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Study of *LATERAL ORGAN BOUNDARIES (LOB)* Genetic Pathways in Boundary Maintenance of *Arabidopsis*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Biology

by

Lifeng Yu

March 2013

Dissertation Committee:
Dr. Patricia S. Springer, Chairperson
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The Dissertation of Lifeng Yu is approved:

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Committee Chairperson

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DEDICATION

This work is dedicated to those whom deeply support me:

families

friends

teachers

Abstract of the Dissertation

Study of *LATERAL ORGAN BOUNDARIES (LOB)* Genetic Pathways in Boundary Maintenance of *Arabidopsis*

by
Lifeng Yu

Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, March 2013
Dr. Patricia S. Springer, Chairperson

The meristem-organ boundary resides between the shoot apical meristem (SAM) and lateral organ primordia, not only providing a physical barrier in the shoot apex, but also actively communicating between the shoot apical meristem and emerging lateral organ primordia. *LATERAL ORGAN BOUNDARIES* (*LOB)* is specifically expressed at the boundary region and functions to suppress growth to ensure boundary integrity. This dissertation focuses on the study of the *LOB* genetic pathway in boundary maintenance of *Arabidopsis*.

In Chapter 1 (together with other previous work - Bell et al., in press), I revealed that *LOB* suppresses brassinosteroid (BR) signaling in the boundary region via directly activating *PHYB ACTIVATION TAGGED SUPPRESSOR1* (*BAS1*) transcription to maintain boundary integrity. LOB directly binds the *BAS1* promoter and activates *BAS1* transcription in the boundary, thus suppressing BR signaling. Boundary-specific overexpression of positive regulators of BR signaling, such as *DWARF4* (*DWF4*) and *BRASSINOSTEROID-INSENSITIVE 1* (*BRI1*), leads to organ fusion, suggesting that BR suppression is required for boundary maintenance.

In Chapter 2, I identified a negative feedback pathway downstream of LOB that involves the BR receptor, *BRI1-LIKE 3* (*BRL3*). LOB directly binds the *BRL3* promoter

and activates *BRL3* transcription in the boundary region. Similar to *BRI1*, mis-expression of *BRL3* in the boundary region led to a boundary fusion phenotype and locally suppressed *LOB* expression. Taken together, I proposed that a negative feedback loop regulates *LOB* expression and boundary maintenance through the *LOB-BRL3* genetic pathway.

Work in Chapter 3 revealed that *LOB* and *LATERAL SUPPRESSOR (LAS)* have a novel synergistic interaction. On one hand, the *lob las* double mutant has dramatic boundary fusion between stem and lateral cauline leaves, demonstrating the synergistic genetic interaction between *LOB* and *LAS* for boundary maintenance. Consistently, overexpressing *LAS* inhibits plant cell growth and reduces plant size. On the other hand, *lob las* double mutants also have a stronger reduction in axillary meristem formation than the *las* single mutant, revealing a novel role for *LOB* in axillary meristem formation. Moreover, *LAS* is required for the normal boundary expression pattern of *LOB* and several other boundary genes such as *LATERAL ORGAN FUSION1* (*LOF1*), *CUP-SHAPED COTYLEDON1* (*CUC1*), suggesting hierarchical interactions between different boundary genes.

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INTRODUCTION

The shoot apical meristem, localized at the apex of the plant shoot, is responsible for both generation of all lateral organs and renewal of itself (Bowman and Floyd, 2008). The SAM consists of different functional zones with unique gene expression patterns and characteristic hormonal signaling. In the central zone of the SAM, *WUS* and *CLV3* are expressed and together with high cytokinin signaling activity form a feedback loop to maintain the undifferentiated cell status (Leibfried et al., 2005; Gordon et al., 2009). In lateral organ primordia, active auxin signaling promotes rapid cell growth and division to form new leaves and flowers (Reinhardt et al., 2003; Heisler et al., 2005).

Functions and properties of the boundary region in plants

In developmental biology, the term boundary region refers to the cells localized to the junction between two different cell types, which have distinct cell identity and functions differently from the organs on both sides (Aida and Tasaka, 2006; Dahmann et al., 2011). In plant development, the meristem-organ boundary is a narrow band of cells localized in the barrier between the SAM and lateral organ primordia (Aida and Tasaka, 2006). The plant M-O boundary has two basic biological functions. On one hand, the boundary region forms a clear physical barrier to stabilize the distinct functional identities of cells at its two sides (Aida and Tasaka, 2006). Plant cells have cell walls, which prevent the cells from moving relative to each other. This feature makes it impossible for the cells at distinct sides of boundary region to mingle. Defective boundary specification leads to a failure of organ separation. For example, in plants containing mutations in two *CUP-SHAPED COTYLEDON* (*CUC*) genes, *CUC1* and *CUC2,* cotyledons fuse together

to form a cup-shaped organ (Aida et al., 1997; Aida et al., 1999). On the other hand, the boundary region is also essential for maintaining SAM identity (Aida et al., 1999; Takada et al., 2001). For example, *CUC1/2* and *SHOOT MERISTEMLESS* (*STM)* have an overlapping expression pattern during early embryogenesis at the globular embryo stage and the *cuc1 cuc2* double mutant lacks *STM* gene expression and consequently fails to maintain the shoot apical meristem (Aida et al., 1999; Takada et al., 2001).

Boundary regions have special characteristics in morphology and cell division dynamics during plant development. Examination of the shoot apex using histology and scanning electron microscopy, it is obvious that the boundary region is a concave "valley" like region between the SAM and leaf or flower primordia (Hussey, 1971; Dumais and Kwiatkowska, 2002; Aida and Tasaka, 2006). Moreover, unlike the round cells in the SAM and primordia, cells in the boundary region are highly polarized along the "valley" region (Dumais and Kwiatkowska, 2002; Reddy et al., 2004). Observation of the shoot apex of tomato treated with the mitosis inhibitor drug colchicine, which arrests dividing plant cells at metaphase, revealed that in the boundary region fewer cells were arrested in metaphase than in the shoot apical meristem and primordia, suggesting cell division in the boundary region is inhibited (Hussey, 1971). Recent advances in microscopy methods make the real time observation of cell growth and division dynamics in the shoot apex of plants possible. One approach is based on real time imaging of replicas of the SAM using Scanning Electron Microscopy (Dumais and Kwiatkowska, 2002). The second approach is based on real time imaging of a fluorescent protein-tagged plasma membrane marker in the SAM, using Confocal Laser Scanning Microscopy (Reddy et al., 2004). Observations of the shoot apex using these two different approaches both revealed that cells in the boundary indeed divide much

more slowly compared to cells in the SAM and primordium (Dumais and Kwiatkowska, 2002; Reddy et al., 2004). From a biomechanical perspective, local polarized cell extension and suppression of cell division would lead to a concave surface (Dumais and Kwiatkowska, 2002). Thus in theory, these two properties of boundary region may be the cause of the typical valley like geometry formation. Once this valley-like boundary is formed, it stabilizes the size of SAM center and allows the separation and outgrowth of lateral organs such as leaf and flower from SAM.

Boundary genes are key regulators for boundary specification

Many previous studies focused on developing an understanding of the genetic mechanisms underlying boundary region specification. Several key genes have been found to contribute to boundary region specification. These boundary genes have the following common features: (1) Boundary genes are often specifically expressed in the boundary region; (2) Loss-of-function mutants of boundary genes usually show organ fusions; (3) Loss-of-function mutants of boundary genes sometimes show defects in meristem maintenance.

The *CUP-SHAPED COTYLEDON (CUC)* genes are members of the NAC-domain containing transcription factor family. *CUC* genes are specifically expressed in the boundary region between the SAM and lateral organ primordia throughout development (Aida et al., 1997; Raman et al., 2008). In the *cuc1 cuc2* double mutant background, the cotyledons are fused together (Aida et al., 1997). In a *cuc2 cuc3* double mutant, boundary fusions can be found between the rosette leaves, between the cauline leaves and stem, and between the stem and axillary shoot (Hibara et al., 2006). These findings suggest that *CUC* genes are required for boundary formation throughout plant

development. In addition to the function in boundary specification, *CUC* genes are required for SAM formation and axillary meristem formation. *CUC1* and *CUC2* are expressed during early embryogenesis in the location of the future SAM and their expression is required for *STM* expression and specification of SAM identity (Aida et al., 1999; Takada et al., 2001; Vroemen et al., 2003), while *CUC3* is required for axillary meristem initiation (Raman et al., 2008). Interestingly, the expression pattern of *CUC1* and *CUC2* is also dynamically regulated by microRNA *miR164* (Laufs et al., 2004). Overexpression of *miR164* causes partial to complete cotyledon fusion, which is similar to the phenotype of the *cuc1 cuc2* double mutant (Laufs et al., 2004). On the other hand, ectopic expression of miR164-resistant *CUC2* causes enlarged boundary domain and altered phyllotactic pattern (Laufs et al., 2004).

LATERAL SUPPRESSOR (LAS) encodes a transcription factor in the GRAS family (Greb et al., 2003). *LAS* was first identified in tomato plants and the loss of function tomato *ls* mutant has a defect in axillary meristem formation (Schumacher et al., 1999). In *Arabidopsis*, *LAS* is specifically expressed in the boundary region between the SAM and leaf primordia before formation of axillary meristems (Greb et al., 2003). After the formation of an axillary meristem, *LAS* expression is specified at the boundary between the axillary meristem and lateral leaf (Greb et al., 2003). While upstream regulators that confer the boundary expression pattern to *LAS* are unknown, *LAS* transcriptional control involves an enhancer element 3.2 kb downstream of the coding region. A recent study based on promoter swap experiments revealed that this enhancer sequence is required and sufficient to specify the boundary-specific expression pattern (Raatz et al., 2011). The *LAS* gene has two important functions. First, the *las* loss-of-function mutant lacks vegetative axillary meristems, indicating that *LAS* controls

vegetative axillary meristem formation (Greb et al., 2003). Second, in short-day growth conditions, *las* loss-of-function mutants exhibit moderate boundary fusion, suggesting that *LAS* also contributes to the control of boundary cell specification (Greb et al., 2003).

The *REGULATOR OF AXILLARY MERISTEMS (RAX)* genes encode MYB family transcription factors. *RAX1* is specifically expressed in the boundary region between the SAM and leaf primordium, predicting the future site of axillary meristem initiation (Keller et al., 2006; Muller et al., 2006). Loss-of-function mutants of RAX have a reduced number of axillary meristems, coinciding with a reduction in expression of the meristem marker *STM* (Muller et al., 2006).

LATERAL ORGAN FUSION1 (LOF1) and LATERAL ORGAN FUSION2 (LOF2) encode two transcription factors of the MYB family. *LOF1* and *LOF2* are specifically expressed in boundary regions as revealed by both GUS fusion assay and *in situ* hybridization studies (Lee et al., 2009; Gomez et al., 2011). *lof1* loss-of-function mutants have fusion between the axillary stem and cauline leaf (Lee et al., 2009). *lof1* mutants also have defects in axillary meristem formation (Lee et al., 2009). Expression of the shoot apical meristem marker *STM* is reduced in the *lof1* mutant background and the *lof1* mutant enhances the meristem defect in a weak loss-of-function allele of *STM*, *stm-10* (Lee et al., 2009), indicating that *LOF1* has a function in maintaining the primary SAM. The *LOF2* gene is closely related to *LOF1* and the two share redundant functions. The *lof1 lof2* double mutant has stronger defects in axillary meristem formation and organ separation than the *lof1* single mutant (Lee et al., 2009). Additionally, a plant overexpressing *LOF1* is dwarfed, which is consistent with a possible function of *LOF1* in inhibiting plant cell growth (Gomez et al., 2011).

