric blade characteristics

Unlike *kan1 kan2* floral organs, which can largely be viewed as adaxialized, the blade outgrowths on the abaxial side of leaves represent a novel phenotype. To address their nature, anatomical, morphological and molecular characterizations were carried out. The outgrowths appeared first in a row on the lower third of the leaf, with additional outgrowths continuing to initiate and expand for as long as the leaf differentiates (Fig. 1G-H, Fig. 4A). The epidermis of these outgrowths in mature leaves had a high density of stomata interspersed amongst long

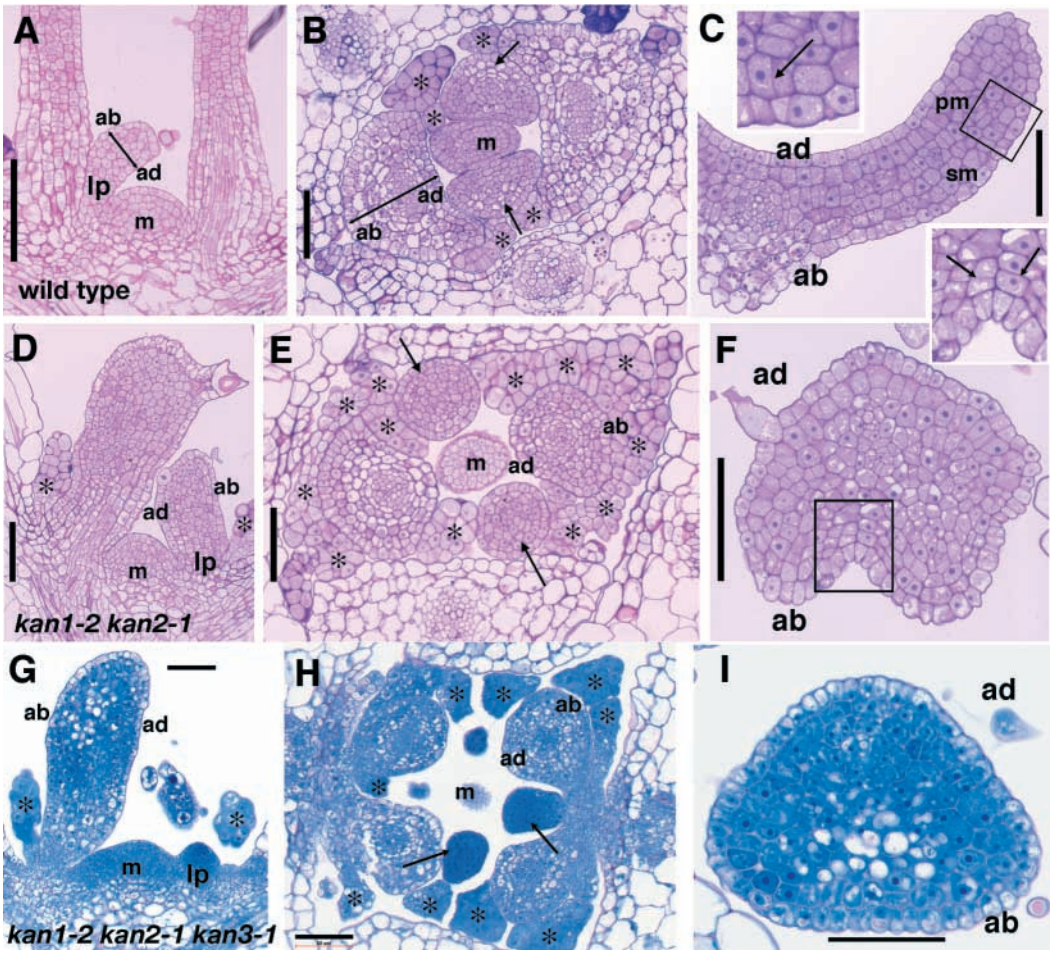

Fig. 2. KANADI loss-of-function anatomical phenotypes. (A,B) Longitudinal and transverse sections of 12-day-old wild-type seedlings demonstrate that young leaf primordia exhibit polarity along the ab/ad axis immediately after their separation from the shoot apical meristem. Polarity is evident both by their crescent shape and the appearance of vacuolated cells (arrows) on the abaxial side first (B). (C) In differentiating leaves, asymmetric anatomy along the ab/ad axis is evident in the shape of the adaxial palisade mesophyll versus the abaxial spongy mesophyll. Anticlinal cell divisions characterize the L1 and L2 cell layers both adaxially and abaxially (insert; arrow). (D,E) In contrast, leaf primordia of *kan1-2 kan2-1* 12-day-old seedlings appear radial (arrows), with all cells maintaining their densely cytoplasmic appearance for a prolonged period. (F) Many more cell layers are found in differentiating leaves, resulting from abnormal periclinal divisions at the abaxial side (insert; arrows). (G-I) Leaf primordia of *kan1-2 kan2-1 kan3-1* seedlings (G,H) are also radial at inception (arrows), but do exhibit some asymmetric growth later in development such that the leaves are also thicker than those of wild type (I). The transverse sections of the leaves in C, F, and I are from the proximal region of expanding leaves of 12-day-old seedlings. *, stipules; ab, abaxial; ad, adaxial; lp, leaf primordia; m, meristem; pm, palisade mesophyll; sm, spongy mesophyll. Scale bars: 50 μm.

rectangular cells similar to those found typically at leaf margins. In wild-type seedlings, the unique leaf marginal cells exhibited specific GUS staining in the enhancer trap marker line YJ158 (Fig. 4B). The outgrowths of *kan1 kan2* young leaves showed GUS expression of this marker throughout their circumference (Fig. 4C), suggesting acquisition of radial blade identity.

