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Transcriptomic Analyses Indicate That Maize Ligule Development Recapitulates Gene Expression Patterns That Occur during Lateral Organ Initiation[W][OPEN]

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Development of multicellular organisms proceeds via the correct interpretation of positional information to establish boundaries that separate developmental fields with distinct identities. The maize (*Zea mays*) leaf is an ideal system to study plant morphogenesis as it is subdivided into a proximal sheath and a distal blade, each with distinct developmental patterning. Specialized ligule and auricle structures form at the blade-sheath boundary. The auricles act as a hinge, allowing the leaf blade to project at an angle from the stem, while the ligule comprises an epidermally derived fringe. Recessive *liguleless1* mutants lack ligules and auricles and have upright leaves. We used laser microdissection and RNA sequencing to identify genes that are differentially expressed in discrete cell/tissue-specific domains along the proximal-distal axis of wild-type leaf primordia undergoing ligule initiation and compared transcript accumulation in wild-type and *liguleless1-R* mutant leaf primordia. We identified transcripts that are specifically upregulated at the blade-sheath boundary. A surprising number of these "ligule genes" have also been shown to function during leaf initiation or lateral branching and intersect multiple hormonal signaling pathways. We propose that genetic modules utilized in leaf and/or branch initiation are redeployed to regulate ligule outgrowth from leaf primordia.

INTRODUCTION

Morphogenesis proceeds from positional determinants that establish the body axis. Plant morphogenesis relies on positional information in part because plant cells do not move, but instead respond in place to diverse morphogenetic signals. Shoot apical meristems (SAMs), which contain populations of plant stem cells that produce lateral organs such as leaves, are the primary starting points for establishing positional information in the plant shoot. Once initiated from the SAM, leaf primordia acquire distinct polarity relative to the meristem: Cells in the leaf initial are either proximal or distal to the SAM and are either adjacent to (adaxial) or away from (abaxial) the meristem. Cell and tissue differentiation proceeds along these proximal-distal and adaxial-abaxial axes to generate anatomically and functionally discrete regions of the leaf. The positional cues required to initiate and maintain these axial domains and their boundaries during plant development are largely unknown.

The maize (*Zea mays*) leaf is particularly useful to study positional information because it is subdivided into unique regions along its relatively linear axis. Maize leaves have a proximal sheath and distal blade, separated by a specialized fringe-like ligule adjacent to an auricle (Figure 1A). Together, the ligule and auricle act as a hinge, extending the leaf blade at an angle at the blade-sheath boundary. The first morphological sign of ligule and auricle development is the formation of the preligule band (PLB), a uniquely linear band of smaller cells that runs perpendicular to the proximal-distal axis of the developing leaf (Figures 1C to 1H). The PLB forms by localized anticlinal divisions (new cell walls perpendicular to the existing cell walls) in the adaxial epidermis. PLB cells accumulate the ZmPIN1a auxin efflux transporter at high levels, suggesting a role for auxin in ligule positioning (Moon et al., 2013) (Figures 1I and 1J). Cell divisions associated with the PLB are likely to occur in response to signals that occur earlier in development. Ligule outgrowth then proceeds via periclinal divisions, which are parallel to the leaf surface (Sharman, 1941; Sylvester et al., 1990).

Genes that play a role in leaf proximal-distal patterning have been identified by analyses of mutations that alter the ligule and leaf angle. One such gene, *liguleless1* (*lg1*) encodes SQUAMOSA PROMOTER BINDING PROTEIN (SBP), which is required for developmental patterning of the blade-sheath boundary (Moreno et al., 1997). Recessive *lg1* mutations delete the ligule and auricle; *lg1-R* leaves are narrower and more upright than wild-type siblings, and the blade-sheath boundary is less distinct (Figure 1B) (Emerson, 1912; Becraft et al., 1990; Sylvester

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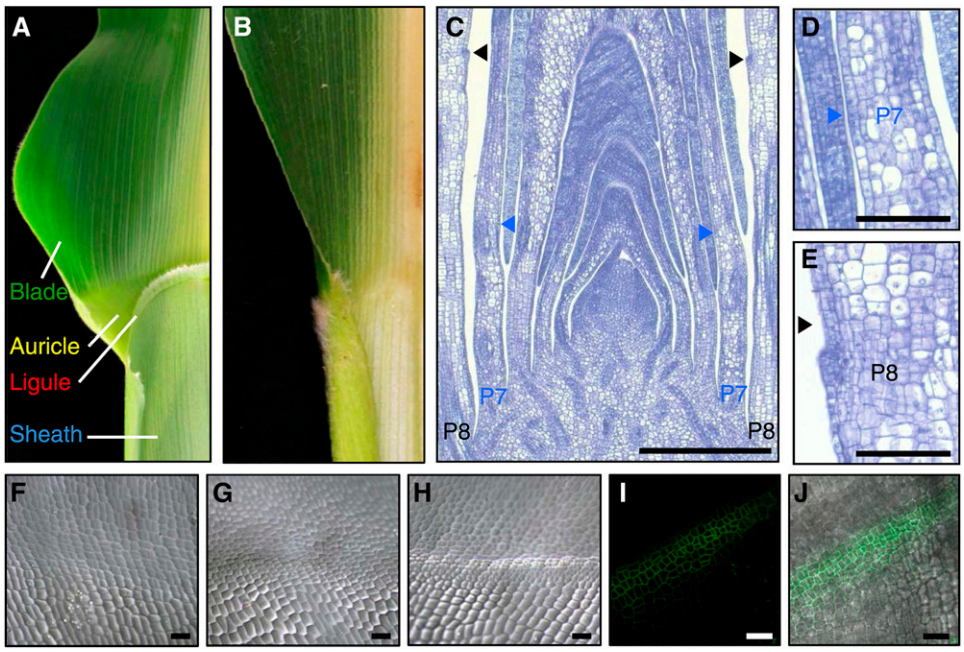

Figure 1. Morphology and Development of the Maize Ligule.

(A) Mature wild-type maize leaf showing auricle and ligule structures.
(B) Mature *lg1-R* leaf. Leaves in **(A)** and **(B)** have been cut in half along the midrib.
(C) to **(E)** Lateral longitudinal section through maize shoot apex showing early stages of ligule development. In this example, the PLB is apparent as a band of smaller cells in the adaxial epidermis of the P7 primordium **(D)**. The initiating ligule is apparent as a bump projecting from the plane of the leaf at P8 **(E)**. Arrowheads indicate position of PLB. P indicates plastochron number.
(F) to **(H)** Scanning electron micrographs of leaf primordia adaxial surfaces showing early stages of ligule development. **(F)** P5, **(G)** P6, and **(H)** P7.
(I) and **(J)** Confocal images of ZmPIN1a-YFP localization in PLB. **(I)** ZmPIN1a-YFP fluorescence; **(J)** bright-field overlay.
Bars = 500 μm in **(C)**, 100 μm in **(D)** and **(E)**, and 25 μm in **(F)** to **(J)**.

et al., 1990; Foster et al., 2004). *knotted1*-like homeobox (*knox*) genes encode transcription factors that function in meristem maintenance and leaf initiation. *knotted1* (*kn1*) transcripts accumulate at high levels in the maize SAM but are restricted from incipient and emerging leaf primordia where auxin levels are high (Jackson et al., 1994; Bolduc et al., 2012a; O'Connor et al., 2014). Dominant maize mutants that ectopically express *knox* genes displace proximal sheath tissue into the distal blade, whereas mutants that are defective in auxin transport exhibit ectopic accumulation of KNOX proteins and similar proximal-distal leaf patterning defects (Freeling and Hake, 1985; Sinha and Hake, 1990; Fowler and Freeling, 1996; Foster et al., 1999; Tsiantis et al., 1999; Scanlon et al., 2002; Ramirez et al., 2009). These observations support a model whereby KNOX accumulation specifies the proximal sheath compartment of very young primordia, and auxin restricts KNOX protein accumulation from distal leaf domains (Bolduc et al., 2012a). Such antagonism between auxin and KNOX is a module that acts in multiple contexts during plant development (Scanlon, 2003; Hay et al., 2006; Gallavotti et al., 2008; Hay and Tsiantis, 2010).

