YABBYs and the Transcriptional Corepressors LEUNIG and LEUNIG_HOMOLOG Maintain Leaf Polarity and Meristem Activity in Arabidopsis

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In Arabidopsis thaliana, FILAMENTOUS FLOWER (FIL) and YABBY3 (YAB3) encode YABBY domain proteins that regulate abaxial patterning, growth of lateral organs, and inflorescence phyllotomy. In this study, we show that YABs physically interact with components of a transcriptional repressor complex that include LEUNIG (LUG), LEUNIG_HOMOLOG (LUH), the LUG-associated coregulator SEUSS, and related SEUSS-LIKE proteins. Consistent with the formation of a LUG-YAB complex, we find that lug mutants enhance the polarity and growth defects of fil yab3 mutant leaves and that this enhancement is due to a loss of LUG activity from the abaxial domain. We performed a more extensive genetic analysis, which included the characterization of yab triple and quadruple mutants, lug luh/+ (heterozygous only for luh) mutants, and plants expressing artificial microRNAs targeting LUG or LUH. These analyses showed that the LUG-YAB complex also promotes adaxial cell identity in leaves as well as embryonic shoot apical meristem (SAM) initiation and postembryonic SAM maintenance. Based on the likely formation of the LUG-YAB complex in the abaxial domain of cotyledons and leaves, we propose that this complex has numerous non-cell-autonomous functions during plant development.

INTRODUCTION

Leaves of higher plants are typically polar structures with an asymmetric distribution of cell types along the proximodistal, lateral and adaxial (dorsal), and abaxial (ventral) axes. While adaxial and abaxial cells become morphologically distinct during later stages of leaf development, differences in the patterns of cell divisions and gene expression occur much earlier. This shows that organ polarity is established at, or just prior to, primordium emergence from the flanks of the shoot apical meristem (SAM).

In Arabidopsis thaliana, several families of transcription factors promote adaxial or abaxial cell identity in leaves and other lateral organs. The adaxial-promoting PHABULOSA (PHB), PHAVOLUTA, and REVOLUTA Class III homeodomain/leucine zipper transcription factors (HD-ZIPIII)s are expressed in the adaxial domain of lateral organs and the central region of the SAM. By contrast, members of the abaxial-promoting KANADI family (KAN1-3) are expressed in the abaxial domain of organs in a pattern that is complementary to the HD-ZIPIIIls (Eshed et al., 2001; Kerstetter et al., 2001; McConnnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003). Based on the analysis of gain- and loss-of-function mutations in the HD-ZIPIIIls and KANs, it has been proposed that mutual antagonism between these classes of genes establishes organ polarity (McConnell and Barton, 1998; Eshed et al., 2001; McConnell et al., 2001; Emery et al., 2003).

In addition to the KANs, several other factors promote abaxial cell identity. These include two members of the AUXIN RESPONSE FACTOR (ARF) family of transcription factors, ETTIN (ETT) and ARF4 (Pekker et al., 2005) and the plant-specific YABBY (YAB) transcription factors (Sawa et al., 1999; Siegfried et al., 1999). Of the six members of YAB family in Arabidopsis, three (FILAMENTOUS FLOWER [FIL], also called YAB1, YAB2, and YAB3) are expressed in the abaxial domain of all lateral organs, whereas the other two (CRABS CLAW [CRC] and INNER NO OUTER) are restricted to the abaxial domains of carpels and the outer integument of ovules, respectively (Bowman and Smyth, 1999; Sawa et al., 1999; Siegfried et al., 1999; Villanueva et al., 1999). Due to the low levels of YABs expression, transcript distribution has not been analyzed in detail. Loss of both FIL and YAB3 results in narrow leaves and a partial loss of abaxial cell identity, whereas ectopic expression of FIL, YAB3, or CRC in leaves and petals causes partial abaxialization (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 2001). YABs also promote abaxial cell identity in other eudicot species, as mutations in GRAMINIFOLIA, the Antirrhinum majus ortholog of Arabidopsis FIL, result in pronounced adaxialization along the abaxial margin of leaves (Golz et al., 2004). In addition to a role in organ polarity, YABs are also thought to promote lamina growth, perhaps by...
mediating communication between the adaxial and abaxial domains of the leaf (Eshed et al., 2004; Golz et al., 2004). Although the precise regulatory relationship between the YABs and the other gene families involved in polarity determination is poorly understood, it is likely that the YABs function as repressors based on their physical and genetic interactions with the Antirrhinum transcriptional corepressor STYLOSA (STY) (Navarro et al., 2004). While the STY ortholog in Arabidopsis, LEUNIG (LUG), is best known for its role in suppressing AGAMOUS expression during flower development (Liu and Meyerowitz, 1995; Conner and Liu, 2000), a recent study found that LUG and its associated coregulator SEUSS (SEU) also promote adaxial-abaxial patterning and growth of petals (Franks et al., 2002, 2006). This raises the possibility that LUG and SEU have a more general role in lateral organ patterning, possibly through interactions with the YABs.

