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YABBY Polarity Genes Mediate the Repression of *KNOX* Homeobox Genes in Arabidopsis

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The *YABBY* (*YAB*) genes specify abaxial cell fate in lateral organs in Arabidopsis. Loss-of-function mutants in two early-expressing *YAB* genes, *FILAMENTOUS FLOWER* (*FIL*) and *YAB3*, do not exhibit vegetative phenotypes as a result of redundancy. Mutations in these genes result in the derepression of the *KNOX* homeobox genes *SHOOTMERISTEMLESS* (*STM*), *BREVIPEDICELLUS*, and *KNAT2* in the leaves and in the partial rescue of *stm* mutants. Here, we show that *fil yab3* double mutants exhibit ectopic meristem formation on the adaxial surfaces of cotyledons and leaf blades. We propose that in addition to abaxial specification, lateral organ development requires *YAB* function to downregulate *KNOTTED* homeobox genes so that meristem initiation and growth are restricted to the apex.

INTRODUCTION

In seed plants, lateral organs are initiated from the peripheral regions of the shoot apical meristem (SAM), a dome of self-perpetuating and self-organizing cells. Genes of the *KNOTTED1* homeodomain, or *KNOX*, family are required for the maintenance and growth of the SAM (Long et al., 1996; Kerstetter et al., 1997; Bowman and Eshed, 2000; Vollbrecht et al., 2000). Initiation of lateral organs from the apex coincides with the downregulation of *KNOX* genes as organ primordia develop. Subsequently, *KNOX* gene expression remains off as lateral organs differentiate, ensuring that these organs display determinate growth, as opposed to the indeterminate growth of the shoot apex.

Recently, it was demonstrated that the *YAB* family of abaxially expressed genes, which encode presumptive transcription factors with high-mobility group and zinc-finger domains, promote abaxial cell fates in the lateral organs of Arabidopsis (Eshed et al., 1999; Sawa et al., 1999; Siegfried et al., 1999; Villanueva et al., 1999; Bowman, 2000). One member of the *YAB* gene family, *FILAMENTOUS FLOWER* (*FIL*) (Chen et al., 1999; Kumaran et al., 1999; Sawa et al., 1999; Siegfried et al., 1999), is required for normal flower development. *FIL* is thought to act redundantly with *YAB2* and *YAB3* because of their overlapping expression pattern and sequence homology (Siegfried et al., 1999), consistent with the absence of aberrant vegetative phenotypes in *fil* loss-of-function mutants. Siegfried et al. (1999) reported the analysis of the *fil-5 yab3-1* double mutant in which an enhanced floral phenotype and a vegetative phenotype were observed. However, the yab3-1 allele, in which a T-DNA insertion is located upstream of the YAB3 promoter, is not a null mutation; rather, it results in a low level of delocalized YAB3 expression. Thus, some functions of FIL and YAB3 may have been obscured.

Here, we report the isolation of a null allele of *yab3* (*yab3-2*) and the generation of double-mutant plants with *fil-8*. We show that *SHOOTMERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*; also known as *KNAT1*), and *KNAT2*, which are members of the *KNOX* gene family, are expressed ectopically in the leaves of *fil-8 yab3-2* double mutants. Furthermore, the loss of *YAB* activity is able to compensate partially for the lack of meristematic activity in *stm-1* mutants. In addition, ectopic vegetative and inflorescence meristems are produced on the margins of cotyledons, rosettes, and cauline leaves in *fil-8 yab3-2* double mutants. These results suggest that *FIL* and *YAB3* act, directly or indirectly, to downregulate meristematic genes during lateral organ development in wild-type plants in addition to promoting abaxial cell fate in the lateral organs.

RESULTS

Identification of *yab3* Mutant Alleles

We reported previously the cloning of the *Abnormal Floral Organs* (*AFO*) gene, which is required for normal flower

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development in Arabidopsis (Kumaran et al., 1999). Because *AFO* is identical to *FIL* and *YAB1*, we renamed the *afo-1* mutant allele *fil-8*. *fil-8* results in partially radialized floral organs but has no effect on vegetative development, as is the case with other *fil* alleles. To address the possibility of overlapping functions of *FIL* with other *YAB* genes during vegetative development, we screened for more knockouts in this family from the *Ds* transposant insertion lines that we generated (Sundaresan et al., 1995; Parinov et al., 1999). Based on the flanking sequence analysis, we isolated two alleles of *YAB3* called *yab3-2* and *yab3-3*, which correspond to *Ds* insertions in the coding region (Figure 1). Both mutants are indistinguishable from wild-type plants under normal growth conditions. For this study, we used the *yab3-2* allele, which carries a gene-trap *Ds* insertion in the first exon, resulting in undetectable levels of *YAB3* transcript by reverse transcriptase–mediated (RT) PCR (data not shown) and in β-glucuronidase (*GUS*) reporter gene expression. In seedlings heterozygous for *yab3-2*, GUS expression was observed in the young leaves, and as the leaf matured, expression was restricted to the abaxial tissues of leaves (Figure 2A). Apart from the abaxial tissue expression pattern, we observed GUS expression on either side of the leaf margin in the younger tissues of leaf blades (Figure 2B). *YAB3*::*GUS* also had GUS expression in floral organs (data not shown). These data are consistent with the results of previous in situ hybridization studies (Siegfried et al., 1999).

