Arabidopsis LATERAL ORGAN BOUNDARIES negatively regulates brassinosteroid accumulation to limit growth in organ boundaries

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Leaves and flowers begin life as outgrowths from the edges of shoot apical meristems. Stem cell divisions in the meristem center replenish cells that are incorporated into organ primordia at the meristem periphery and leave the meristem. Organ boundaries, regions of limited growth that separate forming organs from the meristem, serve to isolate these two domains and are critical for coordination of organogenesis and meristem maintenance. Boundary formation and maintenance are poorly understood processes, despite the identification of a number of boundary-specific transcription factors. Here we provide genetic and biochemical evidence that the *Arabidopsis thaliana* transcription factor LATERAL ORGAN BOUNDARIES (LOB) negatively regulates accumulation of the plant steroid hormone brassinosteroid (BR) in organ boundaries. We found that ectopic expression of *LOB* results in reduced BR responses. We identified *BAS1*, which encodes a BR-inactivating enzyme, as a direct target of LOB transcriptional activation. Loss-of-function *lob* mutants exhibit organ fusions, and this phenotype is suppressed by expression of *BAS1* under the *LOB* promoter, indicating that BR hyperaccumulation contributes to the *lob* mutant phenotype. In addition, *LOB* expression is BR regulated; therefore, LOB and BR form a feedback loop to modulate local BR accumulation in organ boundaries to limit growth in the boundary domain.

Leaves and flowers are produced from the periphery of the shoot apical meristem, a self-perpetuating structure containing a population of self-renewing stem cells. Stem cell divisions in the meristem center replenish the cells that are incorporated into organ primordia at the meristem periphery and exit the meristem (1). The balance between organogenesis and meristem maintenance is essential for continued organ formation, and the boundary between the meristem and organ primordia plays a key role in maintaining the integrity of the meristem and differentiating organs. Boundary cells are small and divide infrequently relative to cells in the adjacent regions; thus, the boundary is a discrete domain that is distinct from the meristem and organ primordia (2–4). During organ formation, inhibition of growth in the boundary allows formation of a cleft, which results in separation of the forming organ from the meristem. A number of boundary-specific transcription factors in several families act redundantly to specify organ boundary cell fate and meristem maintenance (5–11). Few targets of boundary-specific transcription factors have been identified, and little is known about the physiological and biochemical processes they regulate.

Arabidopsis LATERAL ORGAN BOUNDARIES (*LOB*) encodes a member of the plant-specific LOB-domain transcription factor family and is expressed specifically in organ boundaries (12). To investigate the developmental function of *LOB*, we examined the consequence of increased and decreased LOB activity and used expression profiling to identify targets of LOB transcriptional regulation. We show that LOB negatively regulates accumulation of the plant steroid hormone brassinosteroid (BR) in organ boundaries. Loss-of-function *lob* mutants exhibit organ fusions, whereas ectopic expression of *LOB* results in reduced BR responses. Microarray analyses demonstrate that LOB regulates expression of *BAS1*, which encodes a BR-inactivating enzyme, and we demonstrate that BR hyperaccumulation contributes to the *lob* mutant phenotype. In addition, *LOB* expression is BR regulated; therefore, LOB and BR form a feedback loop to modulate local BR accumulation to limit growth in the boundary domain.

Results

Loss-of-Function *lob* Mutants Exhibit Organ Separation Defects. *lob* mutants exhibited normal seedling and vegetative development as previously reported (12). Inspection of cauline leaf axils, a domain where *LOB* is expressed (Fig. 1*A*), revealed that wild-type axillary and accessory branches were well separated from the subtending cauline leaf (Fig. 1*B*), whereas these structures were fused in loss-of-function *lob::DsE* mutant plants (Fig. 1*C*). Three different loss-of-function *lob* alleles displayed similar fusion phenotypes (Fig. 1*F*; Fig. S1). The fusion in *lob::DsE* was significantly more severe than in the other alleles, possibly because of the presence of modifying loci in the L*er* ecotype. Introduction of the wild-type *LOB* gene complemented the *lob::DsE* mutation (Fig. S1*G*), demonstrating that the observed fusion was the result of defects in LOB activity.

In cross sections of cauline leaf axils, wild-type cauline leaves and axillary stems were completely separated (Fig. 1*D*), whereas axillary stem and cauline leaf tissues appeared as a single structure in *lob::DsE* mutants, with continuity between cortex and vascular tissues in the stem and leaf (Fig. 1*E*). Cells in the fused region appeared larger than in wild type. We examined expression of boundary markers *LATERAL ORGAN FUSION1* (11) and *ORGAN BOUNDARY1* (13) in *lob* mutants and observed expansion of *beta-glucuronidase (GUS)* reporter gene expression into the fused region (Fig. 1 *G–J*). Together these results suggest that *lob* mutants have an expansion or overgrowth of the boundary that results in defects in organ separation.