To address this possibility, we characterized both physical and genetic interactions between the vegetatively expressed YABs, LUG, LEUNIG_HOMOLOG (LUH), and SEU. Using bioluminescence resonance energy transfer (BRET) assays, we show that a LUG-YAB complex is formed in planta. Subsequent mutant analysis in conjunction with a microRNA (miRNA)-based approach to suppress AGAMOUS expression revealed that the LUG-YAB complex promotes both adaxial and abaxial cell identity during leaf development. Defects in SAM initiation and maintenance are also observed when the components of this complex are removed. Based on the likely formation of the LUG-YAB complex in the abaxial domain of developing cotyledons and leaves, we propose that this complex regulates multiple signaling pathways that promote both adaxial cell identity in leaves and SAM initiation and maintenance.

RESULTS

Interactions between YAB Proteins and Components of the LUG Complex

Using the yeast two-hybrid system and a plate-based measure of $\alpha$-Gal activity, we observed significant interactions between LUG and three of the four vegetatively expressed YABs (FIL, YAB3, and YAB5) (Table 1). Similar interactions were also observed between these YABs and LUH, which is consistent with LUG and LUH having similar biochemical properties. Subsequent analysis of FIL truncations revealed that the first 109 residues of the protein are required for LUG/LUH interactions (see Supplemental Table 1 online).

Given the importance of the coregulator SEU for LUG function (Franks et al., 2002; Sridhar et al., 2004), we examined whether YABs also interact with SEU in yeast. While DB-FIL fusions could not be tested due to strong autoactivation, both DB-YAB3 and DB-YAB5 induced $\alpha$-Gal expression when coexpressed with AD-SEU. To extend this observation, we looked for interactions between the YABs and the three SEU-like proteins of Arabidopsis (SLK1-3; Franks et al., 2002), which also associate with LUG and LUH (see Supplemental Table 2 online). Consistent with biochemical redundancy among SEU and the SLKs, we find extensive interactions between all three SLKs and the YABs (Table 1), raising the possibility that SEU and SLKs are part of the LUG-YAB complex.

Finally, we examined whether YABs form homodimers and YAB-YAB heterodimers in yeast as previous work has shown that FIL aggregates when expressed in vitro (Kanaya et al., 2002). In each self-self combination tested, a colorimetric change was detected, indicating homodimerization. Similarly, heterodimerization was also observed, although only in some combinations (Table 1).

In Planta Detection of Protein–Protein Interactions

We next used the BRET assay to determine whether protein–protein interactions identified in yeast also occur in planta (Subramanian et al., 2004, 2006). BRET relies on the spectral shift from blue light emission to yellow light emission that occurs when the blue light–emitting Renilla reniformis luciferase (RLUC) is brought into close proximity to the yellow fluorescent protein (YFP). Coexpressing RLUC-LUG and either FIL-YFP or YFP-FIL in onion cells yielded an elevated yellow-to-blue (Y:B) ratio when compared with cells expressing RLUC-LUG and YFP alone, indicating interaction (Figure 1A). Finding that LUG is in close physical contact with FIL supports the hypothesis that LUG is in a complex with the YABs. An elevated Y:B ratio was also detected between RLUC-LUG and the YFP-SLK1 fusion protein, consistent with proposed biochemical redundancy between SEU and the SLKs (Figure 1B).

Interactions were also detected in cells coexpressing FIL-RLUC/YFP-FIL or YAB5-RLUC/YAB5-YFP, confirming that FIL and YAB5 form homodimers. Likewise, detection of interactions between YAB5-RLUC/FIL-YFP and YAB5-RLUC/YFP-FIL is consistent with heterodimerization (Figure 1C).

While elevated Y:B ratios were detected in some protein combinations, others such as those between YAB5 and either SLK1 or SLK2 did not generate elevated ratios (Figures 1A and 1B). While the BRET assay can be a robust technique for

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$\alpha$-gal assay measuring activity of the LacZ reporter in three separate samples. Color change after 4 h (++), color change after 24 h (+), and no color change after 24 h (−).

Table 1. Interactions between the YABs, Corepressors, and Associated Proteins in Yeast
detecting protein–protein interactions in vivo, there are instances where BRET does not occur between interacting proteins (Subramanian et al., 2006), particularly if steric hindrance prevents the RLUC and YFP tags from coming within the critical distance of ~5 nm necessary for efficient energy transfer. Thus, negative BRET results do not rule out the possibility that these proteins interact. In summary, results from BRET assays largely support protein interactions detected in yeast.

lug Mutations Enhances the Vegetative Defects of yab Mutants

To determine the extent to which YAB function is dependent on LUG activity, we looked for synergistic interactions among lug-1, seu-2, and fil-8 yab3-2 mutants. We found that the size of mature fil yab3 lug and fil yab3 seu rosette leaves was noticeably reduced when compared with fil yab3 (Figures 2A to 2D; see Supplemental Table 3 online). As reduced growth was detected soon after leaf emergence (Figures 2E and 2F) and correlated with reduced activity of the cell cycle reporter CYCB<sub>p</sub>-CYCB<sub>β</sub>-GLUCURONIDASE (GUS) (Figures 2G and 2H), we inferred that lug enhances the loss of cell proliferation in fil yab3 plants.