KNAT6 is a member of *KNOX*-type homeobox transcription factor family, which includes the important gene STM, which functions in SAM maintenance (Belles-Boix et al., 2006). Unlike the SAM-specific *STM* gene, *KNAT6* is specifically expressed in the boundary region between SAM and organ primordia (Belles-Boix et al., 2006). The *knat6* loss-of-function mutant enhances the weak *stm-2* allele such that the *knat6 stm-2* double mutant has a severe meristem defect and cotyledon fusion. Moreover, the expression pattern of *KNAT6* is dependent on *STM* (Belles-Boix et al., 2006). Overall, *KNAT6* and *STM* synergistically control both SAM formation and boundary specification between cotyledons.

ORGAN BOUNDARY1 (OBO1) encodes a small protein of 133 amino acids expressed specifically in boundary region between SAM and lateral organs (Cho and Zambryski, 2011; Takeda et al., 2011). *OBO1* is a target of *CUC1*, since *CUC1* directly activate *OBO1* transcription (Takeda et al., 2011).

LBD gene family members control boundary specification

Members of the *LATERAL ORGAN BOUNDARIES* (*LBD*) gene family are important for multiple plant developmental processes, including leaf shape and adaxial-abaxial polarity control, embryogenesis, root development, and boundary specification (Lin et al., 2003; Borghi et al., 2007; Okushima et al., 2007; Xu et al., 2008; Majer and Hochholdinger, 2011). For example, the LBD transcription factor ASYMMETRIC LEAVES2 (AS2) is important for control of leaf shape and adaxial-abaxial polarity. Overexpression of *AS2* suppresses expression of several *KNOX* homeobox genes, *BP, KNAT2* and *KNAT6. 35S:AS2* plants also exhibit alterations in leaf shape and adaxial-abaxial polarity, making the leaf cells adaxialized (Lin et al., 2003).

JAGGED LATERAL ORGANS (JLO) is a member of the *LBD* transcription factor family that is expressed in the embryo and vasculture of leaves in postembryonic stage (Rast and Simon, 2012). Overexpression of a dominant negative version of *JLO* causes fusion of cotyledons in seedlings, suggesting its role in boundary specification (Borghi et al., 2007).

LOB is the founding member of the *LBD* family transcription factors and is the focus of this dissertation. *LOB* encodes a protein containing a LBD domain of around 100 amino acids, which is conserved throughout the *LBD* family (Shuai et al., 2002). Two predicted conserved blocks are found in the LBD domain, namely the C block and the GAS block. The C-terminal 75 amino acids of the LOB protein are a unique sequence. Several experiments confirmed that LOB indeed functions as a transcription factor. First, GFP tagged LOB protein is localized to nucleus, typical for any transcription factor (Husbands et al., 2007). Second, selection and amplification binding assay (SAAB) and electrophoretic mobility shift assay (EMSA) identified a 6-bp DNA motif that LOB selectively binds with (Husbands et al., 2007). Furthermore, when fused to the GAL4 DNA binding domain, LOB can activate transcription in yeast (Husbands et al., 2007).

LOB is important for boundary region specification. First, *LOB* was initially identified as a gene specifically expressed in the boundary region, between the SAM and lateral organs, through a gene trap screening of GUS reporter gene expression pattern (Shuai et al., 2002). Second, loss-of-function *lob* mutations result in moderate fusion between secondary stem and cauline leaf (Bell et al., 2012). Since cells in the boundary region are small and divide slowly, we hypothesized that LOB may inhibit cell expansion and/or cell division. Consistent with this hypothesis, *35S:LOB* overexpression greatly reduces growth in various tissues, making plants dwarf (Shuai et al., 2002).

Taken together, *LOB* is specifically expressed at boundary region and is thought to function to locally suppressed growth to ensure boundary cell integrity.

Hormone signaling and boundary specification

Plant development requires intricate spatial-temporal regulation of hormone signaling, mediated by important transcriptional regulators, hormone receptors and genes required for synthesis and degradation of hormones (Bowman and Floyd, 2008; Veit, 2009). In the shoot apex, differential hormone signaling pathways in the SAM and forming lateral organs, including auxin, brassinosteroids, gibberellins, and cytokinin, meet at the boundary region, contributing to the specification of boundary identity and/or maintenance (Rast and Simon, 2008; Veit, 2009).

Auxin is a growth-promoting hormone necessary for almost all important plant developmental patterning events (Bowman and Floyd, 2008; Veit, 2009). In the shoot apex, auxin accumulates at the site of primordium initiation and promotes lateral organ outgrowth (Reinhardt et al., 2003; Heisler et al., 2005). PIN1 is an auxin efflux carrier that actively transports auxin in a polar manner. In the SAM, PIN1 transports auxin towards future primordium sites from the adjacent older primordium site (Heisler et al., 2005). Primordium outgrowth is one important pre-requirement for the meristem-organ boundary formation (Furutani et al., 2004). If polar auxin transport is inhibited in either the *pin1* loss of function mutant or by treatment of wild-type plants with the polar auxin transport inhibitor NPA, a naked shoot apex forms without any new primordium and boundary region (Furutani et al., 2004). PIN1 is localized to the two longer sides of boundary cells, so PIN1 activity is predicted to deplete auxin from boundary region (Heisler et al., 2005). Consistently, the auxin response is suppressed in the boundary

region and central zone of the SAM, based on expression of the auxin-responsive marker DR5 (Heisler et al., 2005). This local suppression of auxin response is predicted to contribute to the slow growth and division cell properties at boundary region and central zone of SAM (Reddy et al., 2004).

Brassinosteroids (BRs) are plant steroid hormones that promote cell expansion and division. BR signaling is critical for normal plant size and vascular differentiation (He et al., 2000; Wang et al., 2001; Gudesblat and Russinova, 2011). BR signaling can be controlled in three different ways: BR synthesis, BR inactivation and BR perception. BR biosynthesis is regulated by the action of *DWARF4,* a member of cytochrome P450 gene family (Azpiroz et al., 1998; Choe et al., 1998). The DWARF4 protein catalyzes the C22 hydroxylation of campesterol, a critical rate-limiting step for BR synthesis (Choe et al., 1998). BR inactivation is regulated by the activity of *PHYB ACTIVATION-TAGGED SUPPRESSOR1* (*BAS1),* another member of the cytochrome P450 gene family that encodes a negative regulator of active BRs. BAS1 activity catalyzes C26 hydroxylation of brassinolide and castasterone, to inactivate them (Neff et al., 1999). In order to function as a signaling molecule in plants, BR is sensed by membrane-bound receptors. Four BR receptor genes have been identified in *Arabidopsis*: *BR INSENSITIVE1 (BRI1)* and *BRI1-Like 1-3 (BRL1-3)* (He et al., 2000; Wang et al., 2001; Cano-Delgado et al., 2004). BRI1 is a Leucine-rich-repeat receptor like kinase (LRR-RLK) that is localized to the plasma membrane (Wang et al., 2001). BRI1 binds with BR via the extracellular LRR domain (He et al., 2000; Hothorn et al., 2011; She et al., 2011). The binding with BR causes conformational changes in the intracellular kinase domain, which can trigger downstream cell signaling events (Hothorn et al., 2011; She et al., 2011). *BRI1* is expressed almost uniformly in different plant tissues such as meristem, flower, leaf and

root (He et al., 2000; Wang et al., 2001; Cano-Delgado et al., 2004). The sequences of the *BRL* genes are similar to that of *BRI1*, and BRL proteins can also bind BR (Cano-Delgado et al., 2004). Moreover, if expressed under the control of *BRI1* promoter, *BRL1 and BRL3* genes can rescue the dwarf phenotype in a *bri1* loss-of-function mutant, while *BRL2* cannot complement the dwarf phenotype (Cano-Delgado et al., 2004). The expression of *BRL* genes in *Arabidopsis* is more confined to special cell types such as vascular tissue, indicating they may have specific function in vascular tissue formation (Cano-Delgado et al., 2004). Different from auxin, BR is not transported in a polarized manner. As a result, local BR level control largely depends on BR synthesis/degradation and perception. So far the relationship between BR signaling and boundary specification is not well studied. However, since BR is a growth promoting hormone and directly controls cell growth/division (Gudesblat and Russinova, 2011), and the cell growth/division is suppressed in the boundary region (Dumais and Kwiatkowska, 2002; Reddy et al., 2004), it is very tempting to hypothesize that BR level/perception in the boundary region is also suppressed to enhance the boundary cell identity specification.

Gibberellins (GA) are also a type of growth-promoting plant hormone, whose level is regulated by different oxidases: GA20 oxidase is a critical enzyme for GA biosynthesis, while GA2 oxidase is an enzyme important for GA turnover (Sakamoto et al., 2001a; Sakamoto et al., 2001b; Hay et al., 2002; Jasinski et al., 2005). The maintenance of the polarity of GA signaling is achieved by a KNOX transcription factor mediated pathway (Sakamoto et al., 2001a; Sakamoto et al., 2001b; Hay et al., 2002; Jasinski et al., 2005). In the center of SAM, a high level of KNOX transcription factors inhibits the transcription of *GA20* oxidase, which encodes the rate-limiting step in GA biosynthesis, leading to a suppression of GA synthesis (Sakamoto et al., 2001a; Hay et

al., 2002). In the boundary region between the lateral leaf and the SAM, KNOX transcription factors promote the transcription of *GA2* oxidases, decreasing GA level (Sakamoto et al., 2001b; Jasinski et al., 2005). GA suppression in the boundary region is thought to degrade any GAs that diffuse from the leaf into the meristem, maintaining a low GA signaling in SAM.

Cytokinin is the plant hormone that primarily promotes cell division. The biosynthetic isopentenyl transferase *(IPT)* genes are up-regulated by the important SAM expressing KNOX transcription factor STM (Jasinski et al., 2005; Yanai et al., 2005). Moreover, cytokinin application or *STM* promoter driven *IPT* expression in the SAM can partially rescue the loss-of-function *stm* mutant phenotype, suggesting STM functions at least partially via control of the cytokinin signaling pathway (Jasinski et al., 2005; Yanai et al., 2005). *WUSCHEL (WUS)* encodes another important transcription factor for SAM maintenance (Mayer et al., 1998; Schoof et al., 2000). Cytokinin and *WUS* form a positive feedback loop in the SAM. Application of cytokinin results in an increase in *WUS* expression and consequently an enlarged SAM (Gordon et al., 2009). WUS activity upregulates cytokinin responses by directly suppressing transcription of a Type A ARR gene *ARR5*, a negative regulator of cytokinin response (Leibfried et al., 2005). In maize, a Type A ARR *ABNORMAL PHYLLOTAXY1 (ABPHYL1)* is expressed at the base of the leaf to prevent mis-activation cytokinin response in the border of the SAM (Giulini et al., 2004). The loss-of-function *abphyl1* mutant has a larger SAM and altered phyllotactic pattern (Giulini et al., 2004). These data are consistent with a model in which cytokinin is important for SAM identity and size by upregulation of *WUS* and promotion of cell division.

In summary, the boundary region is a very special tissue with low response to all the known growth promoting or cell division promoting hormones, including auxin, BR, GA and cytokinin. Maintenance of this minimum response region in the shoot apex may be critical for specifying boundary cell identity.

Overview of the research in this dissertation

Although it has been revealed that *LOB* encodes a LBD family transcription factor important for boundary region specification, the mechanism underlying LOB function is not well understood. The research in this dissertation focused on addressing two outstanding questions: (1) What is the relationship between LOB and BR hormonal signaling in boundary specification? (2) What are the upstream regulators of *LOB* gene expression?