In this study, we analyzed the transcriptome associated with ligule formation using laser microdissection RNA-sequencing (LM-RNAseq). We quantified transcript accumulation in the PLB and adjacent preblade and presheath regions of wild-type leaf primordia in order to identify candidate genes involved in proximal-distal patterning at the blade-sheath boundary. We also compared transcript accumulation in *lg1-R* mutants and wild-type siblings to identity genes acting downstream of LG1. We discovered that a suite of genes specifically upregulated in the preligule region is also expressed at developmental boundaries of leaves and branches. These results suggest that the genetic network used to initiate lateral organs is redeployed to make the ligule.

RESULTS AND DISCUSSION

LM-RNAseq of the Primordial Blade-Sheath Boundary

To identify transcripts that are differentially expressed (DE) along the leaf proximal-distal axis, we employed LM-RNAseq of the PLB and adjacent preblade and presheath regions of Plastochron 7 (P7; which describes the primordium that is seven leaves from the SAM) stage leaf primordia (Figure 2A; Supplemental Figure 1, red, green, and blue boxes, respectively). Given that the ligule arises from periclinal divisions within the L1-derived epidermis, we also performed LM-RNAseq of just the adaxial epidermal cell layer in the initiating ligule, preblade, and presheath regions.

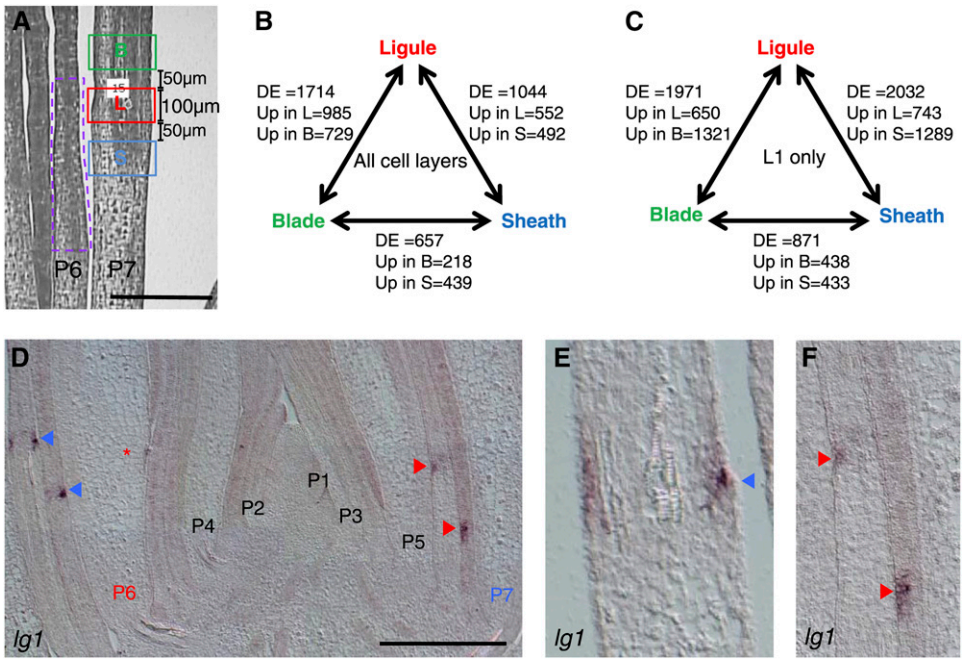

Figure 2. Laser Microdissection of Leaf Primordial Domains.

(A) Scheme for laser microdissection of leaf primordial domains. Preligule tissue (red box) was microdissected from 100-μm-high rectangles, centered on the PLB. Preblade (green) and presheath (blue) tissue was microdissected from 100-μm rectangles 50 μm above and below the preligule selection, respectively. For the comparison of wild-type and *lg1-R* transcriptomes, tissue between 400 and 900 μm from the base of P6 leaf primordia (purple dashed line) was microdissected from lateral sections.
(B) and **(C)** Number of DE genes in pairwise comparisons between leaf regions.
(B) Microdissection of all cell layers.
(C) Microdissection of adaxial L1 only.
(D) to **(F)** *lg1* in situ hybridization. Arrowheads indicate PLB at margins. Asterisk indicates preligule region at midrib. P indicates plastochron number. Bars 300 μm in **(A)** and 500 μm in **(D)**. B, blade; L, ligule; S, sheath.

An analysis of transcript accumulation within all the microdissected tissue layers found a total of 2359 DE genes, with a false discovery rate of <0.05 (Supplemental Data Set 1). Specifically, 1714 genes were DE between ligule and blade, 1044 genes were DE between ligule and sheath, and 657 genes were DE between blade and sheath (Figure 2B; Supplemental Data Set 2). *lg1*, which is expressed specifically at the ligule (Moon et al., 2013), served as a useful control to verify tissue specificity of the microdissections. In both epidermal LM as well as microdissections of all cell layers, *lg1* transcript accumulation was significantly higher in ligule tissue than in either blade or sheath (all-cell-layers analysis; log fold-change ligule versus sheath = 5.925, log fold-change ligule versus blade = 6.615), indicating that our tissue sampling was accurate (Supplemental Figure 2A). In situ hybridizations confirmed that accumulation of *lg1* transcript is restricted to the PLB and the emerging ligule regions (Figures 2D to 2F, arrowheads).

To verify the specificity of our epidermal microdissections, we compared transcript accumulation for several layer-specific genes in epidermis-only microdissections to the data from all cell layers (Supplemental Data Set 3). The genes *brown midrib1*, *Ran BINDING PROTEIN2* (specific to vascular tissues), and *Rubisco small subunit* are predicted to be expressed only in subepidermal cell layers (Humphreys and Chapple, 2002; Takacs et al., 2012). In our data sets, these transcripts were not represented in epidermis-only captures or were present at low levels relative to the all-cell-layers captures, thus supporting the accuracy of the captures. Epidermis-specific genes are expected to be higher in the epidermis-only microdissections but also represented in the all-cell-layers data, since these microdissections included the epidermis as well as internal cell layers. As predicted, accumulation of epidermal gene transcripts such as *Outer Cell Layer 1* (Ingram et al., 2000), *Protodermal Factor2-like* (Abe et al., 2003), and *AT Meristem L1-like* (Lu et al., 1996) was higher in the epidermis-only microdissections than in the all-cell-layers microdissections.

An analysis of transcript accumulation in the adaxial epidermis found a total of 3128 DE genes; 1971 genes were DE between ligule and blade, 2032 genes were DE between ligule and sheath, and 871 genes were DE between blade and sheath (Figure 2C; Supplemental Data Set 4). Thus, in both data sets there were more genes DE between ligule and blade and ligule and sheath than between blade and sheath. This might be due to the fact that the ligule is a specialized group of cells that is undergoing more rapid cell division and growth than surrounding cells. Based on these combined data, we constructed lists of genes that are specifically enriched in each leaf region; preblade, preligule, and presheath (Table 1; Supplemental Data Sets 5 and 6).

Table 1. Number of Genes Significantly Upregulated or Downregulated in Specific Leaf Primordial Domains

	Up in Blade	Up in Ligule	Down in Ligule	Up in Sheath
All cell layers	218	373	246	439
Adaxial epidermis	438	287	778	433

To identify genes that are DE in *lg1-R* mutant leaf primordia, we conducted LM-RNAseq and compared transcript accumulation in wild-type and *lg1-R* P6 leaf primordia (dashed box, Figure 2A). Ninety-six genes were DE with a false discovery rate of <0.05, 59 were downregulated in *lg1-R* mutants, and 37 were upregulated (Supplemental Data Sets 7 and 8). Of these 96 genes DE in *lg1-R*, 34 were also significantly upregulated in preligule tissue in our analysis of all cell layers and thereby comprise an especially interesting subset of ligule-enriched transcripts (Figure 3). All 34 genes that were upregulated in preligule tissue and DE in *lg1-R* were downregulated in *lg1-R* mutants.

