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Members of the *YABBY* gene family specify abaxial cell fate in *Arabidopsis*

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SUMMARY

Lateral organs produced by shoot apical and flower meristems exhibit a fundamental abaxial-adaxial asymmetry. We describe three members of the *YABBY* gene family, *FILAMENTOUS FLOWER*, *YABBY2* and *YABBY3*, isolated on the basis of homology to *CRABS CLAW*. Each of these genes is expressed in a polar manner in all lateral organ primordia produced from the apical and flower meristems. The expression of these genes is precisely correlated with abaxial cell fate in mutants in which abaxial cell fates are found ectopically, reduced or eliminated. Ectopic expression of either *FILAMENTOUS FLOWER* or *YABBY3* is sufficient to specify the development of ectopic abaxial tissues in lateral organs. Conversely, loss of polar expression of these two genes results in a loss of polar differentiation of tissues in lateral organs. Taken together, these observations indicate that members of this gene family are responsible for the specification of abaxial cell fate in lateral organs of *Arabidopsis*. Furthermore, ectopic expression studies suggest that ubiquitous abaxial cell fate and maintenance of a functional apical meristem are incompatible.

Key words: *Arabidopsis*, Meristem, Abaxial-adaxial polarity, *YABBY*, *FILAMENTOUS FLOWER*, *CRABS CLAW*

INTRODUCTION

In higher plants, post-embryonic organogenesis proceeds with lateral organs emerging from the flanks of the shoot apical meristem (SAM). One common feature of most lateral organs is that they are polar in nature, with distinct proximodistal and abaxial-adaxial (abaxial is away from the meristem and adaxial is adjacent to the meristem) axes of asymmetry. In the lateral organs of most species these asymmetries are manifested in a broad distal blade and a narrower proximal petiole in the proximodistal axis and abaxial-adaxial asymmetry, evident at the cellular level. Since all lateral organs exhibit proximodistal and abaxial-adaxial asymmetries, it is likely that common genetic programs are responsible for establishing these polarities. Polarities are evident at or just subsequent to organ primordium formation, and thus the genes involved in establishing asymmetry are expected to act at this time.

It is clear from clonal analyses that positional information plays a more important role than cell lineage in determining cell fate in plant development (reviewed in Meyerowitz, 1997). Because lateral organs develop from the flanks of meristems, there exists a fundamental positional relationship between lateral organ primordia and the meristems from which they are derived: the adaxial side of the primordium is directly adjacent to the cells of the meristem, whereas the abaxial regions of the primordium are at a distance from the meristem. Experiments performed on *Solanum*, in which incipient leaf primordia were separated by incisions from the SAM, suggested that the apical meristem may be a source for a signal required for proper abaxial/adaxial development of the leaf (Sussex, 1954, 1955). In these experiments, if an incision was made directly adjacent to incipient leaf primordia separating them from the SAM, the leaf primordia would develop into radially symmetric abaxialized organs (Sussex, 1955). One interpretation is that signals emanating from the SAM promote adaxial cell fate, and in the absence of such signals, abaxial cell fate may be the default pattern of differentiation. Subsequent experiments on other species suggested that this might be typical for dicotyledonous angiosperms in general (Snow and Snow, 1959; Hanawa, 1961).

The genetic basis of lateral organ polarity establishment has been the focus of a number of recent studies. Radially symmetric leaves develop in *phantastica* (*phan*) mutants of *Antirrhinum* (Waites and Hudson, 1995). In this case the leaves were interpreted to be abaxialized, suggesting that *PHAN* normally promotes adaxial cell identity. Based on partial loss-of-function alleles in which ectopic abaxial/adaxial boundaries of tissue resulted in outgrowths of tissue, it was proposed that a juxtaposition of abaxial and adaxial cell fates is required for lamina outgrowth (Waites and Hudson, 1995). In the absence of any adaxial tissue, such as in severe loss-of-function alleles, the abaxialized organs of *phan* mutants thus develop as radially symmetric organs.

In contrast to *phan*, *phabulosa-1d* (*phb-1d*) mutants display

an adaxialization of the lateral organs (McConnell and Barton, 1998). *phb-1d* mutations are semi-dominant, with adaxialization of lateral organs occurring in a dose-dependent manner (McConnell and Barton, 1998). A striking feature of *phb-1d* mutants is that axillary meristems, normally found only adaxially in the leaf axil, develop around the entire circumference at the base of the adaxialized leaves, suggesting that their formation is correlated with adaxial cell fate. Additionally, *phb-1d* mutants exhibit an enlarged SAM, which led McConnell and Barton (1998) to propose that there is a positive influence of adaxial cell fate on meristem formation. In other words, SAMs produce lateral organs and the adaxial regions of the lateral organs in turn promote meristem formation. Consistent with this hypothesis are the observations that in *phan* mutants where the lateral organs are extremely abaxialized, the apical meristem does not function properly (Waites et al., 1998).

The emerging picture from classical and molecular genetic analyses is that as incipient lateral organ primordia develop from the flanks of the SAM, factors both intrinsic and extrinsic to the organ primordia contribute to the specification of cells as adaxial or abaxial. The SAM itself likely provides a signal(s) that promotes adaxial cell fate, whereas abaxial cell fate may be the default in the absence of such signals. To date, genes have been cloned that unambiguously specify abaxial cell fate.

We describe a family of genes, the *YABBY* gene family, at least three members of which are expressed in a polar manner in all lateral organs produced by apical and flower meristems. Their transcripts are detectable only in the abaxial domains of lateral organs when their primordia emerge and begin to differentiate. Loss of polar expression of these genes results in loss of polar differentiation of cell types in lateral organs, and gain-of-function alleles result in abaxialization of lateral organs. The expression of these genes is precisely correlated with abaxial cell fate in mutants in which abaxial cell fates are found ectopically, reduced or eliminated. We propose that members of this gene family act redundantly to specify abaxial identity in lateral organs produced by the apical and flower meristems in *Arabidopsis*. The significance, with respect to meristem maintenance, of establishing asymmetric development of lateral organs will be discussed.

MATERIALS AND METHODS

Plant material

All mutant alleles of *filamentous flower* are in the Landsberg *erecta* (Ler) background (Chen et al., 1999). The *yabby3-1* allele (Wassilewskija background) was isolated from the Feldmann T-DNA lines (ABRC at Ohio State). A left border T-DNA primer (McKinney et al., 1995) and a *YABBY3* gene-specific primer amplified a 2.3 kb product from line 4622. A single T-DNA insertion was shown to reside in *YABBY3*. *fil-5 yab3-1* double mutants were generated by crossing homozygous *fil-5* and *yab3-1* plants and selecting double mutants in the F_2. The *phabulosa-1d*, *petalloss-1*, *apetala3-3* and *pistillata-1* alleles are in Ler background. Plants were grown in 8 hour light:6 hour dark regime at either 16°C (in situ analyses) or 22°C (phenotypic analyses). Wild-type alleles are italicized block capitals; mutant alleles are italicized lower case.

Isolation of the *YABBY* gene family

Genomic hybridizations indicated that *CRC* belongs to a small gene family. A 140 bp probe was generated using primers flanking the conserved YABBY domain of *CRC* and a rice EST (Bowman and Smyth, 1999) using *CRC* as template. 4×10^5 *Arabidopsis* flower cDNA clones (Weigel et al., 1992) were screened at moderate stringency (hybridization: 5×SSPE, 50°C; washes: 2×SSPE, 50°C) (Sambrook et al., 1989). 100 clones comprising five non-crosshybridizing classes corresponding to *CRC*, *YABBY1* (*YAB1*), *YAB2*, *YAB3* and *YAB5* were isolated. *YAB1*, *YAB3*, *YAB4* and *YAB5* genomic sequences were generated by the *Arabidopsis* genome project. Subsequently, *YAB1* was shown to correspond to *FIL* (see Results). Both strands of *YABBY* cDNAs were sequenced using an ABI automated sequencer. *fil* alleles were sequenced from at least two independent PCR products produced by the amplification of genomic DNA using primers such that all coding regions and intron-exon boundaries were sequenced.

In situ hybridization

In situ hybridizations were performed as previously described (Drews et al., 1991; Long et al., 1996; Grossniklaus et al., 1998). *YAB1*, *YAB2* and *YAB3* probes lacking the YABBY domain were obtained by subcloning the 5′ portion of the genes into pBluescript (Stratagene, La Jolla), resulting in subclones of approximately 520 bp, 230 bp and 320 bp, respectively. Subclones were used to synthesize anti-sense RNA probes.