In addition to reduced growth, fil yab3 lug also showed enhanced organ polarity defects. Examining the abaxial surface of fil yab3 lug mutants by scanning electron microscopy revealed larger and less irregular epidermal pavement cells than those of wild-type or fil yab3 leaves (Figures 2I to 2K). Internally, the mesophyll tissue of fil yab3 lug leaves also appeared adaxialized in comparison to wild-type and fil yab3 tissue, as abaxial cells were similar in shape to adaxial cells (Figures 2L to 2N).

Taking advantage of the yab3-2 enhancer trap allele, which has a GUS reporter gene under the control of the YAB3 promoter (Kumaran et al., 2002), we found that YAB3 expression was slightly reduced in fil yab3 leaves compared with morphologically wild-type yab3 leaves, but greatly reduced and abaxially restricted in fil yab3 lug leaves (Figures 2O to 2Q). RNA in situ hybridization also detected an enlarged PHB expression domain in fil yab3 lug leaves relative to the wild type, consistent with adaxialization (Figures 2R and 2S).

Given that LUG is expressed broadly during plant development (Figures 3A and 3C; Conner and Liu, 2000), we next tested whether the observed synergism between fil yab3 and lug mutations is due to a loss of the LUG-YAB complex from the abaxial side of the developing leaf. Using a LUG-targeting artificial miRNA (amiR-LUG; Figures 2T to 2V), we found that either constitutive or abaxial expression of the amiR-LUG in a fil yab3 background produced a fil yab3 lug phenotype (Figures 2W and 2X), indicating that LUG activity in the abaxial domain of developing leaves is important for YAB function.

In addition to these observations, genetic interactions were also observed between lug, fil, and yab3 mutations during flower development, suggesting that the function of the LUG-YAB complex is not restricted to the vegetative organs (see Supplemental Figure 1 online).
Figure 2. Enhancement of fil yab3 Polarity Defects in lug and seu Mutant Backgrounds.

(A) to (D) Sixteen-day-old wild-type (A), fil-8 yab3-2 (B), fil-8 yab3-2 lug-1 (C), and fil-8 yab3-2 seu-2 (D) plants.

(E) and (F) Scanning electron micrographs of fil-8 yab3-2 (E), fil-8 yab3-2 lug-1 (F), and wild-type seedlings showing the first two leaves at an early stage of development.

(G) and (H) Expression of CYCB1;1-GUS reporter in emerging leaves of fil-5 yab3-1 (G) and fil-5 yab3-1 lug-1 (H) mutants.

(I) to (K) Scanning electron micrographs showing the abaxial epidermis of fully expanded wild-type (I), fil-8 yab3-2 (J), and fil-8 yab3-2 lug-1 (K) leaves.

(L) to (N) Transverse sections through fully expanded wild-type (L), fil-8 yab3-2 (M), and fil-8 yab3-2 lug-1 (N) leaves. Arrowheads mark adaxialized spongy mesophyll cells.

(O) to (S) Distribution of YAB3:GUS ([O] to [Q]) and PHB RNA ([R] and [S]) in transverse sections of yab3-2 ([O]), fil-8 yab3-2 ([P]), fil-8 yab3-2 lug-1 ([Q] and [S]), and wild-type ([R]) apices. Asterisks mark the position of the meristem, and arrowhead indicates expanded domain of PHB expression.

(T) lug-444 and wild-type (inset) flowers.

(U) and (V) Representative flowers from 35Spro:amiR-LUG plants displaying an intermediate (U) and strong (V) lug mutant phenotype.

(W) and (X) Representative 16-d-old fil-8 yab3-2 35Spro:amiR-LUG (W) and fil-8 yab3-2 FILpro:amiR-LUG (X) lines.

Bars = 2 mm in (A) to (D), (W), and (X), 1 mm in (E), (F), and (T) to (V), and the inset in (T), 200 μm in (O) to (S), and 100 μm in (G) to (N).
Defects in Leaf Growth and Polarity Are Associated with *lug luh*/+ Mutants

Although LUG physically and genetically interacts with the YABs, there are no obvious leaf polarity defects associated with *lug* mutants. This may reflect redundancy between LUG and LUH, as promoters of both genes exhibited overlapping expression patterns when fused to the reporter GUS (Figures 3A to 3D). For instance, both LUG and LUH expression is detected throughout young developing leaves (Figures 3A and 3B), before being confined to the vasculature of older leaves (Figures 3C and 3D). As both FIL and YAB3 are abaxially restricted, it is likely that the LUG-YAB complex forms in the abaxial domain of developing organs.

We next examined whether loss of both LUG and LUH activity results in leaf polarity defects as might be expected if these proteins regulate the YABs. Using likely null *lug* and *luh* mutant alleles (see Supplemental Table 4 and Supplemental Figure 2 online), we found that *lug luh* double mutants are embryonically lethal (data not shown; Sitaraman et al., 2008). However, leaf growth and patterning defects were apparent in plants homozygous for *lug* and heterozygous for *luh* (*lug luh*/+) (cf. Figures 3E and 3F; see Supplemental Table 3 online; Sitaraman et al., 2008). These defects range from the petiole being displaced to the underside of the leaf to a complete loss of blade growth (Figure 3G, Table 2). Although variable in frequency, the polarity defects are nearly always confined to the first few leaves that arise following germination.

