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Control of Arabidopsis Leaf Morphogenesis Through Regulation of the *YABBY* and *KNOX* Families of Transcription Factors

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ABSTRACT

The patterning of initiating organs along specific axes of polarity is critical for the proper development of all higher organisms. Plant lateral organs, such as leaves, are derived from the shoot apical meristems located at the growing tips. After initiation, the leaf primordia of species such as *Arabidopsis thaliana* differentiate into a polarized structure consisting of a proximal petiole and a distal blade, but the molecular mechanisms that control proximal–distal pattern formation are poorly understood. The transcriptional activators BLADE-ON-PETIOLE1 (BOP1) and BOP2 are known to control Arabidopsis lateral organ differentiation by regulating gene expression along the adaxial–abaxial (dorsal–ventral) and proximal–distal polarity axes. Here, we demonstrate that the development of ectopic blade tissue along *bop1 bop2* leaf petioles is strongly suppressed in a dosage-dependant manner by mutations in either of two closely related *YABBY* (*YAB*) genes, *FILAMENTOUS FLOWER (FIL)* and *YAB3*. Three *KNOTTED-LIKE HOMEOBOX* (*KNOX1*) genes also make lesser, and partially redundant, contributions to ectopic blade development in *bop1 bop2* leaves. Mutation of these *YAB* and *KNOX1* genes together causes nearly complete suppression of *bop1 bop2* ectopic organ outgrowth at the morphological and cellular levels. Our data demonstrate that *BOP1* and *BOP2* regulate leaf patterning by controlling *YAB* and *KNOX1* gene activity in the developing petiole.

In higher plants, lateral organs initiate from the flanks of the shoot apical meristem (SAM), which acts as a pluripotent stem cell reservoir throughout the plant life span. Through molecular and genetic studies in model plants such as Arabidopsis and maize, the regulatory mechanisms that control the organ differentiation and patterning process are beginning to be understood. Still, much remains to be learned about the pathways that distinguish lateral organ identity from SAM identity and how these pathways intersect with each other during morphogenesis.

The four related Arabidopsis class 1 *KNOTTED-LIKE HOMEOBOX* (*KNOX1*) genes *SHOOTMERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*), *KNOTTED-like from Arabidopsis thaliana2* (*KNAT2*), and *KNAT6* encode homeodomain transcription factors with overlapping expression and function (Hake *et al.* 2004). *STM* is expressed throughout the SAM, and loss-of-function *stm* mutations result in loss of the SAM (Barton and Poethig 1993). *BP* has slightly different expression pattern from *STM* in the SAM and is required for stem and internode morphogenesis (Mele *et al.* 2003), yet can act redundantly with *STM* in certain genetic backgrounds (Byrne *et al.* 2002). *KNAT2* plays a role in carpel development (Pautot *et al.* 2001), whereas *KNAT6*, which is most similar to *KNAT2*, acts in root development (Dean *et al.* 2004). *KNAT6,* but not *KNAT2,* functions redundantly with *STM* in embryonic SAM maintenance and boundary establishment (Belles-Boix *et al.* 2006). During inflorescence growth, *BP* restricts *KNAT2* and *KNAT6* expression to achieve normal shoot morphogenesis, and *KNAT2* and *KNAT6* reveal their functional redundancy during inflorescence and pedicel development in the absence of *BP* (Ragni *et al.* 2008).

In simple-leaved species such as Arabidopsis, ectopic *KNOX1* expression causes lobed and rumpled leaf formation (Sinha *et al.* 1993; Lincoln *et al.* 1994; Nishimura *et al.* 2000; Pautot *et al.* 2001; Dean *et al.* 2004; Cole *et al.* 2006), indicating that continuous suppression of *KNOX1* activity is required for proper organ differentiation. *KNOX1* repression during Arabidopsis organ development is mediated by *ASYMMETRIC LEAVES1* (*AS1*), *AS2*, *BLADE-ON-PETIOLE1* (*BOP1*), and *BOP2* (Byrne *et al.* 2000; Ori *et al.* 2000; Semiarti *et al.* 2001; Ha *et al.* 2003; Hepworth *et al.* 2005; Norberg *et al.* 2005; Ha *et al.* 2007). *AS1* encodes a myb-domain transcription factor that forms a protein complex with the LATERAL-ORGAN-BOUNDARIES (LBD) domain-containing protein AS2 (Byrne *et al.*

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2000; Xu *et al.* 2003). The *BOP1* and *BOP2* genes encode BTB/POZ domain and ankyrin repeat-containing proteins (Ha *et al.* 2004; Hepworth *et al.* 2005; Norberg *et al.* 2005). All of these genes are expressed in leaf primordia and when mutated condition a range of leaf developmental phenotypes similar to those of *KNOX1* overexpressing plants (Byrne *et al.* 2000; Ori *et al.* 2000; Semiarti *et al.* 2001; Ha *et al.* 2003; Hepworth *et al.* 2005; Norberg *et al.* 2005).

KNOX1 expression is also controlled by members of the *YABBY* (*YAB*) family of putative transcription factor genes. *KNOX1* transcription is upregulated in the leaves of plants carrying loss-of-function mutations in the *YAB* genes *FILAMENTOUS FLOWER* (*FIL*) and *YAB3* (Kumaran *et al.* 2002), and *fil yab3* plants display ectopic shoot development on their blades similar to *KNOX1* overexpressing plants (Siegfried *et al.* 1999; Kumaran *et al.* 2002). *FIL* and *YAB3* are expressed in the abaxial domain of developing lateral organs and are implicated in the specification of abaxial organ identity (Eshed *et al.* 1999; Sawa *et al.* 1999; Siegfried *et al.* 1999). In addition, *YAB* activity is required for leaf lamina expansion (Eshed *et al.* 2004).

Here we show that the *bop1 bop2* leaf ectopic outgrowth phenotypes are gradually suppressed by the addition of single, double, and triple *bp, knat2*, and *knat6* allele combinations, revealing functional redundancy of these *KNOX1* genes during *bop1 bop2* leaf formation. We also find that *bop1 bop2* ectopic outgrowth formation requires the activity of the *YAB* genes *FIL* and *YAB3*, with *YAB3* playing a more important role than *FIL*. Furthermore, residual ectopic outgrowth in *bop1 bop2 fil yab3* leaves was almost completely abolished by the introduction of the *bp, knat2*, and *knat6* alleles. These results show that *KNOX1* genes and also *YAB* genes contribute to *bop1 bop2* ectopic organ outgrowth formation. We conclude that *BOP1* and *BOP2* play a key role in organ morphogenesis by suppressing both *KNOX1* and *YAB* activity at the leaf base, thereby maintaining the proper cellular environment for normal leaf differentiation.

MATERIALS AND METHODS

Plant materials and genetics: *A. thaliana* plants were grown as described (Ha *et al.* 2004). All mutant alleles were in the Landsberg *erecta* (L*er*) accession except for *yab3-1* (Wassilewskija), *bop1-4 bop2-11*, and *knat6-5*, which were introgressed from Columbia-0 into L*er* three times before analysis. *knat2-1* (GT 7953) seeds were obtained from the Cold Spring Harbor Laboratory and *knat6-5* (SALK_149322) seeds from the Arabidopsis Biological Resource Center. *fil-8* and *yab3-2* seeds were kindly provided by Venkatesan Sundaresan (University of California, Davis, CA), *fil-5* and *yab3-1* seeds by John Bowman (Monash University, Australia), and *BP::GUS* seeds by Sarah Hake (University of California, Berkeley, CA).