Generation and Phenotypic Analysis of Double-Mutant Plants for *fil yab3*

The single-mutant alleles of *fil-8* and *yab3-2* do not have any visible aberrant vegetative phenotype under normal growth conditions. They produce leaves that are similar to wild-type leaves in shape, size, and epidermal cell types on both adaxial and abaxial surfaces (data not shown). By contrast, *fil-8 yab3-2* double-mutant plants show an abnormal vegetative phenotype, including thin, elongated cotyledons and partially radialized leaves, suggesting a partial loss of leaf polarity. In addition, leaves are sometimes bifurcated near the base (Figure 2C). The floral organs show stronger defects than in *fil* mutants and exhibit a much greater degree of radialization. These phenotypes are very similar to those of the *fil-5 yab3-1* double mutants (Siegfried et al., 1999). We also examined the adaxial and abaxial cell types of *fil-8 yab3-2* mutant leaves by scanning electron microscopy and found that the cells on the abaxial and adaxial leaf surfaces were clearly distinguishable, indicating that polarity had not been eliminated (data not shown).

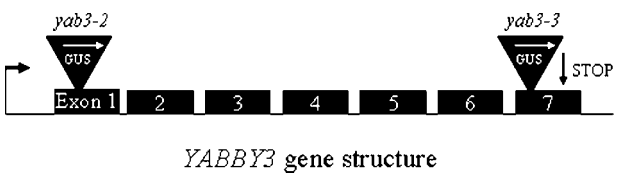

Figure 1. Different Alleles of *YAB3* Generated by *Ds* Transposable Elements.

The *Ds* gene-trap insertions in the *yab3-2* and *yab3-3* alleles are at nucleotide positions 191 and 755, respectively, with reference to the *YAB3* mRNA sequence (ATG at 109).

The absence of abaxial trichomes on the first rosette leaves is a useful marker for the correct specification of abaxial cell types and the maintenance of leaf polarity. For example, seedlings homozygous for the *kanadi1* mutation, which affects abaxial specification, have trichomes on the abaxial leaf surfaces of the first two rosette leaves (Kerstetter et al., 2001). We examined the number of trichomes on the abaxial leaf surfaces of wild-type, *fil-8*, *yab3-2*, and *fil-8 yab3-2* seedlings. The first four rosette leaves were selected for analysis from 15 10-day-old seedlings from each mutant class. Neither the wild-type control nor any of the *fil-8*, *yab3-2*, and *fil-8 yab3-2* leaves produced trichomes on their abaxial surfaces. Subsequently, all seedlings produced three to five abaxial trichomes on the fifth leaf, with none of the *fil-8 yab3-2* seedlings producing more than three abaxial trichomes at this stage, perhaps as a result of their smaller leaves. These results indicate that abaxial polarity is not abolished in the early rosette leaves of the mutants.

Formation of Ectopic Meristems on the Lateral Organs of Double-Mutant Plants

A striking phenotype observed in *fil-8 yab3-2* double-mutant plants was the production of ectopic vegetative and inflorescence meristems on the adaxial surfaces of the cotyledons and leaf blades (Figure 3). Ectopic meristem emergence on cotyledons and leaves occurred in 5 to 7% of double-mutant plants. Ectopic inflorescence meristems arose mainly from the rosette leaves (Figures 3A to 3E), with a few exceptions. This finding could be attributable to the fact that these double-mutant plants produced very few cauline leaves. The ectopic inflorescence meristems had mutant cauline leaves and flowers with the same phenotype as the primary inflorescence meristems of *fil-8 yab3-2* plants (Figure 3A and data not shown).

Even though the emergence of ectopic meristems can occur anywhere on the leaf blade, they developed most frequently toward the base and near the leaf margins. It is interesting that *YAB3* expression also was more intense near the leaf margins (Figure 2B). Similar to the previous results with the *fil-5 yab3-1* mutants (Siegfried et al., 1999), these double-mutant plants also produced bifurcated leaves and cotyledons, with the bifurcation often induced near the margins of the lateral organs. As noted by Siegfried et al. (1999), in some cases bifurcated leaves and cotyledons gave rise to ectopic meristems at the point of bifurcation (Figures 3B and 3F). Here, the *YAB3*::*GUS* fusion in the *yab3-2* allele

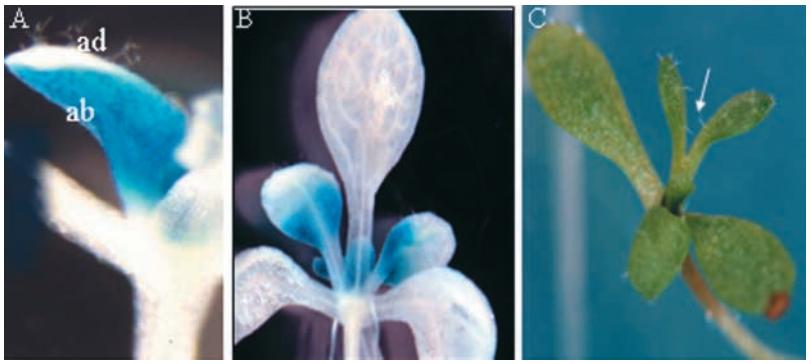

Figure 2. *YAB3*::*GUS* Expression in Heterozygotes, and Phenotype of *fil-8 yab3-2* Plants.