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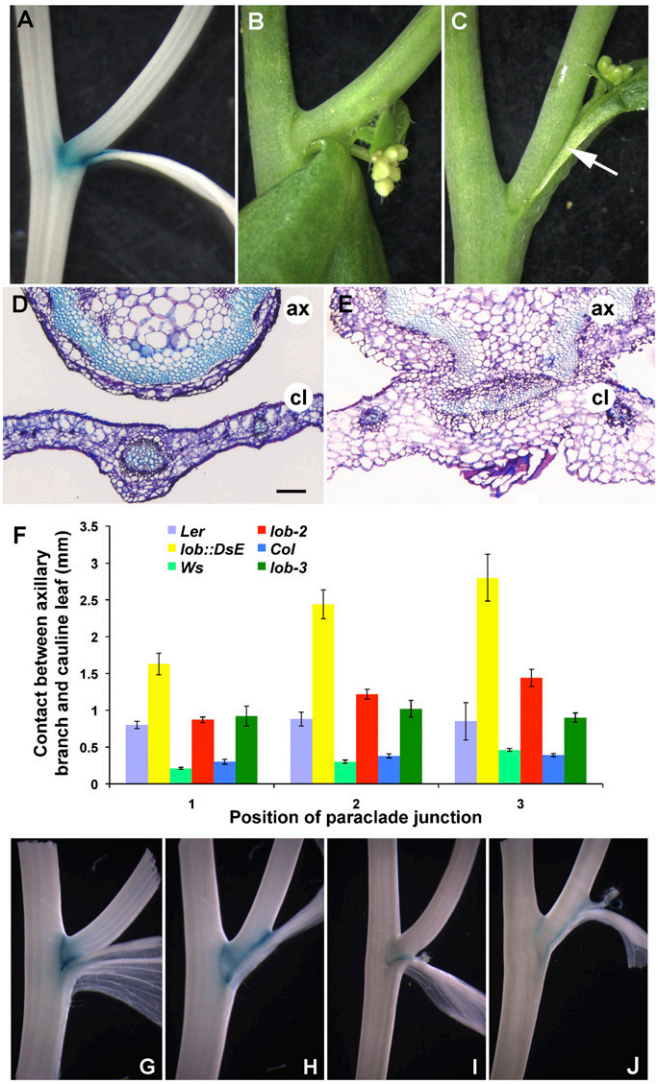


Fig. 1. Mutations in *LOB* result in organ fusion. (*A*) GUS activity in *pLOB:GUS* between the main stem and axillary stem and between the axillary stem and cauline leaf. (*B* and *C*) Paraclade junction between main stem, axillary stem, and cauline leaf in wild-type (*B*) and *lob::DsE* (*C*) plants. The axillary stem and cauline leaf are separated in wild type and fused in *lob::DsE* (white arrow). (*D* and *E*) Cross sections through junction between axillary stem (ax) and cauline leaf (cl) in wild type (*D*) and *lob::DsE* (*E*). (*F*) Length of fused region in wild-type Landsberg *erecta*, *lob::DsE*, wild-type Wassilewskija, *lob-2,* wild-type Columbia, and *lob-3*. Position 1 corresponds to lowest cauline leaf axil on stem. SEs are indicated; $n \geq 11$ for positions 1 and 2, and $n \geq 5$ for position 3 (not all plants have three paraclade junctions). (*G–J*) *GUS* expression in boundary marker lines ET4016 (11) (*G* and *H*) and GT185 (34) (*I* and *J*) in wild type (*G* and *I*) and *lob* (*H* and *J*). ET4016 reports expression of *LOF1* (11) and GT185 reports expression of *ORGAN BOUNDARY1* (13). Expression of both markers is extended throughout the fused region in *lob* mutants, indicating an expansion or overgrowth of the boundary domain. (Scale bar in *D,* 100 μm for *D* and *E*.)

Ectopic *LOB* Expression Affects BR Responses. To identify pathways downstream of LOB activity, we constructed an inducible form of LOB, *35S:LOB-GR,* generated by fusing LOB to the hormone-binding domain of the glucocorticoid receptor (14), under control of the ubiquitously expressed *35S* promoter. Treatment of *35S:LOB-GR* plants with the synthetic steroid dexamethasone (DEX) resulted in an overall reduction in growth in a dose-dependent manner, whereas in the absence of DEX, these plants were phenotypically normal (Fig. 2*A*). *35S:LOB-GR* plants grown in the presence of 3 μM DEX resembled *35S:LOB* plants, which are dwarf and sterile (12).