Transgenic plants

Full-length cDNAs of *YAB1* and *YAB3* lacking poly(A) tails were cloned in a sense orientation into a unique *Bam*H1 site flanked 5′ by the CaMV 35S promoter and 3′ with a nopaline synthase transcription termination signal (Gleave, 1992; pART7). These constructs were introduced into the plant transformation vector pMLBART. Transgenic plants were generated by the infiltration method of Bechtold et al. (1993) in a wild-type Ler background, with selection on soil by resistance to the herbicide BASTA.

Morphological analyses

Scanning electron microscopy was performed on a Hitachi S-3500N SEM with sample preparation as previously described (Alvarez et al., 1992). Digital images were captured at 5 kV and assembled in Adobe Photoshop. Histological analyses were performed as described in Baum and Rost (1996) and Fuchs (1963).

RESULTS

Isolation of the *YABBY* gene family

CRABS CLAW (*CRC*), a gene involved in carpel and nectary development in *Arabidopsis* (Alvarez and Smyth, 1999), was previously shown to be a member of a small gene family, dubbed the *YABBY* gene family (Bowman and Smyth, 1999). Based on genomic DNA hybridization experiments, we estimated that this gene family comprises 6-7 members in *Arabidopsis*. Four additional members of the family (*YABBY1* [*YAB1*], *YAB2*, *YAB3* and *YAB5*) were isolated from a cDNA library, and a fifth additional member (*YAB4*) has been sequenced by the *Arabidopsis* genome project (Bowman and Smyth, 1999). Thus, it is likely we have identified most, if not all, members of this family in *Arabidopsis*. Members of the *YABBY* gene family are characterized by two conserved domains, a C_2C_2 zinc finger-like domain towards the amino terminus and a helix-loop-helix, which we have called the YABBY domain, with sequence similarity to the first two helices of the HMG box towards the carboxyl end of the protein (Fig. 1; Bowman and Smyth, 1999). The presence of

these domains suggests these proteins function as transcription factors. Database searches suggest that this family may be plant-specific, as genes with a similar arrangement of zinc finger and YABBY domains do not occur in either *Caenorhabditis elegans* or *Saccharomyces cerevisiae*.

In addition to the conserved YABBY and zinc finger domains, *YAB1*, *YAB2*, *YAB3* and *YAB5* display some sequence similarity on the carboxyl side of the YABBY domain (Fig. 1A). The region between the YABBY and zinc finger domains is variable amongst family members, although similarities can be noted between *YAB1* and *YAB3*. These data suggest *YAB1* and *YAB3* represent the most recent gene duplication within the family, and *YAB2* and *YAB5* are more closely related to *YAB1/3* than either are to *CRC* or *YAB4*. *YAB1* was shown to correspond to *FILAMENTOUS FLOWER* (*FIL*) (Sawa et al., 1999; Chen et al., 1999; see below) and will be referred to as such hereafter. The present analysis is focused on *FIL*, *YAB2* and *YAB3* because these genes are expressed in a similar manner, suggesting they might encode proteins with similar biological functions.

FIL, *YAB2* and *YAB3* expression is restricted to abaxial regions of lateral organs

FIL, *YAB2* and *YAB3* are expressed in a qualitatively similar manner at the mRNA level, although expression levels differ dramatically. Each is expressed in abaxial regions of above ground lateral organ primordia: cotyledons, leaves, flower meristems, sepals, petals, stamens and carpels. The exception to this generalization is that detectable levels of expression are not observed in ovule primordia. No expression was detected in embryonic or post-embryonic roots. *FIL* is expressed at a high level, *YAB3* at a moderate level, and *YAB2* at a low level. In situ analyses using ^{35}S-UTP required exposure times 5- to 10-fold longer for *YAB3* to yield a signal equivalent to that of *FIL*. Similarly, expression of *YAB2* is approximately an order of magnitude lower than that of *YAB3*.

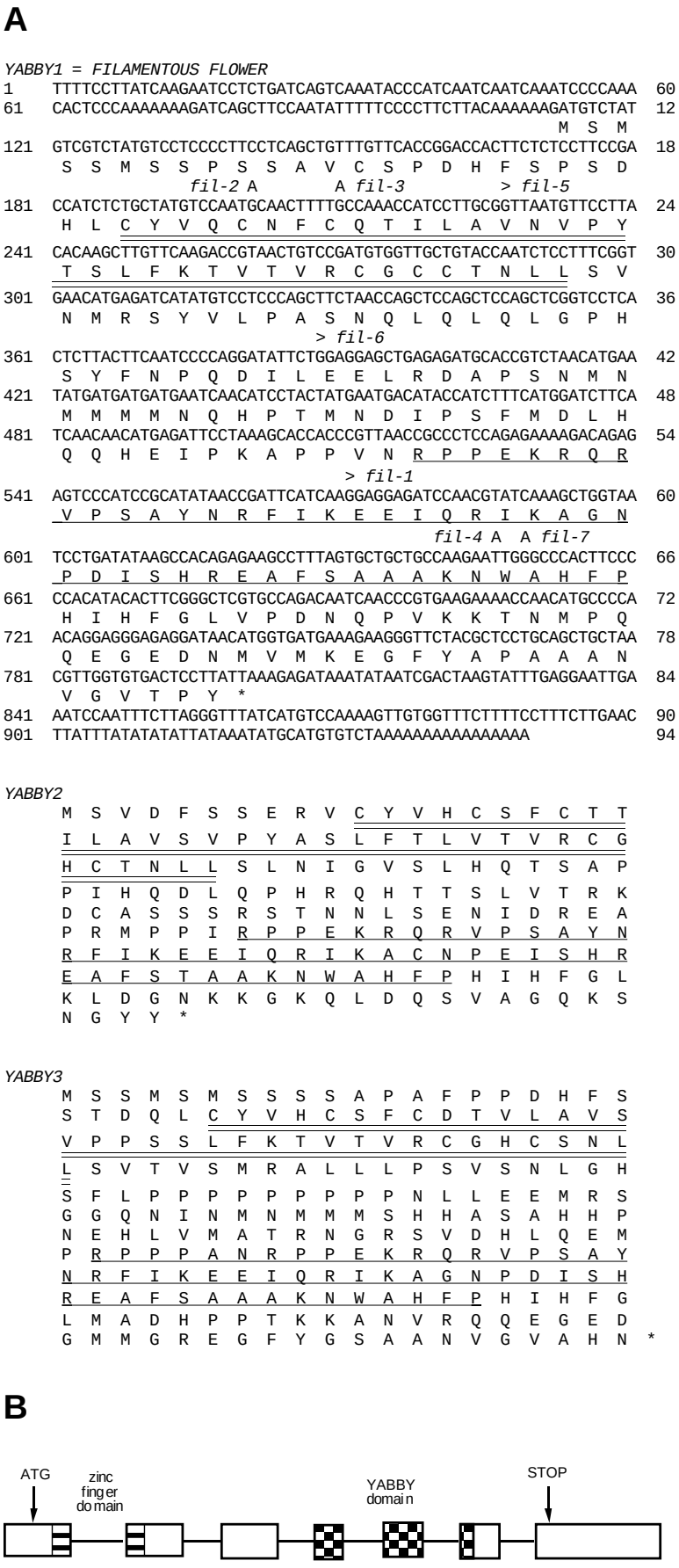

Fig. 1. *YABBY* gene family. (A) Nucleotide sequence of *FIL* and deduced amino acid sequences of *FIL*, *YAB2* and *YAB3*. The zinc finger-like domain (double underline), the YABBY (helix-loop-helix) domain (single underline), and the locations and changes of *fil* mutations are indicated. The *fil-1*, *-5* and *-6* mutations are shown above the intron (indicated by > over the first nucleotide of the following exon); they are the result of a G to A change in splice site acceptor or donor sequences. (B) Cartoon of a generic *YABBY* family member, with zinc finger (stripes) and YABBY (checked) domains indicated. Boxes represent exons and lines represent introns; five genes (*CRC*, *FIL*, *YAB3*, *YAB4*, *YAB5*), consist of seven exons, whereas *YAB2* consists of six exons. GenBank accession numbers: *FIL* (AF136538), *YAB2* (AF136539), *YAB3* (AF136540).