bp-1 knat2 and *bp-1 knat6* plants were generated by crossing *bp-1* with *knat2-1* or *knat6-5*, respectively. *bp-1* F2 plants were then genotyped for the *knat2-1* or *knat6-5* allele, respectively. To generate *bp-1 knat2-1 knat6-5* plants, F1 plants from a cross between *bp-1 knat2-1* and *bp-1 knat6-5* were self-pollinated. *bp-1* F2 plants were grown on plates containing kanamycin to select for the *knat2-1* allele and were genotyped for the *bp-1*, *knat2-1*, and *knat6-5* alleles. To generate *bop1-4 bop2-11 bp-1 knat2-1 knat6-5* plants, F1 plants from a cross between *bop1-4 bop2-11 bp-1 knat2-1* and *bop1-4 bop2-11 knat2-1 knat6-5* were self-fertilized and the F2 plants genotyped for the *bp-1*, *knat2-1*, and *knat6-5* alleles. *bop1-4 bop2-11 fil-8* and *bop1-4 bop2-11 yab3-2* plants were generated by crossing *bop1-4 bop2-11* with *fil-8* or *yab3-2*, respectively. *bop1-4 bop2-11* F2 plants were then genotyped for the *fil-8* and *yab3-2* alleles. *bop1-4 bop2-11 fil-8 yab3-2* plants were generated by crossing *bop1-4 bop2-11 fil-8* plants with *bop1-4 bop2-11 yab3-2* plants. *bop1-4 bop2-11* F2 plants were genotyped for the *fil-8* and *yab3-2* alleles. Phenotypic segregation of *fil-8 yab3-2* plants in a 1:3 ratio was observed in the F3 generation. To generate *bp-1 knat2-1 knat6-5 fil-8 yab3-2* plants, F1 plants from a cross between *bp-1 knat2-1 knat6-5* and *fil-8 yab3-2* were self-pollinated. *bp-1* F2 plants were then genotyped for the *knat2-1*, *knat6-5*, *fil-8*, and *yab3-2* alleles. Phenotypic segregation of *fil-8 yab3-2* plants in a 1:3 ratio was observed in the F3 generation. To generate *bop1-4 bop2-11 bp-1 knat2-1 knat6-5 fil-8 yab3-2* plants, *bop1-4 bop2-11 bp-1 knat2-1 knat6-5* plants were crossed to *bp-1 knat2-1 knat6-5 fil-8 yab3-2* plants. *bop1-4 bop2-11* F2 plants were then genotyped for the *fil-8* and *yab3-2* alleles. Phenotypic segregation of *fil-8 yab3-2* plants in a 1:3 ratio was observed in the F3 generation. Primer sequences for genotyping are listed in supporting information, Table S1.

Histological analysis: Tissue preparation and sectioning were carried out as described (Ha *et al.* 2003).

Expression analysis: RNA was isolated from developing young leaves of 21-day-old plants. Quantification by real-time PCR was performed using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and the MyIQ system (Bio-Rad, Hercules, CA). Primer sequences for mRNA amplification are listed in Table S1. Real-time PCR experiments were repeated using three biological replicates.

GUS analysis: Thirteen-day-old seedlings were fixed in 90% acetone at −20° for 30 min and washed briefly with 100 mm phosphate buffer. Samples were vacuum infiltrated for 10 min in fresh 100 mm sodium phosphate buffer (pH 7.0) containing 0.5 mm potassium ferrocyanide (Sigma, St. Louis), 0.5 mm potassium ferricyanide (Sigma), 1 mm 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (Sigma), 1% DMSO, 1% triton X-100, and 10 mm EDTA, and incubated at 37° for 4 hr. The reactions were terminated with 50% ethanol and the samples cleared in a graded ethanol series to 100% ethanol. Cleared samples in 50% ethanol were fixed in FAA solution (50% ethanol, 5% glacial acetic acid, and 3.7% formaldehyde), vacuum infiltrated for 10 min, and mounted in 50% glycerol. Leaves were dissected and viewed using dark-field microscopy.

RESULTS

***KNOX1* gene activity conditions the *bop1 bop2* leaf phenotype:** Wild-type Arabidopsis rosette leaves are characterized by an ovate blade attached to the main plant stem by a narrow petiole (Figure 1A). *bop1 bop2* rosette leaves form extensive outgrowths of blade tissue along the petioles (Figure 1B), indicating that *BOP1* and *BOP2* are required for proper leaf morphogenesis (Ha *et al.* 2003, 2004). Misexpression of the *KNOX1* genes *BP*, *KNAT2*, or *KNAT6* in leaves causes lobed and dissected leaf formation, and in extreme cases ectopic shoot initiation on the leaf (Sinha *et al.* 1993; Lincoln *et al.*

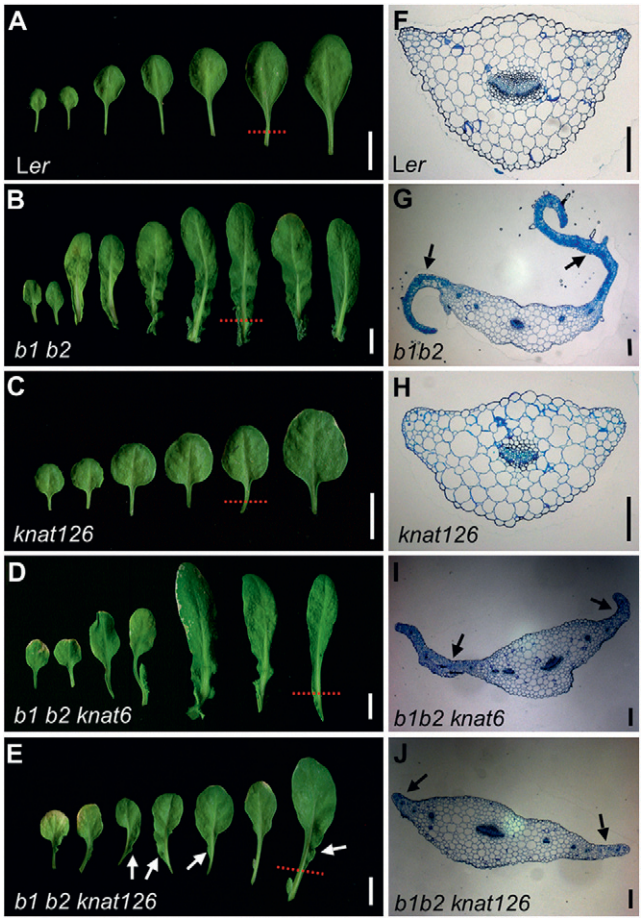

Figure 1.—Genetic interactions between *bop1 bop2* and *knox1* alleles. Photographs of dissected rosettes from 38-day-old (A) wild-type L*er*, (B) *bop1-4 bop2-11* (*b1 b2*), (C) *bp-1 knat2-1 knat6-5* (*knat126*), (D) *bop1-4 bop2-11 knat6-5*, and (E) *bop1-4 bop2-11 bp-1 knat2-1 knat6-5* plants. Transverse sections of (F) L*er*, (G) *bop1 bop2*, (H) *bp-1 knat2-1 knat6-5*, (I) *bop1-4 bop2-11 knat6-5*, and (J) *bop1-4 bop2-11 bp-1 knat2-1 knat6-5* leaf petioles. Dotted lines in A–E indicate the sectioned regions in F–J. Bars, 10 mm (for A–E) and 200 μm (for F–J).

1994; Chuck *et al.* 1996; Pautot *et al.* 2001; Dean *et al.* 2004; Cole *et al.* 2006). The phenotypes of *KNOX1* misexpressing plants are reminiscent of the *bop* phenotypes, and indeed *KNOX1* genes are misexpressed in *bop1 bop2* mutants (Ha *et al.* 2007). Therefore we used genetic analysis to investigate whether the *bop* leaf phenotype is caused by *KNOX1* gene misexpression during organogenesis.

We first obtained loss-of-function alleles of *BP, KNAT2*, and *KNAT6* and examined their phenotypes. We used the *bp-1* and *knat2-1* alleles that had previously been shown to be null alleles (Byrne *et al.* 2002; Venglat *et al.* 2002). We were unable to detect *KNAT6* transcripts from homozygous *knat6-5* plants, indicating that it is also a null allele (Figure S1). To determine the genetic relationship between these *KNOX1* genes we constructed *bp knat2 knat6* plants. These plants showed partial rescue of the downward-oriented fruit phenotype caused by the *bp-1* mutation (Figure S2; Ragni *et al.* 2008), but had normal leaf development (Figure 1C).

Next we introduced the *knox* null alleles, singly and in combination, into the *bop1 bop2* background. Because rosette leaf morphology varied substantially between the different genetic backgrounds, we quantified the phenotypes by classifying them into five classes on the basis of the extent of suppression of the *bop1 bop2* phenotype (Figure S3). Compared to wild-type leaves (Figure S3A), class I plants had leaves with extensive ectopic outgrowths characteristic of *bop1 bop2* leaves (Figure S3B). Class II plants had leaves with a slight reduction in ectopic outgrowths compared to class I plants (Figure S3C), and class III plants had serrated leaves with weak petiole outgrowths (Figure S3D). Class IV plants had nearly normal leaves that displayed occasional ectopic outgrowths (Figure S3E), and class V plants had leaves that resembled those of wild-type plants (Figure S3F). Thus, class I plants showed the least suppression of the ectopic outgrowth phenotype, whereas class V plants displayed complete suppression.