Ectopic *LOB* expression resulted in reduced growth, similar to that caused by defects in BR accumulation or response. BRs are plant steroid hormones that regulate cell expansion and cell division (15). Because the boundary cells where *LOB* is expressed undergo limited division and expansion (2, 3), we considered the possibility that LOB activity might limit growth in organ boundaries by regulating BR responses. We examined a number of BR responses in *35S:LOB-GR* plants grown in the presence or absence of DEX. Dark-grown *Arabidopsis* seedlings produce elongated hypocotyls and form an apical hook, developmental events that require BR signaling (15). When germinated in the dark, DEX-treated *35S:LOB-GR* seedlings failed to make an apical hook (Fig. 2*B*) and had shorter hypocotyls than seedlings grown in the absence of DEX (Fig. 2*D*). Germination on medium containing epi-brassinolide (BL), a biologically active BR, did not restore hook formation or cause cotyledon expansion as it did in non–DEX-induced plants (Fig. 2*C*). Furthermore, germination on increasing concentrations of BL resulted in an increase in hypocotyl length in dark-grown *35S:LOB-GR* seedlings on DEX, whereas mock-treated seedlings exhibited inhibition of hypocotyl

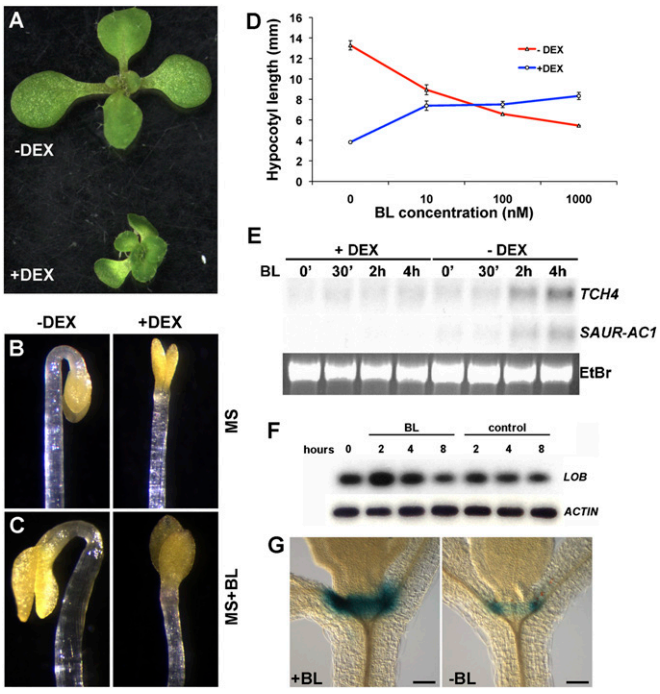


Fig. 2. Ectopic *LOB* expression disrupts brassinosteroid responses. (*A*) *35S:LOB-GR* plants are dwarfed when grown on 3 μM DEX. (*B* and *C*) Dark-grown, 4-d-old *35S:LOB-GR* seedlings produce an apical hook when grown on MS medium in the absence of DEX (–DEX) and lack an apical hook when grown in the presence of 3 μM DEX (+DEX). Apical hook formation is not restored by addition of 100 nM epi-brassinolide in the medium (*C*; MS+BL). (*D*) Hypocotyl lengths of *35S:LOB-GR* seedlings grown in the dark on increasing concentrations of epi-brassinolide (BL) in the presence or absence of 3 μM DEX. SEs ($n \geq 15$) are indicated. (*E*) Northern blot analyses of *TCH4* and *SAUR-AC1* transcripts. Seven-day-old *35S:LOB-GR* seedlings were pretreated overnight in the presence or absence of 3 μM DEX and then incubated in the presence of 1 μM epi-brassinolide (BL) for the indicated times. (*F*) RT-PCR analysis of *LOB* transcript levels in 7-d-old wild-type seedlings following treatment with 1 μM epi-brassinolide (BL) for 2, 4, or 8 h. RT-PCR products were detected by blotting and probing with gene-specific probes, following either 15 (*LOB*) or 12 cycles (*ACTIN*) of amplification. (*G*) GUS activity in *pLOB:GUS:LOB-3′IGR* seedlings after 3-h incubation in liquid MS supplemented with (*Left*) or without (*Right*) 1 μM BL. (Scale bar in *G* and *H*, 100 μm.)

growth (Fig. 2*D*), similar to that reported for wild type (16). Apical hook formation was not restored by treatment with the phytohormones gibberellic acid (GA), auxin (IAA), or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Fig. S2). DEX treatment of *35S:LOB-GR* plants also resulted in a diminished induction of the BR-response genes *TCH4* and *SAUR-AC1* (Fig. 2*E*). Thus, ectopic LOB activity resulted in reduced sensitivity to BR.