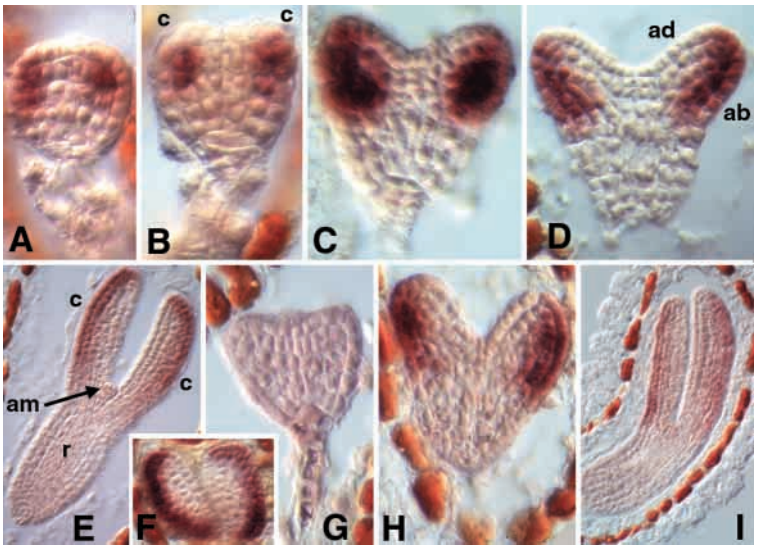

Fig. 2. Embryo mRNA expression patterns of *FIL* (A-F) and *YAB3* (G-I). *FIL*: transition stage (A); triangle stage (B); early heart stage (C); late heart stage (D); torpedo stage (E); torpedo stage, transverse section (F). *YAB3:* early heart stage (G); late heart stage (H); torpedo stage (I). ab, abaxial, ad, adaxial; am, apical meristem; c, cotyledon; r, radicle.

Embryos

FIL is initially expressed in the transition-stage embryo (between the late globular and heart stages) in a small number of subepidermal cells in the central region of the cotyledon anlagen (Fig. 2A,B). By early heart stage expression has expanded to include cells on the abaxial side of the emergent cotyledon primordia, but does not immediately extend to the tip of the cotyledon primordia (Fig. 2C). By mid-heart stage expression is throughout the abaxial domain of the cotyledon primordia (Fig. 2D). This pattern continues through torpedo, walking-stick and U-shaped stages (Fig. 2E-F), but gradually fades as the embryos mature.

YAB3 expression in developing embryos parallels that of *FIL* (Fig. 2G-I). However, one qualitative difference is that initial detection of *YAB3* is at the early heart stage rather than the transition stage (Fig. 2G-H). *YAB2* also appears to be expressed abaxially in cotyledon primordia; however, the low level of expression precluded a detailed analysis.

Leaves

FIL expression in leaves parallels that described for cotyledons. Expression is initially in a small group of subepidermal cells in the central region of leaf anlagen prior to any morphological sign of primordium formation (Fig. 3A,B). It appears that *FIL* expression is detectable in at least two incipient leaf primordia prior to their emergence from the flanks of the apical meristem. Thus, in cross sections, *FIL* expression reflects the spiral phyllotaxy of the SAM (Fig. 3B). As leaf primordia emerge, expression becomes restricted to the abaxial regions (Fig. 3A,B). In leaves, *FIL* is more broadly

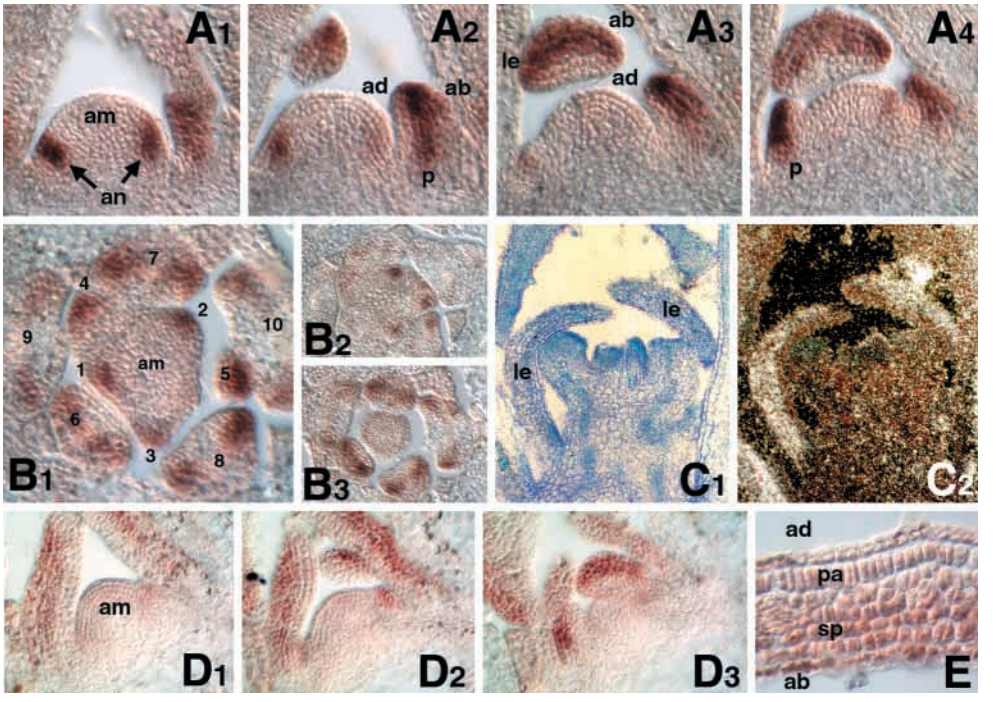

Fig. 3. Vegetative mRNA expression patterns of *FIL* (A-B), *YAB2* (C) and *YAB3* (D-E). Abaxial and adaxial are indicated on developing leaves in A2 and A3. *FIL*: serial longitudinal sections 8 μm apart through a vegetative apex (A1-A4); serial transverse sections (8 μm apart) through a vegetative apex (B1-B4). B2 is below B1 and B3 is above B1. The ten youngest leaf primordia are indicated in B1; 1 = youngest. (C) *YAB2*: longitudinal section, bright field (C1) and dark field (C2). (D1-D3) *YAB3:* serial longitudinal sections (8 μm apart) through a vegetative apex; (E) close-up of a differentiating leaf. ab, abaxial; ad, adaxial; am, apical meristem; an, leaf anlagen; le, developing leaf; p, leaf primordia; pa, palisade mesophyll; sp, spongy mesophyll.

expressed than in cotyledons, with expression extending through more than half of the cell layers in leaf primordia (Fig. 3A,B). Early in leaf primordium growth, cells in the central abaxial domain elongate and become highly vacuolated causing the primordium to arch over the SAM. As the central cells begin differentiating, *FIL* expression declines, but remains high in the marginal abaxial regions (Fig. 3B). No signal is detected in differentiated leaves.

Arabidopsis leaves consist of six cell layers: an abaxial epidermis, three layers of abaxial spongy mesophyll, one layer of adaxial palisade mesophyll and an adaxial epidermis (Pyke et al., 1991). In differentiating leaves *FIL* mRNA can be detected in the abaxial epidermis and in layers destined to become spongy mesophyll, whereas in the palisade mesophyll and adaxial epidermis expression is very low or is undetectable (Fig. 3A,B).

Expression of *YAB2* and *YAB3* is detected in the abaxial regions of the developing leaves (Fig. 3C-E). *YAB3* expression parallels that of *FIL*, with initial expression occurring in leaf anlagen. As leaf primordia emerge from the apical meristem, expression is confined to the abaxial regions of developing primordia. Expression appears to be in at least four cell layers: the abaxial epidermis and three layers of spongy mesophyll (Fig. 3E). As leaves fully differentiate, *YAB3* expression declines to an undetectable level.