(A) *YAB3*::*GUS* in a leaf showing abaxial expression. ab, abaxial surface; ad, adaxial surface.
(B) Strong *YAB3*::*GUS* expression at the leaf margins.
(C) Bifurcated leaf of a *fil-8 yab3-2* seedling (arrow).

also served as a marker to identify the emerging lateral organs arising from ectopic meristems on cotyledons and leaves (Figures 3F and 3G). The ectopic meristems that we observed in *fil yab3* plants arose only from the adaxial surface of the mutant leaves, consistent with the retention of some polarity in the leaves of *fil yab3* plants. As seen in Figure 2, although the leaves appeared partially radialized, they retained leaf lamina, with distinguishable cell types on the leaf surfaces and trichomes only on the adaxial surface of the early rosette leaves.

The vasculature of the ectopic shoots arose from a single vein of the leaf, and the shoots appeared to be associated with the vasculature of the leaf of origin (Figure 3H). The leaves originating from the ectopic shoots had well-differentiated procambial strands that were linked with the vein of the leaf from which the ectopic meristem arose (Figure 3I). Therefore, it seems that a normal pattern of cell differentiation occurs within the tissues and organs derived from the ectopic meristems, despite their abnormal positions.

Derepression of *KNOX* Genes in *fil-8 yab3-2* Plants

fil-8 yab3-2 plants appear to phenocopy plants overexpressing the *KNOX* homeobox genes in that ectopic meristems arise from the leaves. In transgenic lines that overexpress *BP* (known previously as *KNAT1*) using the 35S promoter, ectopic meristems emerge from the sinuses of severely lobed leaves (Chuck et al., 1996). To test for the misexpression of genes that are expressed normally only in meristems, we performed RT-PCR with primers specific for *STM*, *BP*, *KNAT2*, *WUSCHEL* (*WUS*), and *CUP-SHAPED COTYLEDONS2* (*CUC2*) on mRNA isolated from lateral organs of *fil-8*, *yab3-2*, and *fil-8 yab3-2* plants (Figure 4). Importantly, the leaves selected for these experiments had no detectable ectopic meristems. We observed the expression of *STM* in the leaves of double mutants but not in wild-type plants. Interestingly, *STM* leaf expression was detectable even in *fil-8* and *yab3-2* single-mutant plants that did not show any vegetative abnormality. Similarly, *BP* was expressed in leaves of the *fil-8* and *yab3-2* single mutants as well as in *fil-8 yab3-2* double mutants, but not in wild-type plants. We detected low-level expression of *KNAT2* in the leaves of wild-type plants, and an enhanced level of expression was detected in the leaves of both the *fil-8* and *yab3-2* single mutants as well as the *fil-8 yab3-2* double mutants. We did not detect the expression of *WUS* and *CUC2* in any of these genotypes, confirming that the leaves selected for analysis did not contain ectopic meristems.

We also confirmed the derepression of *KNOX* gene expression by crossing to plants carrying a *BP*::*GUS* fusion. In a wild-type background, *BP*::*GUS* expression is detected in the meristematic zone and in lateral root primordia (Ori et al., 2000). As lateral organs emerged from the apical meristem, the expression of *BP*::*GUS* was downregulated (Figure 5A). Because the *yab3-2* allele itself had reporter GUS activity, the *fil-8 yab3-2* mutants could not be used in combination with *BP*::*GUS* to study the expression of the *BP* gene. Instead, we looked for *BP*::*GUS* expression in the emerging lateral organs of *fil-8* mutant plants, because the RT-PCR results showed that they also misexpressed *KNOX* genes in the lateral organs. In most of the *fil-8* seedlings, *BP*::*GUS* was detectable in emerging leaves and was especially prominent in the leaf blade margins, corresponding to the positions at which ectopic meristems most often arose in the *fil-8 yab3-2* plants (Figures 5B and 5C), even though the *fil-8* mutants had normal vegetative development. In some *fil-8* seedlings, *BP*::*GUS* expression appeared to be restricted to the abaxial domains of the emerging leaves (Figure 5D).