To examine the contribution of reduced BR responses to the *35S:LOB-GR* phenotype, we crossed *35S:LOB-GR* plants to *bzr1-1d*, a mutant that exhibits constitutive BR responses due to the stabilization of BZR1, a positive regulator of BR signaling (17). Whereas dark-grown *35S:LOB-GR bzr1-1d* seedlings lacked an apical hook when grown on DEX, similar to *35S:LOB-GR* plants, their hypocotyls were longer than DEX-grown *35S:LOB-GR* seedlings (Fig. S3 *A–F*). Light-grown *35S:LOB-GR bzr1-1d* plants were also larger than *35S:LOB-GR* plants grown on DEX (Fig. S3 *G–I*), indicating that the *bzr1-1d* mutation partially suppressed the growth defects of *35S:LOB-GR* plants. That some aspects of the *LOB* misexpression phenotype were ameliorated by increased BR signaling indicates that reduced BR responses contribute to the phenotype and are consistent with *LOB* acting upstream of *BZR1*.

To further examine the relationship between *LOB* and BR signaling, we investigated the possible regulation of *LOB* expression by BRs. Treatment of seedlings with exogenous BRs resulted in a transient increase in *LOB* transcript levels (Fig. 2*F*). BR treatment also caused an increase in the intensity of GUS staining in *pLOB:GUS* seedlings; however, no change in the staining pattern was seen (Fig. 2*G*), indicating that BR influences the level of *LOB* expression in boundaries.

Identification of LOB-Responsive Transcripts. We performed microarray experiments to identify genes that were differentially expressed in response to LOB activation. *p35S:LOB-GR* and wild-type Col-0 seedlings were exposed to DEX or were mock treated for 4 h, and RNA samples from three biological replicates per treatment were hybridized to Affymetrix ATH1 arrays. Following statistical analyses, we identified genes that were differentially expressed in DEX-treated *p35S:LOB-GR* plants compared with mock treated and not differentially expressed in Col-0 DEX-treated plants compared with mock treated. A total of 288 unique transcripts showed significant changes in response to LOB activation (fold-change $\geq$2; false discovery rate $\leq$0.001) (Dataset S1). Differentially expressed genes may be directly or indirectly regulated by LOB activity. LOB and related proteins were shown to bind in vitro to a 6-bp consensus sequence GCGGCG termed the LBD motif (18). Partial LBD motifs (missing the first or last G: GCGGC or CGGCG) are present nearby or within a majority (269/288) of the LOB-regulated genes; therefore, some may be direct LOB targets.

Strikingly, about 60% (175) of the LOB-regulated transcripts were BR modulated in one or more experiments (reviewed in ref. 19), consistent with the hypothesis that LOB contributes to regulation of BR responses (Dataset S1). LOB-regulated genes were also enriched in Gene Ontology (GO) term categories associated with various stimulus responses and cell wall modifications (Dataset S2).

To investigate the significance of LOB regulation of BR-response genes, we characterized one such gene in detail. *PHYB ACTIVATION TAGGED SUPPRESSOR1* (*BAS1;* At2g26710) encodes a cytochrome P450 enzyme that inactivates BRs by C-26 hydroxylation (20) and showed a 9.3-fold increase in transcript abundance in response to DEX induction. In a time-course experiment, an increase in *BAS1* transcript levels was observed within 60 min of LOB-GR activation by DEX (Fig. S4). *BAS1* transcripts were elevated by DEX treatment in the presence of the protein synthesis inhibitor cycloheximide (Fig. 3*A*), indicating likely direct regulation by LOB. Additionally, *BAS1* transcript levels were reduced in *lob* mutants (Fig. 3*B*), consistent with LOB regulation of *BAS1* expression.

LOB Associates with the *BAS1* Promoter In Vivo and In Vitro. Examination of the *BAS1* sequence revealed four potential LBD motifs. Two partial sites, separated by 5 bp and in inverted orientation relative to one another, were located 306 nucleotides upstream of the *BAS1* ATG, and two sites were located in the third exon (Fig. 3*C*). To determine whether LOB binds to these sites in vivo, we examined the chromatin fragments that immunoprecipitated with a LOB antibody in DEX- and mock-treated *35S:LOB-GR* seedlings. In DEX-treated compared with mock-treated samples, we detected enrichment of a fragment spanning the LBD motifs in the *BAS1* promoter (Fig. 3*D*, region A). A lower level of enrichment was observed for a fragment spanning the binding sites in exon 3 (Fig. 3*D*, region B). No enrichment of a control fragment 3′ to the *BAS1* coding sequence (CDS) that lacked LOB binding sites was detected (Fig. 3*D*, region C). Using EMSAs, we demonstrated that the DNA-binding LOB domain (LD) (18) bound specifically to a fragment from the *BAS1* promoter that contained the two partial LBD motifs (Fig. 3*E*). LD did not bind to fragments in which the two central G residues in either site were mutated to A residues (Fig. 3*E*, lanes 5 and 6), nor did mutated fragments efficiently compete for LD binding to the wild-type fragments (Fig. 3*E*, lanes 9 and 10). Thus, both sites are required for LD binding to the *BAS1* promoter, consistent with the finding that LOB binds DNA as a homodimer (18). Taken together, these data indicate that LOB associates with LBD motifs in the *BAS1* gene to directly regulate *BAS1* expression.