In *Arabidopsis* leaves, ab/ad polarity is also evident by the arrangement of the different tissues in the vascular bundles. Xylem is located adaxially, while phloem is positioned abaxially (Fig. 4D,G). In a transverse section of *kan1 kan2* leaf blades, the numerous bundles present displayed varied xylem/phloem arrangements. In most bundles, xylem elements were closer to the adaxial leaf surface as in wild type, yet, skewed orientations, in which the phloem/xylem axis is perpendicular to the ab/ad leaf axis, were quite common (not shown), possibly the result of the prolonged period of cell division in these leaves. While mesophyll cell anatomy was ambiguous, the vascular organization provided a clue to the identity of the blade outgrowths. Several vascular strands connected each outgrowth to the main leaf bundles (Fig. 4E). These strands formed enlarged, centrally located bundles composed of central xylem tissue surrounded by multiple clusters of phloem tissue (Fig. 4F,H). On the periphery of the older outgrowths, secondary vascular bundles developed with the xylem usually closer to the external surface and phloem cells closer to the phloem of the central bundle (not shown).

Similar amphicribral vascular arrangement has been found in the abaxialized radial leaves of *Antirrhinum phantastica* mutants (Waites and Hudson, 1995) and the abaxialized cotyledons of the *phb phv rev* triple mutant (Emery et al., 2003). An opposite, amphivasal arrangement was found in

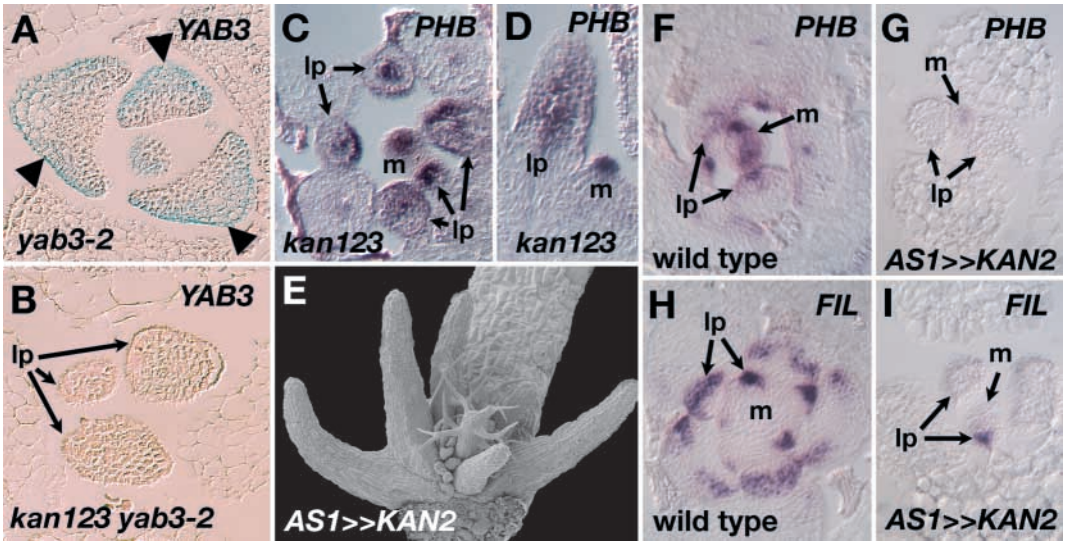

Fig. 3. Gene expression patterns in *kan1 kan2 kan3* and *AS1>>KAN2* leaves. (A) GUS activity, due to an enhancer trap in the *YAB3* gene, is localized to the abaxial regions of developing leaves (arrowheads) in *yab3-2* plants. (B) In contrast, no detectable GUS activity is detected in *kan1-2 kan2-1 kan3-1 yab3-2* plants. (F) In wild-type plants *PHB* expression is localized to the apical meristem and the adaxial regions of lateral organs. (C,D) In a *kan1 kan2 kan3* background, *PHB* expression is no longer adaxially confined in developing leaves, but still displays a gradient with the highest level adaxially. (E) When KANADI activity is expressed uniformly throughout leaves, such as when *KAN2* is driven by the *AS1* promoter, the leaves are both radialized with no lamina formation and abaxialized. (G,F) *PHB* expression is not detected in *AS1>>KAN2* leaves (G) whereas *PHB* is expressed adaxially in wild-type leaves (F). (H) *FIL* is expressed abaxially in wild-type leaf primordia, and its expression becomes localized to the abaxial marginal regions as leaves differentiate. (I) In contrast, *FIL* is only transiently expressed in the radial abaxialized *AS1>>KAN2* leaf primordia. ab, abaxial; ad, adaxial; lp, leaf primordium; m, meristem.