To further characterize the ectopic *KNOX* gene expression,

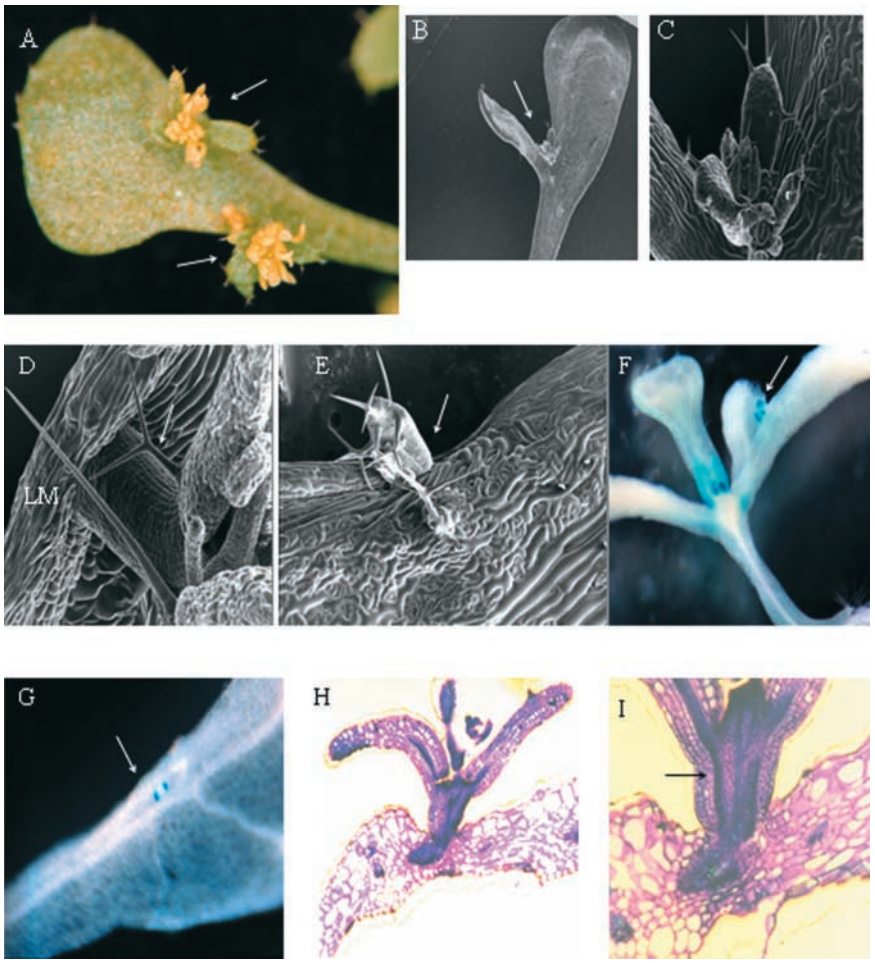

Figure 3. Induction of Ectopic Meristems on the Lateral Organs of *fil-8 yab3-2* Plants.

Arrows indicate the positions of ectopic meristems.
(A) Ectopic inflorescence meristems on both sides of the leaf margin.
(B) Ectopic meristem at the bifurcation point on a leaf.
(C) Higher magnification of the meristem shown in **(B)**.
(D) Ectopic inflorescence meristem on a rosette leaf margin (LM).
(E) Emergence of an ectopic meristem on the adaxial surface of a rosette leaf.
(F) and **(G)** A *YAB3*::*GUS* marker was used to identify the emergence of ectopic meristems from cotyledon **(F)** and leaf **(G)**.
(H) Section of a leaf that has ectopic meristems reveals that they emerge from the vasculature tissues.
(I) Differentiation of the procambium strand from the vein of a leaf producing ectopic meristems.

we examined *STM* expression in *fil-8 yab3-2* mutants during leaf initiation, because *ASYMMETRIC LEAVES1* (*AS1*) expression at this stage can repress *BP* but not *STM* (Byrne et al., 2000; Ori et al., 2000). In wild-type plants, *STM* expression was excluded from leaf anlagen, being restricted to the apical meristem and developing vasculature (Figure 5E) (Long et al., 1996). Likewise, in *fil-8 yab3-2* plants, *STM* expression was excluded from leaf anlagen and was restricted to the apical meristem (Figure 5F). Thus, either *STM* is downregulated properly at this stage, or if it is expressed ectopically, it is below the level of detection in our experiments.

Because *AS1* shows an altered expression pattern in *stm* mutants (Byrne et al., 2000), we also tested for alterations in the *YAB* expression pattern in *stm* mutant plants using the *YAB3*::*GUS* insertion. However, we detected no differences in the *YAB* gene expression pattern in the cotyledons of *stm* mutants and wild-type plants (data not shown). We also tested, by RT-PCR, the levels of *AS1* transcripts in *fil yab3*

leaves, and these appeared unchanged compared with those in wild-type leaves (data not shown).

Partial Suppression of the *stm-1* Mutant Phenotype in *yab* Mutant Plants

In plants homozygous for *stm-1*, a strong but nonnull allele, a functional SAM, is not produced (Barton and Poethig, 1993). Thus, *stm-1* homozygotes lack structures at the site typically occupied by the SAM (Figure 6A). Because *fil-8 yab3-2* plants induce ectopic meristems that are correlated with the misexpression of class-1 *KNOX* genes, we tested for genetic interactions by crossing *stm-1* to the *fil-8* and *fil-8 yab3-2* mutant backgrounds. Analysis of a segregating population from F2 progeny of *stm-1*/+ crossed with *fil-8 yab3-2*/*fil-8 yab3-2* showed that the loss of SAM activity in the *stm-1* mutant was suppressed partially by mutations in *YAB* genes. For example, leaves were produced from the shoot apex in *fil-8 stm-1* plants (Figure 6B, Table 1). However, the morphology of leaves could be abnormal, because lobed and butterfly-shaped leaves were found frequently (Figures 6C and 6D). Vegetative shoots of *fil-8 stm-1* plants were distinguishable from those of *fil-8* plants in that the former exhibited reduced apical dominance, with the formation of multiple shoots with short internodes that initiated leaves, resulting in an abnormal arrangement of leaves. By contrast, *stm-1* plants produced few leaves and no shoots. The *fil-8 stm-1* plants failed to form flowers. In place of inflorescence meristems, leaf-like organs developed, indicating that a reduction in *FIL* activity alone could not rescue the *stm-1* phenotype during flower formation, suggesting that the meristems that are initiated cannot be maintained (Figure 6E).