***BAS1* and LOB Have Overlapping Expression Patterns.** If *BAS1* is a direct target of LOB transcriptional regulation, then *BAS1* and *LOB* expression should partially overlap. *BAS1* expression was previously shown to be light regulated (21), and publicly available microarray data indicated that *BAS1* transcripts were enriched in shoot apices, roots, and immature seeds (22, 23). To characterize *BAS1* expression in more detail, we examined expression in transgenic plants carrying a *pBAS1:BAS1-GUS* reporter construct. *pBAS1:BAS1-GUS* expression was observed in the basal region of young leaves and in developing seeds (Fig. S5 *A* and *B*), consistent with previously reported microarray data (22, 23). In addition, *pBAS1:BAS1-GUS* expression was detected in the boundary between primary and axillary shoots and weakly between axillary shoots and cauline leaves (Fig. S5*C*). Thus, expression of *BAS1* and *LOB* overlaps in boundary regions, consistent with LOB functioning in regulation of *BAS1* expression.

***BAS1* Suppresses Organ Fusion in *lob* Mutants When Expressed in Organ Boundaries.** Given that *BAS1* is a direct target of LOB transcriptional regulation, *35S:LOB-GR* plants are predicted to ectopically express *BAS1*. The reduced BR sensitivity in *LOB-GR* plants is consistent with an increase in BAS1 activity. If LOB regulates *BAS1* expression in organ boundaries, we hypothesized that reduction in *BAS1* expression in the *lob* mutant may contribute to the fusion phenotype. To test this, we expressed *BAS1* under control of the *LOB* promoter in wild-type and *lob-3* mutant plants. In a wild-type background, *pLOB:BAS1* plants produced longer pedicels but were otherwise morphologically normal. In the *lob* mutant background, the fusion between cauline leaves and axillary stems was suppressed by the *pLOB:BAS1* construct (Fig. 4). Thus, expression of *BAS1* in the *LOB* domain is sufficient to rescue the *lob* mutant phenotype, indicating that enhanced BR signaling in *lob* mutants results in organ fusion. *bas1* loss-of-function mutants do not exhibit fusion defects (24); therefore, reduction in *BAS1* activity is not the sole cause of fusion in *lob* mutants. Nearly 300 genes were differentially expressed following DEX induction of *35S:LOB-GR* plants, many of them also BR