Functional Category Enrichment

All expressed genes were assigned to MapMan functional categories (Thimm et al., 2004); tests for enrichment of functional categories in proximal-distal leaf regions and in *lg1-R* mutant primordia were performed (Figures 4A to 4C). Genes that are DE in *lg1-R* mutants are enriched for hormone metabolism and RNA MapMan categories (Figure 4A). Blade and ligule genes are also enriched for hormone metabolism, and lists for blade, ligule, and sheath were all enriched for RNA (Figure 4A). The ligule gene list was also enriched for cell wall genes, possibly reflecting cell wall modifications required for ligule outgrowth (Figure 4A).

A breakdown of transcription factor families revealed that the ligule gene list is enriched for seven transcription factor families, including *MADS* box, *MYB*, and *SBP* (Figure 4C). LG1 is itself a SBP expressed specifically in the PLB and emerging ligule (Figures 2D to 2F), and our LM-RNAseq data reveal that several *SBP* gene family paralogs exhibit domain-specific transcript accumulation during patterning of the blade-sheath boundary (Figure 4A; Supplemental Figures 2A and 2F). Both the ligule and sheath gene lists are enriched for homeobox transcription factors (Figure 4C). Intriguingly, whereas the blade and ligule DE gene lists are enriched for *AUXIN RESPONSE FACTOR* (*ARF*) transcription factor family members (Figure 4F), the ligule and epidermal sheath show significant enrichment for *Aux/IAA* genes that encode repressors of ARF activity (reviewed in Hagen and Guilfoyle, 2002; Figure 4C).

Transcripts Implicated in Maize Leaf Initiation Are Redeployed during Patterning of the Primordial Blade-Sheath Boundary

A surprising number of transcription factor transcripts and hormonal genes differentially accumulating at the blade-sheath boundary of maize leaf primordia were first described as key developmental regulators of early stages in leaf initiation from the SAM. The most highly upregulated homeobox gene in the ligule region of P7-staged leaf primordia is *narrow sheath1* (*ns1*) (GRMZM2G069028) (Figure 4G). *ns1* encodes a WUSCHEL-LIKE HOMEOBOX3 protein that is required for recruitment of lateral leaf founder cells during early stages of maize leaf development (Scanlon et al., 1996; Nardmann et al., 2004). At least two Class III *HOMEODOMAIN LEUCINE ZIPPER* (*HD-ZIPIII*) genes with homology to the *Arabidopsis thaliana* leaf polarity genes *REVOLUTA* (GRMZM2G469551) and *PHABULOSA* (GRMZM2G469551) also display specific expression patterns in both emerging leaf primordia and in developing ligules (Supplemental Figures 2B and 3) (Juarez et al., 2004). Two additional transcription factor genes implicated in abaxial patterning of initiating maize leaf primordia (the *ARF3* paralogs GRMZM2G030710 and GRMZM2G441325) are likewise upregulated in the preligule region epidermis of maize

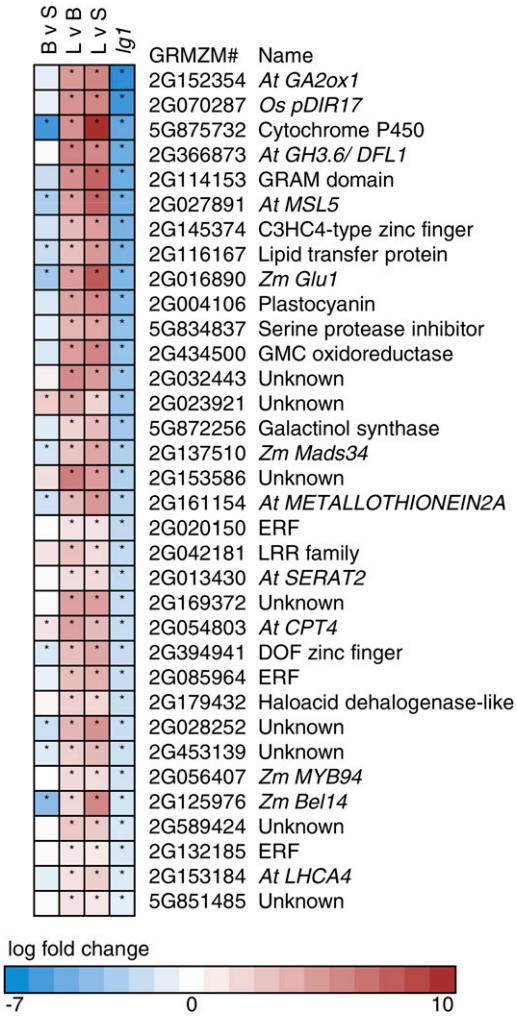

Figure 3. Genes Upregulated in the Preligule Region and DE in *lg1-R* Mutants.

BvS, blade relative to sheath; LvB, ligule relative to blade; LvS, ligule relative to sheath; *lg1*=*lg1-R*, mutant relative to wild-type sibling. Asterisk indicates P < 0.05. "*Zm*" indicates maize gene name, "*At*" indicates name of closest *Arabidopsis* gene, and "*Os*" indicates name of closest rice gene.

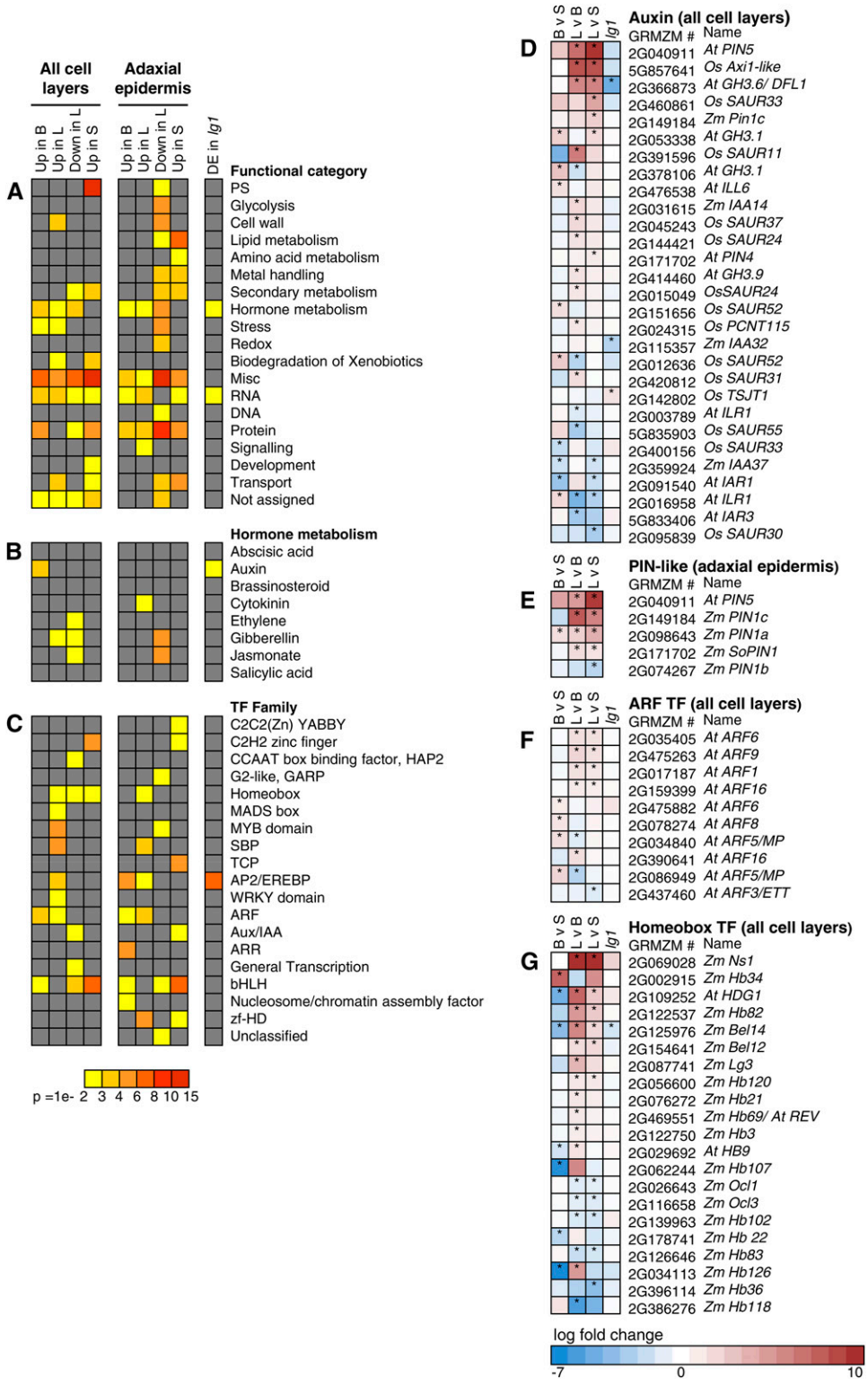

Figure 4. Enrichment of Functional Categories and Expression Profiles for DE Genes.