In *fil-8 yab3-2 stm-1* triple-mutant plants, the *stm-1* phenotype was suppressed to an even greater extent. A single leaf structure was produced initially at the site that normally is occupied by the SAM during germination, and leaf initiation continued such that a large number of leaves were formed before the plants flowered (Figure 6F, Table 1). However, the leaves of *fil-8 yab3-2 stm-1* plants differed from those of *fil-8 stm-1* plants in that they displayed the *fil-8 yab3-2* phenotype, and the leaves produced later often were radialized (Figure 6G). The triple-mutant plants also exhibited reduced apical dominance and excess leaf proliferation, as was observed in *fil-8 stm-1* plants. Eventually, flower-like organs developed, indicating that the loss of *FIL* and *YAB3* was able to partially rescue the *stm* phenotype during flower formation (Figure 6H). The differences in the degree of rescue of the reproductive phenotype may reflect differences in the levels of ectopic *KNOX* gene expression.

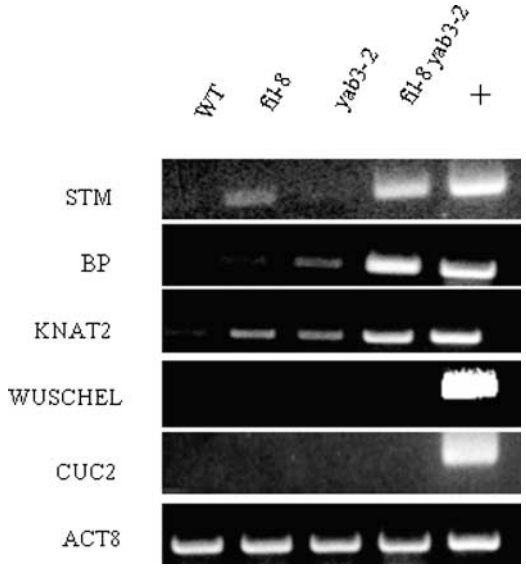

Figure 4. RT-PCR Analysis of Meristematic Genes in Mutant Leaves.

RT-PCR analysis using gene-specific primers for *STM*, *BP*, *KNAT2*, *WUS*, and *CUC2* on RNA from wild-type (WT), *fil-8*, *yab3-2*, and *fil-8 yab3-2* leaves and control wild-type whole seedlings (+). *ACTIN8* (ACT8) primers were used as an internal control.

DISCUSSION

Derepression of *KNOX* Genes Is Not a Direct Consequence of the Loss of Dorsoventral Polarity

Previous studies have demonstrated a correlation between adaxialization and the promotion of meristematic growth. For example, the *phabulosa* (*phab*) mutation, which results in completely adaxialized leaves, leads to new meristems arising from any position on the leaf axil (McConnell and Barton, 1998; McConnell et al., 2001). Conversely, the *phantastica* (*phan*) mutant of *Antirrhinum*, which results in the complete abaxialization of leaves, also can result in meristem arrest (Waites and Hudson, 1995; Waites et al., 1998). Similarly, the ectopic expression of *FIL* or *YAB3* produces seedlings with abaxialized leaves and arrested meristems (Sawa et al., 1999; Siegfried et al., 1999). Therefore, the ectopic meristems observed in *fil yab3* mutants could be interpreted as a consequence of the adaxialization of the lateral organs caused by the loss of genes conferring abaxial cell identity, resulting in the promotion of meristem formation via *KNOX* gene derepression. However, we do not favor this interpretation, because mutations such as *phab* and *kanadi*, which result in more extreme adaxialization than was observed in the *fil yab3* double mutants, do not produce ectopic meristems from leaf lamina (McConnell and Barton, 1998; Eshed et al., 2001; Kerstetter et al., 2001). By contrast, the degree of adaxialization observed in *fil yab3* double mutants was only partial, as seen in Figure 2. The partial retention of polarity can be explained by the activity of other genes that

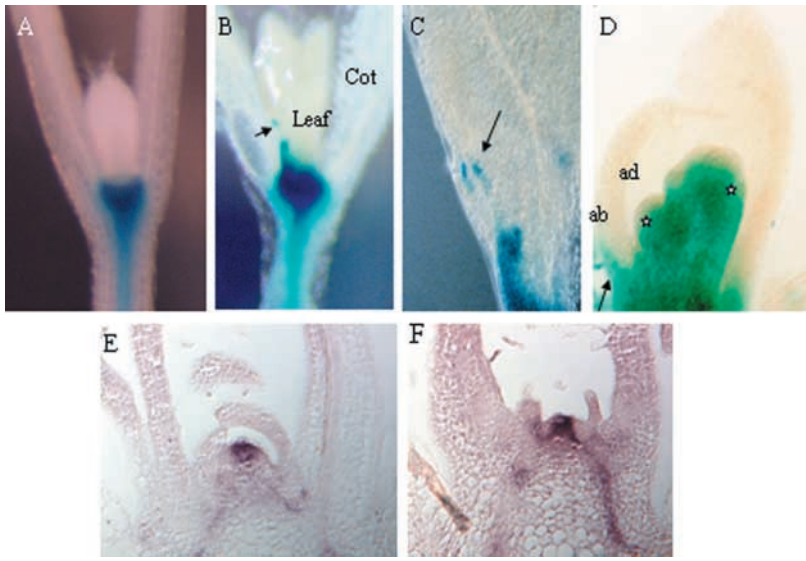

Figure 5. Misexpression of *KNOX* Genes in *yab* Mutants.