Introduction of the *bp-1*, *knat2-1*, or *bp knat2* alleles into the *bop1 bop2* background had no effect on the *bop* leaf phenotype. In contrast, *bop1 bop2 knat6* leaves developed reduced ectopic blade outgrowths in their petiole region (Figure 1D) compared to *bop1 bop2* leaves. Whereas all *bop1 bop2 bp*, *bop1 bop2 knat2*, and *bop1 bop2 bp knat2* plants displayed a class I leaf phenotype, ~28% of *bop1 bop2 knat6* plants displayed a class II leaf phenotype (Figure 3A), showing that mutating *KNAT6* slightly suppresses the *bop1 bop2* phenotype. *bop1 bop2 bp knat6* and *bop1 bop2 knat2 knat6* leaves had slightly milder phenotypes than *bop1 bop2 knat6* leaves. Class II leaf phenotypes appeared in 41% of *bop1 bop2 bp knat6* plants and in 57% of *bop1 bop2 knat2 knat6* plants (Figure 3A). In *bop1 bop2 bp knat2 knat6* leaves the extent of ectopic outgrowth was further reduced (Figure 1E), with 67% of *bop1 bop2 bp knat2 knat6* leaves displaying class II phenotypes (Figure 3A) and some developing ectopic outgrowths along only one side of the petiole (Figure 1E, arrows).

We also characterized the effects of the *KNOX1* alleles on *bop1 bop2* ectopic blade organogenesis at the cellular level. Developing wild-type rosette leaf petioles displayed a fully differentiated cellular morphology (Figure 1F), as did *bp knat2 knat6* petioles (Figure 1H). In contrast, *bop1 bop2* petioles had small and undifferentiated cells at the margins, where the ectopic outgrowths formed (Figure 1G, arrows). The introduction of the *knat6* allele into the *bop1 bop2* background caused a slight suppression of ectopic outgrowth at the cellular level (Figure 1I, arrow), whereas the introduction of the *bp knat2 knat6* allele combination caused a more dramatic suppression of the *bop1 bop2* leaf phenotype (Figure 1J, arrows). Thus *BP, KNAT2*, and *KNAT6* are combinatorially responsible for conditioning some of

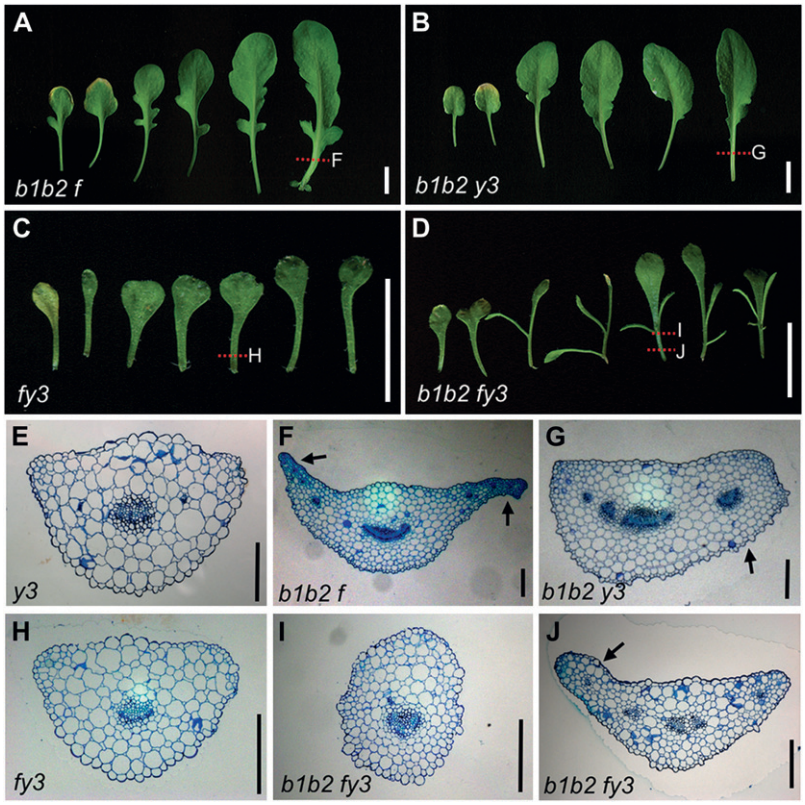

Figure 2.—Genetic interactions between *bop1 bop2* and *yab* alleles. Photographs of dissected rosettes from 38-day-old (A) *bop1-4 bop2-11 fil-8* (*b1b2f*), (B) *bop1-4 bop2-11 yab3-2* (*b1b2y3*), (C) *fil-8 yab3-2* (*fy3*), and (D) *bop1-4 bop2-11 fil-8 yab3-2* (*b1b2fy3*) plants. Transverse sections of (E) *yab3-2*, (F) *bop1-4 bop2-11 fil-8*, (G) *bop1-4 bop2-11 yab3-2*, (H) *fil-8 yab3-2*, and (I and J) *bop1-4 bop2-11 fil-8 yab3-2* leaf petioles. Dotted lines in A–D indicate the sectioned regions in F–J. Bars, 10 mm (for A–D) and 200 μm (for E–J).

the ectopic blade outgrowth that occurs in *bop1 bop2* rosette leaves, with *KNAT6* playing a more important role in conditioning the *bop* phenotype than either *BP* or *KNAT2*. These data indicate that an important function for *BOP1* and *BOP2* during organogenesis is to repress *KNOX1* gene activity at the leaf base to ensure proper leaf formation.

***YAB* gene activity conditions the *bop1 bop2* leaf phenotypes:** Because moderate ectopic blade outgrowth still occurred along *bop1 bop2 bp knat2 knat6* petioles, we hypothesized that other factors in addition to the *KNOX1* genes also contribute to outgrowth production. Two candidates were *FIL* and *YAB3*, which promote leaf lamina expansion (Eshed *et al.* 2004) and are required to repress *KNOX1* expression in leaves (Siegfried *et al.* 1999; Kumaran *et al.* 2002). Previously we had shown that *FIL* is ectopically expressed in the adaxial domain of *bop1 bop2* leaves, and we proposed that the juxtaposition of ectopic *FIL* expression with normal class III *HD-ZIP* expression might lead to ectopic blade outgrowth along new adaxial–abaxial boundaries in *bop1 bop2* plants (Ha *et al.* 2007). We therefore predicted that mutation of *FIL*, and potentially of *YAB3*, which has overlapping expression and function (Siegfried *et al.* 1999), would suppress the *bop1 bop2* ectopic outgrowth phenotype.

We tested this hypothesis by performing a genetic analysis between the *bop* alleles and loss-of-function *yab* alleles. Because compared to wild-type rosette and cauline leaves (Figure S3A and Figure S4A) *fil-8 yab3-2* leaves are small and narrow (Figure S3G and Figure S4B), we separately grouped the variable leaf phenotypes of plants carrying *fil* and/or *yab3* alleles into five classes. Class I plants had four or five leaves that each developed five to eight proximal ectopic organs (Figure S3H). Class II plants had four or five leaves that each developed three to four proximal ectopic organs (Figure S3I). Class III plants had two or three leaves with one or two proximal ectopic organs (Figure S3J). Class IV plants had one rosette and/or cauline leaf with a single ectopic organ (Figure S3K), whereas the leaves of class V plants lacked ectopic outgrowths (Figure S3L).