Flower meristems and floral organs

FIL expression is detected in flower meristems and floral organs in a pattern reminiscent of its expression in leaf primordia (Fig. 4). *FIL* mRNA is first detected in subepidermal cells of flower anlagen (Fig. 4A). By stage 2 (stages according to Smyth et al., 1990), when flower meristems become distinct from the inflorescence meristem, *FIL* expression is confined to their abaxial domain (Fig. 4B). Expression is subsequently observed in sepal anlagen (Fig. 4C-E). Initial expression is subepidermal, with later expression being confined to the abaxial regions (Fig. 4D,E). Expression of *FIL* extends through several cell layers of the sepals, with expression excluded from

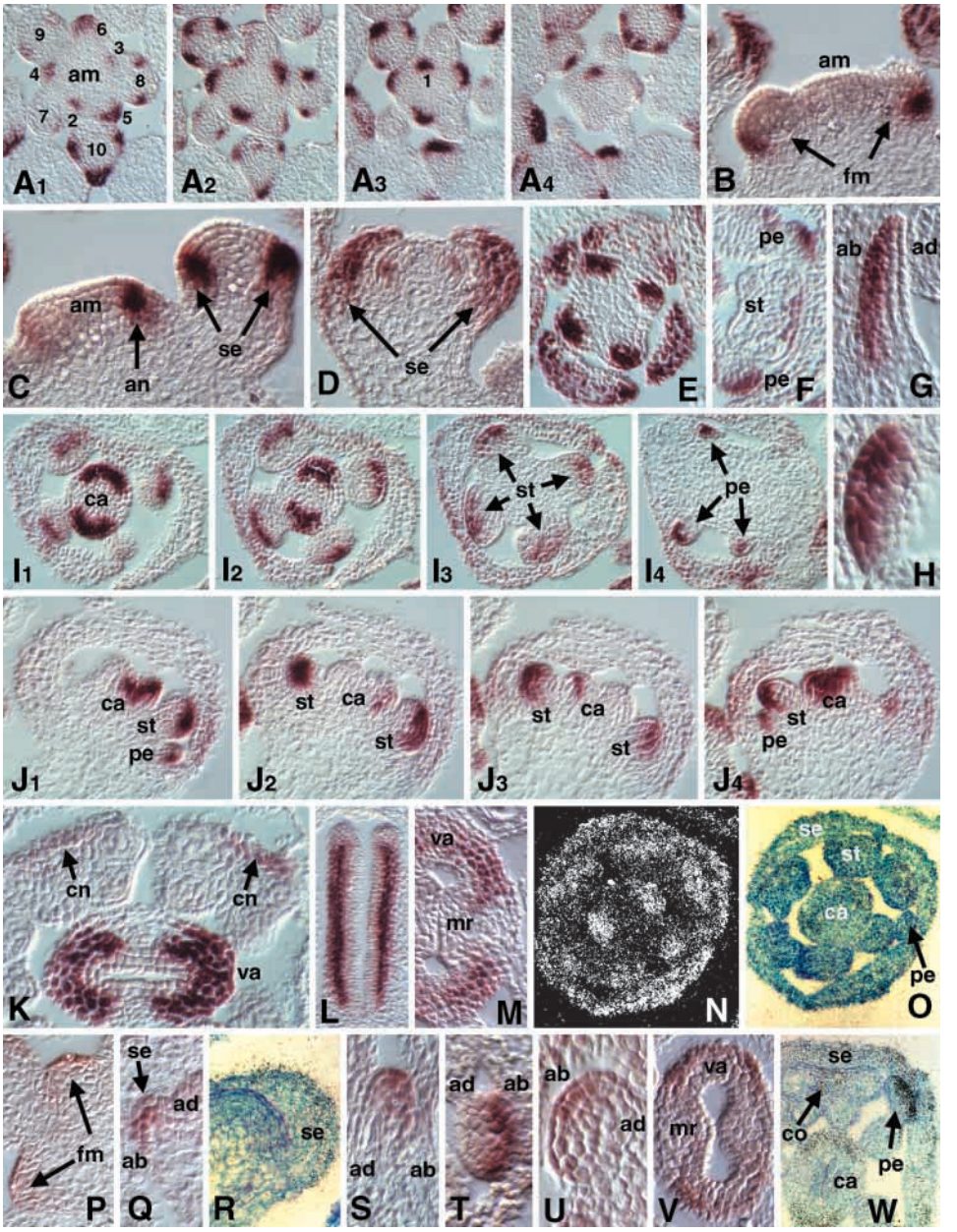

Fig. 4. Floral mRNA expression patterns of *FIL* (A-M) *YAB2* (N, O) and *YAB3* (P-W). *FIL*: (A1-A4) serial transverse sections (8 μm apart) through an inflorescence apex. The ten youngest flower primordia are indicated in A1 and A3; 1 = youngest. (B) Longitudinal section through an inflorescence apex. (C) Longitudinal section through an inflorescence apex and a stage 3 flower. (D) Longitudinal section through a stage 5 flower. (E) Transverse section through a stage 5 flower. (F) Transverse section through a stage 7 flower. (G,H) Longitudinal and transverse sections of a stage 9 petal. (I1-I4) Serial transverse sections (8 μm apart) through a stage 7 flower. (J1-J4) Serial longitudinal sections (8 μm apart) through a stage 7 flower. (K) Transverse section of a stage 8 flower. (L) Longitudinal section of a stage 9 gynoecium. (M) Transverse section of a stage 9 gynoecium. *YAB2*: (N,O) Transverse section of a stage 7 flower, dark field (N) and bright field (O). *YAB3:* (P) Transverse section of an inflorescence apex. (Q-R) Longitudinal sections, respectively, of stage 3 (Q) and stage 5 (R) sepals. (S-T) Longitudinal (S) and transverse (T) sections of stage 8 petals. (U) Transverse section of a stage 6 stamen primordium. (V) Transverse section of a stage 8 gynoecium. (W) Transverse section of a stage 9 flower. ab, abaxial; ad, adaxial, am, apical meristem; an, flower meristem anlagen; ca, carpel; cn, connective; fm, flower meristem; mr, medial ridge; pe, petal, se, sepal; st, stamen; va, valve.

only one or two cell layers on the adaxial side of the sepals. *FIL* expression is detected in the abaxial regions of petal and stamen primordia (Fig. 4F-J). In the case of stamens, expression commences prior to primordial emergence in a pattern similar to that described for other primordia. The small size of petal primordia precluded observation of whether a similar pattern holds in petals. Expression in the stamen primordia becomes restricted to those regions that will differentiate into the connective (the central abaxial domain), and is excluded from the developing locules and filaments (Fig. 4F,K). In each of the floral organs expression declines as the organs differentiate.

FIL expression in the gynoecium is in several abaxial cell layers in two horseshoe shaped domains whose descendents will differentiate into the valves (Fig. 4I,K). The valves of the *Arabidopsis* carpels consist of six cell layers: an abaxial epidermis, three layers of mesophyll, an adaxial subepidermal layer and an adaxial epidermis. Expression of *FIL* is high in the three cell layers destined to differentiate into mesophyll, while a much lower level of expression is detected in the adaxial subepidermal layer (Fig. 4K,M). *FIL* is initially expressed in the abaxial epidermis (Fig. 4I,K) but subsequently becomes restricted from this cell layer (Fig. 4L,M). *FIL* expression is not detected in the adaxial epidermis or the marginal regions of the carpel (Fig. 4L,M).

YAB2 and *YAB3* are expressed in sepal, petal, stamen and carpel primordia in a similar spatial pattern to that described for *FIL* (Fig. 4N-O,P-S). We did not detect *YAB2* expression in flower meristem anlagen, but this may be due to a lack of sensitivity in our experiments.

YABBY gene expression is correlated with cell fate in mutant backgrounds

Semi-dominant *phb-1d* mutations result in adaxialization of leaves and floral organs in a dose-dependent manner (McConnell and Barton, 1998). Organs of *phb-1d* homozygotes are nearly completely adaxialized and radially symmetrical, whereas organs of *phb-1d/+* heterozygotes are mosaics of adaxial and abaxial tissues. To examine whether *YABBY* genes are misregulated in *phb-1d* plants we ascertained the *FIL* expression pattern in *phb-1d* heterozygotes and homozygotes (Fig. 5A-C). In most leaf primordia of *phb-1d* homozygotes, *FIL* expression is undetectable (Fig. 5A). In *phb-1d/+* heterozygotes *FIL* expression is detected in most leaf primordia. However, the *FIL* expression domain is reduced, usually consisting of only a couple of cell layers on the abaxial side of the leaf primordium (Fig. 5B), rather than in four cell layers as observed in wild type. Trumpet-shaped leaves, with an inner surface of abaxial identity and an outer surface of adaxial identity, are characteristic of *phb-1d/+* heterozygotes (McConnell and Barton, 1998). *FIL* expression was observed to be along the inner surface of some branched leaf primordia (Fig. 5C), in a manner consistent with these primordia differentiating into trumpet-shaped leaves. Thus, *FIL* expression correlates with abaxial cell fate in *phb-1d* leaf primordia.