(A) A wild-type seedling showing *BP*::*GUS* expression restricted to the meristematic regions and downregulated during lateral organ emergence.
(B) *BP*::*GUS* expression persists in a *fil-8* mutant in the leaf blades near the margins. The arrow indicates GUS expression in the leaf margin.
(C) Enlarged view of the leaf shown in **(B)**.
(D) *BP*::*GUS* expression is observed in the abaxial tissues of leaves in a *fil* seedling. The arrow indicates GUS expression in the abaxial tissue layer of a *fil-8* seedling leaf. The asterisks indicate younger leaves emerging from the SAM. ab, abaxial surface; ad, adaxial surface.
(E) *STM* expression in wild-type plants is limited to the apical meristem and is excluded from leaf anlagen and primordia.
(F) *STM* expression in *fil-8 yab3-2* plants is not detected from leaf anlagen, as in the wild type.

specify abaxial cell identity, such as *YAB2*, *YAB5*, and members of the *KANADI* gene family, which are still active in these plants. The restriction of the ectopic meristems to the adaxial surface may reflect a requirement for adaxial-specific factors that promote meristematic growth, possibly those encoded by class III homeodomain-zipper genes (e.g., *PHB*) that are expressed adaxially (McConnell et al., 2001).

Therefore, we propose that *FIL/YAB* genes may act as regulators of *KNOX* genes independently of their role in abaxial tissue fate. Consistent with this interpretation is our observation that *KNOX* gene derepression was present even in *fil* single mutants (Figures 3 and 4), in which no abnormal vegetative phenotypes were observed. With this interpretation, the ectopic meristems seen in the *fil yab3* double mutants are more likely to result from further derepression of *KNOX* genes than from adaxialization of the lateral organs. Conversely, it is possible that the downregulation of *KNOX* genes in the shoot meristem could be partially responsible for the arrest of meristem growth observed in plants that overexpress *FIL* or *YAB3* (Sawa et al., 1999; Siegfried et al., 1999).

Both *as1* and *fil yab3* can partially suppress the apical defect in *stm* mutants. In the case of *as1* (Byrne et al., 2000), this effect is mediated through the ectopic expression of *BP*, because apical meristems fail to form in *as1 stm bp* triple mutants (Byrne et al., 2002). Because *BP* also is expressed ectopically in *fil yab3* leaves, the partial rescue of meristem defects in the *fil yab3 stm* background is likely to be mediated by *BP*. Because the KNOTTED homeodomain proteins have been shown to move through plasmodesmata (Lucas et al., 1995), a possible mechanism for the suppression of the *stm* mutation is the misexpression of *BP* in leaf primordia, which results in the transport of BP proteins into the shoot meristem to rescue the *stm* phenotype.

YAB Repression of *KNOX* Genes May Act by a Mechanism Separate from That of the *PHAN* Myb Genes

Three orthologous Myb genes from different plant species, called *AS1*, *PHAN*, and *ROUGH SHEATH2* (*RS2*) in Arabidopsis, *Antirrhinum*, and maize, respectively, have been shown to downregulate *KNOX* genes in lateral organs (Schneeberger et al., 1998; Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000; Ori et al., 2000). Even though mutations in these genes result in the misexpression of *KNOX* genes in the lateral organs, the phenotypes that they exhibit are very different for each species.

The *phan* mutation gives rise to radialized leaves in *Antirrhinum*, *rs2* mutants are defective in proximal-distal polarity in maize, and *as1* mutants produce lobed leaves in Arabidopsis.

In Arabidopsis, *AS1* represses *BP* and *KNAT2*, but not *STM*, during leaf development (Byrne et al., 2000; Ori et al., 2000). The loss of *as1* function can partially phenocopy the *BP* overexpression phenotype, but only in the background of either *serrate* (*se*) or *pickle* (*pkl*) mutants (Ori et al., 2000). *PKL* (also called *GYMNOS*) and *SE* appear to encode chromatin-remodeling factors (Eshed et al., 1999; Ogas et al., 1999; Prigge and Wagner, 2001). Similar results have been obtained with the *as2* mutation (Ori et al., 2000; Semiarti et al., 2001; Iwakawa et al., 2002). However, in *fil yab3* plants, enhanced levels of *KNOX* genes are sufficient to induce meristem activity in the leaves despite the presence of wild-type *PKL* and *SE* gene products, perhaps because *STM* also is derepressed in this background.