(A) Enrichment of functional categories for DE gene lists.
(B) Enrichment of hormone metabolism subcategories.

leaf primordia (Supplemental Figures 2B and 2C). According to a model proposed by Waites and Hudson (1995), the juxtaposition of adaxial (upper leaf) and abaxial (lower leaf) developmental fields generates a new leaf axis that promotes laminar outgrowth (Waites and Hudson, 1995; Timmermans et al., 1998; Nogueira et al., 2007; Candela et al., 2008; Douglas et al., 2010). This mechanism may have been co-opted to generate new growth axes in leaves with nonplanar morphologies, such as the maize prophyll (Johnston et al., 2010). We hypothesize that *ARF3* and *HD-ZIPIII* paralogs also function during ligule outgrowth from later-staged leaf primordia in a manner analogous to laminar outgrowth at the earliest stages of maize leaf initiation.

In support of this hypothesis, a number of transcripts implicated in phytohormone metabolism, transport, and signaling during maize leaf initiation are likewise redeployed during establishment of the blade-sheath boundary. These include four *PIN-like* genes, previously described to function during the earliest known events in lateral organ initiation (Benková et al., 2003; Reinhardt et al., 2003; Carraro et al., 2006; Gallavotti et al., 2008; Lee et al., 2009), that are upregulated in the preligule L1 (Figures 4D and 4E). These data are consistent with previous reports that ZmPIN1a-YFP accumulates in the PLB (Moon et al., 2013) (Figures 1I and 1J). Three paralogous *GRETCHEN HAGEN3* (*GH3*)-like genes, which are predicted to encode IAA-amide-synthetase proteins that function to convert the auxin IAA into biologically inactive conjugates (Zhao et al., 2013), are also highly upregulated in the ligule and downregulated in *lg1-R* mutants (Figure 4D). Intriguingly, misexpression of a rice (*Oryza sativa*) homolog of GH3 conditions mutant phenotypes at the blade-sheath boundary that alter leaf inclination (Zhao et al., 2013).

Two genes implicated in brassinosteroid biosynthesis, *brassinosteroid-deficient dwarf1* (*brd1*; GRMZM2G103773) and a gene with high similarity to rice *Dwarf11* (*ZmD11*; GRMZM2G107199), were significantly upregulated in our LM-RNAseq analyses of preligule tissue (Supplemental Data Set 5). Likely due to their low transcript abundance, neither *brd1* nor *ZmD11* transcripts are detected by in situ hybridization in emerging ligules, although transcripts are observed near the primordial leaf base and in initiating lateral organs, respectively (Figures 5A and 5B). Maize *brd1-m1* mutants exhibit enlarged auricles and an indistinct blade-sheath boundary, whereas rice *d11* mutants have upright leaves (Tanabe et al., 2005; Makarevitch et al., 2012). Our data reinforce previous findings implicating brassinosteroid biosynthesis during patterning of the blade-sheath boundary in maize.

Transcripts of two genes with predicted functions in gibberellin metabolism are also DE during ligule initiation. A maize homolog of the *Arabidopsis GIBBERELLIN2-OXIDASE1* gene (*ga2ox1*), which functions in gibberellin catabolism, was significantly upregulated in the preligule region of wild-type leaves and downregulated in *lg1-R* mutants (Figure 3; Supplemental Figure 2D). In situ hybridization revealed that *ga2ox1* transcripts accumulate on the abaxial domains of the developing ligular/auricle region and at the base of lateral organ primordia in axillary buds (Figures 5C to 5E).

Two cytokinin oxidase genes were upregulated in the preligule epidermis (Supplemental Figure 2E). Cytokinin oxidases degrade cytokinin (CK), suggesting that epidermal-localized CK degradation occurs in preligule cells (Houba-Hérin et al., 1999; Schmülling et al., 2003). A gene with similarity to *ARABIDOPSIS THALIANA RESPONSE REGULATOR9*, a type-A response regulator and negative regulator of CK signaling, was also upregulated at the blade-sheath boundary (Supplemental Figure 2E; Kiba et al., 2003; To et al., 2004). Lateral organ founder cells exhibit low ratios of CK/auxin (Jasinski et al., 2005; Yanai et al., 2005). Our data suggest that reduced CK levels in preligule cells may promote cell expansion and ligule outgrowth in a manner similar to leaf initiation.

Ligule Genes Are Expressed at Multiple Organ Boundaries

In situ hybridization of genes that are DE along the leaf proximal-distal axis revealed that many are also expressed at organ boundaries in the SAM. Several of these genes are putative orthologs of genes that function in boundary specification in other species. One grass-specific gene of unknown function, GRMZM2G101682, was highly upregulated in the preligule region (Supplemental Data Set 5; Figures 5F to 5H). In situ hybridizations reveal that GRMZM2G101682 is expressed in one or a few cells immediately distal to the PLB (Figure 5H). Transcripts accumulate at the emerging ligule cleft and on the adaxial side of the ligule at later stages of development (Figure 5G). Strikingly, accumulation of this unknown transcript is also detected at boundaries between initiating organs and the SAM and at the base of young lateral organs (Figure 5F). Transcript is largely confined to L1-derived cells in all these tissues and developmental stages.

Another gene that is highly upregulated in the ligule region is GRMZM2G393433, similar to *Arabidopsis CUP-SHAPED COTYLEDON2* (*CUC2*) (Supplemental Data Set 5). In *Arabidopsis* embryos, CUC activity represses growth between the cotyledons (Aida et al., 2002). These organ boundaries are associated with low auxin levels, in keeping with models wherein *CUC2*

Figure 4. (continued).

(C) Enrichment of transcription factor subcategories. Gray, not significantly enriched. Up in B, genes significantly upregulated in blade; Up in L, genes significantly upregulated in ligule; Down in L, genes significantly downregulated in ligule; Up in S, genes significantly upregulated in sheath; genes DE in *lg1*, genes DE in *lg1-R* versus the wild type.

(D) Expression profiles for DE auxin-related genes (all-cell-layers LM).

(E) Expression profiles for DE *pin* genes (epidermis-only LM).

(F) Expression profiles for DE ARF transcription factor genes (all-cell-layers LM).

(G) Expression profiles for DE homeobox transcription factor genes (all-cell-layers LM). BvS, blade relative to sheath; LvB, ligule relative to blade; LvS, ligule relative to sheath; *lg1*, *lg1-R* mutant relative to wild-type sibling. Asterisk indicates $P < 0.05$. "*Zm*" indicates maize gene name, "*At*" indicates name of closest *Arabidopsis* gene, and "*Os*" indicates name of closest rice gene.