Mutations in *PETALLOSS* (*PTL*) cause defects in the number, stature and orientation of petals that develop in the second whorl of the flower (Griffith et al., 1999). A fraction of the petals develop in a reverse orientation, 180° from normal, with adaxial cell types now occurring abaxially, and vice versa. Mutations in either *PISTILLATA* (*PI*) or *APETALA3* (*AP3*) enhance this phenotype such that nearly all second whorl organs arise in a reverse orientation (Griffith et al., 1999). Because mutations in either *PI* or *AP3* result in second whorl organs developing as sepals (Bowman et al., 1989), *ptl pi* and *ptl ap3* double mutants have second whorl sepals which are oriented 180° from normal. To determine whether the altered cell-fate specification is reflected in *YABBY* gene expression, we examined *FIL* expression in *pi-1 ptl-1* and *ap3-3 ptl-1* flowers. As seen in Fig. 5D, *FIL* expression is now detected in the adaxial portion of the second whorl organs in *pi-1 ptl-1* flowers; similar results were obtained for *ap3-3 ptl-1* flowers (data not shown). Again, *FIL* expression is correlated with abaxial cell fate.

Ectopic expression of *YABBY* genes induces abaxialization of lateral organs

The expression patterns of *FIL, YAB2* and *YAB3* suggest a function in specification of abaxial cell fate. In order to determine whether ectopic expression of these genes is sufficient to specify abaxial cell fate, we generated transgenic plants in which either *FIL* or *YAB3* was expressed using the 'constitutive' Cauliflower Mosaic Virus (CaMV) 35S promoter (Benfey and Chua, 1990). This promoter drives expression in most plant tissues, although the levels vary between tissues. When either *FIL* or *YAB3* is ectopically expressed in a constitutive manner, two classes of phenotypes are observed in transgenic plants (Figs 6, 7).

The first class of transgenic plants produces both cotyledons and leaves, although their morphology is abnormal; the leaves are epinastic and narrow, likely due to decreased cell expansion (Fig. 6B1). The wild-type adaxial epidermis of cotyledons and leaves is characterized by a flat surface composed of uniformly sized cells and a low density of stomata (Fig. 6E,G). In contrast, the wild-type abaxial epidermis of cotyledons and leaves is characterized by an undulating surface, a high density of stomata and frequent large cells amongst smaller cells (Fig. 6F,H). Additionally, trichomes differentiate from the adaxial surfaces of the first few leaves and both surfaces of subsequently produced leaves. Strikingly, the adaxial epidermises of cotyledons and leaves of plants constitutively expressing *FIL* or *YAB3* have cell types resembling wild-type abaxial epidermises (Fig. 6I-L). Despite the marked transformation of the adaxial epidermis, the leaves are not completely abaxialized since they retain some adaxial character. This is manifested in the adaxial palisade mesophyll of the leaves being largely similar to that of wild type (Fig. 6C,D) and the differentiation of trichomes from the adaxial epidermis in the first produced leaves (Fig. 6K,L). In many cases, the adaxial epidermis of the leaves was not easily recognized as either wild-type abaxial or adaxial cell types, but rather appeared as a mixture of the two. Some transgenic plants of this class make the transition to flowering, although the more severe lines exhibit meristem arrest (as described below) after producing a small number of leaves (Fig. 7E).

The second class of transgenic plants exhibits malformed cotyledons that are purple, likely due to accumulation of anthocyanins (Fig. 6B2). In these seedlings the SAM is disrupted, and in most (Fig. 7E,F), but not all (Fig. 7D) cases, arrested primordia-like structures form around the periphery of the SAM. At least some of these structures appear to be

arrested leaf primordia as trichomes could be observed on their adaxial surfaces (Fig. 7G). Other structures resemble stipules, but without additional morphological or molecular markers; their identity remains enigmatic. In contrast to the densely cytoplasmic cells of wild-type SAMs (Fig. 7A), the arrested meristems are comprised of vacuolated cells (Fig. 7C). The sizes of the arrested meristems are larger than those of wild-type SAMs, apparently due to cell expansion (Fig. 7A-F). Apical meristems of the transgenic plants did not exhibit the typical tunica-corpus architecture found in wild-type meristems (Fig. 7A,C).

Although both of the classes were observed with either transgene, on average, ectopic expression of *YAB3* resulted in more severe phenotypes. Of 75 transgenic plants expressing ectopic *FIL*, 4 had no aberrant phenotype, 68 produced leaves and 3 produced only cotyledons; of 51 transgenic plants expressing ectopic *YAB3*, 1 had no aberrant phenotype, 11 produced leaves and 39 produced only cotyledons. We interpret the increasing severity of transgenic phenotypes to correspond to increasing levels of ectopic *FIL* or *YAB3* expression: while moderate levels of *FIL* or *YAB3* activity are sufficient to specify abaxial cell fate, high levels result in SAM arrest. From our analyses it is not clear that lines of the second class represent the most severe phenotype that might be attained by ectopic expression of these genes, since selection of transgenic plants was performed post-germination.

Loss of polar expression results in loss of polar differentiation of lateral organs

Mapping experiments demonstrated that *YAB1* mapped to the same BAC (F4L23) as *FIL*, and sequencing of seven *fil* mutant alleles confirmed that *YAB1* encodes *FIL* (Fig. 1A). Three alleles (*fil-1*, *fil-5* and *fil-6*) are due to splice site acceptor or donor site changes, two alleles (*fil-2* and *fil-3*) are missense mutations altering conserved zinc finger domain cysteines, and two alleles (*fil-4* and *fil-7*) are missense and nonsense mutations, respectively, in the YABBY domain (Fig. 1).

Despite a high level of *FIL* expression in leaves and cotyledons, no mutant phenotype is observed in these organs suggesting redundant activities may mask the loss of *FIL*. This could confound interpretations, based on the *fil* single mutant phenotype, of the function of *FIL* (Sawa et al., 1999; Chen et al., 1999). Based on sequence similarity, *YAB3* is a candidate for a redundant activity. Using a reverse genetic approach (McKinney et al., 1995), we isolated a T-DNA insertional allele of *YAB3*, *yab3-1*. *yab3-1* is the result of a T-DNA insertion approximately 450 bp 5′ to the transcription start site. However, the *yab3-1* allele is not null since *YAB3* mRNA is detected at a low level throughout lateral organ primordia and the SAM, rather than being restricted to the abaxial domains of lateral organs (data not shown). Homozygous *yab3-1* plants do not display a phenotype different from wild-type plants, indicating that in an otherwise wild-type background the delocalization of *YAB3* activity has little phenotypic consequence. *FIL* expression is not altered in embryos, leaves or flowers of *yab3-1* mutants and neither *FIL* nor *YAB3* expression is altered in embryos or leaves of *fil* mutants (data not shown).

To determine whether *FIL* and *YAB3* encode functionally redundant activities, we generated *fil-5 yab3-1* double mutants. In contrast to the single mutant phenotypes, double mutants exhibit a striking vegetative phenotype. Cotyledons and leaves of the double mutant are linear rather than the wild-type ovate and are occasionally bifurcated. In wild type the vasculature has a reticulate pattern whereas in the double mutant it usually consists of central unbranched strands (Fig. 6P,Q). While the vasculature still retained a normal adaxial-abaxial polarity (xylem, adaxial; phloem, abaxial) in the double mutants, the mesophyll appeared uniform with little evidence of polarity (Fig. 6O). The adaxial leaf surfaces are relatively normal, but the abaxial leaf surfaces may be mosaics of abaxial and adaxial tissues (Fig. 6M,N). In some cases, the epidermal leaf surfaces of double mutants are not identical to either wild-type adaxial or wild-type abaxial surfaces, but instead resemble a mixture of characters from both. Occasionally, an ectopic SAM arises from the adaxial surface of a sinus of a bifurcated leaf (Fig. 7H,I).

Flowers and floral organs are affected in *fil-5* mutants (Sawa et al., 1999; Chen et al., 1999). Sepals and carpels are least affected, although their number may be increased. Petals are usually missing, but when present are abnormal. Stamens are variably affected, the most severe being radially symmetric structures. In some regions of the inflorescence flowers are replaced by filamentous organs (Sawa et al., 1999; Chen et al., 1999). In *fil-5 yab3-1* double mutants, nearly all floral organs are radialized to some extent. Sepals are either radial structures with little evident polarity or linear structures that retain an abaxial-adaxial polarity as indicated by distinctive epidermal cell types (Fig. 8B). Petals fail to develop. Stamens are usually absent, and those that develop are severely radialized, with basal filament and apical anther domains (Fig. 8B). Carpels exhibit an enlarged style domain, and the ovary walls often lack distinct differentiation of adaxial epidermis and adaxial subepidermal cell layers. Additionally, a large fraction of positions normally occupied by flowers are replaced with filamentous structures. In at least some cases these filamentous structures are bract-like organs with axillary meristems (Fig. 8C).