In contrast to maize, Arabidopsis, and *Antirrhinum*, in tomato, which forms compound leaves, *PHAN* alone is insufficient to repress *KNOX* gene expression as a result of overlapping domains of expression (Koltai and Bird, 2000). Because the observed *KNOX* gene expression is restricted to the adaxial region, abaxial-specific factors may be more important for the downregulation of *KNOX* genes in tomato. It is interesting that *BP*::*GUS* was misexpressed in the abaxial tissues of *fil-8* plants, indicating the role played by *YAB* genes in the repression of *KNOX* genes in the abaxial tissues of the lateral organs. The complementary nature of *KNOX* and *AS1* gene expression may not be maintained in Arabidopsis in all contexts. For example, levels of *AS1* transcript

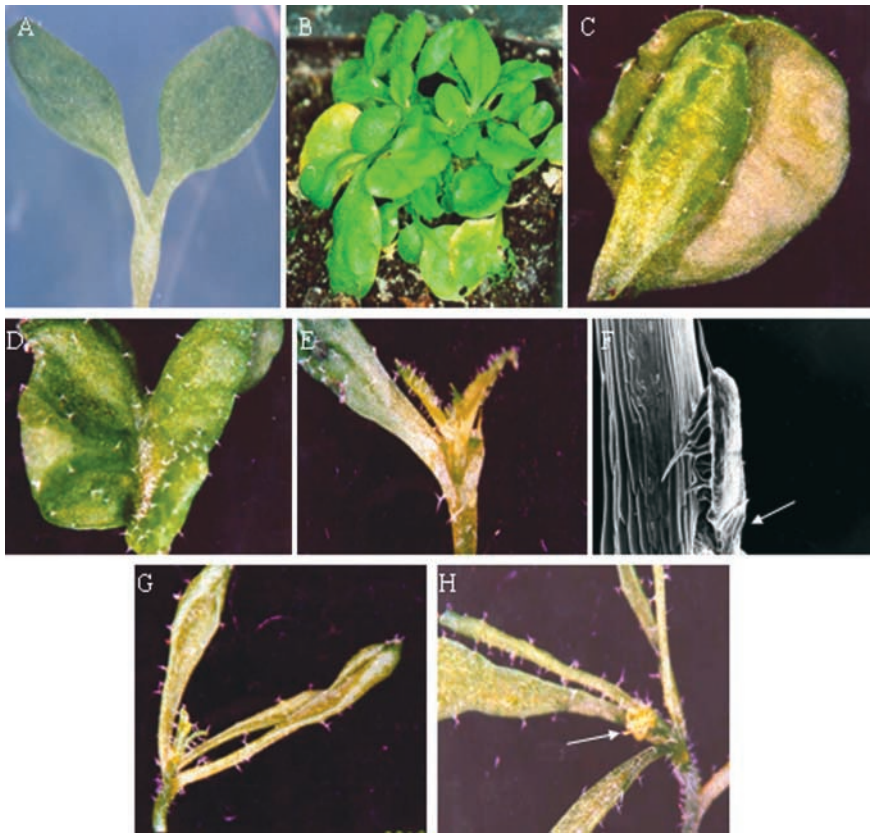

Figure 6. Suppression of the *stm-1* Phenotype in *fil* and *fil yab3* Mutant Backgrounds.

(A) The *stm-1* mutant fails to initiate leaves.
(B) *stm-1 fil-8* double-mutant plants showing leaf proliferation but failure to produce flowers.
(C) and **(D)** Lobed **(C)** and butterfly-shaped **(D)** leaves of *stm-1 fil-8* double-mutant plants.
(E) Proliferation of leaves from the apex of *stm-1 fil-8* double-mutant plants.
(F) Scanning electron microscopy analysis showing the emergence of a leaf (arrow) from the meristem in a *fil-8 yab3-2 stm-1* triple-mutant seedling.
(G) Formation of radialized leaves in *fil yab3 stm* triple mutants.
(H) The arrow indicates the filamentous-like organs from the inflorescence apex of *fil yab3 stm* triple mutants.

Table 1. Number of Leaves in *stm-1* Plants in the *yabby* Mutant Background

Genotype	Number of Plants Analyzed	Number of Plants Producing Leaves	Number of Leaves after 5 Weeks
stm-1/stm-1	12	3	4 to 6
fil-8 stm-1/fil-8 stm-1	10	10	18 to 24
fil-8 yab3-2 stm-1/fil-8 yab3-2 stm-1	4	4	14 to 17

appeared unchanged in *fil yab3* leaves despite the ectopic expression of *STM* in these tissues. This finding suggests that *AS1* is not likely to function downstream of *YAB* genes and that the repression of *AS1* by *STM* may require other factors that are restricted to the apical meristems.

Although *KNOX* gene overexpression phenotypes are observed in both *as1 pkl* and *fil yab3* plants, their leaf phenotypes differ in many respects. Some differences could be attributed to the differences in the levels of expression of specific *KNOX* genes (e.g., *STM*), although most are more likely attributable to the other roles that these genes play in leaf development. For instance, unlike *as1 pkl* plants, leaf blades do not display serration in *fil yab3* plants, and the positions and frequencies of ectopic meristems vary between the two genotypes. In addition, the formation of ectopic stipules is prominent in *as1 pkl* and *BP*-overexpressing plants, but it is not observed in *fil yab3* plants, perhaps because *fil yab3* mutants do not produce stipules, even on leaves initiated at the SAM (Siegfried et al., 1999). Finally, the phenotype observed in the *as2-14 fil-8 yab3-2* triple mutant appears to be additive, which is consistent with the independent operation of the *AS* and *YAB* pathways (Y. Eshed and J.L. Bowman, unpublished results).