adaxialized *phb-1d/+* leaves, where a cluster of phloem tissue is surrounded by a ring of xylem (McConnell and Barton, 1998). Combined with the formation of leaf-margin-specific cell types around the blade outgrowths, the outgrowths can be viewed as ectopic filamentous leaves, consisting primarily of marginal circumference and abaxial internal tissues. These observations are consistent with the ectopic boundaries of *FIL* expression detected in these outgrowths as they temporally persist beyond the normal abaxial *FIL* expression (Fig. 4J) and are associated with prolonged cell divisions (Fig. 2F). Earlier in leaf development, *FIL* expression appeared to be at highest levels in the region in which outgrowths will soon develop, and at lower levels throughout the abaxial regions of the majority of the leaf (Fig. 4K).

Residual apolar morphology and abaxial outgrowths of *kan1 kan2* leaves are dependent upon YABBY activity

Since the blade-like outgrowths were associated with strong and localized *FIL* expression, we tested whether *FIL* is required for their formation. While *kan1 kan2 fil* leaves still developed outgrowths, their formation was reduced and delayed (not shown). However, when the activity of *YAB3*, which is molecularly and functionally redundant with *FIL* (Siegfried et al., 1999; Kumaran et al., 2002) is also compromised, leaves of quadruple mutants, *kan1 kan2 fil yab3*, exhibited almost no traces of focal outgrowths. The two different quadruple mutant genotypes, *kan1-2 kan2-1 fil-8 yab3-2* or *kan1-2 kan2-1 fil-5 yab3-1*, exhibited similar phenotypes; the first two leaves appeared nearly radial, with trichomes, which are normally found only adaxially on the first few leaves, present around their entire circumference (Fig. 5A). The quadruple mutant plants were greatly reduced in overall size, and organ expansion was severely decreased. Subsequently produced leaves lacked a clear plane of blade symmetry and expanded at random orientations, resulting in short and thick structures (Fig. 5B,I). As in *phb-1d* homozygotes, the quadruple mutant plants lacked stipules entirely, a feature seen in *fil yab3* mutants as well (Fig. 5A,B). Unlike the abaxial epidermal surface of *kan1 kan2* cotyledons, which has a clear abaxial appearance, the abaxial epidermis of the quadruply mutant cotyledons and leaves resembled those of the wild-type adaxial epidermis (Fig. 5C-E). In the quadruple mutant all floral organs except carpels were completely radialized, reduced in number and lacked defining cell types (Fig. 5F). Overall, these flowers closely resembled those of severely adaxialized plants homozygous for *phb-1d* (Fig. 5K).

Anatomical analyses of the *kan1-2 kan2-1 fil-5 yab3-1* leaves revealed a dramatic loss of tissue asymmetry (Fig. 5G-I). Subepidermal cells on both the abaxial and adaxial sides resemble each other, and exhibited an overall similarity to adaxial cell types. In the first two leaves, vascular bundles were greatly reduced with normal phloem/xylem orientation. In the later produced leaves, there were 10-12 cell layers and vasculature was often lacking. As *FIL* is normally expressed in *fil-5* mutants (data not shown), it could serve as an abaxial marker in the quadruple mutant background. Overall, *FIL* expression was greatly reduced, and found only at the margins of the leaves where expansion occurs (Fig. 5J).

The YABBY genes were previously suggested to promote abaxial cell fate on the basis of their expression pattern and gain-of-function alleles. Yet, the loss-of-function phenotypes in either *fil* or *fil yab3* mutant backgrounds do not provide clear