fil-8 and *yab3-2* plants displayed almost wild-type rosette leaf morphology, and at the anatomical level the cellular morphology of *fil* and *yab3* petioles was undistinguishable from that of wild-type petioles (Figure 2E). Both *bop1 bop2 fil* and *bop1 bop2 yab3* leaves showed significantly decreased ectopic outgrowth compared to *bop1 bop2* leaves (Figure 2, A and B). The *bop1 bop2 fil* phenotypes ranged from class I to class III, with 3% of *bop1 bop2 fil* plants forming leaves with class III character (Figure 3A). *bop1 bop2 yab3* leaves displayed even stronger suppression of the ectopic outgrowth phenotype. These plants developed leaves with phenotypes ranging from class I to class IV, with 78% of plants displaying either class III or class IV leaf morphology (Figure 3A). However, undifferentiated marginal cells were still evident in *bop1 bop2 fil* and *bop1 bop2 yab3* petioles (Figure 2, F and G, arrows), although they formed reduced ectopic outgrowths compared to *bop1*

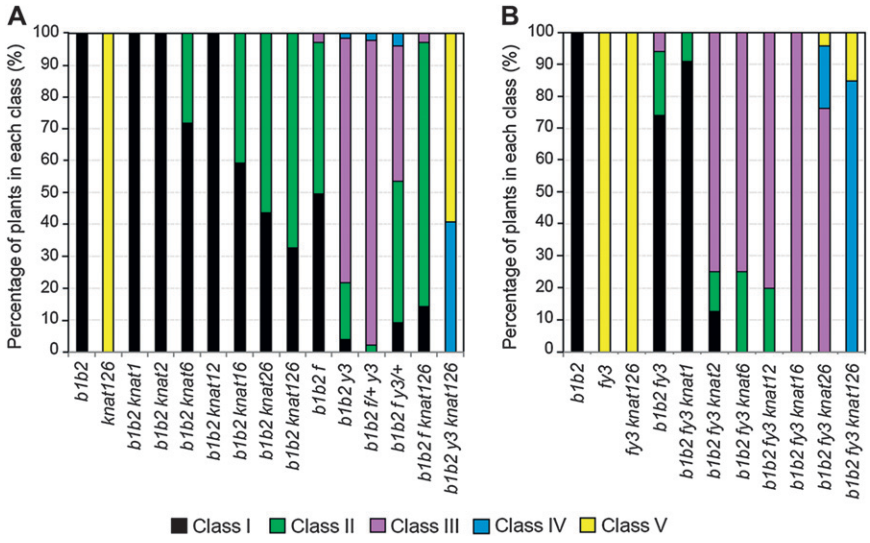

FIGURE 3.—Comparision of leaf ectopic outgrowth phenotypes. (A) Graphical representation of the percentage of plants in the *bop1 bop2* background displaying rosette and cauline leaf phenotypes of differing severities, grouped from class I (most severe) to class V (least severe). (B) Graphical representation of the percentage of plants in the *fil yab3* background displaying rosette and cauline leaf phenotypes of differing severities, grouped from class I (most severe) to class V (least severe). $n \geq 85$ plants per genotype.

bop2 petioles. These data indicate that *FIL* and *YAB3* each play an important role in conditioning the *bop1 bop2* leaf phenotypes, with *YAB3* making a more significant contribution.

In addition, we noted a dosage-dependent effect of the *fil* and *yab* alleles on the *bop1 bop2* ectopic outgrowth phenotype. Compared to 3% of *bop1 bop2 fil* plants that displayed class III leaf morphology, 46% of *bop1 bop2 fil yab3/+* plants displayed class III or class IV leaf morphology (Figure 3A). Similarly, compared to 78% of *bop1 bop2 yab3* plants, 98% of *bop1 bop2 fil/+ yab3* plants displayed class III or class IV leaf morphology.

fil-8 yab3-2 plants were small and formed narrow leaves with reduced blades (Figure 2C and Figure S4B) as previously reported (SIEGFRIED *et al.* 1999; KUMARAN *et al.* 2002). Like *bop1 bop2* leaves, *fil yab3* leaves misexpress *KNOX1* genes at high levels and infrequently display ectopic meristem formation on their blades characteristic of *KNOX1* overexpressing plants (KUMARAN *et al.* 2002). Thus we tested whether the absence of both *FIL* and *YAB3* expression would affect the *bop* ectopic outgrowth phenotype. *bop1 bop2 fil yab3* plants developed two to four organ outgrowths discontinuously along their rosette leaf petioles (Figure 2D) and four to seven organ outgrowths at the base of their cauline leaves (Figure S4C). The outgrowths were longer and narrower than those produced by *bop1 bop2* leaves. The main blade region was also narrow (Figure 2D). The *bop1 bop2 fil yab3* phenotypes ranged from class I to class III, with 74% of *bop1 bop2 fil yab3* plants forming leaves with class I character (Figure 3B).

We also examined the effects of the *fil yab3* alleles on the cellular morphology of rosette leaf petioles. *fil yab3* leaves had small petioles with normal cellular morphology (Figure 2H). In contrast, *bop1 bop2 fil yab3* petioles exhibited two types of anatomy depending on the region analyzed: petiole regions lacking ectopic outgrowths were nearly radialized, whereas other regions clearly showed ectopic outgrowths at the margins (Figure 2, I and J, arrow). Thus, although mutations in either *FIL* or *YAB3* partially suppressed the *bop* leaf phenotype, *bop1 bop2 fil yab3* leaves still developed substantial ectopic outgrowths potentially because of high-level misexpression of *KNOX1* genes through either a common or a converging genetic pathway.

***KNOX1* expression analysis:** Our study shows that *KNOX1* gene activity is partially responsible for conditioning the *bop1 bop2* leaf phenotypes, and both the *BOP* and *YAB* genes repress *KNOX1* expression during leaf morphogenesis (KUMARAN *et al.* 2002; HA *et al.* 2007). To determine whether the *BOP* and *YAB* genes regulate *KNOX1* transcription via the same or different pathways, we analyzed *KNOX1* expression in the young, developing leaves of plants carrying various combinations of *bop* and *yab* alleles.

We first confirmed that the steady-state expression levels of *BP*, *KNAT2*, and *KNAT6* were higher in *bop1 bop2* leaves than in wild-type leaves (Figure 4A), as previously reported. *KNOX1* expression levels were also higher in *fil yab3* leaves than in wild type and *bop1 bop2* leaves. *KNOX1* expression levels in *bop1 bop2 fil* and *bop1 bop2 yab3* leaves were similar to those in *bop1 bop2* leaves (Figure 4A), demonstrating that suppression of the *bop1 bop2* ectopic outgrowth phenotype by *fil* or *yab3* is not caused by a reduction in ectopic *KNOX1* expression. In contrast, the expression of all three *KNOX1* genes, in particular *BP*, was much higher in *bop1 bop2 fil yab3* leaves than either *bop1 bop2* or *fil yab3* leaves (Figure 4A). This result indicates that the *BOP* and *YAB* genes act separately to repress *KNOX1* transcription in leaf primordia.

We next characterized the spatial aspect of *KNOX1* misexpression in *bop* and *yab* leaves by examining the expression pattern of a *BP::GUS* reporter gene. Here we used the *fil-5* and *yab3-1* alleles rather than the *fil-8* and *yab3-2* alleles, because both *fil-8* and *yab3-2* plants