In trumpet-like leaves, disruptions to cell patterning were limited to the midrib, where the normal colateral vascular arrangement was replaced by phloem encircling xylem (Figures 3H and 3I). However, epidermal and mesophyll cell identity was
more severely affected in needle-like leaves (Figures 3J and 3K), although defects in vascular patterning were more variable (Figure 3L). These observations suggest that lug luh/+ leaves are partially abaxialized, an interpretation that is supported by the observed ectopic expression of YAB3:GUS in the adaxial domain of lug luh/+ yab3-2 needle-like leaves (Figure 3M).

**YABs Act Redundantly in Promoting Adaxial Cell Identity**

While the lug luh/+ leaves are partially abaxialized, it is not clear that these defects arise from altered YAB activity. We therefore investigated whether YABs have a broader role in leaf patterning by examining the phenotypes of plants lacking not only FIL and YAB3 activity, but also YAB2 and YAB5. yab2 and yab5 mutants were obtained from the Arabidopsis stock center and their characterization showed that both mutant alleles encode likely nonfunctional proteins (Figure 4A; see Methods). While neither single mutant has noticeable organ polarity defects, yab5 mutants have considerably smaller leaves than the wild type (see Supplemental Table 3 online).

Of the mutant combinations examined, only fil yab3 yab5 triple mutants and fil yab2 yab3 yab5 quadruple mutants displayed significant differences from fil yab3 (see Supplemental Figure 3 online for fil yab2 yab3 mutants; note that when the triple and quadruple mutants showed identical phenotypes, only one of them is shown). For instance, 12% (n = 99) of yab triple mutants and 46% (n = 192) of yab quadruple mutants had three cotyledons (Figure 4B), whereas 9% (n = 192) of quadruple mutants had four cotyledons (Figure 4C). As the vasculature of each cotyledon joins the central vasculature of the hypocotyl, it is likely that the cotyledons have arisen separately during embryogenesis.

Triple and quadruple mutants also had short leaves that are either narrow or completely radialized (Figures 4D and 5E). Internally, spongy mesophyll tissue of narrow leaves extends around the leaf margin into the adaxial domain, and the adaxial palisade mesophyll tissue is centrally confined (Figure 4E). Consistent with abaxialization, phloem is adaxially displaced in some veins (Figure 4F). Surprisingly, vascular patterning is not severely affected in needle leaves, even though these organs lack adaxial/abaxial distinctions in the mesophyll (Figure 4G). Presence of YAB3:GUS staining throughout young needle-like leaves and restriction of PHB expression to the leaf vasculature (Figures 4H and 4I) are also consistent with abaxialization of triple mutant leaves. Similar defects are observed in yab quadruple mutants and thus are not shown. Given these similarities, it is likely that the polarity defects observed in lug luh/+ mutants reflect disruptions in the YAB pathway.

**SAM Defects in Mutant Lines**

yab mutants also display a range of meristem defects, with 10-d-old fil yab3 meristems appearing flatter than wild-type meristems (Figures 5A and 5B). By contrast, SAMs of triple and quadruple yab mutants at a similar age lacked the distinctive anticlinal L2 cell layer that is normally seen in wild-type and fil yab3 apices (Figure 5C shows the quadruple mutant; the triple mutant is identical). Unlike wild-type plants, both triple and quadruple yab mutants form ectopic axillary meristems in the axils of cotyledons (Figure 5D, arrow), which then go on to initiate secondary shoots as the plant matures (observed in 88% of yab quadruple mutants, n = 48; Figure 5E). Consistent with meristem dysfunction, approximately one-third of the primary and secondary shoots become noticeably fasciated (Figure 5E, arrowhead). As well as secondary shoots, additional shoots also arise from the adaxial surface of triple and quadruple yab mutant leaves (Figure 5F, asterisks), a phenomenon observed at low frequency in fil yab3 mutants (Kumaran et al., 2002).

While presence of secondary shoots from cotyledon axils was not a feature of lug luh/+ mutants, SAM defects were also apparent in this line. In comparison to lug mutant apices, which are similar to the wild type, 14-d-old lug luh/+ SAMs were broader and lacked strict anticlinal cell divisions in the L2 layer (Figures 5G and 5H). Consistent with an increased size of lug luh/+ apices, STM expression was expanded when compared with wild-type apices (Figures 5I and 5J) and fasciation often occurred following the transition to flowering (Figures 5K and 5L; lug-444 luh-3/+ 61%, n = 61; lug-444 luh-4/+: 45%, n = 51). Internally, fasciated inflorescences were significantly broader and flatter than lug apices (Figures 5M and 5N).