To search for putative LOB target genes, a microarray experiment was carried out to study the effect of Dex-induced transient *LOB* overexpression on gene transcription (Bell et al., 2012). This experiment revealed that *LOB* transient overexpression strongly up-regulates several putative downstream targets, including a BR-inactivation gene, *BAS1*. This observation leads to a refined hypothesis that LOB specifies the boundary region by activating BAS1-mediated BR suppression. The predicted LOB-binding DNA motif is present in the promoter region of *BAS1*, strongly suggesting the possibility that *BAS1* is a direct target of LOB. To test this possibility, the ChIP-PCR experiment was carried out. Quantitative ChIP-PCR and yeast one hybrid experiments in this study confirmed the binding between LOB and *BAS1* promoter DNA *in vivo*. To gain further insights into the relationship between BR signaling and boundary formation, the consequence of upregulating BR signaling in the boundary region was

tested. Over-expressing the BR-biosynthesis gene *DWARF4* or BR receptor genes *BRI1* and *BRL3* under the control of *LOB* promoter leads to different degrees of boundary fusion between secondary shoots and cauline leaves, strongly supporting the hypothesis that BR signaling needs to be suppressed in the boundary region for local inhibition of cell growth and normal organ separation.

To invesgate other regulators involved in boundary specification and maintenance, an EMS mutagenesis mutant screen to identify enhancers of the *lob::DsE* loss-of-function mutant was carried out previously (unpublished data). Several putative *lob* enhancers have been selected from this screen. Characterization of one enhancer of *lob::DsE*, *las-13,* reveals a link between LAS and *LOB* expression. Characterization of *las lob* double mutants revealed synergistic interactions. Moreover, *LOB* gene expression at the boundary is significantly reduced in *las* loss of function mutants, suggesting LAS is an upstream regulator of *LOB*.

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Chapter 1 LOB-mediated BR suppression is required for boundary cell specification

Abstract

The plant shoot apical meristem produces all lateral organs while also maintaining itself by regulating a self-renewing population of stem cells. Cells between shoot apical meristem and lateral organ primordia are slow growing and dividing, forming a boundary region to separate the meristem and lateral organs. The mechanisms that control boundary specification and maintenance are not well understood. In this chapter, we studied the role of the boundary-specific gene *LOB* in boundary function. LOB directly binds with its target gene *BAS1* promoter and activates *BAS1* transcription in the boundary. BAS1 is an important BR signaling suppressor and LOB activity serves to increase *BAS1* levels in the boundary region, which in turn reduces the BR levels to ensure boundary integrity. Moreover, mis-expression of the BR activators *BRI1* and *DWF4* in the boundary region enhanced the boundary fusion phenotype in the loss-of-function mutant *lob-3.* This research not only revealed a LOB-dependent genetic pathway for boundary maintenance, but also highlighted the importance of BR suppression in boundary cells.

Introduction

The boundary region in plants, where cells undergo slow division and expansion, functions to separate different organs (Reddy et al., 2004; Aida and Tasaka, 2006; Rast and Simon, 2008). Many boundary-specific genes specify the cellular identity in the boundary region and loss-of-function mutants of boundary specific genes lead to defects in lateral organ separation and meristem maintenance (Aida et al., 1997; Aida et al., 1999; Schumacher et al., 1999; Greb et al., 2003; Vroemen et al., 2003; Dean et al., 2004; Aida and Tasaka, 2006; Belles-Boix et al., 2006; Borghi et al., 2007; Rast and Simon, 2008; Bureau et al., 2010; Rast and Simon, 2012). The general biological function of the boundary and the mechanisms for boundary maintenance have been reviewed in the Introduction chapter.

Members of the *LBD* gene family have been shown to have versatile functions in different aspects of plant development, including embryo development, flower development and lateral root formation (Majer and Hochholdinger, 2011). *LOB*, the founding member of the *LBD* gene family of transcription factors, is an important gene for boundary specification. *LOB* appears to function by suppressing cell growth in the junction between two different organs (Shuai et al., 2002). Previous studies revealed that *LOB* encodes a transcription factor and is specifically expressed in the boundary regions of *Arabidopsis* (Husbands et al., 2007; Shuai et al., 2002). Moreover, LOB binds to a conserved DNA sequence motif and its binding affinity can be regulated by its putative cofactors such as bHLH048 (Husbands et al., 2007). Loss-of-function *LOB* mutants show organ fusion and boundary overgrowth phenotypes, suggesting a critical role of LOB in limiting boundary growth.

To answer the question of how LOB controls boundary cell identity, we studied the genetic interaction between *LOB* and its downstream targets. Using a dexamethasone (DEX)-inducible *35S:LOB-GR* line, a microarray experiment was carried out previously in our lab to identify putative target genes of LOB (Bell et al., 2012). After transient DEX induction, several putative target genes were significantly up-regulated, including the important negative regulator of brassinosteroid (BR), *BAS1* (Neff et al., 1999). Since *35S:LOB* overexpression plants are very similar to BR-signaling defective mutants and *BAS1* is rapidly induced by transient LOB activation, we hypothesized that LOB may function in boundary maintenance via *BAS1*-mediated local suppression of BR signaling (Shuai et al., 2002; Gudesblat and Russinova, 2011).

BR is a plant steroid hormone that is essential for promoting cell growth (Wang et al., 2004). BR signaling is regulated by multiple mechanisms, including biosynthesis, perception and inactivation. Local BR synthesis and perception controls the strength of BR signaling and consequent cell growth. *DWARF4* (*DWF4*), a member of the cytochrome P450 gene family, encodes a protein that catalyzes the C22 hydroxylation of campesterol, a critical rate-limiting step for BR synthesis (Azpiroz et al., 1998; Choe et al., 1998; Kim et al., 2006). The BRASSINOSTEROID INSENSITIVE1 (BRI1) protein is a Leucine-Rich-Repeat receptor like kinase (LRR-RLK) that is localized to the plasma membrane (He et al., 2000; Wang et al., 2001). BRI1 binds with BR via the extracellular LRR domain (He et al., 2000; Wang et al., 2001; Hothorn et al., 2011; She et al., 2011). The binding to BR triggers downstream cell signaling events by causing conformational changes in the intracellular kinase domain (Hothorn et al., 2011; She et al., 2011). Consistent with the function of BR in promoting plant growth, loss-of-function mutants of *DWF4* and *BRI1* are both severely dwarfed (Choe et al., 1998; He et al., 2000; Wang et

al., 2001). *BAS1 (CYP72B1)* is a member of cytochrome P450 gene family and catalyzes C26 hydroxylation to inactivate BR (Neff et al., 1999). *BAS1* overexpressing plants have over accumulation of 26-hydroxybrassinolide (Neff et al., 1999; Turk et al., 2003). *BAS1* overexpressing plants have shorter hypocotyls due to reduced BR level in plants, whereas the loss-of-function *BAS1* mutant plants have longer hypocotyls due to increased BR level in plants (Turk et al., 2003). In summary, biosynthesis, degradation and perception act together to maintain normal BR signaling activity and plant cell growth (Neff et al., 1999; He et al., 2000; Kim et al., 2006; Gudesblat and Russinova, et al., 2011).

In this chapter, I focused on further characterization of the role of the LOB-*BAS1* signaling pathway in boundary maintenance. We first showed that LOB indeed binds with the *BAS1* promoter and activates *BAS1* transcription in the boundary region. Moreover, we found that specific mis-activation of BR signaling in the boundary region leads to boundary fusion phenotypes, which further supported the importance of LOB-mediated BR suppression in boundary maintenance.

Results

BAS1 expression is positively regulated by LOB in the boundary region

To study the possible downstream targets of LOB transcriptional regulation, a microarray-based study was carried out in our lab (Bell et al., 2012). Since *35S:LOB* plants, which ectopically express high levels of *LOB*, are dwarf and male sterile (Shuai et al., 2002), we used a chemically inducible line *35S:LOB-GR* in this experiment. Seedlings of *35S:LOB-GR Arabidopsis* plants were treated with a mock control or DEX solution for 4 hours before RNA isolation for microarray analysis. The microarray data

indicated that several gene transcripts rapidly accumulated in response to transient LOB activation, including the negative regulator of BR signaling, *BAS1*. *BAS1* transcript levels were increased up to 4 fold in the *35S:LOB-GR* treated with DEX solution compared with that treated with Mock.

Since LOB is a boundary specific transcription factor (Figure 1.1 A-D), (Shuai et al., 2002) and *BAS1* transcription is upregulated by LOB overexpression (Bell et al., 2012), we tested whether *BAS1* is also expressed in the boundary region using two sets of experiments. First, we used Reverse Transcription (RT)-PCR analysis to examine *BAS1* expression in paraclade junctions. *BAS1* transcripts were detected in RNA isolated from dissected paraclade junctions of wild-type plants (Figure 1.1 I). Because dissected paraclade junctions contain accessory meristems in addition to boundary tissue, we also examined *BAS1* transcript accumulation in paraclade junctions from the *lof1* mutant, which does not produce accessory meristems (Lee et al., 2009). RT-PCR results showed that *BAS1* transcripts were clearly amplified from paraclade junctions of the *lof1* mutant (Figure 1.1 I), consistent with expression in boundary cells. In addition, we examined the *BAS1* expression pattern using a reporter line containing the GUS reporter gene driven by the *BAS1* promoter. We first examined *GUS* expression in a reporter line containing *BAS1* genomic sequences 1846 bp upstream of the *BAS1* coding sequence and the *BAS1* coding region fused to *GUS* (Turk et al., 2003). Transgenic plants containing this construct showed *GUS* expression in the SAM and in flower buds. However, we failed to detect GUS activity in boundaries. One reason may be that the essential factors for control of *BAS1* transcription in the boundary, other than LOB, would bind to a region further upstream of *BAS1*. Therefore, we generated a new *pBAS1:BAS1-GUS* construct that includes genomic sequences from 6084 bp upstream

of the *BAS1* ATG and the *BAS1* gene, including introns, fused to *GUS*. In the *Col* wild-type seedlings carrying the new *pBAS1:BAS1-GUS* construct, *GUS* expression was readily detected in the SAM, young true leaves, and emerging lateral roots (Figure 1.1 E, H). In adult *Col* wild-type plants, *pBAS1:BAS1-GUS* expression was observed in flower organs (petals and carpels) (Figure 1.1 F). In addition, we detected GUS activity in the boundary between the primary stem and axillary shoots and the boundary between pedicels and flowers (Figure 1.1 F, G). This expression overlaps with that of *LOB*, which is expressed in all boundaries (Shuai et al., 2002). In *Col* wild-type seedlings, *pLOB:GUS* expression was specifically detected in the junction between the SAM and hypocotyls, the base of leaves, and the junction between the primary root and lateral roots (Figure 1.1 A, D) (Shuai et al., 2002). After flowering, the *pLOB:GUS* signal was exclusively detected at the boundary of nodes of the stem, the base of pedicels and the base of flowers (Figure 1.1 B, C) (Shuai et al., 2002). Therefore, although *GUS* expression controlled by the *BAS1* promoter has a broader expression than *LOB* in seedlings and flowering plants, it showed partial overlap with the expression pattern of *LOB* in the boundary regions.