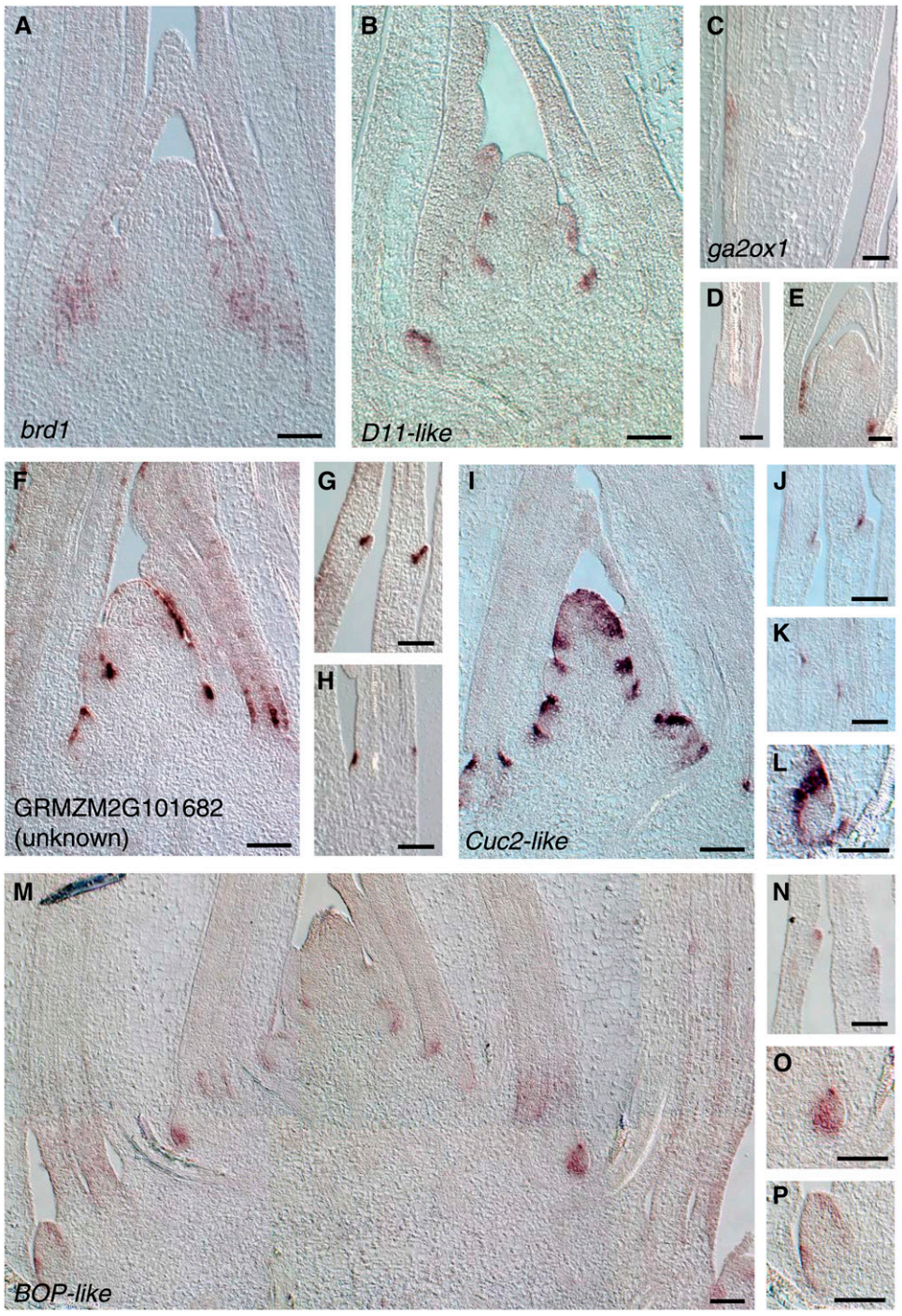

Figure 5. Hormonal Signaling Pathways and Organ Boundary Genes in Leaf Proximal-Distal Patterning.

In situ hybridization of maize wild-type B73 seedlings using the indicated probes. Bars = 100 μm.
(A) *brd1*, lateral longitudinal section through shoot apex.
(B) *D11-like*, longitudinal section through shoot apex.
(C) to **(E)** *ga2ox1*.
(C) Longitudinal section through leaf primordium at midrib.
(D) Longitudinal section through leaf primordium near margin.
(E) Axillary bud.
(F) to **(H)** GRMZM2G101682 (unknown gene).

expression is negatively regulated by auxin (Furutani et al., 2004; Bilsborough et al., 2011; Q. Wang et al., 2014; Y. Wang et al., 2014). In situ hybridization revealed that transcripts of the maize *CUC2-like* gene accumulate in the PLB region, prior to ligule outgrowth, and in the cleft above developing ligules (Figures 5J and 5K). Also expressed in the SAM and in the boundaries between initiating organs and the shoot apex, *CUC2-like* transcripts are not detected in initiating leaves per se (Figure 5I). A band of *CUC2-like* accumulation is also seen between initiating leaf margins and axillary meristems, presumably demarcating the boundary between the primary shoot and lateral branches (Figure 5L). These results suggest that common mechanisms specify the blade-sheath boundary and other developmental boundaries in the maize shoot.

A homolog of *Arabidopsis BLADE-ON-PETIOLE1/2* (*BOP1/BOP2*) (GRMZM2G039867) is upregulated in sheath relative to blade (Supplemental Data Set 5). *Arabidopsis BOP* genes function in leaf proximal-distal patterning (Ha et al., 2003, 2004, 2007), and our results suggest that this role is conserved in maize. In situ hybridization of the maize *BOP-like* gene reveals transcript accumulation at organ boundaries, the base of leaf primordia (presheath domain), in axillary meristems, and in developing ligules (Figures 5M to 5P). A second *BOP-like* gene (GRMZM2G060723) was also upregulated in sheath and ligule relative to blade, but transcripts of this paralog accumulate at much lower levels (Supplemental Data Set 5). These results suggest that BOPs may specify the proximal (sheath) portion of the leaf primordium, contributing to specification of the blade-sheath boundary and later ligule outgrowth.

The correct establishment of developmental boundaries is required for leaf initiation and lateral branching. Mutants that are defective in boundary specification, such as *Arabidopsis knox* and *bell* mutants, exhibit aberrant branching patterns (Byrne et al., 2003; Smith and Hake, 2003; Ragni et al., 2008). In maize, the male inflorescence (tassel) has long branches that develop from indeterminate meristems at the base of the inflorescence (Tanaka et al., 2013). In contrast, axillary meristems initiated by female inflorescence meristems are determinate, resulting in ears that lack long branches. Maize *ramosa1* (*ra1*) mutants are characterized by abnormal branching in the ear, a phenotypic anomaly that mimics the branching patterns normally observed in the developing tassel (Gallavotti et al., 2010). Recent data show that LG1 accumulates in the axils of tassel branches (Eveland et al., 2014; Lewis et al., 2014). *lg1* mutants exhibit reduced tassel angle and three *SBP* genes, including *lg1*, are implicated in tassel branching, suggesting that LG1 functions in boundary specification in the tassel (Brown et al., 2011; Eveland et al., 2014; Lewis et al., 2014). Our finding that genes such as *CUC2-like* have specific expression patterns at the blade-sheath boundary and other developmental boundaries prompted us to ask if other genes that are DE at the blade-sheath boundary are also implicated in lateral branching. Thus, we looked for overlap between our transcriptomic data sets and genes implicated in lateral branching (Eveland et al., 2014). Genes that are common in multiple data sets are likely to play evolutionarily conserved roles during regulation of developmental processes.

We compared transcripts that are DE specifically in preligule tissue and *lg1-R* mutants to genes that are either DE between branched tassel and unbranched ear primordia or DE in *ra1* mutant ears compared with the wild type (Supplemental Figure 4) (Eveland et al., 2014). Of 619 genes that are DE in preligule tissue, 227 were also DE in *ra1* ear primordia relative to wild-type ears and 151 were DE in tassel primordia relative to ear primordia (Supplemental Figures 4A and 4B). Of 96 genes that are DE in *lg1-R* mutant primordia, 45 were also DE in *ra1* ears and 26 were DE in tassel versus ear primordia ($P < 1e\text{-}2$ for all comparisons) (Supplemental Figures 4C and 4D). Given that *lg1* is ectopically expressed in the highly branched *ra1* mutant ear primordia, but not in wild-type ears (Eveland et al., 2014), it is likely that DE genes common to both *lg1-R* and *ra1* mutants act downstream of LG1 and are involved in branching.