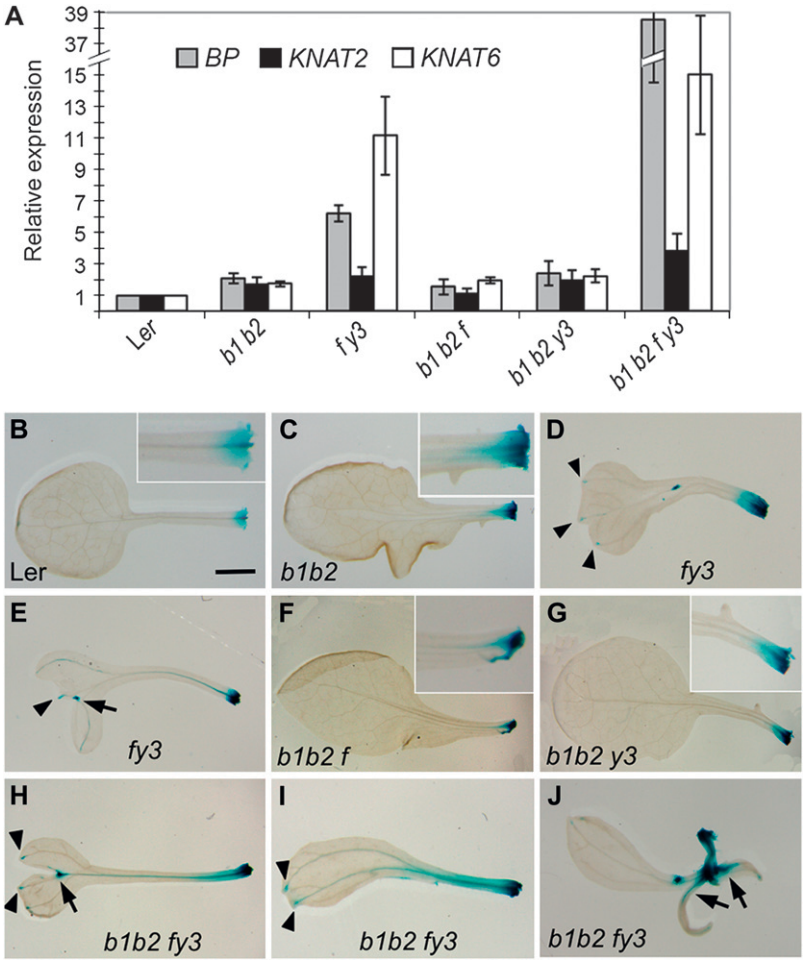

FIGURE 4.—Analysis of class 1 *KNOX* gene expression. (A) *BP*, *KNAT2*, and *KNAT6* expression in the developing young leaves of 21-day-old seedlings. Mean transcript levels were determined by real-time quantitative PCR analyses and normalized to EF1α. Error bars represent SD. *b1 b2*, *bop1-4 bop2-11*; *f y3*, *fil-8 yab3-2*. *pBP::GUS* activity in (B) L*er*, (C) *bop1 bop2*, (D and E) *fil-5 yab3-1*, (F) *bop1 bop2 fil-5*, (G) *bop1 bop2 yab3-1*, and (H–J) *bop1 bop2 fil-8 yab3-2* rosette leaves. Insets in B, C, F, and G are magnified views of the leaf base. Arrowheads indicate *BP::GUS* activity in hydathodes. Arrows in E and H indicate *BP::GUS* activity in bifurcated leaves. Arrows in J indicate *BP::GUS* misexpression in developing ectopic organs.

already carry a GUS reporter cassette in their genomes (KUMARAN *et al.* 2002). The phenotypes of *fil-5 yab3-1* plants were indistinguishable from those of *fil-8 yab3-2* plants (Figure S5A; SIEGFRIED *et al.* 1999). Moreover, the morphological phenotypes of *bop1 bop2 fil-5*, *bop1 bop2 yab3-1*, and *bop1 bop2 fil-5 yab3-1* plants were indistinguishable from those of *bop1 bop2 fil-8*, *bop1 bop2 yab3-2*, and *bop1 bop2 fil-8 yab3-2* plants, respectively (Figure S5, B–D).

It has been reported that in wild-type plants *BP* is expressed in the shoot apex but not in leaf primordia or developing leaves (ORI *et al.* 2000). However, we found that the base of wild-type leaves showed weak *BP::GUS* activity (Figure 4B), likely due either to direct transcription of *BP* in these cells or to GUS diffusion from the neighboring SAM. Compared to wild-type leaves, *bop1 bop2* leaves exhibited stronger and more distally expanded *BP::GUS* activity in the petiole (Figure 4C). In *fil yab3* leaves, *BP::GUS* activity was detected strongly in the petiole and more weakly in the vasculature and hydathodes (Figure 4, D and E, arrowheads), and in the distal region of bifurcated leaves (Figure 4E, arrow). *bop1 bop2 fil* and *bop1 bop2 yab3* leaves had strong *BP::GUS* activity restricted to the proximal region of the petiole (Figure 4, F and G), similar to *bop1 bop2* leaves. In *bop1 bop2 fil yab3* quadruple mutant leaves, *BP::GUS* activity was detected in a broader domain than in double- and triple-mutant leaves. In addition to the proximal petiole, the petiole and blade vasculature, the hydathodes and the distal region of bifurcated leaves (Figure 4, H and I), *bop1 bop2 fil yab3* leaves also showed strong *BP::GUS* activity in the ectopic organs along the petiole (Figure 4J, arrows). Thus *KNOX1* expression is both strongly elevated and spatially expanded in *bop1 bop2 fil yab3* leaves, and thus may play an important role in conditioning the extensive ectopic organ development seen in *bop1 bop2 fil yab3* leaves.

***KNOX1* and *YAB* genes together condition the *bop1 bop2* leaf phenotypes:** Although both *KNOX1* and *YAB* mutations were able to partially suppress ectopic organogenesis in *bop1 bop2* leaves, the *bop* phenotypes were not completely rescued by either *KNOX1* or *YAB* mutations. To examine the combinatorial effect of both *KNOX1* and *YAB* genes on leaf morphogenesis, we first combined various *knox1* and *yab* alleles. Despite the fact that *KNOX1* gene expression was highly elevated in *fil yab3* leaves (KUMARAN *et al.* 2002), the *fil yab3* leaf patterning phenotypes were unaffected by the introduction of any combination of the three *knox1* alleles (Figure S4D), as was the ectopic shoot formation phenotype (data not

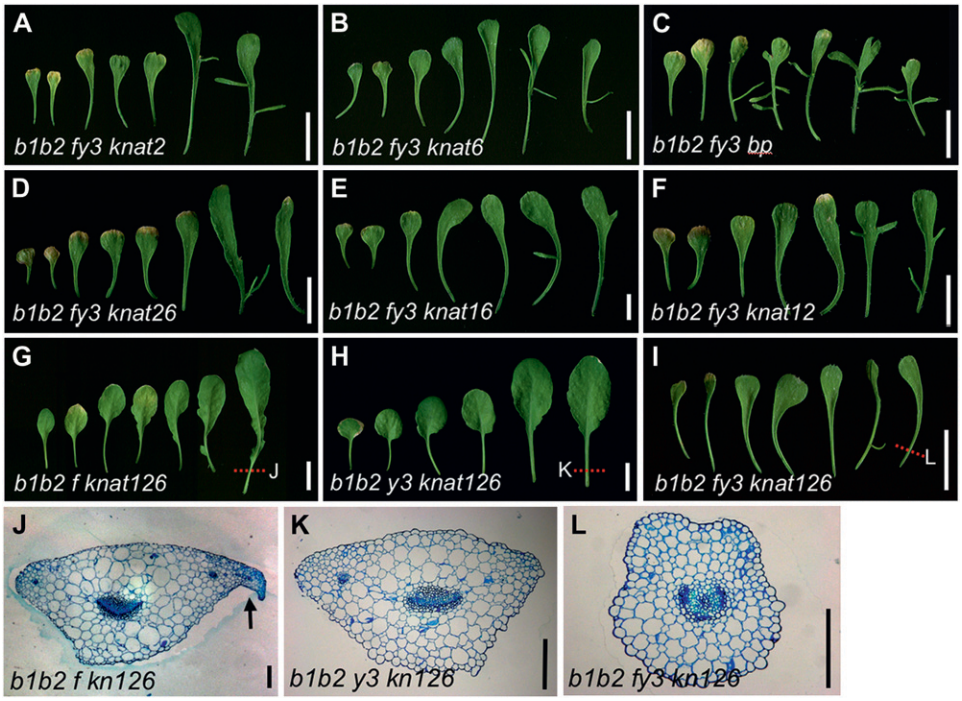

FIGURE 5.—Genetic interactions between *yab* and *knox1* alleles. Photographs of dissected rosette leaves from 38-day-old (A) *bop1-4 bop2-11 fil-8 yab3-2 knat2-1*, (B) *bop1-4 bop2-11 fil-8 yab3-2 knat6-5*, (C) *bop1-4 bop2-11 fil-8 yab3-2 bp-1*, (D) *bop1-4 bop2-11 fil-8 yab3-2 knat2-1 knat6-5*, (E) *bop1-4 bop2-11 fil-8 yab3-2 bp-1 knat6-5*, (F) *bop1-4 bop2-11 fil-8 yab3-2 bp-1 knat2-1*, (G) *bop1-4 bop2-11 fil-8 bp-1 knat2-1 knat6-5*, (H) *bop1-4 bop2-11 yab3-2 bp-1 knat2-1 knat6-5*, and (I) *bop1-4 bop2-11 fil-8 yab3-2 bp-1 knat2-1 knat6-5* plants. Transverse sections of (J) *bop1-4 bop2-11 fil-8 bp-1 knat2-1 knat6-5*, (K) *bop1-4 bop2-11 yab3-2 bp-1 knat2-1 knat6-5*, and (L) *bop1-4 bop2-11 fil-8 yab3-2 bp-1 knat2-1 knat6-5* leaf petioles. Dotted lines in G–I indicate the sectioned regions in J–L. Bars, 10 mm (for A–I) and 200 μm (for J–L).

shown). Our results demonstrate that derepression of *KNOX1* expression does not condition the *fil yab3* leaf phenotypes.