Our previous microarray experiment using DEX-inducible *35S:LOB-GR* plants revealed that the LOB protein may activate *BAS1* expression. To confirm this finding, we carried out two additional experiments. We carried out RT-PCR analysis to examine whether *BAS1* transcript levels changed in response to LOB activation in the boundary region. Flowering *35S:LOB-GR* plants were sprayed with 15 μM DEX or mock treated. Four hours after treatment, the boundary region was dissected for RNA extraction and cDNA synthesis, followed by RT-PCR analysis. RT-PCR results revealed that *BAS1* transcripts were significantly increased in the boundary of *35S:LOB-GR* plants after DEX

treatment compared to that in mock-treated plants (Figure 1.2 A, upper lane). In this RT-PCR experiment, *ACTIN2* (*ACT2*) served as an internal control (Figure 1.2 A, lower lane). To quantify *BAS1* transcript accumulation in response to induction of LOB protein accumulation in the nucleus, I also carried out quantitative RT-PCR with the same samples. Again, *ACT2* was used as a normalization control. The amount of *BAS1* transcripts in the boundary region of *35S:LOB-GR* following DEX treatment was approximately 2.6-fold higher than that in the mock-treated *35S:LOB-GR* plants (Figure 1.2 C). In addition to RT-PCR, I examined *GUS* expression in *pBAS1:BAS1-GUS* plants in response to ectopic *LOB* expression. Five independent *pBAS1:BAS1-GUS* transgenic lines were crossed to the *35S:LOB-GR* line. To avoid detection of changes in GUS activity in response to the severe phenotype caused by long-term induction of *35S:LOB-GR*, we used a short-term induction. Ten-day-old *pBAS1:BAS1-GUS 35S:LOB-GR* seedlings were treated with 15 μM DEX or mock treated for 24h followed by GUS staining for 12h. The *pBAS1:BAS1-GUS 35S:LOB-GR* plants treated with DEX had a significant increase in GUS activity in hypocotyls, expanded leaves, and young leaves compared with that in mock-treated *pBAS1:GUS 35S:LOB-GR* plants (Figure 1.3 C, D). In contrast, *GUS* expression in DEX-treated *pBAS1:BAS1-GUS* plants was unchanged compared mock-treated (Figure 1.3 A, B). Furthermore, RT-PCR confirmed that both *BAS1* and *GUS* transcript levels were increased in DEX-treated *pBAS1:GUS 35S:LOB-GR* plants (Figure 1.3 E). Taken together, both RT-PCR and promoter:*GUS* fusion results confirmed that LOB activity indeed up-regulates *BAS1* expression.

To further examine the relationship between *LOB* and *BAS1*, I tested whether *BAS1* expression at the boundary region is altered in the *LOB* loss-of-function mutant. I examined *BAS1* transcript accumulation in two *LOB* loss-of-function mutants, *lob-3* and

lob::DsE. Dissected paraclade junctions of one-month-old *lob-3* and *lob::DsE* plants were isolated for RT-PCR analysis, with the *Col* and *Ler* wild-types serving as the respective controls. *BAS1* transcript levels were moderately decreased in the *lob-3* mutant compared with that in *Col* wild-type plants (Figure 1.2 B). Similarly, quantitative RT-PCR showed *BAS1* transcripts were decreased by 50% in *lob-3* compared to *Col* and by 60% in *lob::DsE* compared to *Ler*, respectively (Figure 1.2 C). Moreover, we found that *pBAS1:BAS1-GUS* expression in the boundary region was decreased in the *lob-3* mutant background compared to *Col* wild-type control plants (Figure 1.4). In *Col* wild-type plants, *pBAS1:BAS1-GUS* expression was detected in flowers (Figure 1.4 A) and the boundary region between the axillary meristem and stem (Figure 1.4 B). In the *lob-3* mutant background, *pBAS1:BAS1-GUS* expression was significantly reduced in the boundary between axillary meristem and stem (Figure 1.4 D), but not changed in flowers (Figure 1.4 C).

In brief summary, our collective data derived from multiple independent experiments all support the conclusion that LOB activates *BAS1* gene expression in the boundary region.

LOB protein binds to *BAS1* promoter

LOB is a transcription factor that controls downstream gene expression via binding to individual promoters. LOB binds specifically to the DNA sequence motif (G)CGGC(G) (Husbands et al., 2007). In the *BAS1* promoter, we found that LOB binds to two partial LBD sites separated by 5 bp, 306 bp upstream of the *BAS1* ATG (Figure 1.5 A) (Bell et al., 2012). We hypothesized that LOB binds to the *BAS1* promoter and activates *BAS1* transcription. To test this hypothesis, we carried out two independent

experimental tests: chromatin immunoprecipitation (ChIP) and yeast-one-hybrid experiments. In the ChIP experiment, crude chromatin was extracted from 10-day-old seedlings of *35S:LOB-GR* treated with mock or 15 μM DEX. The LOB antibody (Husbands et al., 2007) was used to immunoprecipitate the LOB protein and associated DNA, while an antibody against glutathione S-transferase (GST) was used as a negative control. To investigate the association of LOB with the *BAS1* promoter, primers flanking the conserved motifs in the *BAS1* promoter were used to amplify DNA that co-purified with immunoprecipitated LOB. Primers that amplified *LOB* or *ACT2*, DNA sequences not expected to be bound by LOB, served as normalization controls. The *BAS1* fragment was enriched in DNA purified with the LOB antibody from DEX-treated *35S:LOB-GR* seedlings compared to that from the mock-treated samples and the input samples; while the *BAS1* promoter was not enriched in *35S:LOB-GR* treated samples immunoprecipitated with the GST-antibody compared with the input samples (Figure1.5 B). Quantitative PCR showed that the *BAS1* fragment was enriched by 4-fold in the DEX treated ChIP samples purified with LOB antibody (Figure 1.5 C), indicating that LOB protein binds to this *BAS1* promoter region in vivo.

Next we investigated whether LOB binds the *BAS1* promoter using a yeast-one-hybrid assay, based on the GAL4 activation system. The *BAS1* promoter, including the LOB binding motif, was cloned upstream of the reporter gene *HIS3* in *pMW#2* vector and upstream of the reporter genes β-galactosidase and *URA* in *pMW#3* (Figure 1.6 A) (Deplancke B. et al. 2006). These two constructs were integrated into the genome of yeast reporter strain YM4271 sequentially. To identify yeast that had the lowest level of background reporter gene expression, cells containing these two constructs were selected on minimal medium lacking histidine and uracil (SD/-His/-Ura) and containing a

range of concentrations of 3-Amino-1,2,4-trizaole (3-AT), a competitive inhibitor of the product of the *HIS3* gene. In addition to activation of the *HIS3* reporter gene, I also examined activation of β-galactosidase with an assay for X-gal activity. Yeast clones that were most sensitive to 3-AT and did not show X-gal activity were used as recipients for introduction of constructs containing LOB fused to the activation domain of Gal4 (AD). The yeast clones containing both a *BAS1*-reporter gene construct and the LOB-AD construct were screened on minimal media lacking histidine, uracil, and tryptophan plus varying concentrations of 3-AT. The yeast clones transformed with an empty AD vector served as negative control. Yeast cells carrying *BAS1* and LOB constructs grew on SD medium -His/-Ura/-Trp containing up to 20 mM 3-AT, while yeast cells carrying the empty constructs only exhibited weak growth on SD/-His/-Ura/-His plus 5mM 3-AT and their growth was completely inhibited by the higher concentrations of 3-AT (Figure 1.6 B). Taken together, the ChIP assay and yeast-one-hybrid experiments support our hypothesis that LOB directly binds with *BAS1* promoter.

Interaction between *BAS1* and *LOB* genes

In the boundary, cell size is relatively small and the rate of cell division is slow (Reddy et al., 2004). *LOB* is specifically expressed in boundary cells and suppresses cell growth and elongation (Shuai et al., 2002). *lob* mutants show defects in the boundary region, with the base of cauline leaves fused with the axillary shoot (Bell et al., 2012). Because LOB regulates the BR inactivating enzyme, *BAS1*, BR levels are likely to be increased in the boundary of *LOB* loss-of-function mutants. We showed that expression of *BAS1* under the *LOB* promoter can suppress the fusion phenotype of *lob* mutants, consistent with this hypothesis (Bell et al, 2012).

To further investigate the genetic interaction between *LOB* and *BAS1*, I generated the *bas1-2 lob-3* double mutant. *bas1-2* contains a T-DNA insertion in the third intron of *BAS1* (Figure 1.7 A). I tested the mRNA level of *BAS1* in *bas1* mutants using RT-PCR analysis (Figure 1.7 F). In the excised boundary region of *bas1-2*, *BAS1* mRNA was undetectable, indicating the *bas1-2* is a loss-of-function mutant. The *bas1-2* mutant did not exhibit an organ fusion phenotype (Figure 1.7 C). The *lob-3* mutant shows a moderate fusion phenotype (Figure 1.7 D). The *bas1-2 lob-3* double mutant shows a similar fusion length as that in the *lob-3* mutant (Figure 1.7 D, E). This result suggests that *BAS1* level in *lob-3* mutant may be already decreased below the minimum level required for suppressing BR and further reduction in *BAS1* level does not lead to further increase in BR level in boundary region.

Overexpression of BR activators cause fusion in the boundary

To investigate the function of BR regulation in the boundary, I mis-expressed the BR positive regulators *BRI1* and *DWF4* driven by boundary specific *LOB* promoter. *BRI1* encodes a BR receptor kinase located on the plasma membrane and *DWF4* encodes the rate-limiting step in BR biosysthesis (Choe et al., 1998; He et al., 2000). In wild-type plants, the cauline leaves and axillary shoots are separated completely, with the base of the leaf attached to the stem at the internode. In some paraclade junctions, accessory meristems form between cauline leaves and axillary shoots. *pLOB:BRI1* mis-expression in both *Col* and *Ler* backgrounds did not result in a visible phenotype in the boundary (Figure 1.8 B, D). However, *pLOB:BRI1* mis-expression enhanced the boundary fusion phenotype of *lob::DsE* (Figure 1.8 F). In some cases the cauline leaves also fuse with the primary inflorescence stem at the base of cauline leaves. This results in an extension

of the cauline leaf below, a feature known as a decurrent strand, and anincrease in the angle between stem and secondary shoot to more than 90 degrees (Figure 1.8 F) (Emery et al., 2003). Similarly, *pLOB:DWF4* mis-expression in the *Col* background did not result in a visible phenotype in the boundary (Figure 1.8 H), however *pLOB:DWF4* mis-expression dramatically enhanced the organ fusion phenotype of *lob::DsE* (Figure 1.8 J). Mis-expression of *pLOB:BRI1* and *pLOB:DWF4* alone did not cause a boundary fusion phenotype, possibly due to the fact that in these lines native LOB protein is still functional and locally upregulates *BAS1* to suppress BR level in boundary region. In the *lob::DsE* background, the LOB protein is no longer functional in boundary region and cannot upregulate *BAS1* to suppress BR level. As a result, mis-expression of *pLOB:BRI1* and *pLOB:DWF4* significantly enhanced the fusion phenotype of *lob::DsE* alone. These data provide evidence that a relatively low level of BR signaling is critical for organ separation and boundary formation.

Discussion

Rather than a passive quiescent tissue that it might appear to be, the boundary has versatile roles in active control of plant development (Aida and Tasaka, 2006; Rast and Simon, 2008). First of all, the boundary region cells form a physical barrier for separation of distinct cell populations at two sides, such as shoot apical meristem and lateral organ primordia (Aida and Tasaka, 2006). Because plant cells cannot migrate relative to each other, the physical barrier provided by the boundary would prevent the mixing of two different cell populations. Secondly, the boundary region is slow growing and slow dividing, thus making growth at the boundary region very limited compared to the SAM and lateral organ primordia (Reddy et al., 2004). This difference of surface

expansion rate would eventually lead to the narrow “valley” like structure separating lateral organs and meristem (Dumais and Kwiatkowska, 2002). Thirdly, the boundary region also communicates with the SAM to maintain SAM identity (Rast and Simon, 2008). Several boundary specific genes, including *LOB,* have been identified and their loss-of-function mutants showed defects in SAM maintenance and lateral organ separation, the downstream pathways below these genes are not well understood.