Interactive Networks: KN1-Bound and Modulated

KNOX accumulates at the base of wild-type leaf primordia and *knox* misexpression in the leaf blade causes cells to differentiate as proximal tissues, such as sheath. These observations form the basis of a model in which KNOX specifies the presheath domain during early leaf development (Bolduc et al., 2012a). Given the proposed role of KNOX in leaf proximal-distal patterning, we investigated genes that are DE in our data and are also bound and modulated by KN1 protein (Bolduc et al., 2012b). In *Arabidopsis*, BEL1-like homeodomain (BELL) proteins form heterodimers with KNOX proteins in combinations that determine target selection and subcellular localization (Bellaoui

Figure 5. (continued).

(F) Longitudinal section through shoot apex.
(G) Longitudinal section through developing ligule near leaf margins.
(H) Longitudinal section through PLB.
(I) to **(L)** *CUC2-like*.
(I) Longitudinal section through shoot apex.
(J) Longitudinal section through developing ligule near leaf margins.
(K) Longitudinal section through PLB.
(L) Axillary meristem.
(M) to **(P)** *BOP-like*.
(M) Longitudinal section through shoot apex.
(N) Longitudinal section through developing ligule near leaf margins.
(O) Axillary meristem.
(P) Axillary bud.

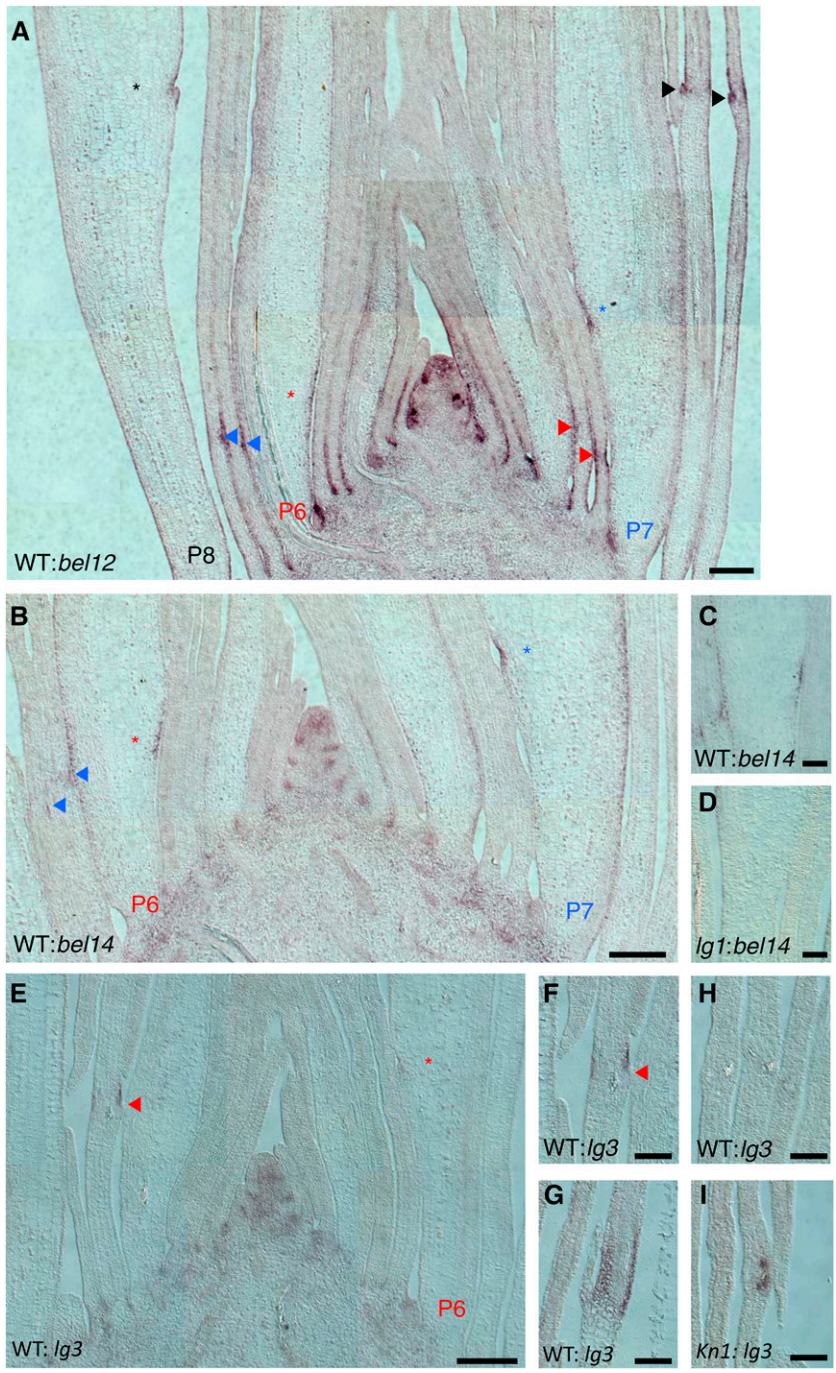

Figure 6. In Situ Hybridization of KN1-Bound and Modulated Genes.

(A) *bel12*, longitudinal section through shoot apex.
(B) to **(D)** *bel14*.
(B) Longitudinal section through shoot apex.
(C) Longitudinal section through wild-type midrib showing transcript accumulation in preligule region.
(D) Longitudinal section through *lg1-R* midrib at equivalent stage and position to "C" showing absence of transcript accumulation.
(E) to **(I)** *lg3*.
(E) Longitudinal section through shoot apex.
(F) Longitudinal section through PLB.
(G) Longitudinal section through ligule.

et al., 2001; Smith et al., 2002; Bhatt et al., 2004; Hackbusch et al., 2005; Cole et al., 2006). Our data show that maize *bel12* (GRMZM2G154641) is significantly upregulated in the preligule region, whereas *bel14* (GRMZM2G125976) is expressed at low levels in the blade, moderate levels in the sheath, and most highly in the ligule region (Figure 4G; Supplemental Table 4). Both *bel12* and *bel14* are bound by KN1 and are upregulated in *Kn1-N*/*Kn1-N* leaves compared with the wild type, although the difference is not statistically significant in the case of *bel14* (Bolduc et al., 2012b).

In situ hybridization analyses of *bel12* reveal transcript accumulation at lateral organ boundaries in the SAM and where older leaf primordia insert at the stem (Figure 6A). The in situ analyses confirm increased transcript accumulation in the preligule region of P6 and P7 primordia, as identified in our LM-RNAseq, and also reveal transcript accumulation in the proximal domain of younger leaf primordia. Similar to the pattern observed for *bel12*, *bel14* transcripts accumulate within lateral organ boundaries in the SAM and stem and in the preligule region of P6 and P7 leaf primordia (Figures 6B and 6C). Intriguingly, both our *lg1-R* RNAseq data and in situ hybridizations of *lg1-R* mutant leaf primordia revealed decreased accumulation of *bel14* in the preligule region of P6 mutant leaves (Figures 3A, 6C, and 6D). The finding that *bel14* transcript is significantly lower in *lg1-R* mutants suggests that LG1 function may be required to maintain *bel14* expression specifically in the ligule region. The dynamic expression of *bel12* and *bel14*, and the finding that *bel14* is DE in *lg1-R* mutants, suggest that they may be important factors in leaf proximal-distal patterning.