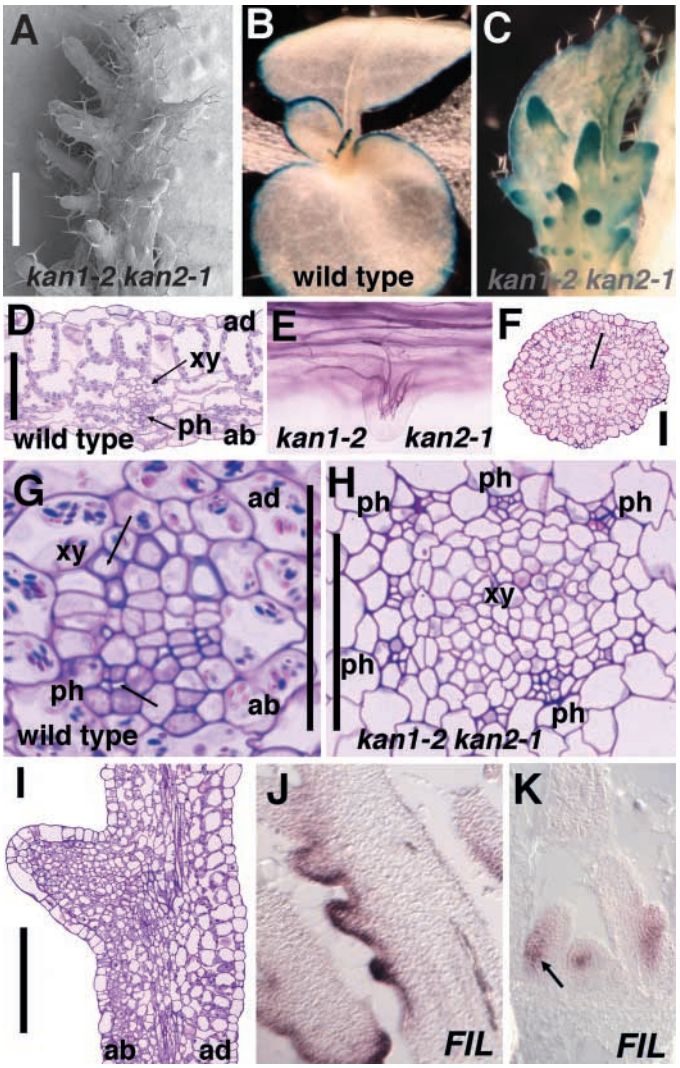

Fig. 4. The nature of the *kan1 kan2* blade outgrowths. (A) The outgrowths developing on the abaxial side of *kan1-2 kan2-1* leaves appear nearly radial, with cell types normally found on leaf margins positioned around their entire circumference. (B) In wild-type seedlings, these cells show blue staining when the GUS reporter is driven by the enhancer trap YJ158. (C) This reporter drives GUS throughout the epidermis of the *kan1 kan2* blade outgrowths and in scattered cells on the abaxial leaf surface. (D) Transverse section through a mature wild-type leaf displaying an asymmetric anatomy both within the leaf and in the vascular bundle. (E,F) In mature *kan1 kan2* leaves, numerous vascular bundles are formed, connecting the outgrowths to leaf main bundles (E). The radial outgrowths have nearly radial anatomy, with a large bundle (arrow) found in their center (F). (G) Close-up of a wild-type minor leaf bundle showing xylem vessel members (arrow) positioned adaxially, while phloem sieve tube elements (arrow) are located abaxially. (H) In *kan1 kan2* the central bundles of the outgrowths have clusters of phloem tissue surrounding xylem vessel members. (I,J) The prolonged period of cell division in the abaxial region of *kan1 kan2* leaves is responsible for the formation of outgrowths, reflected by the maintenance of the densely cytoplasmic appearance of these cells (I), and correlated with prolonged localized *FIL* mRNA expression (J). (K) Earlier in leaf development, high levels of *FIL* expression demarcate presumptive outgrowths (arrow) while a lower level of *FIL* expression is throughout the abaxial region of the leaves. ab, abaxial; ad, adaxial; ph, phloem; xy, xylem. Scale bars: 1 mm (A), 50 μm (B-K).

support for this assumption, as replacement of abaxial cell types by adaxial ones is limited. For example, trichomes were not found on the abaxial surfaces of *fil yab3* leaves until the 5th or 6th leaf as in wild type (Fig. 5L inset). However, when *KANADI* activity is partially compromised the role of the YABBYs as promoters of abaxial identity is revealed. Although *kan1-2/+ kan2-1* plants resembled wild type, *fil-5 yab3-1 kan1-2/+ kan2-1* plants had short and narrow leaves, with trichomes on both sides of the first produced leaves (Fig. 5L).

Previous analyses of mutations of genes involved in establishing the ab/ad axis suggested an association between this axis and proper development along the proximodistal axis (Waites and Hudson, 1995). While no clear morphological markers define the *Arabidopsis* proximodistal axis beyond petiole and blade tissues, neither the *kan1 kan2* nor the *fil yab3* plants have leaves that grow to the normal length. Leaves of the quadruple mutants only grew to 20% of the normal length of wild-type leaves, similar in size to homozygous *phb1-d* leaves (Fig. 5M), corroborating the concept that proper establishment of adaxial-abaxial and proximodistal axes are related.

Other YABBY associated lamina growth

SPLAYED (*SYD*) encodes a SWI/SNF homolog required for proper B class gene expression during flower development (Wagner and Meyerowitz, 2002). In a screen for genetic enhancers of *gym kan1* mutations, we identified two alleles of *syd* that in the *gym kan1 syd* triple mutants resulted in ovules developing from the abaxial regions of the carpels (Eshed et al., 2001). To examine whether *syd* enhances other aspects of the *kanadi* mutant phenotype, we constructed the *kan1-2 kan2-1 syd-2* triple mutant. The leaves of *kan1 kan2 syd* were radially symmetric and lacked outgrowths (Fig. 6A). Consistent with their adaxialized appearance, *PHB* was expressed throughout the developing leaves (Fig. 6B). *FIL* expression was greatly reduced in this background (Fig. 6C-D). Thus, the suppression of abaxial outgrowths by *syd* mutations in a *kan1 kan2* background is associated with a loss of YABBY gene expression in this background.