Introducing combinations of *KNOX1* and *YAB* mutations into the *bop1 bop2* background led to gradually increased suppression of the *bop* leaf phenotype. The addition of either the *knat2* or the *knat6* allele into the *bop1 bop2 fil yab3* background slightly suppressed the leaf phenotype (Figure 5, A and B, and Figure S4F), with 87% of *bop1 bop2 fil yab3 knat2* plants and 100% of *bop1 bop2 fil yab3 knat6* plants displaying more suppressed class II or class III leaf phenotypes compared to 5% of *bop1 bop2 fil yab3* plants (Figure 3B). In contrast, the addition of the *bp* allele had little effect, with *bop1 bop2 fil yab3 bp* leaves displaying phenotypes similar to those of *bop1 bop2 fil yab3* leaves (Figure 3B, Figure 5C, and Figure S4E).

We then introduced the *knat2 knat6*, *bp knat2*, and *bp knat6* alleles into the *bop1 bop2 fil yab3* background. All hextuple mutants showed suppression of the *bop1 bop2 fil yab3* leaf phenotypes (Figure 3B, Figure 5, D–F, and Figure S4G). Whereas 75% of *bop1 bop2 fil yab3 knat6* plants exhibited class III character, 80% of *bop1 bop2 fil yab3 bp knat2* and 100% of *bop1 bop2 fil yab3 bp knat6* plants had class III phenotypes (Figure 3B). The *bop1 bop2 fil yab3 knat2 knat6* allelic combination generated the greatest degree of suppression, with all plants displaying class III, class IV, or even class V leaf character (Figure 3B).

Introduction of the *fil* or *yab3* alleles into *bop1 bop2 bp knat2 knat6* plants induced further suppression of the leaf phenotypes (Figure 5, G and H). *bop1 bop2 fil bp knat2 knat6* plants exhibited phenotypes ranging from class I to class III, indicating a reduction in the severity of the class I and class II *bop1 bop2 bp knat2 knat6* phenotypes (Figure 3A). *bop1 bop2 yab3 bp knat2 knat6* plants developed almost wild-type leaves displaying either class IV or class V morphology (Figure 3A). These data confirm that *YAB3* plays a more important role than *FIL* in conditioning the *bop1 bop2* leaf phenotypes. Finally, the *bop1 bop2 fil yab3 bp knat2 knat6* septuple mutant combination conditioned nearly complete suppression of the *bop1 bop2* leaf phenotype (Figure 5I and Figure S4H). *bop1 bop2 fil yab3 bp knat2 knat6* leaves exhibited only class IV or class V character (Figure 3B), with an occasional single ectopic outgrowth observed along the petiole or proximal blade region.

At the cellular level, the extent of ectopic outgrowth reduction was striking in *bop1 bop2 fil bp knat2 knat6* petioles, which developed far smaller ectopic organs than *bop1 bop2* petioles (Figure 5J). The anatomy of *bop1 bop2 yab3 bp knat2 knat6* petioles was very similar to wild-type petioles (Figure 5K). *bop1 bop2 fil yab3 bp knat2 knat6* petioles were radialized in shape, although approximately 80% (33/41) showed normal polarity in their central vasculature (Figure 5L). The remaining 20% (8/41) of *bop1 bop2 fil yab3 bp knat2 knat6* petioles had vasculature consisting of xylem surrounded by phloem, indicating abaxialization. These histological data clearly show at the cellular level that the extent of ectopic outgrowth gradually decreases with the stepwise addition of mutations in *KNOX1* and *YAB* genes. In sum, our data demonstrate that three *KNOX1* genes, *BP*, *KNAT2*, and *KNAT6*, and two *YAB* genes, *FIL* and *YAB3*, are together responsible for ectopic lamina development in *bop1 bop2* leaves.

DISCUSSION

Leaf morphogenesis in Arabidopsis to form a narrow proximal petiole and a wide distal blade requires carefully regulated patterning coupled with controlled cell proliferation. *BOP1* and *BOP2* play key roles in the patterning process because *bop1 bop2* leaves display ectopic outgrowth of blade tissue along the petiole (Ha *et al.* 2004). This phenotype is associated with ectopic *KNOX1* and *YAB* expression (Ha *et al.* 2003, 2007), yet whether these genes mediate *bop* outgrowth formation had not previously been determined.

Here we demonstrate that *BP, KNAT2,* and *KNAT6* make a modest contribution to the *bop* ectopic outgrowth phenotype. The expression of all three genes is elevated in *bop1 bop2* leaves, and *BP::GUS* activity expands more distally in *bop1 bop2* than wild-type leaves (Figure 4). Removal of *KNAT6,* but not *BP* or *KNAT2,* activity alone slightly rescued the *bop* leaf phenotype (Figure 1D). However, introduction of the *bp* or *knat2* allele into the *bop1 bop2 knat6* background further attenuated the *bop* phenotype, and inactivation of all three *KNOX1* genes produced the greatest extent of rescue (Figure 1E and Figure 3A). Thus *BP* and *KNAT2* function redundantly with each other, and overlap with *KNAT6,* to condition the *bop* ectopic outgrowth phenotype. These data confirm that an important function for BOP1 and BOP2 during leaf morphogenesis is to repress *KNOX1* activity to permit proper petiole differentiation.

FIL and *YAB3* also make a significant contribution to ectopic outgrowth formation in *bop1 bop2* leaves. We found that compromising either *FIL* or *YAB3* activity reduced *bop* ectopic outgrowth, with *YAB3* playing a more important role than *FIL* (Figure 2, D and E). These genes also suppressed the *bop* phenotype in a dose-dependent fashion, with *bop1 bop2 fil yab3/+* and *bop1 bop2 yab3 fil/+* plants showing more complete suppression than *bop1 bop2 fil* and *bop1 bop2 yab3* plants, respectively (Figure 3A). Thus another key role for the *BOP* genes during leaf development is to repress *YAB* expression in the adaxial leaf domain.

Interestingly, the complete loss of both *FIL* and *YAB3* did not cause the disappearance of *bop1 bop2* leaf outgrowths. Instead, *bop1 bop2 fil yab3* leaves developed extensive ectopic outgrowths compared to *bop1 bop2 fil* and *bop1 bop2 yab3* leaves (Figure 2). This result is explained by strong ectopic expression of *KNOX1* genes in *bop1 bop2 fil yab3* plants (Figure 4), because the removal of *BP, KNAT2,* and *KNAT6* function strongly suppressed the *bop1 bop2 fil yab3* phenotype, such that the leaves of all *bop1 bop2 fil yab3 bp knat2 knat6* plants either formed a single ectopic outgrowth or lacked them entirely (Figure 3B and Figure 5I). We conclude that *BOP1/BOP2* and *FIL/YAB3* act through separate pathways to suppress *KNOX1* transcription during leaf morphogenesis.

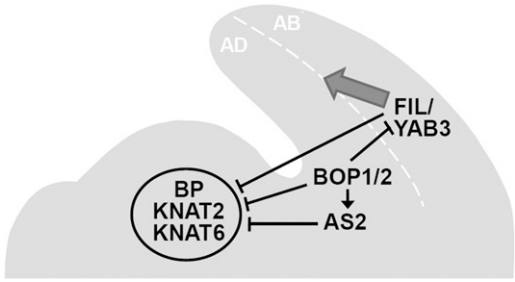

Figure 6.—Model for Arabidopsis gene function during lateral organ development. The *BOP1* and *BOP2* genes directly induce *AS2* expression and also negatively regulate *FIL* and *YAB3* activity in developing leaves. In addition, the *BOP* genes repress *KNOX1 (BP, KNAT2, KNAT6)* expression via an *AS2*-independent pathway. *AS2* and *FIL/YAB3* play separate roles in negatively regulating *KNOX1* transcription during lateral organ growth. *FIL/YAB3* also promote lamina outgrowth along the adaxial–abaxial boundary (shaded arrow) through a *KNOX1* independent pathway. AD, adaxial; AB, abaxial.