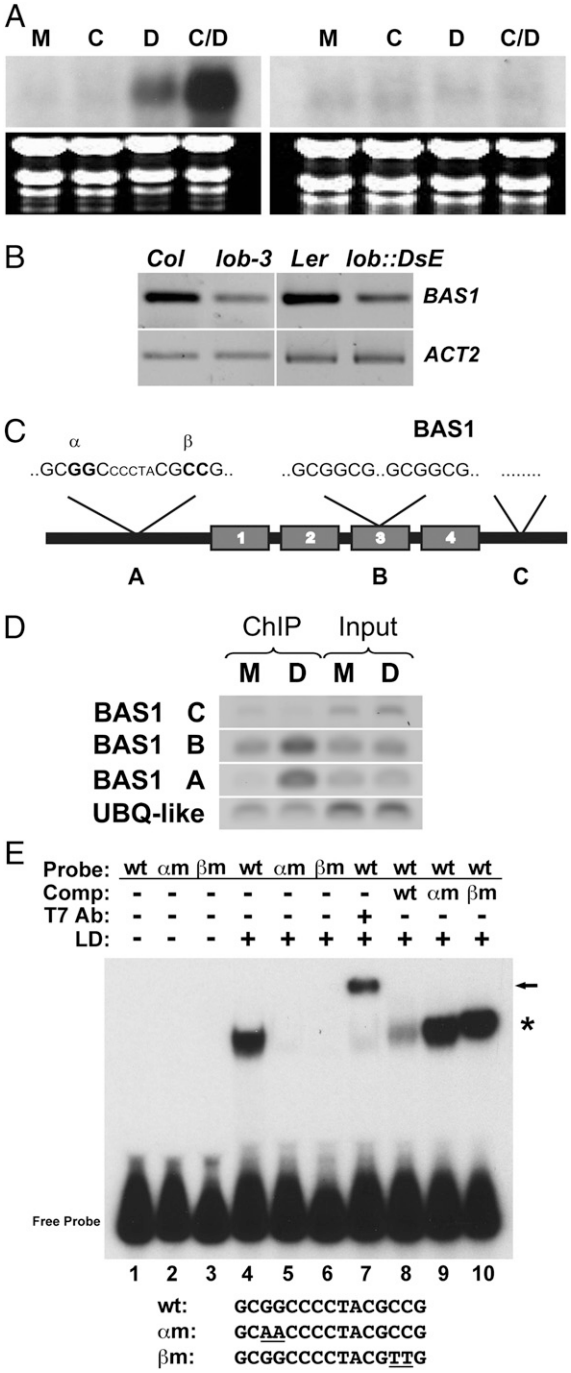


Fig. 3. *BAS1* is a direct target of LOB. (*A*) Northern blot analyses of *BAS1* transcript levels in *35S:LOB-GR* (*Left*) and Columbia wild-type (*Right*) 8-d-old seedlings following 4-h mock (M), cycloheximide (C), DEX (D), or cycloheximide plus DEX (C/D) treatment. (*B*) RT-PCR analysis of *BAS1* transcript levels in dissected cauline leaf-axillary stem junctions of Col, *lob-3*, Ler, and *lob::DsE*. RT-PCR products were detected by blotting and probing with gene-specific probes, following either 15 (*BAS1*) or 12 cycles (*ACT2*) of amplification. (*C*) Cartoon of the genomic structure of *BAS1* showing the locations of LBD motifs and regions tested for enrichment after ChIP. *BAS1 A* contained two partial LBD sites separated by five nucleotides, 306 bp upstream of the ATG. *BAS1 B* contained two full LBD sites separated by 46 nucleotides, 1,700 bp downstream of the ATG in the third exon. The *BAS1 C* region contained no LBD sites and was 2,900 bp downstream of the ATG. (*D*) PCR products were amplified from DNA obtained before (Input) and after (ChIP) collection of specific LOB-DNA complexes by a LOB 1° antibody. DEX (D) and mock-treated (M) *35S:LOB-GR* plants were used in ChIP experiments. *BAS1 A* and *B* regions were amplified for 27 cycles, and *BAS1 C* was amplified for 38 cycles. Thirty cycles of amplification were performed with the control gene *UBQ-LIKE* (*At3g26980*). (*E*) The LOB domain (LD) of LOB was incubated with a 133-bp radiolabeled probe generated from the *BAS1 A* region and separated on a native polyacrylamide gel. Probes contained unmodified LBD motifs (wt; lanes 1, 4, and 7–10), a mutation in the 5′-most motif in which the central GG residues were mutated to AA (αm; lanes 2 and 5), or a mutation in the 3′-most motif in which the central GG residues were mutated to AA (βm; lanes 3 and 6). Inclusion of T7 Ab against the tag on LD results in a supershift, demonstrating that recombinant LD protein bound the wild-type probe (lane 7). An excess of cold wild-type αm or βm DNA was used in competition experiments to demonstrate specificity of binding (lanes 8–10). The sequence of the central motif in the wt and mutant probes is shown.

regulated (Dataset S1). Boundary defects in *lob* mutants likely result from alteration in expression of a suite of genes.

Discussion

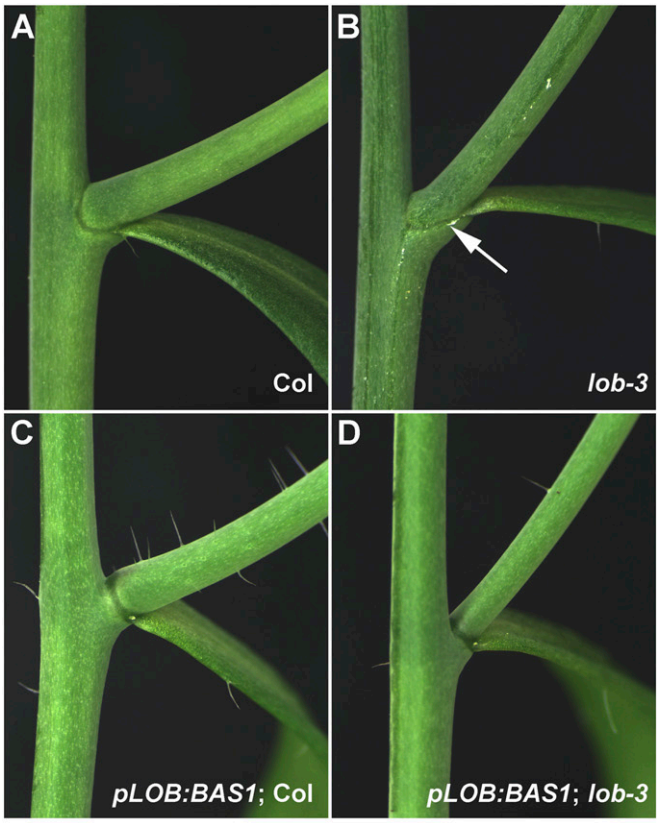
The establishment and maintenance of adjacent populations of cells with distinctly different cell fates are critical problems in developmental biology. Boundaries between domains play an important role in this process, but the mechanisms controlling boundary formation are relatively poorly understood (25). In plants, cells in the boundary between the meristem and organ primordia are small and divide infrequently. Reduced growth in the boundary allows the forming organ to separate from the meristem. Organ boundaries also play a role in meristem maintenance and are the site of axillary meristem formation; therefore, they contribute to the regulation of overall plant form (4). Despite the identification of a number of boundary-specific transcription factors (5–12), an understanding of the pathways they regulate to specify and maintain boundaries has not been developed.