Plant hormones are important regulators for plant organ development. In the center of SAM, cytokinin signaling is highly activated and forms a positive feedback to maintain *WUS* gene expression and cell division (Leibfried et al., 2005; Gordon et al., 2009). In the lateral organ primordia, auxin signaling is highly activated and promotes outgrowth of primordia (Reinhardt et al., 2003; Heisler et al., 2005). Interestingly, in the boundary region, auxin signaling is inactive (Reinhardt et al., 2003; Heisler et al., 2005), suggesting the boundary region has special characteristics in terms of hormonal signaling control. BR is one important growth-promoting hormone controlling plant shoot architecture (Gudesblat and Russinova, 2011). Previous studies revealed that active BR signaling in the epidermal cell layer is critical for cell size and plant growth control (Savaldi-Goldstein et al., 2007). In the boundary region, plant cell expansion is severely suppressed, suggesting local BR signaling might be inhibited (Dumais and Kwiatkowska, 2002; Reddy et al., 2004).

Through this study, we established a direct link between the boundary specific gene *LOB* and downstream BR hormonal signaling. We found that the *LOB* activity suppresses BR signaling in the boundary region, a process that is critical to limit growth in the boundary. The local control of BR levels and BR receptor concentration on the plasma membrane are critical for tuning BR signaling activity. In this research, we

revealed that LOB directly activates the transcription of *BAS1* via binding to the *BAS1* promoter. *BAS1* protein is an important enzyme critical for BR inactivation (Neff et al., 1999), thus the increased *BAS1* expression in the boundary region would lead to suppression of BR signaling. Importantly, previous study in our lab revealed that boundary specific overexpression of *BAS1* driven by the *LOB* promoter suppressed boundary fusion phenotype in *LOB* loss-of-function mutant (Bell et al., 2012). Moreover, through this study, we found that boundary specific overexpression of positive regulators for BR signaling, *DWF4* and *BRI1*, leads to boundary fusion phenotype, similar to *LOB* loss-of-function phenotype. These results further supported the importance of BR signaling suppression in boundary specification process.

This research revealed one important pathway downstream of LOB activity, however, it is not the only *LOB* downstream pathway involved in boundary maintenance. BR signaling strength in plants is a combined result of BR level control and BR perception control in plant cells. It is highly possible that *LOB* may also regulate gene expression pattern of BR receptor to make the boundary specification more robust. In agreement with this hypothesis, in Chapter 2 of this dissertation, I studied another downstream pathway of *LOB*, mediated by a special BR receptor BRL3.

In a broader sense, suppression of signaling strength of growth promoting hormones in the boundary region might be a conserved mechanism in plant boundary development. The suppressed hormone is not only limited to BR. For example, in the valve region of the *Arabidopsis* silique, it has been reported that auxin signaling is suppressed and this local auxin level minimum leads to limited cell growth in the valve margin (Sorefan et al., 2009).

In a summary, this study provided a good example of how boundary gene *LOB* controls boundary cell maintenance via suppression of the plant growth promoting hormone BR. It is also a good starting point to further understand the mechanism of the *LOB*-mediated genetic pathways in boundary specification. Moreover, this study, together with other research on auxin signaling suppression in valve region of fruit (Sorefan et al., 2009), supported a common mechanism for plant boundary specification via local suppression of growth promoting hormones.

Materials and methods

Plant materials

35S:LOB-GR transgenic plants in the *Col* ecotype are reported in Bell et al., 2012. T-DNA insertional mutants *lob-3* (SALK_042599) and *bas1-2* (SALK_006781) isolated from the SALK Institute Genomic Analysis Laboratory mutant *col*lection (Turk et al., 2005). *lob-3* mutants contains a T-DNA fragment inserted at the 3' UTR of *LOB* (Figure 1.7 A). *bas1-2* mutants contains a T-DNA fragment inserted at the exon of *BAS1* (Figure 1.7 A). *lob-3* homozygous mutants were identified by PCR-based genotyping with gene-specific primers lob-3-LP and lob-3-RP together with primer SALK_LBa1 (Table 1). *bas1-2* homozygous mutants were identified by PCR-based genotyping with gene-specific primers bas1-2F and bas1-2R together with primer SALK_LBa1 (Table 1). The *lob bas1* double mutant was generated by crossing *lob-3* with *bas1-2* and the homozygous mutants were identified in the F2 generation by genotyping with above primers. The *lob::DsE* mutant contains an enhancer insertion in the *LOB* coding region in the *Ler* background (Figure 1.7 A) (Shuai et al, 2002). Homozygous *lob::DsE* insertion

plants were confirmed by genotyping with specific primers SET223' and ET22-1 together with primer DS3-4N (Table 1).

Growth Conditions

Arabidopsis thaliana wild-type *Col* and *Ler* plants were used for all transgenic experiments. For growth in soil, seed were surface-sterilized with 95% ethanol for 5 min, then sowed in soil and grow under long-day (16h light, 8h dark) growth conditions at 22°C. For growth on sterile medium, seeds were sterilized with 20% bleach and 0.1% Tween-20 for 5 min, followed by washes with sterile water, then sowed on Murashige and Skoog (MS) medium including respective antibiotics (50 µg/ml kanamycin, 25 µg/ml phosphinothricin (PPT), 25 µg/ml hygromycin) (Murashige et al., 1962). For the ChIP experiments, 10-day-old *35S:LOB-GR* seedlings growing on MS plates were flooded with 30 ml of 0.05% EtOH or 0.05% EtOH+15 µM Dexamethasone (DEX) solution in liquid MS medium and incubated for 4 hours with shaking. Treated seedlings were harvested and processed for ChIP.

Plasmid constructs

To generate the *pBAS1:BAS1-GUS* construct, we modified pLET-18-*BAS1*, which contains *BAS1* genomic sequences from 1846 upstream of the *BAS1* ATG to the last codon fused to *GUS* (Turk et al,2003). Additional upstream *BAS1* promoter sequence (-6084 bp to +96bp) was amplified from *Col* wild-type genomic DNA with primers pBAS1-F and pBAS1-R (Table 1), cloned into the TOPO II vector, sequenced to confirm integrity. The part of *BAS1* promoter fragment (-6084bp to -1847bp) was

subcloned to the binary vector pLET-18-*BAS1* with restriction enzymes sites *Bam*HI and *Sal*I.

pLOB:DWF4 and *pLOB:BRI1* constructs were made using the GATEWAY cloning system. The *LOB* CDS was excised from plasmid *pLOB:LOB-3'-UTR* (Bell et al., 2012) with *Nco*I and *Bam*HI. The sticky ends of *pLOB:3'-UTR* fragment were filled in using DNA polymerase I and the resulting blunt ends ligated to a Gateway cassette frame A fragment to generate the plasmid *pLOB:Frame A-3'UTR*. Coding sequences of *AtDWF4* (AT3G50660) and *AtBRI1* (AT4G39400) were amplified from *Col* cDNA with primers DWF4-F together with DWF4-R and BRI1-F together with BRI1-R, respectively. The CDS fragments were ligated into pENTR-TOPO (Invitrogen) to make *DWF4-pENTR* and *BRI1-pENTR* constructs, and were sequenced to confirm their integrity. *pLOB:DWF4* and *pLOB:BRI1* were generated using a recombination reaction between *DWF4-pENTR* or *BRI1-pENTR* and *pLOB:Frame A-3'-UTR* using LR Clonase (Invitrogen), respectively. The generated constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 introduced to *Col* and *Ler* wild-type *Arabidopsis* plants using the floral-dip method (Clough et al., 1998).

CHIP assay

35S:LOB-GR seeds were grown on MS plates for 10 days before incubation with 15 μM DEX or MOCK for 4h. Plants were cross-linked with 1% formaldehyde for 10 min at room temperature and the reaction was stopped by the addition of glycine to a final concentration of 0.125 mM. Tissues were ground under liquid nitrogen and put into a 50ml Falcon tube with 30ml Buffer 1 [0.4 M sucrose, 10 mM Tris–HCl (pH 8.0), 1 mM PMSF, 5 mM beta-mercaptoethanol (BME), 1% Plant protein inhibitor cocktail (PIs)].

After filtering through two layers of miracloth, the filtrate was centrifuged at 4,000 rpm for 20 min at 4°C. The pellet was washed twice with 1 ml Buffer 2 [0.25 M sucrose, 10 mM Tris–HCl (pH 8.0), 1% Triton X-100, 10 mM $MgCl_2$, 1 mM PMSF, 5 mM BME, 1% PIs] and then resuspended in 600 μl Buffer 3 [(1.7 M sucrose, 10 mM Tris–HCl (pH 8.0), 0.15% Triton X-100, 2 mM $MgCl_2$, 1 mM PMSF, 5 mM BME, 1% PIs]. The suspension in Buffer 3 was spun for 1 h at 14,000rpm at 4 °C. The pellet was resuspended in 300 μl Nuclei Lysis Buffer [1% SDS, 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1 mM PMSF, 1% PIs] as crude chromatin. Crude chromatin was solubilized with 4 cycles of sonication (each cycle includes 10 seconds sonication plus 1 minute pause to cool down solution). The samples were centrifuged at 14,000 rpm for 10 min and the supernatant was diluted with ChIP Dilution Butter [1.1% Triton X-100, 16.7 mM Tris–HCl (pH 8.0), 167 mM NaCl, 1.2 mM EDTA, 1 mM PMSF, 1% PIs]. The chromatin was then pre-cleared by adding 40 μl Protein A beads equilibrated with ChIP Dilution Butter and incubated for 1 h at 4°C. The precleared chromatin was transferred to a new tube with 10 μl antibody (anti-LOB or anti-GST at 200 ng/μl) and incubated with gentle shaking overnight at 4°C. The antibody was captured by incubation with 50 μl protein A-Sepharose for 1h at 4°C. Protein A beads were washed with low salt butter (150 mM NaCl, 0.2% SDS, 20 mM Tris-HCl pH 8.0, 0.5% Triton X-100, 2 mM EDTA), high salt buffer (500 mM NaCl, 0.2% SDS, 20 mM Tris-HCl pH 8.0, 0.5% Triton X-100, 2 mM EDTA), LiCl buffer (0.25 M LiCl, 10 mM Tris-HCl pH 8.0, 0.5% NP-40, 1 mM EDTA, 0.5% deoxycholic acid) and TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA). The DNA was reverse crosslinked by boiling with 100 μl 10% Chelex resin and recovered by treatment with 1 μl 10mg/ml RNase A (37°C, 30min) and 1 μl 20mg/ml Proteinase K (55°C, 30min) followed by boiling for 10 min. The supernatant was collected after centrifuging for 5 min at 14,000 rpm at room temperature.

The DNA was used for PCR with the specific primers flanking the LOB binding motif. The *BAS* promoter fragment was amplified with BAS1 ChIP 1 F together with BAS1 ChIP 1 R primers, *LOB* amplified with QLOB-F and QLOB-R primers was used as an equal loading control for semi-quantitative PCR, *ACT2* amplified with QACT2-F and QACT2-R primers was used as an equal loading control for quantitative PCR (primers are on Table 1.1).