Previously, YABBY expression was associated with lamina expansion of *phb-1d/+* plants, and this association was maintained even when YABBY activity was detected on the upper side of the trumpet shaped leaves (Siegfried et al., 1999). To examine whether YABBY gene expression is tightly correlated with regions of active cell division and blade expansion in developing leaves of other species as well, we isolated YABBY gene family members from *Solanum tuberosum*. The leaves of *S. tuberosum* facilitated examination of gene expression patterns in greater detail than the small and rapidly differentiating leaves of *Arabidopsis* (Fig. 6E). *StYABBY1* of *S. tuberosum* is orthologous to *FIL* and *YAB3* of *Arabidopsis* and *NbYABBY* of *Nicotiana benthamiana* (Foster et al., 2002). *StYABBY1* was found to be expressed abaxially in young developing potato leaf primordia. As leaves differentiate, it continues to be expressed abaxially, but only in regions that consist of cytoplasmically dense cells that presumably retain the capacity to divide (Fig. 6F). These cells are located primarily towards the margins, and along the ab-ad boundary of the leaf. In conclusion, expression of *StYABBY1* appears abaxial at early stages of leaf development and parallels regions associated with extensive future lamina expansion.

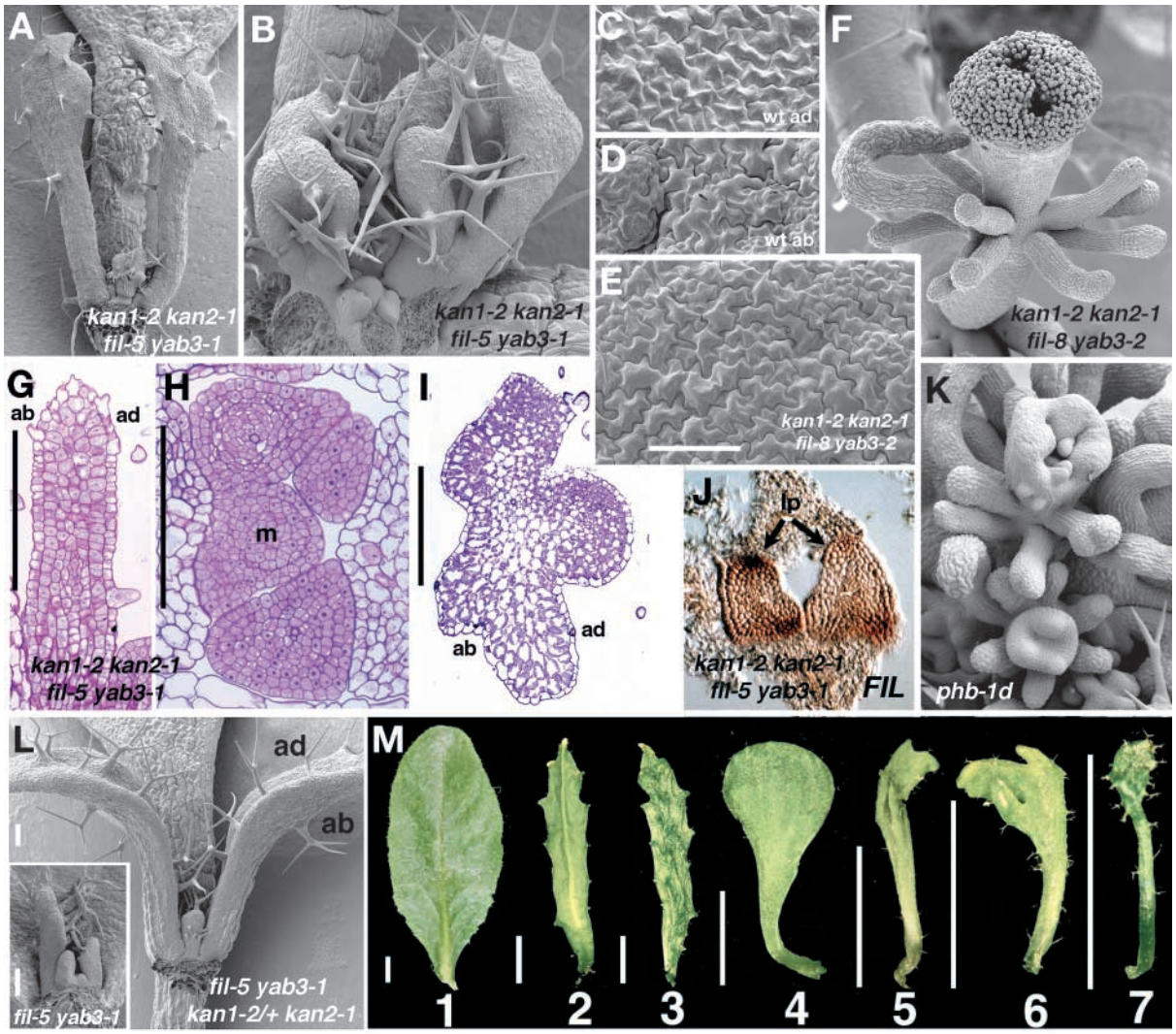

Fig. 5. Simultaneous loss of both KANADI and YABBY functions. (A) In contrast to the clear asymmetric development of *kan1 kan2* leaves, *kan1 kan2 fil yab3* quadruple mutants initiate nearly radial first leaves. The two genotypes, *kan1-2 kan2-1 fil-5 yab3-1* and *kan1-2 kan2-1 fil-8 yab3-2*, are indistinguishable in morphology. (B) The leaves have trichomes on all sides, an adaxial characteristic of the first leaves, and expand laterally only at their distal end. Later-formed leaves are short, thick and lack organized plane of expansion. (C-E) The epidermal surfaces of these leaves (E) appear similar on both sides, and resemble those of adaxial leaf surfaces (C), rather than the abaxial leaf surfaces (D) of wild-type leaves. (F,K) Flower organs of the quadruple mutants are completely radialized (F), lack epidermal characteristics of wild-type floral organs, and