**Role of the YAB-LUG Pathway in Regulating Early SAM Development**

We reasoned that more profound defects in SAM development were likely to arise if the activity of the LUG-YAB complex was severely compromised. To test this possibility, we generated fil lug luh/+ and fil yab3 lug luh/+ mutant combinations. Despite
moderate linkage between FIL and LUH, both mutant classes were identified at low frequency in segregating populations and, consistent with a greater affect on SAM development, had extremely narrow cotyledons and inactive SAMs (Figure 6A shows the triple mutant). Unlike lug luh/+ apices, which only have defective cell division in the L2 layer of the SAM, divisions in both L1 and L2 cell layers of fil lug luh/+ and fil yab3 lug luh/+ apices were disrupted, resulting in a loss of SAM organization (Figures 6B to 6D). Some cytoplasmically dense cells were still present in these meristems, although they were arranged randomly within the central region of the apex (Figure 6D). As these defects were apparent in 4-d-old meristems, it is likely that they reflect alterations in embryonic SAM formation.

By ~10 d after germination, meristem activity was restored in all fil lug luh/+ and most fil yab3 lug luh/+ apices, and the structure of these reestablished SAMs was indistinguishable from that of lug luh/+ mutants (Figure 5H). Leaves arising from these meristems had more severe growth and polarity defects relative to fil yab3 and lug luh/+ mutants, with many fil yab3 lug luh/+ leaves being radial (Figure 6E). Analysis of the polarity defects in these leaves suggested disruptions to both adaxial and abaxial cell identities (see Supplemental Figure 4 online).

Given the obvious enhancement of SAM defects when mutations in YABs and corepressors mutants are combined, we hypothesized that the LUG-YAB complex regulates SAM development via a signaling pathway active in developing cotyledons and leaves. Consistent with this model, we found coexpression of LUG, LUH, and FIL in developing cotyledons as well as leaves (see Supplemental Figure 5 online). To test this model directly, we examined the effect on SAM development when the activity of both LUG and LUH is removed from developing leaves. Transgenic plants expressing amiR-LUG under the control of the organ-specific AS1 promoter were crossed to luh mutants and their progeny examined for SAM defects. luh AS1pro>amiR-LUG seedlings identified in segregating populations had broad SAMs that produced one or two leaf primordia before becoming inactive (Figure 6F). Internally, the structure of these SAMs was similar to the wild type, although cells lacked the dense cytoplasm that is characteristic feature of actively dividing cells of wild-type meristems (Figure 6G).
finding shows that LUG and LUH function non-cell-autonomously and is consistent with the LUG-YAB complex promoting SAM development via a leaf-based signaling pathway.

Embryonic Defects of seu slk2 Mutants

Further characterization of the embryonic SAM defects of fil lug luh/+ and fil yab3 lug luh/+ mutants was not possible due to rarity of such seeds in siliques segregating for these mutations. To circumvent this problem, we examined whether mutations in other components of the LUG-YAB complex have embryonic SAM defects. We focused on SEU and the SLKs, as yeast two-hybrid assays and expression analyses suggest that these genes may function redundantly (see Supplemental Table 2 online; Figure 7A).

Given that protein alignments and phylogenetic analysis suggested that SLK2 is more closely related to SEU than either SLK1 or SLK3 (Figure 7B), we introgressed slk2 T-DNA mutants
into a seu background (see Methods; see Supplemental Figure 2 online). Consistent with functional redundancy, we found that seu mutants heterozygous for slk2 (called seu slk2+/+) had enhanced floral defects when compared with seu mutants. This included a reduction in floral organ number and size and the formation of prominent stigmatic horns, all characteristic features of lug mutant flowers (Figures 7C to 7E). This phenotypic enhancement shows that SLK2 functions with SEU in LUG-regulated processes during flower development.

Genotyping showed that all seu slk2 double mutants lacked a SAM and had cotyledons that were both reduced in size and frequently fused (Figure 7F). Internally, there was a complete absence of cytoplasmically dense cells and no evidence of SAM formation (Figure 7G). Using differential interference contrast (DIC) optics to follow embryo development in developing seu+/+ slk2 siliques, we found that ~25% of heart-staged embryos had reduced cotyledons and lacked the stereotypical pattern of cell divisions that occur in the apical regions of the phenotypically wild-type slk2 mutant embryos (Figures 7H and 7I). Both the frequency of occurrence and the cotyledon defects suggested that these aberrant embryos are seu slk2 mutants. We next examined expression of the meristem-specific gene CLV3 in seu slk2 embryos by introgressing a CLV3pro:YFP-ER reporter into the seu+/+ slk2 mutant background. Consistent with a lack of SAM formation, we were unable to detect CLV3 expression in heart-stage embryos with a seu slk2 phenotype (Figures 7J and 7K).

Finally, we introduced a constitutively expressed amiRNA that targets LUH (35Spro:amiR-LUH) into a lug+/+ background and found meristemless progeny in population derived from six independent lines. Genotyping confirmed that these seedlings were lug 35Spro:amiR-LUH (Figure 7L). Sections through the apex of these seedlings were similar to those of seu slk2 (Figure 7M) in that there was a complete absence of cytoplasmically dense cells and no evidence of SAM formation, supporting the notion that the LUG-YAB pathway is required for the initiation of embryonic SAM development.