Gene Expression Analysis

Adult *35S:LOB-GR* plants were sprayed with 0.05% EtOH or 0.05% EtOH+15 μM DEX plus 0.02% [v/v] Silwet L-77. After 4h treatment total RNA was isolated from dissected paraclade junction tissue of *Arabidopsis* plants. *pBAS1:BAS1-GUS 35S:LOB-GR* seedlings were flooded with 0.05% EtOH or 0.05% EtOH+15 μM DEX for 24h before harvesting. RNA was isolated using Trizol extraction buffer and quantified by the NanoDrop spectrophotometer. RNA was treated with DNase at 37°C for 1h before cDNA synthesis. The cDNA was synthesized from 3 μg of total RNA with Superscript III following the manufacturer's proto*col*s (Invitrogen). For Reverse Transcription (RT)-PCR, *BAS1* was amplified with BAS1F together with BAS1R primers. GUS was amplified with GUS plus-F and GUS plus-R. *ACT2* was amplified with ACT2-F and ACT2-R and was used as an equal loading control in RT-PCR. For Quantitative PCR, *BAS1* was amplified with QBAS1-F together with QBAS1-R primers. *ACT2* amplified with QACT-F and QACT-R was used as an equal loading control in Q-PCR experiment (primers are shown in Table 1.1).

Yeast one hybrid assay

BAS1p:pMW#2 and *BAS1p:pMW#3* were generated using GATEWAY cloning technology (Invitrogen). A fragment of the *BAS1* promoter (-500bp to -8bp) was amplified from *Col* wild-type genomic DNA using primers *BAS1* Y1H1-attB4 together with *BAS1* Y1H1-attB1r, which contained introduced attB4 and attB1R sites, and cloned into *pDONR4R-P1R* using BP recombinase. *BAS1p:pMW#2* and *BAS1p:pMW#3* were generated using the LR recombination reaction between *BAS1-pDONR4R-P1R* and *pMW#2* or *pMW#3*, respectively (Deplancke B. et al. 2006). The *LOB* CDS was amplified from *Col* wild-type cDNA with primers LOBgateway-F2 and LOB-R and introduced to *pENTR-TOPO*. *LOB-pDEST*^{AD}, generated using an LR reaction with *LOB-pENTR* and *pDEST*^{AD}, encoded a fusion protein between LOB and the Gal4 activation domain (AD). The plasmids *BAS1p:pMW#2* and *BAS1p:pMW#3* were transformed into yeast strain YM4271 sequentially following previously described proto*col*s (Deplancke B. et al, 2006). The autoactivation was examined by patching positive clones on minimal yeast medium lacking histidine and uracil (-His, -Ura) with 0, 5, 10, 20, or 40 mM 3-AT and also with beta-Galactosidease Assay (Clonetech). The yeast clones that were most sensitive to 3-AT and did not show X-gal activity were used as recipients for introduction of *LOB-pDEST*^{AD} constructs, and selected on SD-His-Ura-Trp with appropriate concentration of 3-AT. All primers are shown in Table 1.1).

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Turk, E.M., Fujioka, S., Seto, H., Shimada, Y., Takatsuto, S., Yoshida, S., Wang, H., Torres, Q., Ward, J.M., Murthy, G., Zhang, J.Y., Walker, J.C., Neff, M.M. (2005). *BAS1* and *SOB7* act redundantly to modulate *Arabidopsis* photomorphogenesis via unique brassinosteroid inactivation mechanisms. The Plant Journal **42**, 23–34

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Figure 1.1 *BAS1* and *LOB* have partially overlapping expression patterns.

(A-D) GUS activity in *pLOB:GUS* plants. GUS activity in the boundary at the base of cotyledons (A), base of floral organs and pedicels (B), paraclade junction between primary stem, axillary stem, and cauline leaf (C), base of lateral root (D). (E-H) GUS activity in *pBAS1:BAS1-GUS* plants. GUS activity in young leaves and the boundary at the base of cotyledons (E), carpels, base of floral organs, stem, and pedicel (F), paraclade junction (G) and emerging lateral root (H). (I) RT-PCR analysis of *BAS1* transcript accumulation in dissected paraclade junctions of wild type and *lof1* mutants. *lof1* mutants lack accessory meristems. 35 cycles of amplification was performed with primer BAS1 F RE probe and primer BAS1 cds RP (Table 1.1)

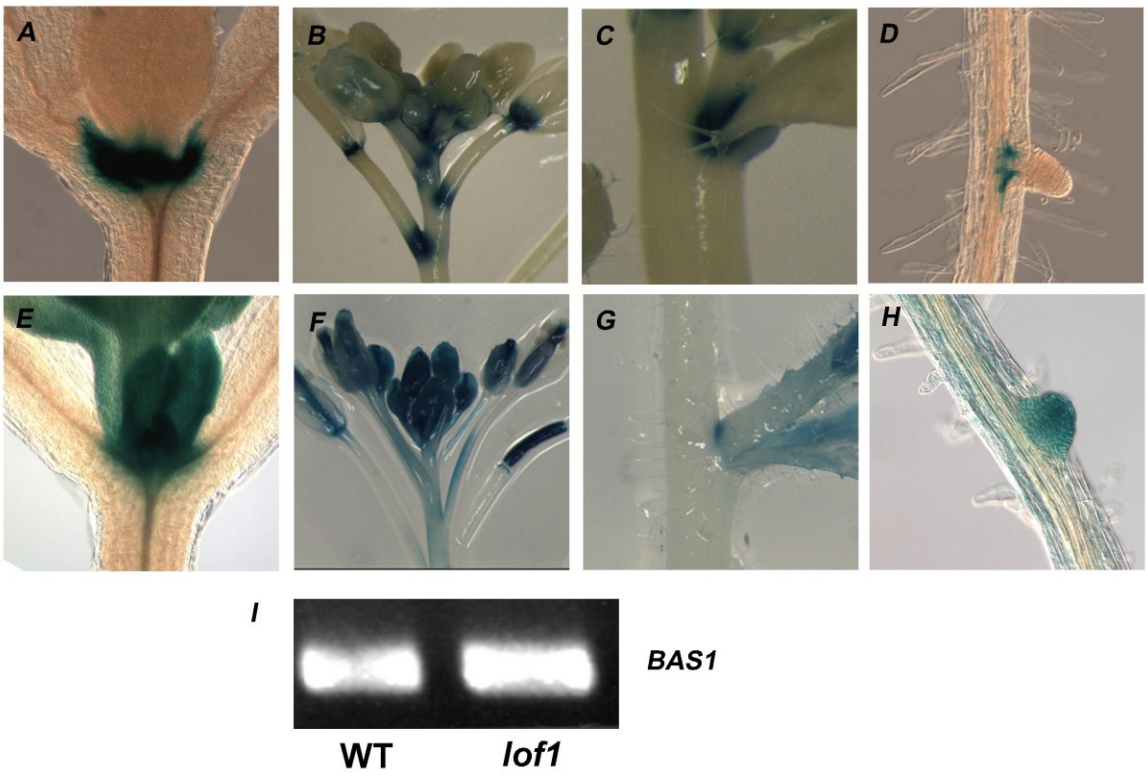

A
B
C
D
E
F
G
H
I
BAS1
WT
lof1

Figure 1.2 *BAS1* transcripts accumulation is positively regulated by LOB activity.

(A) RT-PCR analysis of *BAS1* transcript levels in dissected paraclade junctions of *35S:LOB-GR* plants that were mock-treated or induced with DEX. *BAS1* transcript levels increased in response to DEX treatment relative to the mock treatment. RT-PCR products were detected after 30 cycles of amplification for *BAS1* transcripts. 20 cycles of amplification for *ACTIN2* (*ACT2*) served as a loading control.

(B) RT-PCR analysis of *BAS1* transcript levels in dissected paraclade junctions of *Col* wild-type and *lob-3* plants. *BAS1* transcripts were significantly decreased in *lob-3* mutant compared to that in *Col* wild-type control. RT-PCR products were detected after 30 cycles of amplification for *BAS1* transcripts. 20 cycles of amplification for *ACT2* served as a loading control.

(C) Quantitative RT-PCR analysis of *BAS1* transcripts in dissected paraclade junctions of *Ler*, *lob::DsE*, *Col*, *lob-3*, and *35S:LOB-GR* sprayed with mock or DEX. *BAS1* transcript levels were increased approximately 2.5-fold in the DEX treated *35S:LOB-GR* plants compared to mock treated control. *BAS1* transcripts were decreased approximately 40% in the *lob-3* mutant compared to the *Col* wild-type control. And approximately 50% in the *lob::DsE* mutant compared to the *Ler* wild-type control. n means biological replicates, n=3. Error bar indicates S.D.

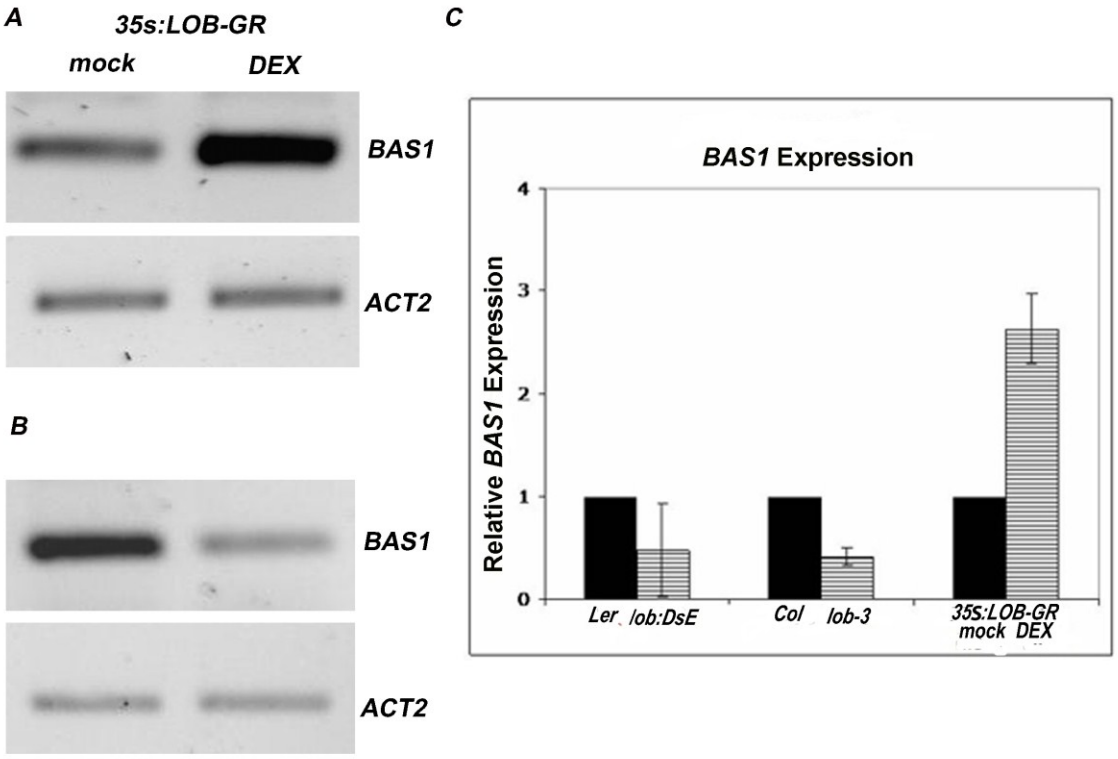
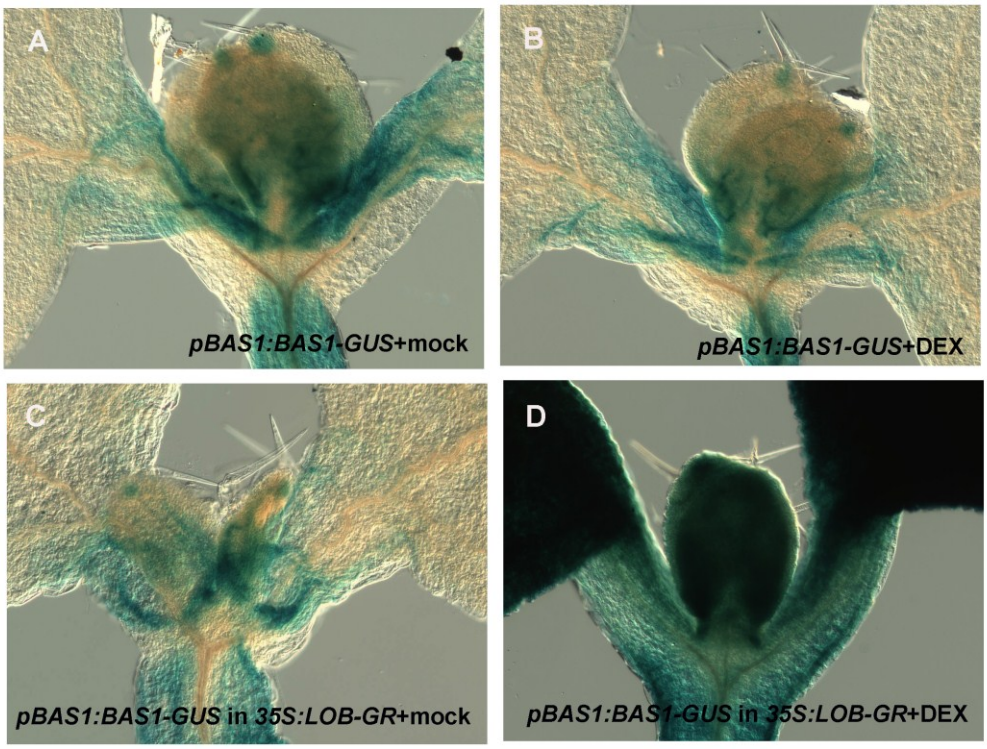