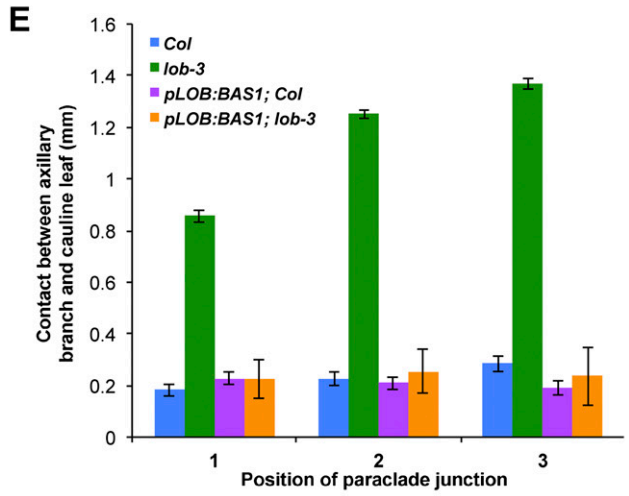
Here we show that the *Arabidopsis* transcription factor LOB negatively regulates accumulation of BR in organ boundaries. Our results show that 60% of LOB-regulated genes are also regulated by BR. Furthermore, LOB and BR signaling form a feedback loop involving BR regulation of LOB accumulation and LOB repression of BR accumulation, which functions to limit growth in organ boundaries (Fig. S6). Loss of LOB function results in overgrowth of the boundary region and organ fusions, demonstrating the importance of regulating growth in this domain. Similar fusion defects have been observed in other BR signaling mutants such as *bzr1-d* (26). In addition, Gendron et al. (26) reported that a BZR1-YFP fusion protein accumulated to low levels in the boundary region compared with the adjacent meristem and primordia, consistent with reduced BR levels in these cells. Moreover, 60% (106) of the 175 genes that are regulated by both LOB and BR are putative BZR1 targets (Dataset S1), raising the possibility that the combined action of LOB and BZR1 influence the expression of a subset of BR-regulated genes.

BRs have long been known to have important functions in plant growth, primarily by promoting cell expansion (15), and have recently been implicated in cell cycle regulation (27–29). Our findings indicate that reduced local BR accumulation and response are critical for patterning shoot architecture. Formation of a region of reduced auxin accumulation has recently been reported to be important for specification of valve margins in the *Arabidopsis* fruit (30), indicating that the formation of local hormone minima may be a general mechanism for organ patterning in plants.

Materials and Methods

Plant Material, Growth Conditions, and Transformation. *Arabidopsis thaliana* plants were grown under standard conditions as previously described (12). Binary T-DNA vectors were introduced into *Agrobacterium tumefaciens* GV3101, and *Arabidopsis* plants were transformed by floral dip (31). Transformed plants were selected on Murashige and Skoog medium (32) supplemented with 50 μM





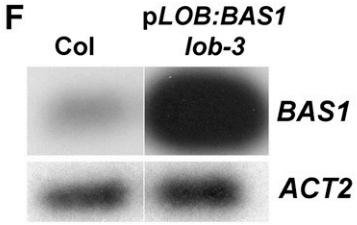


Fig. 4. Expression of *BAS1* under the *LOB* promoter suppresses fusion in the *lob* mutant. (*A*–*D*) Cauline leaf/axillary stem junctions of Columbia wild type (*A*), *lob-3* (*B*), transgenic *pLOB:BAS1* in Col (*C*), and *pLOB:BAS1* in *lob-3* (*D*). Arrow in *B* indicates fused region. (*E*) Length of fused region in Col, *lob-3*, *pLOB:BAS1* Col, and *pLOB:BAS1 lob-3*. Position 1 corresponds to lowest cauline leaf axil on stem. Expression of *pLOB:BAS1* suppresses the fusion in *lob-3*. SEs ($n \geq 10$) are indicated. (*F*) RT-PCR analysis of *BAS1* transcript levels in isolated paraclade junctions of Col and *pLOB:BAS1 lob-3* plants. RT-PCR products were detected by blotting and probing with gene-specific probes, following either 15 (*BAS1*) or 12 cycles (*ACT2*) of amplification.

kanamycin or 50 μM phosphinothricin. *lob::DsE* and *lob-2* have been previously described (12); *lob-3* (SALK_042599) is from the Salk T-DNA collection and is in Columbia-0 (33). Boundary marker lines ET4016 (11) and GT185 (34) are in the Landsberg *erecta* background.