resemble the flowers of adaxialized homozygous *phb-1d* mutants (K). (G) Longitudinal section through a developing third leaf of a *kan1-2 kan2-1 fil-5 yab3-1* quadruple mutant reveals anatomical symmetry. (H,I) This symmetry is also apparent in by transverse sections of either young leaf primordia (H) or a differentiating leaf (I). (J) In a similar fashion to its expression in wild-type leaves, *FIL* mRNA is detected in the margins of the quadruple mutant leaf where prolonged cell divisions occurs, yet this expression is no longer abaxial as seen in this transverse section through two leaves above the level of the meristem. (L) Leaves of *fil-5 yab3-1* show dramatic loss in their polarity as expressed by trichome distribution on both sides of the first two leaves, when the activity of the KANADI proteins is partially compromised. This phenotype, is in sharp contrast to the normal distribution of trichomes in *fil-5 yab3-1* alone (inset; first three leaves removed). (M) Besides the distribution of cell types along the ab/ad leaf axes, all the described mutations have a strong effect on growth along the proximal/distal axis. In general, increasing losses of asymmetric development are accompanied by reduced leaf elongation. All leaves shown, except for no. 5 are fully expanded 4th or 5th leaves of the respective genotypes. (1, wild type; 2, *kan1 kan2* adaxial surface; 3, *kan1 kan2* abaxial surface; 4, *fil5 yab3-1*; 5, *kan1-2 kan2-1 fil-5 yab3-1* first leaf; 6, *kan1-2 kan2-1 fil-5 yab3-1* 5th leaf; 7, *phb-1d*. (Bars to the left of each leaf: 5 mm.) ab, abaxial; ad, adaxial; lp, leaf primordium; m, meristem; wt, wild type. Scale bars: 100 μm (C-E and G-I).

Discussion

Loss of KANADI function results in loss of most, but not all polarity

Although four KANADI genes are present in the *Arabidopsis* genome, simultaneous inactivation of three of them is enough to transform most abaxial features of leaves into adaxial features. This transformation includes adaxialization of vasculature and mesophyll and the generation of nearly radial leaf primordia accompanied by occasional production of abaxially placed axillary buds. Compromising most of the activity of both KANADI and YABBY genes, as in the *kan1-*