Several lines of evidence suggest that the roles of the *YAB* genes in repressing *KNOX1* expression and promoting lamina outgrowth are separable. First, removal of *BP, KNAT2,* and *KNAT6* activity in *fil yab3* leaves did not restore blade growth (Figure S4D). Second, we did not detect a direct correlation between the level of *KNOX1* expression and the extent of ectopic organ outgrowth in the various *bop yab* backgrounds. *bop1 bop2, bop1 bop2 fil,* and *bop1 bop2 yab3* plants all exhibited similar *KNOX1* expression levels and patterns (Figure 4), yet the *bop1 bop2 fil* and *bop1 bop2 yab3* ectopic outgrowth phenotypes were weaker than the *bop1 bop2* phenotype (Figure 3A). In addition, *KNOX1* mRNA levels were much higher in *bop1 bop2 fil yab3* plants than in *bop1 bop2* plants (Figure 4A), although the *bop1 bop2 fil yab3* phenotype was slightly weaker than the *bop1 bop2* phenotype (Figure 3, A and B). We interpret these data in the following way. In *bop1 bop2* plants, ectopic *KNOX1* and *YAB* expression produces ectopic outgrowths along the petiole by sustaining the cells in a proliferative state, while simultaneously the juxtaposition of normal *PHV* and ectopic *KAN1* expression domains (Ha *et al.* 2007) forms new adaxial–abaxial boundaries that trigger lamina outgrowth. In *bop1 bop2 fil* and *bop1 bop2 yab3* plants, ectopic *KNOX1* expression is unaltered but the capacity for *YAB*-mediated lamina outgrowth is reduced, and thus the phenotype is attenuated. In *bop1 bop2 fil yab3* plants, the capacity for lamina outgrowth is further compromised, but is largely compensated for in the proximal leaf region by the dramatic increase in ectopic *KNOX1* expression (Figure 4, A and H–J) that sustains the cells in an excessively proliferative capacity.

A slight amount of residual ectopic organ outgrowth was observed in *bop1 bop2 fil yab3 bp knat2 knat6* leaves, and a higher proportion of *bop1 bop2 yab3 bp knat2 knat6* plants than *bop1 bop2 fil yab3 bp knat2 knat6* plants displayed fully wild-type leaf morphology (Figure 3).

One explanation for these observations is that the residual ectopic organ outgrowth could be conditioned by misregulation of other *YAB* genes, such as *YAB2* and *YAB5,* which show molecular and functional redundancy with *FIL* and *YAB3* (Siegfried *et al.* 1999; Izhaki and Bowman 2007). Alternatively, *STM*, the remaining *KNOX1* gene, might function in this process. *STM* expression is restricted to the SAM and is not detected in developing leaves except infrequently in the sinus (Kawamura *et al.* 2010). Ectopic expression of a nuclear-localizable *STM* construct produced plants with lobed and rumpled leaves similar to the phenotypes of *BP*, *KNAT2*, and *KNAT6* overexpressing plants (Lincoln *et al.* 1994; Pautot *et al.* 2001; Dean *et al.* 2004; Cole *et al.* 2006). We found that ectopic *STM* transcripts were not detectable in the developing young leaves of *bop1 bop2 yab3 bp knat2 knat6* plants, but were highly elevated in those of *bop1 bop2 fil yab3 bp knat2 knat6* plants (Figure S6). These data suggest that *STM* misexpression is likely responsible for the residual ectopic leaf outgrowth observed in the *bop1 bop2 fil yab3 bp knat2 knat6* plants. Unfortunately, it is not feasible to genetically test the contribution of *STM* to the ectopic organ outgrowth phenotype because *stm* null mutants lack leaves (Barton and Poethig 1993) and weak *stm* mutants produce fused leaves with abnormal morphology (Endrizzi *et al.* 1996).

Arabidopsis *as1* and *as2* plants in which *KNOX1* genes are misexpressed also infrequently develop leaflet-like lobed organs along the petioles (Byrne *et al.* 2000; Ori *et al.* 2000; Serrano-Cartagena *et al.* 2000; Semiarti *et al.* 2001). Interestingly, unlike *bop1 bop2* ectopic outgrowth formation, which is partially suppressed by the *bp knat2 knat6* allelic combination, *as1* and *as2*-lobed leaf morphogenesis was not affected by the *bp knat2 knat6* allelic combination (Ikezaki *et al.* 2010). Furthermore, *as1 fil yab3* and *as2 fil yab3* plants showed additive rather than suppressed phenotypic effects (Fu *et al.* 2007).

Our data point toward a complex network of interactions between transcriptional regulators during leaf patterning (Figure 6). We have shown that BOP1 is a transcriptional activator that directly associates with specific sites in the *AS2* promoter to induce its expression at the base of developing leaf primordia (Jun *et al.* 2010). AS2 acts with AS1 to maintain *KNOX1* repression in leaf primordia (Guo *et al.* 2008). We demonstrate that BOP1/2 also repress *KNOX1* expression, but several observations indicate that this effect is not mediated entirely via AS2. First, *bop as2* plants have synergistic phenotypes (Ha *et al.* 2003, 2007), showing that these genes are not part of a strictly linear genetic pathway. Second, *knox1* mutations partially rescue the *bop* but not the *as2* leaf phenotypes (Ikezaki *et al.* 2010). The *FIL* and *YAB3* genes represent a third set of transcriptional regulators that suppress *KNOX1* gene expression in developing leaves (Kumaran *et al.* 2002). Although *FIL* is misregulated in *bop1 bop2* leaves (Ha *et al.* 2007) there is no epistatic relationship between *bop* and *yab* mutations, implying that *BOP1* and *BOP2* negatively regulate *KNOX1* expression through a separate pathway from the *YAB* genes. *BOP1* and *BOP2* also negatively regulate *YAB* expression in proximal, adaxial leaf cells (Ha *et al.* 2007), preventing lamina expansion along the petiole. We conclude that ectopic blade outgrowth along Arabidopsis leaf petioles is prevented by BOP1/2 repression both of ectopic *YAB* gene expression in the adaxial, proximal domain that would promote lamina expansion and of ectopic *KNOX1* gene expression that would sustain the leaf cells in an excessively proliferative state.

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Control of Arabidopsis Leaf Morphogenesis Through Regulation of the *YABBY* and *KNOX* Families of Transcription Factors

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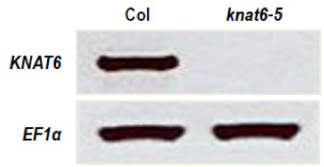

FIGURE S1.—*KNAT6* transcript analysis in *knat6-5* plants. *KNAT6* transcripts are not detected in *knat6-5* plants by RT-PCR. *EF1α* was used as a control.

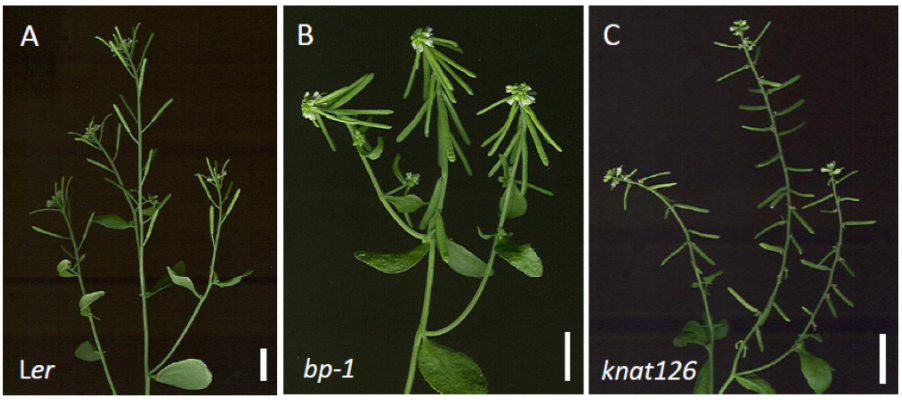

FIGURE S2.—Rescue of the *bp* inflorescence defect in *bp knat2 knat6* plants. Inflorescence development in (A) L*er*, (B) *bp-1* and (C) *bp-1 knat2-1 knat6-5* (*knat126*) plants. Arrows indicate siliques showing rescue of the *bp* downward-oriented morphology. Bars, 10 mm.