Phenotypic Analyses. BR-response assays were conducted on seedlings grown vertically in the dark for 4–7 d on MS medium with or without 3 μM DEX and in the presence of variable concentrations of BL. Hormone concentrations were as follows: BL, 1 nM to 2 μM; IAA, 1 μM; GA_3, 10 μM; ACC, 20 μM. Hypocotyls were measured using MCID Elite 7.0 software (Imaging Research). Measurements of contact length between stems and cauline leaf were made using a digimatic caliper (model 700-113; Mitutoyo).

Constructs. The *LOB* CDS was amplified with primers that contained introduced restriction sites and subcloned into pBIΔGR (35) to generate the *35S:LOB-GR* construct with GR fused in frame to the C terminus of LOB. To construct *pLOB:GUS:3′IGR*, the 2,557-bp intergenic region 3′ to the *LOB* stop codon was amplified from genomic DNA using primers LOButrF and LOBigrR, which contained introduced XbaI and PstI restriction sites (Table S1). The amplified fragment replaced the 3′ Octopine synthase terminator in *pLOB5.0:GUS* (12). To construct *pLOB:LOB:3′IGR* and *pLOB:BAS1:3′IGR,* the *GUS* CDS was replaced with the *LOB* or *BAS1* CDS. The *pBAS1:BAS1-GUS* construct was modified from a previously described construct that contained a shorter *BAS1* promoter (21). *pBAS1:BAS1-GUS* contains 6,084 bp of genomic DNA upstream of the *BAS1* ATG, and the *BAS1* gene, including introns, fused, in frame, to *GUS*.

Expression Analysis. Total RNA was isolated with TRIzol reagent. RT-PCR was performed as described previously (36). RNA gel blot hybridizations were performed as previously described (37) using gene-specific *BAS1*, *TCH4,* and *SAUR-AC1* probes. Primers and amplification conditions for *ACT2* and *LOB* were as described previously (12). Primers for *BAS1* amplification are shown in Table S1. For DEX treatment in the presence of cycloheximide, DEX was used at 5 μM, and cycloheximide was used at 10 μM.

Histology and Microscopy. GUS histochemical staining and image capture were performed as previously described (34). Cross sections were performed as previously described (11). Images were captured as previously described (36).

Microarray Experiment. Nine-day-old *35S:LOB-GR* and Col-0 seedlings, grown on MS plates, were flooded with MS medium containing 5 μM DEX or a mock solution and incubated, with shaking, for 4 h. Three independent biological replicates were performed for each treatment. Total RNA was isolated using TRIzol, followed by purification over RNeasy columns (Qiagen). Labeling and hybridization to *Arabidopsis* ATH1 GeneChips were done at the University of California–Riverside (UCR) Core Instrumentation Facility following the manufacturer's instructions (Affymetrix). Data were analyzed in R using Bioconductor packages. The Affy package was used for robust multi-array average normalization, and differentially expressed genes were identified using the linear models for microarray data package (38). Differentially expressed genes were identified based on a false-discovery rate (FDR)-adjusted P value ≤ 0.001 (39). GO term enrichment analyses were done with the GOHyperGAll script, which uses hypergeometric distribution (40).

ChIP and EMSA. For ChIP, 12-d-old *35S:LOB-GR* seedlings, grown on MS plates, were induced by flooding with MS medium containing 15 μM DEX or a mock solution and incubated with shaking for 3 h. ChIP was performed as described (41) using an anti-LOB antibody. The ChIP DNA was analyzed by PCR, and LOB binding was calculated as the ratio between the DEX-treated and mock-treated samples. Data were normalized to the control gene *At3g26980*. Primer sequences are shown in Table S1.

EMSAs were performed with the LD protein as described (18). Probes were generated from a 133-bp fragment corresponding to the upstream region of *BAS1* (BAS1 A) and a 110-bp fragment corresponding to the third exon region of *BAS1* (BAS1 B). Primer sequences used to generate probes are shown in Table S1. Probe fragments were cloned and sequenced to verify

the integrity of the LBD sites. The QuikChange Site Directed Mutagenesis Kit (Stratagene) was used to create mutant α and β versions of the BAS1 A probe according to the manufacturer's instructions. To create radiolabeled BAS1 A and B probes, fragments were PCR amplified and end-labeled using T4 polynucleotide kinase and 32P-γ-ATP. Cold competition experiments were performed using a 30-fold excess of unlabeled DNA.

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