**DISCUSSION**

**YABs Are Part of a Multicomponent Regulatory Complex**

Based on studies in *A. majus*, it has been proposed that the transcriptional corepressor STY regulates YAB activity (Navarro et al., 2004). Using yeast and BRET assays, we show that these interactions are conserved in *Arabidopsis* and thus are likely to be important for YAB function in other eudicots. As well as interacting with the corepressor LUG and related LUH protein, we show that *Arabidopsis* YABs may also interact with the coregulator SEU and related SLKs proteins. Given that SEU is a known component of the LUG complex (Sridhar et al., 2004; Sitaraman et al., 2008) and that SLKs are likely to share this function (this study), we propose that YABs are part of a multicompartment complex that includes a corepressor (LUG or LUH) and a coregulator (SEU or a SLK protein) (Figure 8A). Furthermore, as YABs are also capable of forming homo- or heterodimers, it is probable that this complex includes more than one YAB protein.
Genetic analysis provides support for the formation of a LUG-YAB regulatory complex. For instance, lug luh/+ mutants display polarity and meristem defects that can be explained, at least in part, by disruptions in the YAB pathway. In addition, lug and seu mutants enhance the growth and abaxial polarity defects of fil yab3 leaves. This enhancement becomes more pronounced when fil yab3 mutants are combined with lug luh+, leading to disruptions in embryonic SAM formation. Finally, we show that reduced LUG activity in the abaxial domain of developing fil yab3 leaves causes a fil yab3 lug leaf phenotype. In addition to regulating leaf polarity and meristem activity, our analysis indicates that LUG functions with the YABs to prevent KNOX expression in leaves (see Supplemental Figure 6 online). Taken together, these data make a compelling case for the formation of a LUG-YAB complex in the abaxial domain of developing cotyledons and lateral organs. Based on the vegetative and SAM defects associated with mutations in LUG and LUH, this study has expanded the known functions of the corepressors and further underscored their importance as general regulators of plant development.

**Function of YABs in the LUG Complex**

Although FIL binds DNA in vitro, it displays no sequence specificity (Kanaya et al., 2002). If this observation applies equally to the other YABs, it is unlikely that YABs recruit LUG or LUH to specific sites within the genome. We therefore favor a model in which the LUG-YAB complex is recruited to promoter elements via interactions with transcription factors bound to SEU or SLKs (Figure 8A). What then is the function of YABs within the LUG complex? One possibility is that the YABs recruit factors involved in long-term transcriptional regulation, such as chromatin-modifying enzymes or, alternatively, the YABs might enhance the stability of the LUG-SEU transcription factor complex when
bound to DNA. Current work is aimed at distinguishing between these possibilities.

The YAB Pathway Promotes Adaxial Cell Identity

The absence of PHB expression in the mesophyll tissue of triple yab mutants leaves suggests that these organs have lost adaxial cell identity. This finding, together with previous work showing a minor loss of adaxial cell identity kan1 kan2 mutants leaves (Ha et al., 2007), raises the intriguing possibility that establishment of adaxial cell identity is dependent on the correct specification of abaxial cell identity. Several lines of evidence argue against such a relationship. First, severe loss of abaxial cell identity in leaves of kan triple mutants is not associated with a loss of PHB expression (Eshed et al., 2004). Second, detecting YAB3 expression in yab triple mutants leaves clearly shows that these organs retain abaxial cell identity. Given this, we propose that YABs play a more direct role in promoting adaxial cell identity, although how this is achieved is currently not understood. As adaxial regulators, YABs are unique in being expressed predominantly in the abaxial domain of lateral organs in divergent eudicots (Bowman and Smyth, 1999; Siegfried et al., 1999; Kim et al., 2003; Eshed et al., 2004; Golz et al., 2004). Furthermore, as YAB proteins accumulate abaxially (Navarro et al., 2004; Goldshmidt et al., 2008), we propose that the YABs promote adaxial cell identity non-cell-autonomously (Figure 8B). Such a function is not unprecedented, as previous studies have hinted at such functions during floral development (Álvarez and Smyth, 1999; Bowman and Smyth, 1999; Villanueva et al., 1999; Goldshmidt et al., 2008; Prunet et al., 2008).

Given the redundant role of FIL and YAB3 in promoting abaxial cell identity, it is perhaps surprising that further loss of YAB activity (loss of YAB2, YAB5, or both) does not cause a more pronounced loss of abaxial cell identity. In fact, the only evidence that YAB2 and YAB5 promote abaxial cell identity comes from mild adaxialization observed in fil/+ yab2 yab3 yab5 leaves (see Supplemental Figure 3 online). There are several possible explanations for this observation. First, it may simply be that YAB2 and YAB5 do not promote abaxial cell identity, perhaps due to a loss of this function over evolutionary time. Equally possible is that the abaxial-promoting activity of the YABs is an evolutionary-derived function that has been acquired by FIL/YAB3 but not YAB2 or YAB5. Consistent with YABs originally being adaxial determinants, at least one YAB gene is expressed adaxially in the basal angiosperm Amborella trichopoda (Yamada et al., 2004).

Alternatively, the abaxial function may be masked by factors that act redundantly with the YABs. Candidates include the KANs, which are known to function with the YABs in leaf development (Eshed et al., 2004), and the related ARFs, ETT and ARF4. One way to distinguish between these possibilities is to examine whether YAB2 and YAB5 share the same or different gene targets as those of FIL/YAB3. If the analysis of gene targets is extended to the KANs and ETT/ARF4, it will be possible to determine the extent of redundancy both within the YAB family and between YABs and other polarity determinants.