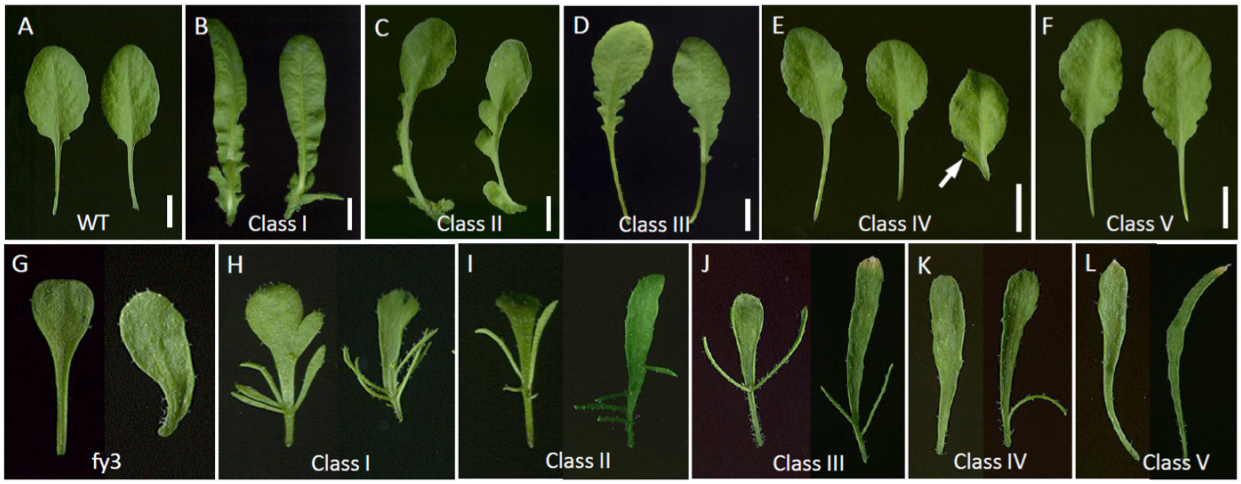

FIGURE S3.—Classification of leaf phenotypes. Detached rosette leaves from (A) wild-type, (B and H) class I, (C and I) class II, (D and J) class III, (E and K) class IV, (F and L) class V plants, and (G) *fil-8 yab3-2* plants. Arrow in (E) indicates mild ectopic outgrowth along the petiole. Bars, 10 mm.

FIGURE S4.—Cauline leaf phenotypes of multiple mutants. Cauline leaves from (A) L*er*, (B) *fil-8 yab3-2* (*fy3*), (C) *bop1-4 bop2-11 fil-8 yab3-2*, (D) *bp-1 knat2-1 knat6-5 fil-8 yab3-2*, (E) *bop1-4 bop2-11 fil-8 yab3-2 bp-1*, (F) *bop1-4 bop2-11 fil-8 yab3-2 knat6-5*, (G) *bop1-4 bop2-11 fil-8 yab3-2 knat2-1 knat6-5* and (H) *bop1-4 bop2-11 fil-8 yab3-2 bp-1 knat2-1 knat6-5* plants. Bars, 10 mm.

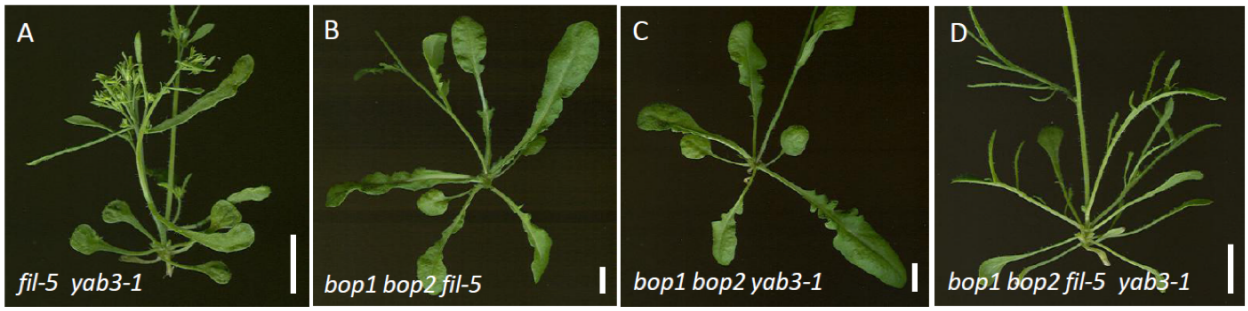

FIGURE S5.—Genetic interaction between and *bop1 bop2* and *fil yab3* alleles. Photographs of (A) *fil-5 yab3-1*, (B) *bop1-4 bop2-11 fil-5*, (C) *bop1-4 bop2-11 yab3-1* and (D) *bop1-4 bop2-11 fil-5 yab3-1* plants. Bars, 10 mm.

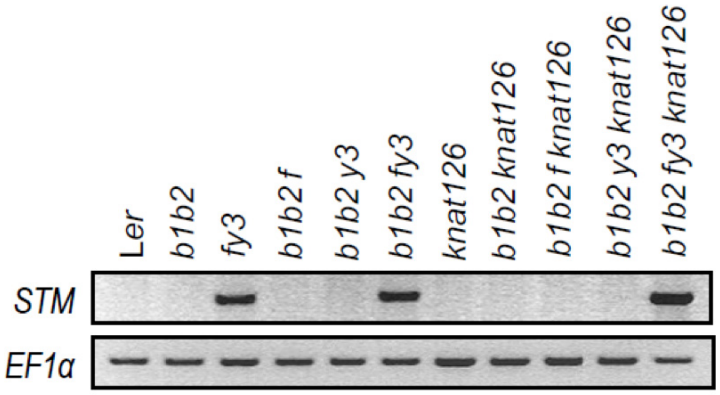

FIGURE S6.—*STM* transcript analysis. mRNA was isolated from developing young leaves of 21-day-old wild-type and mutant plants, and *STM* transcripts detected by RT-PCR. *EF1α* was used as a control. PCR cycles: *STM,* 32 cycles; *EF1α*, 23 cycles. *b1b2*, *bop1-4 bop2-11*, *fy3*, *fil-8 yab3-2*, *knat126*, *bp knat2 knat6*.

TABLE S1

Primer sequences used in this study

Primer	Primer sequences (5'-3')	Reference
For genotyping		
BP-F	GATGATCCCATATTGTCACTCTTCCC	this study
BP-R	ATGGAAGAATACCAGCATGACAAC	this study
KNAT2-F	CCGAAGGCTTCCAATGGCG	this study
KNAT2-R	GCGGCGATCACTGATCGTATC	this study
KNAT6-F	TCATTCCTCGGTAAAGAATGATCCACTAG	this study
KNAT6-R	ATCTACAATTTCCATTCGGCCGGTG	this study
FIL-F	GCTATGTCCAATGCAACTTT	Lin et al., 2003
FIL-R	TTCTTGGCAGCAGCACTAAA	Lin et al., 2003
YAB3-F	ACTTCTCATCTACGGACCAG	Lin et al., 2003
YAB3-R	TCAGCCATGAGTCCAAAGTG	Lin et al., 2003
For qRT-PCR		
BP-qF	CGTAAGCGATGTTGAAGCCA	this study
BP-qR	TCGAGCCTCAAAGTCTTGCC	this study
KNAT2-qF	GACGAACTCGCTACCGCTTT	this study
KNAT2-qR	CCACCTTTTGGCAATCGATG	this study
KNAT6-qF	AAGACCGTTTGACGAGGCAA	this study
KNAT6-qR	TGCTACCTCATGATCACCTCCA	this study

SUPPORTING REFERENCE

Lin, W. C., B. Shuai and P. S. Springer, 2003 The Arabidopsis LATERAL ORGAN BOUNDARIES-domain gene ASYMMETRIC LEAVES2 functions in the repression of KNOX gene expression and in adaxial-abaxial patterning. Plant Cell **15:** 2241-2252.