KN1 accumulation overlaps with *bel12* and *bel14* transcripts in the SAM and proximal portion of early leaf primordia; thus, BEL12 and BEL14 may interact with KN1 in these domains. However, *kn1* transcript is very low in the preligule region of P7 leaf primordia where *bel12* and *bel14* transcripts accumulate, suggesting another KNOX protein may interact with BEL proteins in this domain. One KNOX protein that potentially interacts with BEL12 and BEL14 in the ligule is LG3. Dominant *Lg3* mutants are characterized by leaf patterning defects that include displacement of the ligule over the midrib or deletion of the ligule (Fowler and Freeling, 1996; Muehlbauer et al., 1997, 1999). *lg3* is bound by KN1 and RNAseq data show that *lg3* is highly upregulated in *Kn1-N* leaves (Bolduc et al., 2012b). We found that *lg3* transcript is significantly higher in preligule tissue than in blade tissue and is also higher in preligule than in sheath tissue, although this latter difference was not statistically significant (Figure 4G). In situ hybridization shows *lg3* transcript accumulation in the preligule region and distal to the ligule after ligule outgrowth (Figures 6E to 6G). *Kn1-N* mutant blades were examined to test whether accumulation of *lg3* is also ectopically upregulated in this gain-of-function mutant. In wild-type leaf blades, *lg3* transcript is not detected (Figure 6H); however, ectopic *lg3* transcript accumulates around the veins of *Kn1-N* mutant leaf blades (Figure 6I). These observations further support the upregulation of *lg3* by KN1.

lg3 is most similar to *KNAT6* in *Arabidopsis*. *KNAT6* is expressed at organ boundaries and contributes to boundary establishment via interactions with the KNOX protein SHOOT-MERISTEMLESS1 and with CUC (Belles-Boix et al., 2006). We speculate that LG3 may have a similar function during boundary establishment in maize. The role of KNOX proteins in establishing the blade-sheath boundary may be analogous to the formation of dissected or compound leaves, where reactivation of *knox* gene expression juxtaposed to PIN-induced auxin maxima creates new boundaries that lead to leaflet formation (Hay et al., 2006; Hay and Tsiantis, 2006; Barkoulas et al., 2008; Shani et al., 2009). Interactions between *CUC2*, whose expression is antagonized by auxin (Bilsborough et al., 2011), and its posttranscriptional regulator miR164 are important in the control of leaf serration and lobing (Nikovics et al., 2006). Given that KNOX-BEL-auxin modules act in multiple contexts during plant development, it is possible that different *SBP* family members are expressed depending on context, thus contributing to the elaboration of different developmental boundaries.

Intriguingly, two auxin transport genes, Zm-*PIN1a* and So-*PIN1*, are upregulated in preligule epidermal cells and are also bound by KN1 and upregulated in *Kn1-N* mutant leaves (Bolduc et al., 2012b). This finding suggests that KN1 directly activates Zm-*PIN1a* and *SoPIN1* transcription at ectopic boundaries and suggests a model in which auxin transport by PIN proteins is activated at the KNOX presence/absence boundary at the base of wild-type leaf primordia.

A Model for Ligule Development

We employed LM-RNAseq to identify genes expressed at early stages of ligule formation and compared these with adjacent blade and sheath. Many of the genes we identified are also expressed at lateral organ boundaries or in initiating lateral organs. We hypothesize that patterning mechanisms that operate in the SAM during organogenesis are reiterated at the blade-sheath boundary during ligule initiation. This model is supported by differential coexpression, in both the primordial ligule and initiating lateral organs, of multiple transcription factors involved in lateral organ patterning, including *ns1*, *arf3a*, *HD-ZIPIII* family members, and *CUC2-like* (Figure 4G; Supplemental Figures 2B and 2C). Hormonal signaling networks typically associated with organogenesis and branching are also recapitulated during ligule biogenesis in the young primordium. PIN1 accumulation within the PLB strongly resembles PIN1 maxima at the site of leaf initiation (Figures 7A to 7D) (Carraro et al., 2006; Gallavotti et al., 2008; Lee et al., 2009).

Figure 6. (continued).

(H) Longitudinal section through wild-type preblade showing absence of transcript accumulation.

(I) Longitudinal section through *Kn1-N* preblade showing transcript accumulation around vascular tissue.

P indicates plastochron number. Asterisks indicate height of PLB at the midrib. Arrowheads indicate height of PLB at the leaf margins. Asterisks and arrowheads are color-coded by plastochron number: red, P6; blue, P7; black, P8. Bars = 200 μm in **(A)**, **(B)**, and **(E)** and 100 μm in **(C)**, **(D)**, and **(F)** to **(I)**.

Our data support a model in which KNOX accumulation at the base of the leaf primordium and auxin accumulation and signaling in the distal portion of the primordium provide positional cues that demarcate the blade-sheath boundary. KNOX proteins accumulate throughout the SAM and are excluded from the P0 cells of the incipient leaf primordium (Smith et al., 1992; Jackson et al., 1994). Localization of PIN-mediated auxin transport at the P0 correlates with subsequent *knox* gene downregulation (Figure 7E) (Hay et al., 2006). At P1 and later, KNOX accumulates at the base of the primordium (Figures 7F and 7G), while the distal portion of the primordium becomes an auxin source (Jackson, 2002; Bolduc et al., 2012a). Our data show that the preblade region is enriched for auxin-related transcripts, whereas the preligule and presheath regions are enriched for homeobox transcription factors. We propose that KNOX proteins interact with BEL12 to define the basal presheath domain prior to formation of the PLB. Auxin in the distal portion of the leaf restricts KNOX accumulation to this basal domain, and the resultant boundary between auxin and KNOX signaling specifies the position of the blade-sheath boundary. A presumed reduced concentration of auxin in this basal, presheath domain is supported by the downregulation of *arf* gene expression in the presheath (Figures 4C and 4F). This model is supported by evidence that reduced auxin transport can alter the position and elaboration of the blade-sheath boundary (Tsiantis et al., 1999; Scanlon et al., 2002).

lg1, *lg3*, and other ligule genes are expressed at the boundary between preblade and presheath, thereby activating *bel14* expression and reactivating transcription of *bel12* (Figure 7H). Initially, *lg1* is expressed in a broad domain and activates PIN1a expression at the PLB (Figure 7H). We propose that subsequent antagonism between auxin and boundary genes, such as *CUC2-like* and *lg3*, restricts boundary gene expression to cells that will form the ligule cleft (Figure 7I). LG3 interacts with BEL14 and/or BEL12 during boundary specification. PIN-mediated auxin accumulation promotes ligule outgrowth, while boundary genes restrict cell division/growth in the ligule cleft (Figure 7I'). In mutants with ectopic *knox*

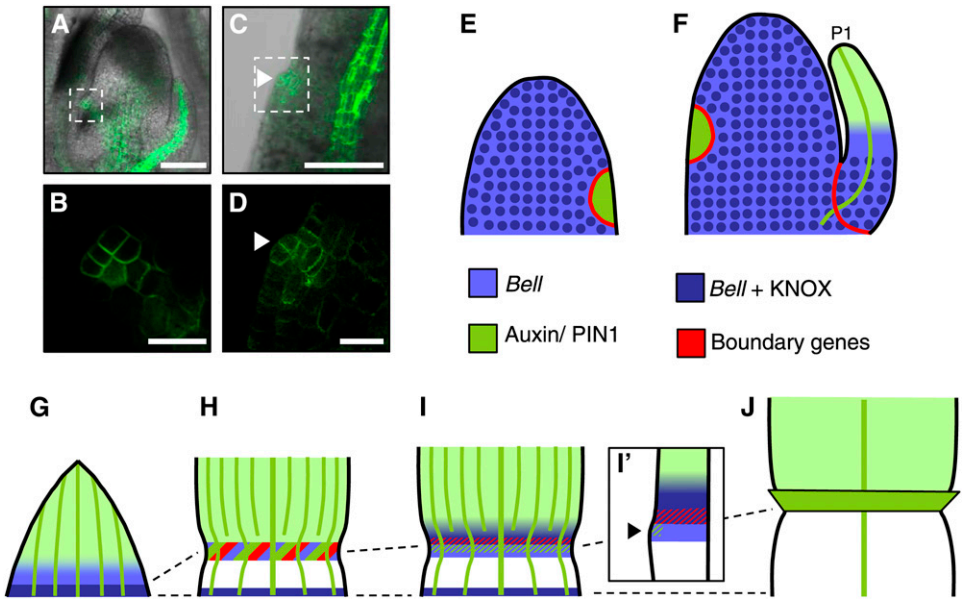

Figure 7. Ligule Initiation Reiterates Leaf Initiation at the SAM.