The Importance of Boundaries for YAB Function

The involvement of the LUG-YAB complex in promoting embryonic SAM development and postembryonic maintenance reflects a further non-cell-autonomous function. This may be related to the recently identified role for short-range YAB signaling in regulating phyllotaxis, primordia growth, and SAM activity (Goldshmidt et al., 2008). Genetic analysis indicates that these functions are largely dependent on LATERAL SUPPRESSOR (LAS), a gene that is expressed at the boundary between lateral organs and the SAM (Greb et al., 2003). The precise relationship between the YABs and the organ boundary is presently not clear, but an attractive scenario is that the YAB signal induces a signaling cascade or secondary messenger upon reaching the organ/SAM boundary (Goldshmidt et al., 2008). Alternatively, the primary function of the YABs may be to promote boundary formation and that the non-cell-autonomous functions ascribed to the YAB signal are in fact those of the boundary. Finding LAS expression is altered in yab mutants and in lines overexpressing FIL is consistent with YABs regulating boundary specification (Goldshmidt et al., 2008). If YABs do indeed perform this function, it might be expected that further disruptions to the LUG-YAB complex might cause phenotypes associated with boundary disruption. The best-studied boundary genes are those belonging
to the **CUP-SHAPED COTYLEDON (CUC)** family of transcription factors; mutations in which cause a loss of embryonic SAM formation and cotyledon fusion (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). Although not a complete phenocopy of **cuc1 cuc2** mutants, presence of fused cotyledons in **seu slk2** mutants, together with the loss of the embryonic SAM, suggests that the LUG-YAB complex may regulate CUC activity. Given that LAS has been placed downstream of the CUCs in the genetic hierarchy regulating axillary meristem formation (Hibara et al., 2006), it is possible that YABs regulate LAS via the CUCs. Future work will therefore need to address the precise regulatory relationship between the LUG-YAB complex and genes involved in boundary formation.

**METHODS**

**Plant Material and Genetics**

*Arabidopsis thaliana* mutants used in this study and their backgrounds are listed (see Supplemental Table 4 online). Where genetic interactions involved a mixed Columbia/Landsberg erecta background, the mutant phenotypes of F2 or F3 plants were shown to be independent of the erecta mutation. Insertion sites for the **lug-444**, **lug-012**, **lugh-4**, **slk2-1**, and **slk2-2** T-DNA insertion alleles were confirmed by PCR using oligonucleotides specific for each line and by sequencing across the insertion site. Genomic DNA spanning the **YAB2** and **YAB5** loci was sequenced to confirm the presence of the ethyl methanesulfonate–induced mutations in **yab2-1** and **yab5-1** plants. The **yab2-1** mutation abolishes the annotated splice acceptor site for the 4th intron, leading to a cryptic site just downstream of the mutation being used for splicing. Sequencing reveals an extra nucleotide the **yab2** mutant transcript, leading to the conserved YABBY DNA binding domain being replaced with 35 unrelated amino acids as a result of the frame shift. Similarly, **yab5-1** is predicted to encode a truncated protein lacking most of the YABBY domain. Double, triple, and higher-order yab mutant combinations were identified in segregating F2 or F3 families and genotyped to confirm identity. Primers and PCR conditions are available upon request.

**Yeast Two-Hybrid Assays**

LUG, LUH, **SEU**, **SLK1-3**, and YAB coding sequences were amplified from vegetative cDNA using a high-fidelity Taq polymerase and cloned into pGBK-T7 and pGAD-T7 (Clontech) vectors. The suffix DB and AD were give to fusions protein produced from pGBK-T7 and pGAD-T7, respectively. FIL truncations were generated by PCR using the FIL cDNA as a template and cloned into pGAD-T7. Constructs were transformed separately into yeast and then mated according to the manufacturer’s instructions (Clontech). Interactions between fusion proteins were assessed by growth and an α-Gal plate-based colorimetric assay according to published protocols. Interactions were assessed after 4, 24, and 36 h.

**BRET Analysis**

Proteins used in the BRET assay were cloned downstream of the 35S promoter and in frame with RLUC or YFP at the N or C terminus. Plasmid DNA containing these fusions constructs were then introduced into adaxial onion epidermal tissue using particle bombardment as described previously (Subramanian et al., 2004, 2006). Following incubation, onion tissue was submerged in 10 μM coelenterazine to excite RLUC and BRET measurements taken with a TD 20/20 luminometer fitted with a dual-color accessory (Turner Designs) using previously described settings (Subramanian et al., 2004, 2006). Expressed fusion proteins showed appropriate RLUC or YFP activity in onion cells and all YFP-tagged proteins localized to the nucleus. The Y:B ratio for each protein RLUC/ YFP combination was calculated from at least three and up to 20 onion samples and statistical analysis performed using a Student’s t test.