DISCUSSION

YABBY gene family members specify abaxial cell fate

Several lines of evidence lead us to conclude that *FIL*, *YAB2* and *YAB3* function to specify abaxial cell fate in lateral organs produced by the SAM. First, expression of each of the genes correlates with abaxial cell fate in lateral organs. Each gene is expressed in abaxial regions of cotyledons, leaves, sepals, petals, stamens and carpels. Furthermore, when abaxial cell fate has been altered, the *FIL* expression altered correspondingly. In plants heterozygous for *phb-1d* mutations, abaxial domains of lateral organs are reduced in size, and in *phb-1d* homozygotes, abaxial domains are almost completely eliminated (McConnell and Barton, 1998). *FIL* expression in *phb-1d* mutants parallels the changes observed in morphology: the spatial domain of *FIL* expression is greatly reduced in *phb-1d* heterozygotes, while in *phb-1d* homozygotes *FIL* expression is essentially absent. Likewise, in *pi ptl* double mutants, where second whorl organs of the flower are oriented 180° from normal such that cell fates usually associated with abaxial positions are now positioned adaxially (Griffith et al.,

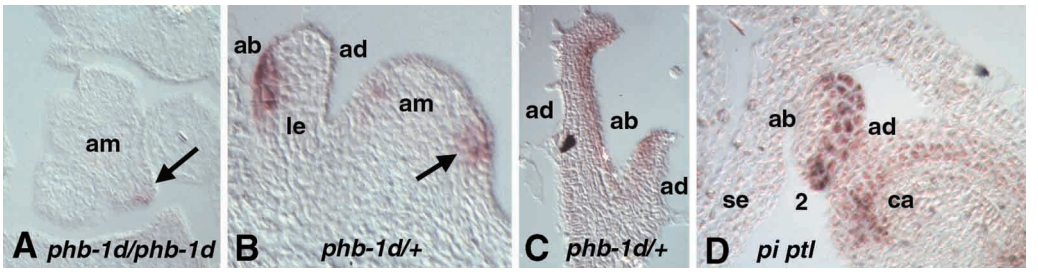

Fig. 5. mRNA expression patterns of *FIL* in *phb-1d* and *pi ptl* mutant backgrounds. (A) *phb-1d* homozygote. (B,C) *phb-1d/+*. (D) *pistillata-1 petalloss-1*. Arrows indicate leaf anlagen; ab, abaxial; ad, adaxial; am, apical meristem; ca, carpel; le, leaf primordia; se, sepal; 2, second whorl sepal.

1999), *FIL* expression again parallels the morphology and is found in adaxial regions of these organs. Thus, in all cases examined, expression of these genes is precisely correlated with abaxial cell fate in lateral organs.

Second, ectopic *FIL* or *YAB3* expression leads to ectopic differentiation of abaxial cell types. Expression of these genes in adaxial regions of developing cotyledons and leaves is sufficient to cause epidermal tissues to differentiate with an abaxial cell fate. Furthermore, ectopic expression of either *FIL* or *YAB3* in flowers promotes ectopic abaxial fates in floral organs (Y. E. and J. L. B., unpublished observations). In other cases where lateral organs have been interpreted as abaxialized or adaxialized, the organs develop as radially symmetrical structures with little evidence of blade outgrowth (Waites and Hudson, 1995; McConnell and Barton, 1998; Lynn et al., 1999). This and the observation that ectopic

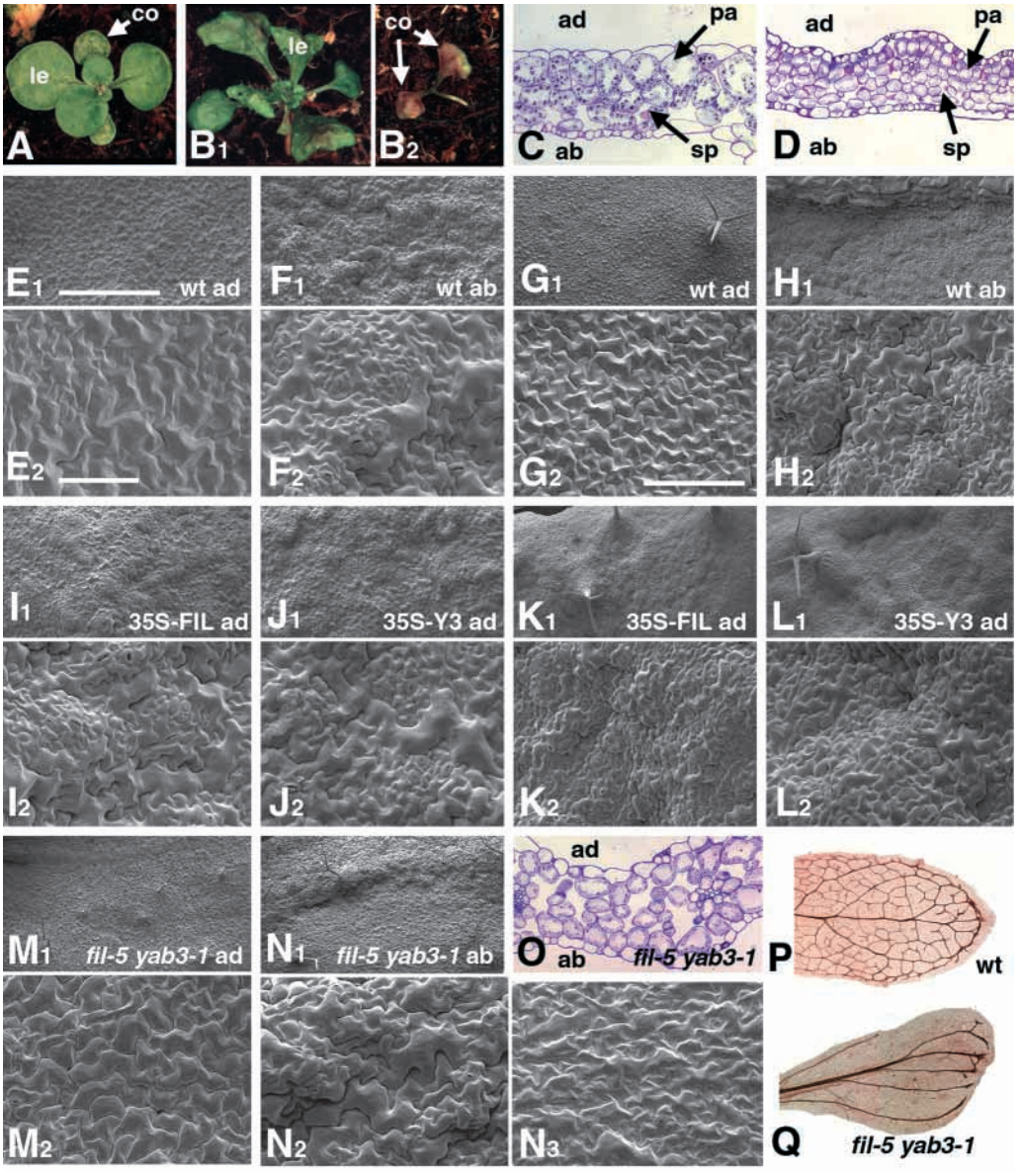

Fig. 6. Vegetative phenotypes of plants resulting from ectopic expression of *FIL* and *YAB3* and of *fil-5 yab3-1* double mutants. In (E-N) two magnifications are given of each surface: the low magnification provides an overview of surface topology whereas the high magnification provides information on cell shape, size and type. (A) Wild-type seedling, 2.5 weeks old. (B) 35S-YAB3 seedlings with mild (B1) and severe (B2) phenotypes; each is 4 weeks old. (C) Transverse section through a wild-type leaf. (D) Transverse section through a 35S-YAB3 leaf. (E,F) Wild-type cotyledons: adaxial (E) and abaxial (F) surfaces. (G,H) Wild-type leaves: adaxial (G) and abaxial (H) surfaces. (I) 35S-FIL cotyledon, adaxial surface. (J) 35S-YAB3 cotyledon, adaxial surface. (K) 35S-FIL leaf, adaxial surface. (L) 35S-YAB3 leaf, adaxial surface. (M) *fil-5 yab3-1* leaf, adaxial surface. (N) *fil-5 yab3-1* leaf, abaxial surface (N1-N3) *fil-5 yab3-1* leaf, abaxial surface: overview showing mixture of cell types (N1), abaxial-like cells (N2), adaxial-like cells (N3). (O) Transverse section of *fil-5 yab3-1* leaf. (P) Vascular pattern of wild-type leaf. (Q) Vascular pattern of *fil-5 yab3-1* leaf. ab, abaxial; ad, adaxial; co, cotyledon; le, leaf; pa, palisade mesophyll; sp, spongy mesophyll; wt, wild-type. Bars, 500 μm (E1-N1); 100 μm (E2,F2,I2,J2); 100 μm (G2,H2,K2,L2,M2,N2,N3).