A
35s:LOB-GR
mock
DEX
BAS1
ACT2
B
BAS1
ACT2
C
BAS1 Expression
Relative BAS1 Expression
0
1
2
3
4
Ler lob:DsE
Col lob-3
35S:LOB-GR
mock DEX

Figure 1.3 LOB activates *BAS1* transcription.

GUS expression pattern in *pBAS1:BAS1-GUS* (A-B) and *pBAS1:BAS1-GUS p35S:LOB-GR* (C-D) plants mock-treated (A,C) or treated with 15 μM DEX (B,D) for 24h. (C-D) In the *35S:LOB-GR* background, DEX treatment resulted in an increase in *pBAS1:BAS1-GUS* expression in leaves and hypocotyls (D).

(E) RT-PCR analysis of *BAS1* and *GUS* transcript levels in mock and DEX treated *pBAS1:BAS1-GUS 35S:LOB-GR* plants. RT-PCR products were detected after 28 cycles of amplification for *BAS1* transcripts or 26 cycles of amplification for GUS transcripts. 20 cycles of amplification for *ACT2* served as a loading control.


A
pBAS1:BAS1-GUS+mock
B
pBAS1:BAS1-GUS+DEX
C
pBAS1:BAS1-GUS in 35S:LOB-GR+mock
D
pBAS1:BAS1-GUS in 35S:LOB-GR+DEX

E
pBAS1:BAS1-GUS in 35S:LOB-GR
mock
DEX
BAS1
GUS
ACT2

Figure 1.4 *BAS1* expression is decreased in the *lob-3* mutant background.

GUS activity in *pBAS1:BAS1-GUS* (A-B) and *pBAS1:BAS1-GUS lob-3* (C-D) inflorescences (A, C) and paraclade junctions (B, D).

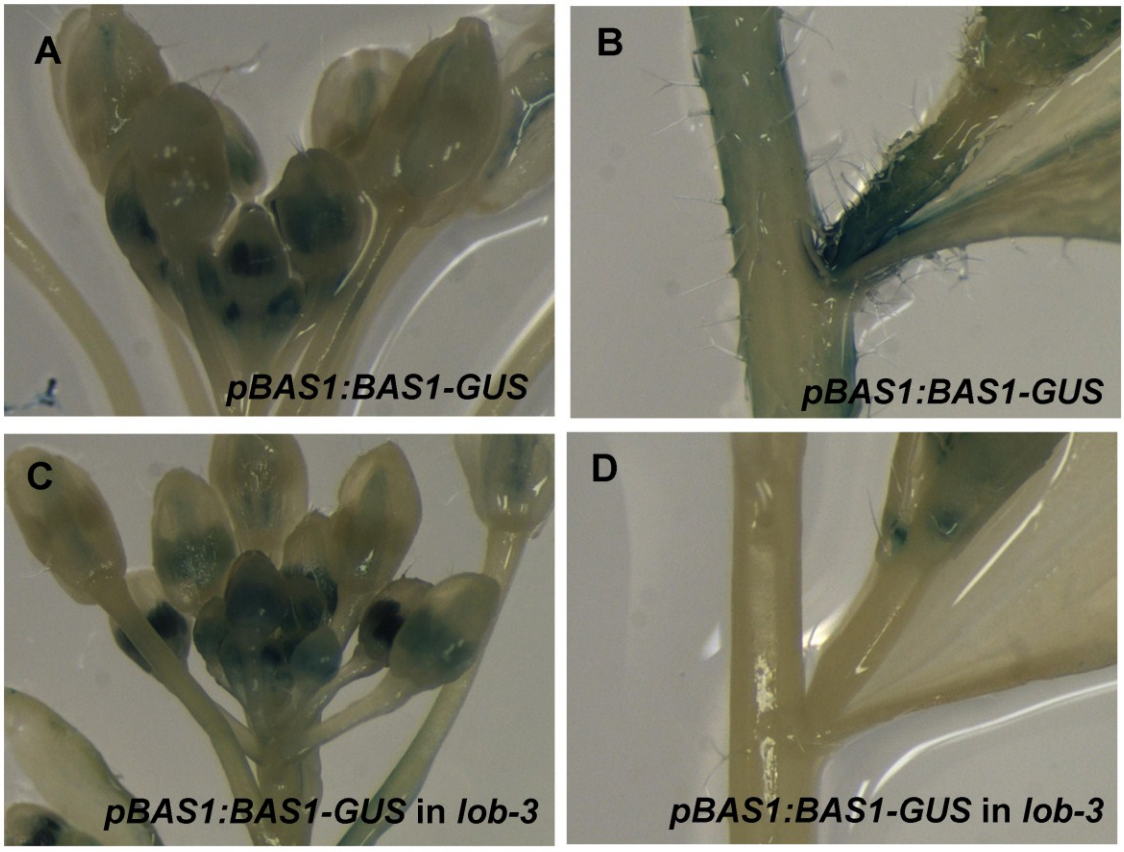
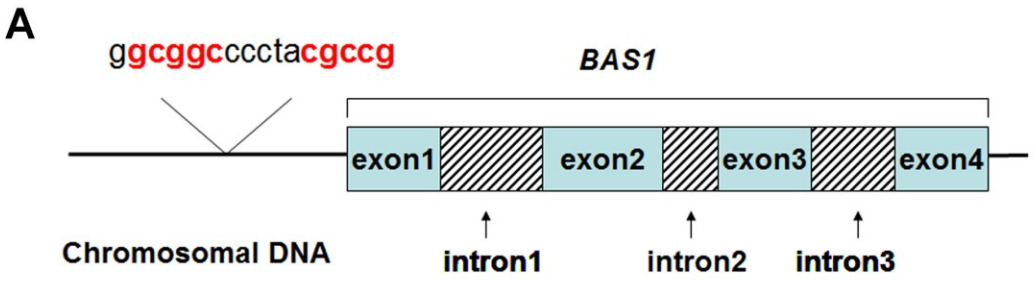
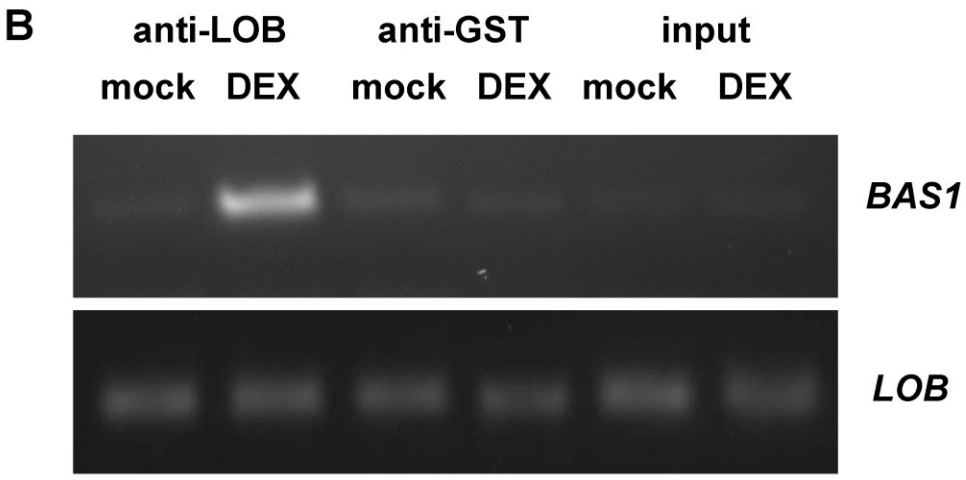

A
pBAS1:BAS1-GUS
B
pBAS1:BAS1-GUS
C
pBAS1:BAS1-GUS in lob-3
D
pBAS1:BAS1-GUS in lob-3

Figure 1.5 LOB binds with *BAS1* promoter in vivo.

(A) Cartoon representing the structure of the *BAS1* gene, comprised of 4 exons interrupted by 3 introns. The putative LOB binding sites CGGCC and CGCCG (marked with red) are located approximately 300 bp upstream of *BAS1* coding region.

(B-C) PCR products obtained from DNA obtained before (input) or after ChIP with anti-LOB or anti-GST antibodies. Ten-day-old *35S:LOB-GR* seedlings treated with mock or 15 μM DEX for 4 hours were used to extract crude chromatin for ChIP assay. The LOB protein and associated DNA were enriched in ChIP samples using anti-LOB antibody. Samples using the GST antibody served as a negative control. PCR products were amplified using semi-quantitative PCR (B) and quantitative PCR (C). Primers spanning the putative LOB binding sites were used to amplify the *BAS1* promoter fragment. For semi-quantitative PCR, 40 cycles of amplification was performed for *BAS1*. 34 cycles of amplification for *LOB* served as a loading control. For quantitative PCR, *ACT2* was used for a normalization control. The *BAS1* fragment including putative LOB binding motifs is enriched by 3-fold in the ChIP sample from DEX-treated *35S:LOB-GR* plants purified with LOB antibody. n means biological replicates, n=3. Error bar indicates S.D.