(A) ZmPIN1a-YFP accumulation in longitudinal section through the SAM.
(B) Enlargement of boxed region in **(A)** showing ZmPIN1a-YFP accumulation in leaf buttress.
(C) ZmPIN1a-YFP accumulation in longitudinal section through initiating ligule.
(D) Enlargement of boxed region in **(C)** showing ZmPIN1a-YFP accumulation in initiating ligule. Arrowheads indicate PLB. Bars = 100 μm in **(A)** and **(C)** and 25 μm in **(B)** and **(D)**.
(E) to **(J)** Model for proximal-distal patterning of the maize leaf.
(E) KNOX proteins accumulate throughout the SAM except for the P0 primordium (green), where PIN-mediated auxin transport is correlated with KNOX downregulation. Boundary genes, such as *CUC2-like*, are expressed between P0 and the SAM.
(F) and **(G)** KNOX accumulates at the base of P1 and older primordia and overlaps with *bel12* transcript, which is expressed more broadly. KNOX and BEL proteins interact to activate target genes. KNOX accumulation defines the presheath domain. Auxin accumulation in the distal portion of the primordium restricts KNOX to the base of the primordium and defines the preblade region.
(H) Initially, ligule genes are expressed at the presumptive blade-sheath boundary in a broad band. LG1 activates PIN1a expression. White portion represents growth of the presheath domain.
(I) *lg3* expression is reactivated at the blade-sheath boundary (indicated by dark purple). Antagonism between auxin and boundary genes restricts boundary genes to cells that will form the ligule cleft.
(I') Illustrates longitudinal section through primordium depicted in **(I)**.
(J) Leaf after ligule outgrowth.

expression, BEL proteins are ectopically expressed and misplaced ligules are formed. In *lg1-R* mutants, *bel14* expression at the PLB is deleted (Figures 6C and 6D) and no ligule is formed.

In summary, our model proposes that elements of meristematic function observed at the SAM and branch meristem are repeated at developmental boundaries throughout the plant shoot, in this particular case at the blade-sheath boundary. Consistent with this model, we found similar patterns of transcript accumulation in the developing ligule and at the boundaries of lateral organs, such as leaves and tassel branches.

METHODS

Plant Material and Growth Conditions

B73 plants were used for the LM-RNAseq analysis of wild-type leaf domains. Homozygous *lg1-R* plants introgressed into the "Freeling B73" background and wild-type siblings were used for LM-RNAseq analysis of *lg1-R* mutants and in situ hybridization in *lg1-R*. B73 was used for wild-type in situ hybridization. *Kn1-N* and *Lg3-O* introgressed into B73 were used for in situ hybridization in these mutant backgrounds. ZmPIN1a-YFP transgenic seed were provided by D. Jackson (Cold Spring Harbor Laboratory).

Plants for LM were grown in growth chambers with a cycle of 15 h light at 25°C and 9 h dark at 20°C. Plants for in situ hybridization were grown under standard greenhouse conditions. All analyses were performed on 14-d-old seedlings.

Laser Microdissection and Sequencing

For analysis of wild-type leaf domains, plants were sectioned along the lateral axis, perpendicular to the midrib-margin axis (Figure 2A; Supplemental Figures 1A and 1B). Sites targeted for microdissection were identified by the appearance of the late PLB as it emerged from the plane of the leaf at about P7. For the preligule region, a 100-μm-high rectangle centered on the PLB was selected (Figure 2A). Preblade and presheath domains were microdissected from similar sized rectangles 50 μm above and below the ligule region, respectively (Figure 2A; Supplemental Figure 1, red, green, and blue boxes, respectively).

To identify genes that are DE in *lg1-R* mutant leaf primordia, we conducted LM-RNAseq and compared transcript accumulation in wild-type and *lg1-R* P6 stage leaf primordia (Figure 2D). LM was used to isolate tissue from a region between 400 and 900 μm from the base of each P6 primordium, which encompasses the domain and developmental stage where *lg1* is first expressed (Figure 2A, dashed box). Three replicates of five to six plants each were used for each tissue type.

Laser microdissection was performed using the Positioning and Ablation with Laser Microbeams system (P.A.L.M. Microlaser Technologies). RNA extraction and amplification, cDNA library preparation, and Illumina sequencing were performed as described previously (Takacs et al., 2012).

Bioinformatics

The sequencing reads were aligned to the maize (*Zea mays*) B73 RefgenV2 using TopHat (v2.0.11) (Trapnell et al., 2009). Reads that were mapped to multiple positions were filtered out by Bamtools (Barnett et al., 2011). The Cuffdiff software (2.1.0) (Trapnell et al., 2010) was used to quantify the raw read count in each gene, using the version 5a gene annotation (working set genes) provided by the maize genome sequencing project . The gene read counts were based on the Cuffdiff output file "genes.read_group_tracking." The Bioconductor edgeR package (Robinson et al., 2010) was used to identify differentially expressed genes. This analysis used the generalized linear model approach. The trimmed means of M-values were used as scaling factors for data normalization, and the Benjamini and Hochberg's algorithm was used to control the false discovery rate (tested on a subset of genes with cpm > 0.1 in at least three samples). MapMan gene annotation (Thimm et al., 2004; Usadel et al., 2009) was used for function enrichment analyses. The binomial test was used to identify enrichment of differentially expressed gene sets compared with all maize gene sets in each of the MapMan function bins, using a P value cutoff of 0.01.

Construction of Domain-Enriched Gene Lists

Lists of genes that are specifically enriched in each leaf domain were constructed. Genes that were DE between blade and sheath and upregulated in the sheath were considered to be sheath-enriched, whereas genes that were upregulated in the blade compared with the sheath were considered to be blade-enriched. Genes that were DE in both ligule versus blade and ligule versus sheath and that were upregulated in ligule tissue in both comparisons were considered to be ligule-enriched (Table 1; Supplemental Data Sets 5 and 6).

In Situ Hybridization and Confocal Imaging

In situ hybridization was conducted as described previously (Jackson et al., 1994). Primer sequences used for probe synthesis are provided in Supplemental Table 1. Confocal imaging was performed as described (Shimizu et al., 2009).

Data Access

All the Illumina RNA-seq raw data (FASTQ) and processed data (read counts per gene) used in this study have been deposited at NCBI Gene Expression Omnibus under accession number GSE61333.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases; all accession numbers for genes mentioned in this study are given in Supplemental Data Set 2.

Supplemental Data

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

Supplemental Figure 1. Scheme for Laser Microdissection of Leaf Primordial Domains.

Supplemental Figure 2. Expression Profiles for DE Genes.

Supplemental Figure 3. In Situ Hybridization of the Zm*PHB* Gene.

Supplemental Figure 4. Ligule Genes Overlap with Genes Implicated in Regulation of Branching.

Supplemental Table 1. Primer Sequences Used for Synthesis of in Situ Hybridization Probes.

Supplemental Data Set 1. Alignment Statistics.

Supplemental Data Set 2. RNAseq Data All-Cell-Layers Analysis, All Expressed Genes.

Supplemental Data Set 3. Transcript Accumulation of Selected Layer-Specific Genes in Epidermis-Only and All-Cell-Layers Microdissections.

Supplemental Data Set 4. RNAseq Data Adaxial Epidermis Only, All Expressed Genes.

Supplemental Data Set 5. Genes Upregulated in Primordial Blade, Ligule, or Sheath (All-Cell-Layers Analysis).

Supplemental Data Set 6. Genes Upregulated in Primordial Blade, Ligule, or Sheath (Adaxial Epidermis-Only Analysis).

Supplemental Data Set 7. RNAseq Data *lg1-R* Mutants versus Wild-Type Siblings.

Supplemental Data Set 8. Genes DE in *lg1-R* Mutants.

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AUTHOR CONTRIBUTIONS

R.J., S.H., A.W.S., and M.J.S. designed the research. R.J. performed the research. R.J., M.W., Q.S., S.H., A.W.S., and M.J.S. analyzed the data. R.J. wrote the article, and S.H., A.W.S., Q.S., and M.J.S. edited the article.

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