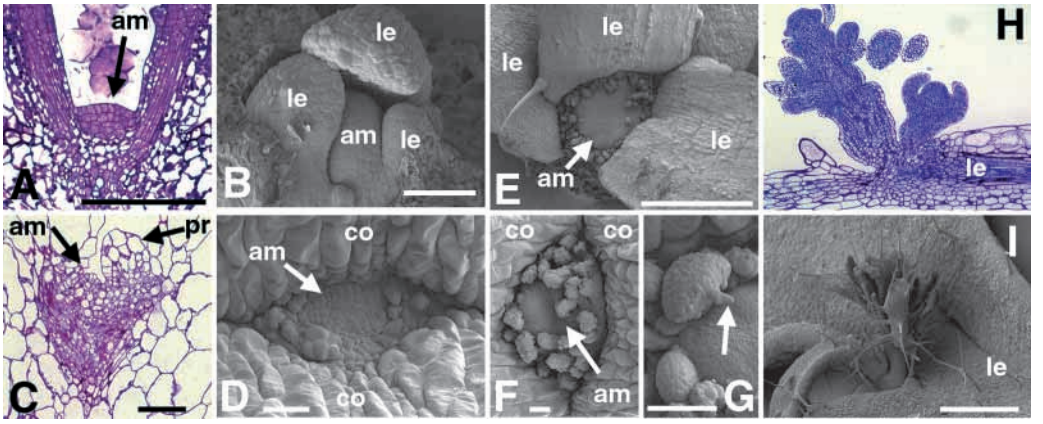

Fig. 7. Meristem phenotypes resulting from ectopic expression of *FIL* and *YAB3* and ectopic meristems of *fil-5 yab3-1* double mutants. (A,B) Longitudinal section and SEM of a wild-type vegetative meristem, respectively. (C,D) Longitudinal section and SEM of a 35S-YAB3 seedling meristem, respectively. (E-G) SEM of 35S-FIL vegetative meristems. The plant in E produced five leaves prior to meristem arrest, whereas those in F and G produced only cotyledons. Arrow indicates a trichome developing from the adaxial surface of a reduced leaf primordium in G. (H,I) Longitudinal section and SEM of an ectopic meristem developing from a sinus of a *fil-5 yab3-1* leaf. am, apical meristem; co, cotyledon; le, leaf; pr, primordium. Bars, 50 μm (B,D,F,G); 100 μm (A,C); 500 μm (E, I).

abaxial/adaxial boundaries of tissue result in outgrowths of tissue, led to the proposal that juxtaposition of abaxial and adaxial cell fates is required for lamina outgrowth (Waites and Hudson, 1995). In contrast, leaves in which *FIL* or *YAB3* is ectopically expressed are usually not radially symmetric, but rather display some laminar outgrowth. However, leaves in these transgenic lines are not completely abaxialized since organs retain some adaxial characters (e.g. numerous trichomes develop from their adaxial epidermis and palisade mesophyll cells are present). It is likely that in those transgenic plants which do produce leaves, the level of ectopic *YABBY* gene expression from the constitutive CaMV 35S promoter is insufficient to overcome the endogenous asymmetric *YABBY* gene expression. Consistent with this hypothesis is the observation that endogenous *FIL* expression is at a high quantitative level, higher than levels usually attained by the CaMV 35S promoter. Additionally, only a fraction of the transgenic plants produce any leaf-like structures, with the more severe phenotype being arrest of the SAM, precluding the analysis of leaf phenotypes in these plants. Our interpretation is that ectopic expression of *FIL* or *YAB3* is sufficient to direct ectopic differentiation of abaxial cell types, but high levels of their gene products result in arrest of the SAM, either directly or indirectly.

Third, loss of polar expression of the *YABBY* genes leads to a loss of polar differentiation of cell types. The interpretations of *fil* and *fil-5 yab3-1* mutant phenotypes are problematic due to redundancy of the gene products and the nature of the *yab3-1* allele. However, in both cases there is a loss in polar expression of one or more *YABBY* gene products and a corresponding loss of polar development of lateral organs. The loss of polarity is manifested in two ways: (1) floral organs and flowers are often filamentous structures with radial symmetry and (2) leaves are often a mixture of ectopic abaxial and adaxial cell types. Taken together, these data suggest that the primary functions of *FIL*, *YAB3*, and probably *YAB2*, are to specify abaxial cell fate in lateral organs produced by apical and flower meristems.

Redundant relationships between the *YABBY* genes

Given the similar expression patterns of these three genes, scenarios involving both redundancy of gene action and combinatorial gene action can be envisaged. FIL and YAB3 are highly homologous, suggesting that they act in a partially redundant manner, and this is supported by the lack of vegetative phenotypes in the single mutants. The phenotypes of *fil-5 yab3-1* double mutants and 35S::*FIL* and 35S::*YAB3* plants indicate that differentiation of cells as abaxial versus adaxial is not mutually exclusive. For example, leaves may be neither abaxial nor adaxial, but rather exhibit a mixture of abaxial and adaxial characteristics at both cellular and tissue levels. One attractive hypothesis is that members of the *YABBY* gene family contribute different functions in the specification of abaxial identity, perhaps acting combinatorially. In this regard, it is of interest to note that while *YAB2* has a similar expression pattern to *FIL* and *YAB3*, its sequence is significantly diverged from that of *FIL* and *YAB3*, suggesting that *YAB2* might act in a pathway not completely redundant with that of *FIL* and *YAB3*. Another member of the gene family, *YAB5*, is also similar in sequence to *FIL*/*YAB2*/*YAB3* (Bowman and Smyth, 1999) and likely shares similar functions. Loss-of-function alleles of *YAB2*,

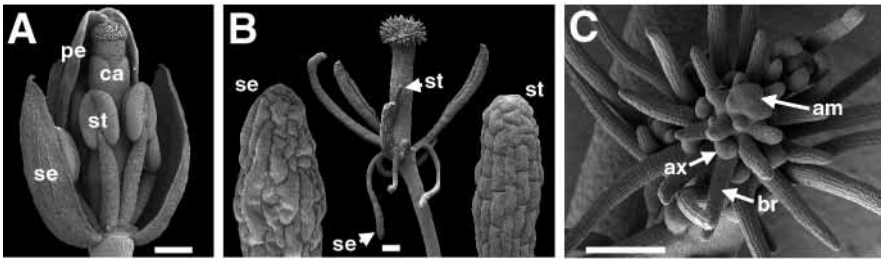

Fig. 8. Flower phenotypes of *fil yab3-1* mutants. (A) Wild-type flower. (B) *fil-5 yab3-1* flower with close-ups of a filamentous sepal (left) and stamen (right). (C) Inflorescence of *fil-5 yab3-1* showing filamentous bracts subtending flower meristems. am, apical meristem; ax, axillary flower meristem; br, bract; ca, carpel; pe, petal; se, sepal, st, stamen. Bar, 200 μm.

YAB3 and *YAB5* are required to investigate these possibilities.

Although in some cases we detect a gradient of *FIL* or *YAB3* expression in developing leaves and cotyledons, the lack of mutant leaf and cotyledon phenotypes in *fil* and *yab3-1* mutants suggest these are likely not critical for specification of cell fate. A more plausible explanation is that cells in the developing primordia assess the relative expression levels of these genes and subsequently differentiate as abaxial or adaxial. Thus, it might not be the absolute level of *YABBY* gene expression, but rather a relative difference in levels between abaxial and adaxial domains that is required to direct appropriate differentiation of cell types. That ectopic expression of *YAB3* in *yab3-1* mutants does not result in phenotypic defects in an otherwise wild-type background but has a dramatic effect in a *fil* mutant background is consistent with this proposal. The high level of ectopic *FIL* or *YAB3* expression required to override endogenous expression of these genes in wild-type plants is also consistent with a relative difference in expression levels being critical.