FIL/*YAB3* Have Two Distinct Functions in Promoting Lateral Organ Development

The region of the leaf at which ectopic meristems arise in *fil yab3* plants appears to correlate with the *YAB3*::*GUS* expression pattern. Most of these meristems originate near leaf margins, where *YAB3*::*GUS* is expressed in heterozygous plants. In general, leaf margins remain densely cytoplasmic for a longer time than the cells in the center of the leaf and are the last to differentiate. In addition, putative marginal meristems have been proposed to be important for lamina outgrowth in many species (Hagemann and Gleissberg, 1996; Donnelly et al., 1999). In the absence of *FIL* and *YAB3* function, *KNOX* gene activity may be derepressed in the marginal regions, leading to the formation of ectopic shoot meristems. Waites and Hudson (1995) have proposed that the juxtaposition of abaxial and adaxial cell fate is required for the laminar outgrowth of the leaves, consistent with the phenotypes of the *phan* and *phab* mutants. In the context of this model, we may link the proposed two functions of *FIL* and *YAB3* (i.e., the promotion of abaxial cell fate and the repression of *KNOX* genes) as reflecting dual functions required for proper leaf outgrowth. Thus, the outgrowth of the Arabidopsis leaf blade, and by extension that of other lateral organs, requires not only the proper specification of abaxial and adaxial cell identities through the action of the *PHAB*, *YAB*, and *KANADI* genes but also the action of the *YAB* genes to repress *KNOX* genes to allow the maintenance of dividing cells at the margins that will not revert to stem cells.

METHODS

Generation of the *yab3* Mutant Allele and Genetic Analysis

The *afo-1/fil-8* mutant (Kumaran et al., 1999) and the *yab3* mutants used in the study are in the *Arabidopsis thaliana* ecotype Landsberg *erecta*. All plants were grown on soil at 22°C under long-day conditions. The mutations in the *yab-3* alleles (Figure 1) were identified by homology search of the *YAB3* gene sequence with the flanking sequences of *Ds* in the transposants collection that we published previously (Parinov et al., 1999). *fil-8 yab3-2* double-mutant plants were generated by crossing *fil-8* as a female plant with pollen from *yab3-2* plants.

The *stm-1* mutation used in this study also is in the Landsberg *erecta* ecotype (Barton and Poethig, 1993). The *fil-8 stm-1* double-mutant plants were identified in F2 progeny segregating for both mutants by phenotypic and PCR analysis. Among 200 plants tested, 8 were confirmed by PCR as homozygous for *fil-8* and *stm-1*. Crossing *fil-8 yab3-2* flowers with pollen from *stm-1* heterozygous plants was used to generate triple mutants of *fil-8 yab3-2 stm-1*. Of 320 F2 plants that were examined, 21, 31, 14, 13, and 4 plants showed *stm-1*, *fil-8*, *fil-8 yab-2*, *fil-8 stm-1*, and *fil-8 yab3-2 stm-1* phenotypes, respectively; the genotypes of the last two classes were confirmed by PCR.

Reverse Transcriptase–Mediated PCR Analysis

Total RNA from cotyledons and leaves of the wild type, single *fil-8* and *yab3-2* mutants, and the *fil-8 yab3-2* double mutant was isolated using the Oligotex mRNA midi kit (Qiagen, Valencia, CA). Reverse transcriptase–mediated PCR was performed on an RNA template using a one-step reverse transcriptase–mediated PCR kit supplied by Qiagen. The primers used for amplification were as follows: *STM*, 5′-TGTCAGAAGGTTGGAGCACCA-3′ and 5′-TTTGTTGCTCCGAAGGGTAA-3′; *BP*, 5′-TCATGGAAGCATACTGTGACA-3′ and 5′-TGACTCAGAAGGATATGGCCA-3′; *KNAT2*, 5′-GATTGCCAAAAGGTGGGAGC-3′ and 5′-TGTCGCCTTCAGTAGGGTA-3′; *ACTIN8*, 5′-ATGAAGATTAAGGTCGTGGCA-3′ and 5′-CCGAGTTTGAAGAGGCTAC-3′;

CUC2, 5′-CAGCCAATATCTTCCACCGGG-3′ and 5′-GGAGAGGTGGGAGTGAGACGGA-3′; and *WUS*, 5′-CAGTCTTGTTCCATAGATCC-3′ and 5′-ATCATCATCATCAAGCCGA-3′.

Microscopy

Scanning Electron Microscopy

Fresh material was mounted on silver tape and viewed using a JOEL JSM-5310 LV microscope, and images were captured using JOEL SEMafore software (JOEL, Sundbyberg, Sweden). Further processing was performed using Adobe Photoshop 3.0 (Adobe Systems, Mountain View, CA).

Light Microscopy

Leaves having ectopic meristems were fixed in formaldehyde (5%), acetic acid (5%), and alcohol (EtOH 90%) under vacuum for 20 min, dehydrated in an ethanol series to 95%, and embedded in resin medium. Sections were cut at 5 μm with glass knives on a rotary microtome, mounted on slides, and stained with periodic acid–Schiff reagent and 0.1% toluidine blue.

In situ hybridizations were performed as described by Eshed et al. (1999) with the *STM* probe as described by Long et al. (1996).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Number

The accession number for the *YAB3* mRNA sequence is AF136540.

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