**RT-PCR**

RNA was isolated from root, whole seedling, and inflorescence tissue using an RNeasy kit (Qiagen) and cDNA generated from 1 μg RNA using an oligo(dT) primer with Superscript III reverse transcriptase (Invitrogen). The sequences of primers used to amplify cDNA are listed (see Supplemental Table 5 online). All cDNAs were amplified using a standard PCR cycle. Due to sequence identity, **SLK1** and **SLK3** PCR products are amplified by the same set of primers. Presence of an Hpal site in the **SLK1** cDNA sequence, however, allows these PCR products to be distinguished following a restriction digest.

**Microscopy**

Scanning electron microscopy and in situ hybridization were performed as described previously (Golz et al., 2004). Digoxigenin-labeled probes were transcribed from a PCR fragment containing a portion of the LUH, LUH, STM, PHB, or FIL cDNA (details available upon request). Histological sections (2 or 4 μm) were made from gluteraldehyde-fixed material embedded in LR-white resin and stained with Toluidine Blue. GUS staining was performed by briefly fixing tissue in 90% acetone and then incubating tissue overnight in a 50 mM phosphate buffer containing 2 mM X-Gluc and a mixture of 3 mM potassium ferricyanide and ferrocyanide at 37°C. Tissue was either examined as whole mounts in 70% ethanol or embedded in Paraplast Plus, before being sectioned at 8 μm and viewed under DIC optics. Embryos within developing seeds were observed under DIC optics following treatment with Hoyer’s solution. For fluorescence microscopy, developing seeds were placed in a 10% glycerol solution and pressure applied to force the embryo out of the seed.

**Construction of Transgenic Plants Expressing amiRNAs**

Primers for amiRNAs targeting LUG and LUH mRNA were designed according to WMD2 Web MicroRNA Designer (http://wmd2.weigelworld.org; Schwab et al., 2006), **amiR-LUG** (targets 2443 to 2463 bp of the LUG mRNA) and **amiR-LUH** (targets 437 to 457 bp of the LUH mRNA) were generated by sequential PCR using **miR-319a** as a template and subsequently placed downstream of the 35S promoter in pART7 (Gleave, 1992). The 35S promoter amiRNA cassette was subcloned into pMLBART and introduced into Arabidopsis plants (Columbia background) using an Agrobacterium floral dip procedure (Clough and Bent, 1998) and identified following treatment with BASTA. Plants were either grown on soil or on 0.5× Murashige and Skoog media in a growth room at 18°C or growth cabinet kept at 21°C under lights for 8 h (short days) or 16 h (long days).
Arabidopsis lines expressing AS1pro:LhG4. This combination was called AS1pro::amiR-LUG.

Phylogenetic Analysis

Inferred protein sequence was aligned using Clustal software and then manually adjusted to optimize alignments. PAUP software was then used to generate an unrooted phylogeny using the neighbor-joining method (Saitou and Nei, 1987) and nodes of the tree supported by bootstrap values generated from 1000 trials.

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ACCESSION NUMBERS

Sequence data of genes described in this article can be found in the Arabidopsis Genome Initiative library under the following accession numbers: FIL (NM_130082, At2g45190), LUG (NM_119407, At4g32551), LUH (NM_128829, At2g32700), SEU (NM_103511, At1g43850), SLK1 (At4g25520), SLK2 (NM_125602, At5g62090), SLK3 (At4g25515), YAB2 (At1g08465), YAB3 (NM_116235, At4g00180), and YAB5 (NM_128215, At2g26580). T-DNA insertion alleles and tilting mutant alleles described in this article were obtained from the ABRC using the following identifiers: SALK_126444 (lug-444), SALK_113012 (lug-012), SALK_097509 (luh-4), SALK_089954 (slk2-1), SALK_038662 (slk2-2), CS93680 (yab2-1), and CS90062 (yab5-1).

Supplemental Data

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

Supplemental Figure 1. Floral Phenotypes of fil lug and fil yab3 lug Mutants.

Supplemental Figure 2. Vegetative Phenotypes of yab Mutants.

Supplemental Figure 3. Polarity Defects of fil yab3 lug luh/+ Leaves.

Supplemental Figure 4. RNA in Situ Hybridization Showing the Pattern of LUG and LUH Expression in Developing Embryos.

Supplemental Figure 5. Structure of T-DNA Insertion Alleles Used in This Study.

Supplemental Figure 6. Ectopic KNOX Expression in Mutants.

Supplemental Table 1. Defining the Dimerization Domain of FIL.

Supplemental Table 2. Interactions between SEU, SLK, and the Corepressors LUG and LUH in Yeast.

Supplemental Table 3. Dimensions of Wild-Type and Mutant Leaves.

Supplemental Table 4. Plant Material Used in This Study.

Supplemental Table 5. RT-PCR Primers Used in This Study.

Supplemental Data Set 1. Text File of the Alignment Used for the Phylogenetic Analysis Shown in Figure 7B.

Arabidopsis thaliana is reduced by EDTA. J. Biol. Chem. 277: 11957–11964.
YABBYs and the Transcriptional Corepressors LEUNIG and LEUNIG_HOMOLOG Maintain Leaf Polarity and Meristem Activity in Arabidopsis
Melissa I. Stahle, Janine Kuehlich, Lindsay Staron, Albrecht G. von Arnim and John F. Golz

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