Establishment of *YABBY* gene expression

The emerging picture from classical and molecular genetic analyses is that as incipient lateral organ primordia develop from the flanks of the SAM, factors both intrinsic and extrinsic to the organ primordia contribute to the specification of cells as adaxial or abaxial (Sussex, 1954; Waites and Hudson, 1995; McConnell and Barton, 1998; Lynn et al., 1999). The SAM itself provides a signal(s) that promotes adaxial cell fate, whereas abaxial cell fate may be the default in the absence of such signals. The establishment of these two domains occurs during the transition from leaf anlagen to leaf primordium because older primordia can develop autonomously into phenotypically normal leaves (Sussex, 1954, 1955; Snow and Snow, 1959; Hanawa, 1961). Subsequently, the juxtaposition of abaxial and adaxial cells results in formation of a laminar structure (Waites and Hudson, 1995).

Based on loss-of-function mutant alleles that exhibit abaxialized lateral organs, three genes (*PHAN*, *ARGONAUTE1* and *PINHEAD*) have been identified that promote adaxial cell fate (Waites and Hudson, 1995; Bohmert et al., 1998; Lynn et al., 1999). Mutations in *PHAN* of *Antirrhinum majus* result in abaxialization of lateral organs (Waites and Hudson, 1995). *PHAN* encodes a myb family transcription factor and is expressed throughout leaf anlagen and developing leaf primordia, suggesting that it must interact with other factors, perhaps including putative factors emanating from the SAM, to promote adaxial cell fate (Waites et al., 1998). *PINHEAD* (*PNH*, also known as *ZWILLE*; McConnell and Barton, 1995; Moussian et al., 1998) and *ARGONAUTE1* (*AGO1*; Bohmert et al., 1998) of *Arabidopsis* encode partially redundant activities that also promote adaxial cell fate. While *ago1* single mutants produce partially abaxialized lateral organs, when *PNH* activity is also compromised, as in *ago1/ago1 phd/+* plants, more severely abaxialized organs are produced (Lynn et al., 1999). Based on the increasingly severe phenotypes as the activities of *PNH* and *AGO1* are reduced, one interpretation of the filamentous organs produced in *ago1 pnh* double mutants is that these organs may be severely abaxialized. While *AGO1* is ubiquitously expressed, *PNH* exhibits an intriguing expression pattern: expression is initially uniform in leaf anlagen, but later adaxially restricted in emerging leaf primordia (Lynn et al., 1999). Thus, the *PNH* expression pattern correlates with the presumed timing of polarity establishment. *AGO1* and *PNH* encode similar proteins with family members in other multi-cellular organisms, and although they share sequence similarity with translation initiation factors, their precise biochemical function is presently unknown (Bohmert et al., 1998; Lynn et al., 1999).

We have shown that members of the *YABBY* gene family promote abaxial cell fate in lateral organs. Are any of the above mentioned genes regulators of *YABBY* gene expression? *FIL* and *YAB3* expression commences in the central region of leaf (and other lateral organs) anlagen. As leaf primordia emerge, expression becomes restricted to abaxial domains. Thus, while *FIL*/*YAB3* and *PNH* are both expressed in a non-polar manner in leaf anlagen, they display complementary expression patterns in emerging leaf primordia. That restriction of *FIL*/*YAB3* and *PNH* to their respective domains within leaf primordia occurs concurrently argues against *PNH* directly regulating *YABBY* expression. The activation of *FIL* and *YAB3* coincides with down-regulation of *SHOOTMERISTEMLESS* (*STM*) (Long et al., 1996), one of the earliest markers for the formation of incipient leaf primordia, and it seems likely that their initial activation is linked to this and other molecular events establishing leaf anlagen. Subsequently, the hypothesized adaxializing signal from the SAM could be responsible for the abaxial restriction of *YABBY* expression. Since the *YABBY* gene family members encode putative transcription factors, they could promote abaxial cell fate by activating downstream effector genes as well as inhibiting genes promoting adaxial cell fate.

In contrast to *ago1* and *pnh*, *phb-1d* mutants exhibit adaxialization of lateral organs (McConnell and Barton, 1998). However, due to the semi-dominant nature of the *phb-1d* allele, it is not clear whether *PHB* promotes abaxial or adaxial cell fates. *PHB* formally regulates *YABBY* expression; however, *PHB* could either act positively (if the *phb-1d* allele is haplo-insufficient) or negatively (if *phb-1d* represents a gain-of-function allele) on *YABBY* expression. Until the molecular identity of *PHB* is known, the directness of its relationship with *YABBY* gene expression will remain ambiguous.

Abaxial cell fate suppresses meristem cell fate

Several lines of evidence suggest that adaxial leaf cell fate influences the competence to form axillary meristems and maintenance of the SAM itself. First, adaxialized lateral organs of *phb-1d* mutants develop axillary meristems around the entire basal circumference of lateral organs (McConnell and Barton, 1998). Additionally, SAMs of *phb-1d* mutants are enlarged relative to those of wild type, suggesting that adaxial domains of leaves can promote SAM formation (McConnell and Barton, 1998). Second, when *phan* mutants are grown at non-permissive temperatures causing lateral organs to develop as severely abaxialized filamentous structures, the SAM arrests, ceasing production of further lateral organs (Waites et al., 1998). Since *PHAN* is expressed in lateral organ primordia, it is required non-cell-autonomously to maintain SAM activity. Third, in *ago1 pnh* double mutants, where lateral organs could be interpreted as being abaxialized, a proper SAM fails to form (Lynn et al., 1999). Furthermore, in *pnh* mutants the SAM is arrested, enlarged and composed of vacuolated cells (Moussian

et al., 1998; McConnell and Barton, 1995). Thus, *AGO1* and *PNH* are partially redundant in promoting adaxial cell fate and competence to form or maintain SAMs (Lynn et al., 1999). These observations lead to the conclusion that either loss of adaxial cell fates or gain of abaxial cell fates in the lateral organs causes arrest of the SAM.

When either *FIL* or *YAB3* is constitutively expressed, the most dramatic phenotype is cessation of SAM development. Most of these arrested meristems produce a number of small primordia that usually suspend their development prior to any signs of differentiation. These primordia are reminiscent of those produced by SAMs in severely affected *pnh* (McConnell and Barton, 1995; Moussian et al., 1998) and *phan* mutants (Waites et al., 1998). One interpretation of the transgenic plants is that severe abaxialization of cotyledons or lateral organs leads to arrest of the SAM. Alternatively, *YABBY* gene expression in cells of the SAM results in loss of proper meristem functioning. We favor the interpretation that abaxialized organs themselves lead to meristem arrest, based on non-cell autonomous effects of ectopic *YABBY* gene expression within flower organs (Y. E. and J. L. B., unpublished observations). Regardless, ubiquitous abaxial cell fate and maintenance of a functional SAM appear to be incompatible. The question arises as to whether adaxial domains of cotyledons themselves promote formation of the SAM during embryogenesis as suggested by enlargement of the SAM in *phb-1d* embryos (McConnell and Barton, 1998) and the failure to form an organized SAM in *ago1 pnh* embryos (Lynn et al., 1999). That plants ectopically expressing *FIL* or *YAB3* fail to produce functional SAMs is consistent with this hypothesis. This adds to a growing body of evidence that communication between meristems and lateral organ primordia they produce involves signals travelling in both directions to ensure their proper maintenance and differentiation, respectively (Sussex, 1954; Fleming et al., 1997; McConnell and Barton, 1998; Waites et al., 1998; Lynn et al., 1999).

The *YABBY* gene family

A general theme is emerging for the function of *YABBY* gene family members: the specification of abaxial cell fate. Six family members are known from *Arabidopsis*, and this is likely to encompass most or all gene family members in this species. The three members (*FIL*, *YAB2* and *YAB3*) described here are expressed in abaxial domains of all above-ground lateral organ primordia (except the ovules) and act to specify abaxial cell fate in lateral organs. A fourth member of the gene family, *CRC*, whose expression is restricted to carpels and nectaries (Bowman and Smyth, 1999), also acts to specify abaxial cell fate in the carpel (Bowman et al., 1999; Y. E. and J. L. B., unpublished). Intriguingly, a fifth member of the gene family, *INO*, functions in ovules to specify abaxial identity in the outer integument (Baker et al., 1997; Jacinto Villanueva and Charles Gasser, personal communication). Since at least five of six family members in *Arabidopsis* are likely to be involved in specifying abaxial cell fate, it is tempting to speculate that the origin and evolution of this gene family was tightly linked to the evolution of abaxial cell fate in lateral organs of plants.

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Note added in proof

While the manuscript was being reviewed, Sawa et al. (**Sawa, S., Watanabe, K., Goto, K., Kanaya, E., Morita, E. H. and Okada, K.** (1999). *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) reported the cloning of *FIL*, its expression pattern and gain of function studies.

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