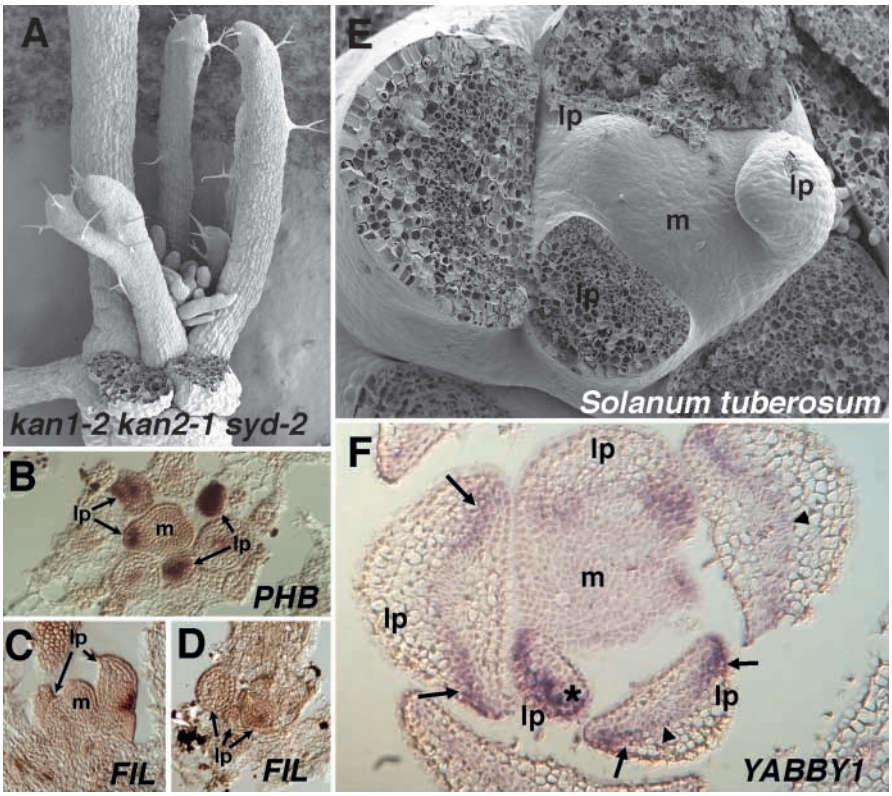

Fig. 6. YABBY and lamina expansion. (A) In *kan1-2 kan2-1 syd-2* leaves, no abaxial outgrowths form, and the leaves are radialized. (B) *PHB*, normally confined to the adaxial regions of the developing leaves, is expressed throughout *kan1 kan2 syd* leaves. (C,D) Consistent with YABBY gene expression being responsible for the abaxial outgrowths on *kan1-2 kan2-1* leaves, *FIL* expression is greatly reduced in *kan1-2 kan2-1 syd-2* triple mutants as seen in both longitudinal (C) and transverse (D) sections of developing leaves. (E,F) In developing *Solanum tuberosum* leaves (E), *YABBY1* expression is associated with abaxial regions in which cells are cytoplasmically dense (F). These regions include the entire abaxial domain in young leaf primordia (*), and in differentiating leaves, the marginal abaxial regions (arrows) and towards the center of the leaf, along the boundary between the abaxial and adaxial domains (arrowhead). lp, leaf primordium; m, meristem.

2 kan2-1 fil-5 yab3-1 quadruple mutants, further reduces the polar nature of lateral organs, as evidenced by formation of trichomes on both sides of the first leaves. However, the leaves of the quadruple mutant do not display all the adaxial characteristics seen in homozygous *phb1-d* plants (McConnell and Barton, 1998). They are not filamentous but rather bulbous and have many more cell layers than *phb-1d* leaves. Expression of the other YABBY and KANADI gene family members may still be promoting abaxial identity and limited random lamina expansion in this background, or alternatively, other abaxial identity genetic programs could be invoked. Other suppressors of PHB-like activity such as the recently identified microRNAs could also account for residual abaxial identity (Reinhart et al., 2002; Tang et al., 2002). Without further understanding of the 'polarity ground state' it will be difficult to predict whether residual abaxial identity stems from failure to activate adaxial factors or from activity of additional abaxial factors.

While *fil yab3* double mutants do not exhibit a conspicuous loss of polarity with respect to differentiation of leaf tissues (Siegfried et al., 1999; Kumaran et al., 2002), we show that *FIL* and *YAB3* contribute to polar differentiation of tissues in the leaf when in the context of a *kan1/+ kan2* background. Trichomes develop on the abaxial sides of the first leaves in *fil yab3 kan1/+ kan2* plants, but not in *kan1/+ kan2* plants, indicating that *FIL*/*YAB3* contribute to the differentiation of cell types on the abaxial sides of these leaves. Thus, these loss-of-function phenotypes are consistent with the hypothesis, based on gain-of-function alelles, that YABBY gene activity promotes abaxial cell fates (Sawa et al., 1999; Siegfried et al., 1999).

Association of polar YABBY activity with lamina expansion

The establishment of polarity is required for lamina expansion, a critical process in the development of nearly all lateral organs. Based on elegant genetic and classic dissection experiments it has been proposed that a juxtaposition of adaxial and abaxial domains is required for lamina development (Sussex, 1955; Waites and Hudson, 1995). Several lines of evidence lead us to propose that a major component of the genetic program directing lamina expansion in leaves is the activity of members of the YABBY gene family. As outlined below, YABBY gene expression is correlated with lamina expansion in several different contexts, ectopic YABBY gene expression leads to ectopic growth, and loss of YABBY gene activity results in a loss of lamina expansion.

YABBY gene expression patterns mirror distributions of cytoplasmically dense cells competent to undergo cell division. In *Arabidopsis*, *FIL* and *YAB3* are initially expressed throughout the abaxial regions of leaves, and later, expression becomes restricted to the laminar marginal domains as the central abaxial domain differentiates into vacuolated cells. Expression ceases in a basipetal manner, paralleling the differentiation of leaf cells and preceding the progression in cell division distributions (Donnelly et al., 1999; Sawa et al., 1999; Siegfried et al., 1999; Kumaran et al., 2002). In *Solanum tuberosum*, the correlation with densely cytoplasmic cells is readily observed, as YABBY expression becomes restricted to the marginal domains of the abaxial-adaxial boundary. Given that the YABBY-expressing cells represent a two dimensional sheet of cells, originally throughout the leaf, but then becoming restricted to two marginal regions of the differentiating leaf, it

would follow that lamina expansion along the proximodistal axis would also be coupled to establishment of the abaxial-adaxial axis. Thus, in each of these cases YABBY gene expression patterns are consistent with activity being linked to lamina expansion.

The reduction in lamina growth, most markedly in the floral organs of *fil yab3* plants is also consistent with YABBY gene activity contributing to lamina expansion (Siegfried et al., 1999). Some lamina expansion does occur in *fil yab3* leaves, but this limited lamina development may be due to two other members of the YABBY gene family, *YAB2* and *YAB5*, which are expressed in leaves (Siegfried et al., 1999). Characterization of plants lacking all YABBY activity will be required to address whether YABBY activity is absolutely required for leaf lamina development. The outer integument is a lateral organ in which expression of only a single YABBY gene family member, *INNER NO OUTER* (*INO*) has been detected (Villanueva et al., 1999). In this case, *INO* is expressed in the abaxial cell layer of the outer integument (Balasubramanian and Schneitz, 2002; Meister et al., 2002) and loss of *INO* activity results in a complete loss of lamina expansion of the outer integument. Thus, loss-of-function alleles of YABBY gene family members are also consistent with a role of YABBY gene expression promoting lamina expansion.

The model of leaf blade development by Waites and Hudson (Waites and Hudson, 1995) proposed that juxtaposition of abaxial and adaxial domains is required for lamina outgrowth. Leaves of *kan1 kan2* plants are mosaic, having both ectopic adaxial and abaxial characteristics. Young leaf primordia in the double mutant are nearly radial, with spatially expanded expression domains of adaxial promoting genes (Eshed et al., 2001). Laminar expansion in these leaves occurs largely as a result of the low level of properly localized YABBY genes since this expansion is lost in the *kan1 kan2 fil yab3* quadruple mutant. However, as *kan1 kan2* leaves differentiate, reactivation of YABBY genes occurs within specific abaxial foci by a presently unexplained mechanism. These foci develop as blade-like outgrowths with abaxial fates based on anatomical characters and vascular organization. YABBY activity is required for lamina expansion associated with the focal outgrowths since this growth disappears in the *kan1 kan2 fil yab3* background, providing positive evidence that YABBY activity can induce lamina expansion.

In *kan1 kan2* leaves, three levels of YABBY gene expression are evident: the adaxial domain has no expression, the abaxial domain has low levels, and the abaxial foci have high levels. Lamina expansion is correlated with the boundaries between each of these expression domains, suggesting that relative levels, rather than absolute levels, of YABBY activity could be responsible for the blade growth in these leaves. The abnormal thickness of *kan1 kan2* leaves can also be seen in a context of prolonged boundaries of YABBY gene expression leading to continued periclinal cell divisions in the abaxial regions of developing leaves. Such boundaries still exist even in *kan1 kan2 fil yab3* quadruple mutants, as reflected by *FIL* expression, and therefore thickness of these leaves could also be attributable to residual YABBY activity, possibly mediated by *YAB2* and *YAB5*. However, when no boundaries (as assayed by *FIL*) are present, such as in *phb-1d*, and *kan1 kan2 syd* leaves, abnormal leaf thickness is not present.

In a *phb-1d* background, radial adaxialized leaves are produced and *FIL* is not expressed at detectable levels in the leaves (Siegfried et al., 1999). Thus, loss of YABBY activity is associated with loss of lamina expansion in this background. In contrast to the adaxialized leaves of the above genotypes, *AS1>>KAN2* plants produce radial abaxialized leaves. If YABBY activity was solely associated with abaxial cell type specification, one might expect YABBY gene expression to be prominent. However, in *AS1>>KAN2* leaves *FIL* expression is transient, consistent with the lack of lamina expansion in this genotype. YABBY gene family expression does not necessarily have to be localized to the abaxial domain to effect lamina expansion. For example, in *phb-1d* heterozygotes, peltate leaves, in which cells with abaxial identity are inside the cup, commonly develop (McConnell and Barton, 1998). In this case, YABBY gene expression is associated with expansion of the cup and is located at the tip of the developing leaf rather than the abaxial domain (Siegfried et al., 1999). Perhaps more remarkably, *FIL* is expressed adaxially in *petal loss pistallata* second whorl floral organs that are morphologically inverted (Siegfried et al., 1999; Griffith et al., 1999). As these organs display normal blade expansion, YABBY gene expression can still exert its role in lamina expansion as long as expression remains polar.

Thus, in each case examined, YABBY gene activity is associated with regions of lamina expansion, and is necessary in at least some contexts to promote lamina development. In these cases it appears that it is a boundary of YABBY gene activity that is associated with lamina formation. Unlike many genes whose products will promote growth per se, via cell division or cell expansion, we suggest that boundaries of YABBY activity act to promote cell division indirectly in regions spanning both sides of the boundary. This would imply that YABBY activity is signaling between domains, and consistent with this hypothesis ectopic expression of *FIL* or *YAB3* results in dramatic non-autonomous phenotypic effects (Y.E. and J.L.B., unpublished observations). Determination of whether YABBY gene activity is sufficient to induce lamina expansion will require the analysis of genetic chimeric plants in which sectors of YABBY gene expression are produced in fields of cells lacking YABBY activity.

Boundaries and organizers

The proposal that boundaries of YABBY gene expression act as mediators of zones of lamina expansion has elements in common with the paradigm of boundaries acting as organizing centers in animal development (Lawrence and Struhl, 1996; Basler, 2000; Irvine and Rauskolb, 2001). In such a developmental paradigm an initial polarity leads to asymmetric short-range signaling creating a third type of cell at or near the boundary that produces long-range organizing signals that pattern the differentiation of surrounding tissues. Such systems provide a powerful mechanism to generate complex patterns from an initial simple asymmetry. While no signaling molecules that pattern differentiation within developing leaves have yet been identified, boundaries of YABBY gene activity appear to promote organ growth in adjacent cells and thus acting as a focus for lamina formation.

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