A
ggcggccctacgccg
BAS1
exon1
exon2
exon3
exon4
Chromosomal DNA
intron1
intron2
intron3


B
anti-LOB
anti-GST
input
mock
DEX
mock
DEX
mock
DEX
BAS1
LOB

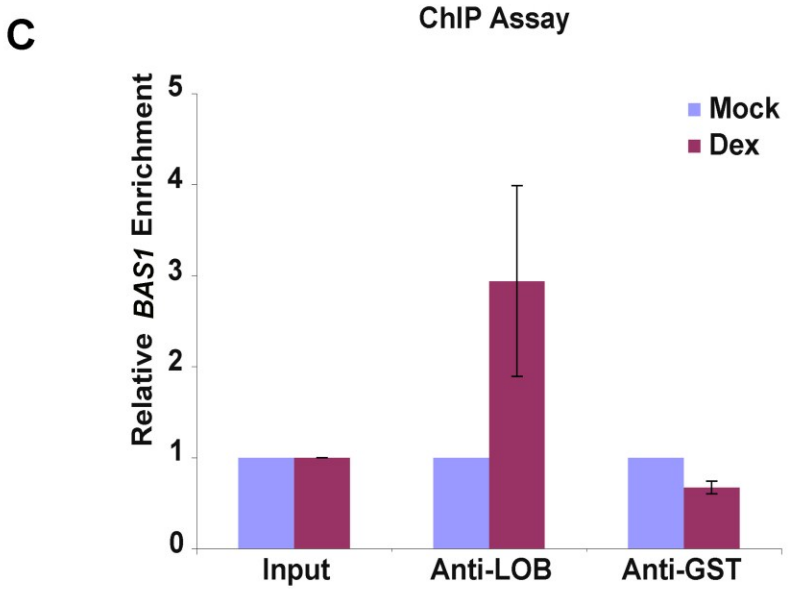
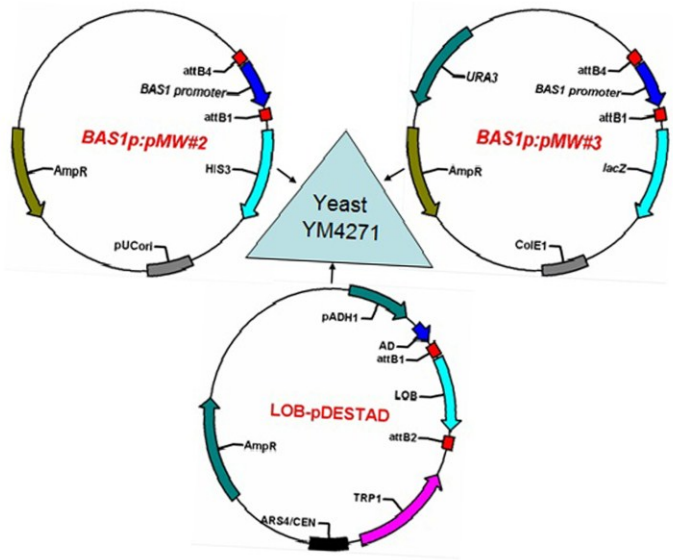

C
ChIP Assay
Relative BAS1 Enrichment
0
1
2
3
4
5
Mock
Dex
Input
Anti-LOB
Anti-GST

Figure 1.6 LOB binds with *BAS1* promoter in a yeast one-hybrid assay.

(A) *BAS1* promoter was integrated into *pMW#2* and *pMW#3* constructs, and transformed into Yeast strain YM4271. LOB is fused to the Gal4 DNA-binding domain in the pDEST^{AD} construct. The yeast clones with *BAS1p:pMW#2* and *BAS1p:pMW#3* constructs that were most sensitive to 3-AT and did not show X-gal activity were used as recipients for introduction of *LOB-pDEST*^{AD} constructs.

(B) Yeast cells carrying the *BAS1* promoter driving the *HIS3* reporter gene and an empty AD control show minimal growth on selective growth medium without 3-AT, but do not grow on medium containing various concentrations of 3-AT. Yeast cells carrying the *BAS1* promoter-reporter construct and the *LOB-AD* construct grow on selective growth medium containing up to 20 mM 3-AT.

A


attB4
BAS1 promoter
attB1
BAS1p:pMW#2
AmpR
HIS3
pUCori
Yeast
YM4271
URA3
attB4
BAS1 promoter
attB1
BAS1p:pMW#3
AmpR
lacZ
ColE1
pADH1
AD
attB1
LOB
LOB-pDESTAD
attB2
AmpR
TRP1
ARS4/CEN

B

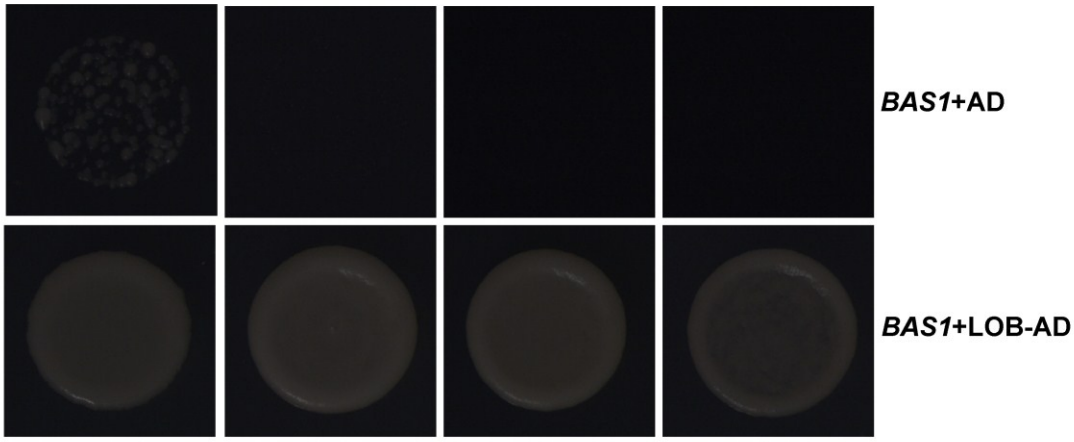

SD/-His/-Ura/-Trp
0mM 3-AT
5mM 3-AT
10mM 3-AT
20mM 3-AT
BAS1+AD
BAS1+LOB-AD

Figure 1.7 Genetic interactions between *bas1-2* and *lob-3* mutants.

(A) Cartoon showing insertion sites the mutants *bas1-2* (top); *lob-3* (middle); and *lob::DsE* (bottom).

(B-E) Phenotype of paraclade junction in *Col* wild-type (B), *bas1-2* (C), *lob-3* (D) and *lob-3 bas1-2* double mutant (E). No organ fusion was detected in *Col* wild-type and *bas1-2* mutant plants. Moderate boundary fusion was detected in *lob-3* and *lob-3 bas1-2* mutant plants. *bas1-2* mutant does not enhance boundary fusion phenotype of *lob-3* mutant.

(F) RT-PCR analysis of *BAS1* transcript levels. *BAS1* transcripts are undetectable in *bas1-2* mutants and significantly reduced in *lob-3* mutant. RNA was extracted from paraclade junctions. RT-PCR products were detected after 30 cycles of amplification for *BAS1* transcripts. 20 cycles of amplification for *ACT2* served as a loading control.

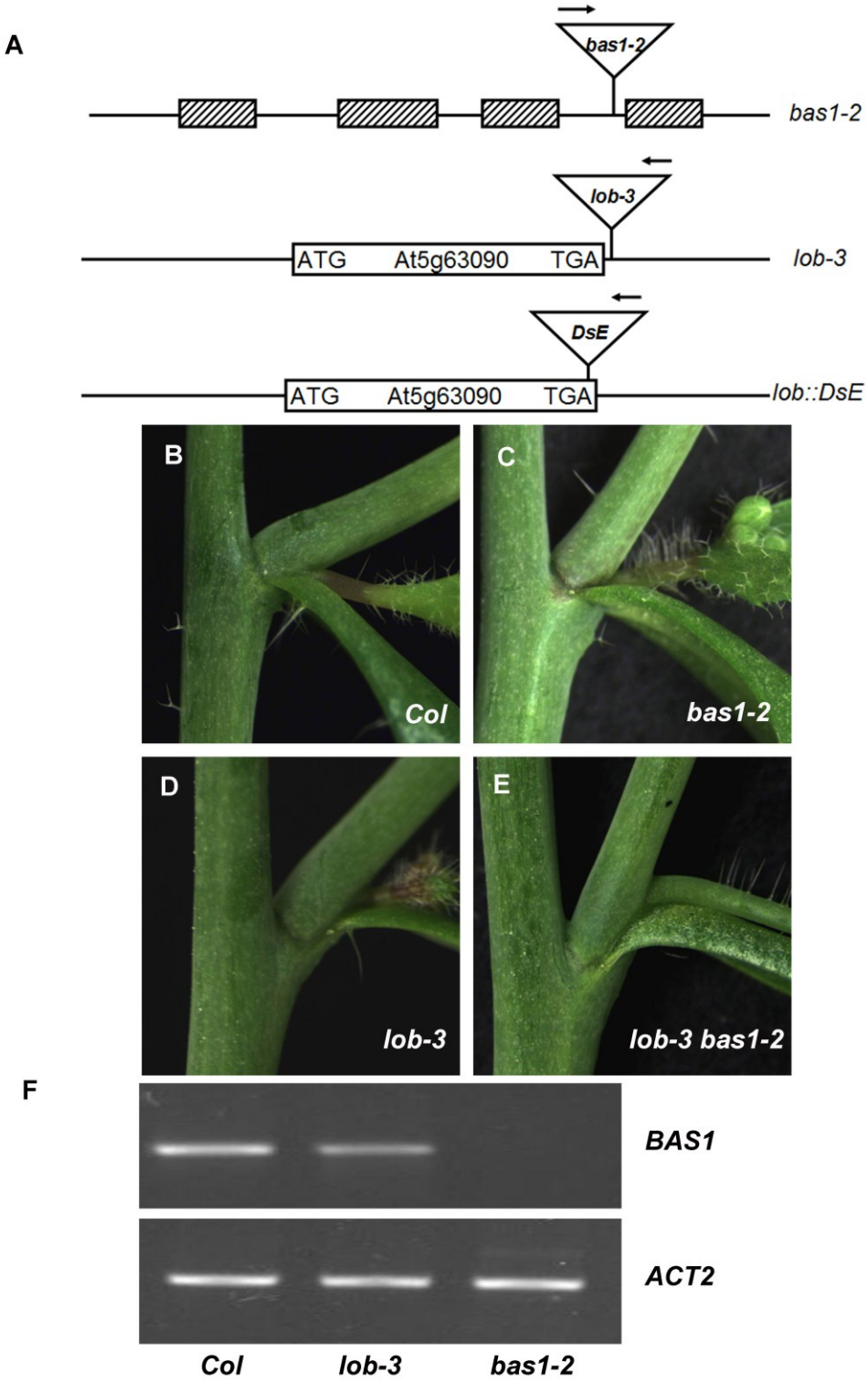

A
bas1-2
bas1-2
lob-3
ATG
At5g63090
TGA
lob-3
DsE
ATG
At5g63090
TGA
lob::DsE
B
Col
C
bas1-2
D
lob-3
E
lob-3 bas1-2
F
BAS1
ACT2
Col
lob-3
bas1-2

Figure 1.8 Overexpression of *BRI1* and *DWF4* in boundary enhances the phenotype of *lob* mutant

(A-F) Overexpression of *BRI1* in boundary driven by *LOB* promoter results in enhanced boundary fusion phenotype in *lob::DsE* mutants. Paraclade junctions of *Col* (A); *pLOB:BRI1* in *Col* (B); *Ler* (C); *pLOB:BRI1* in *Ler* (D); *lob::DsE* (E); *pLOB:BRI1 lob::DsE* (F).

(G-J) Overexpression of *DWF4* in boundary driven by *LOB* promoter enhanced boundary fusion phenotype of *lob::DsE* mutant. *Col* (G) *pLOB:DWF4 Col* (H); *lob::DsE* (I); and *pLOB:DWF4 lob::DsE* (J).

A
Col
C
Ler
E
lob::DsE
B
pLOB:BRI1 in Col
D
pLOB:BRI1 in Ler
F
pLOB:BRI1 in lob::DsE
G
Col
I
lob::DsE
H
pLOB:DWF4 in Col
J
pLOB:DWF4 in lob::DsE

Figure 1.9 Model for *BAS1* mediated BR regulation in boundary region.

Boundary enriched LOB protein upregulates *BAS1* gene expression locally, thus suppressing BR accumulation in the boundary region. Suppressed BR signaling in boundary region is required to limit boundary growth. Positive regulators *DWF4* and *BRI1* overexpression at boundary region enhanced boundary fusion defect in *LOB* loss-of-function mutant.

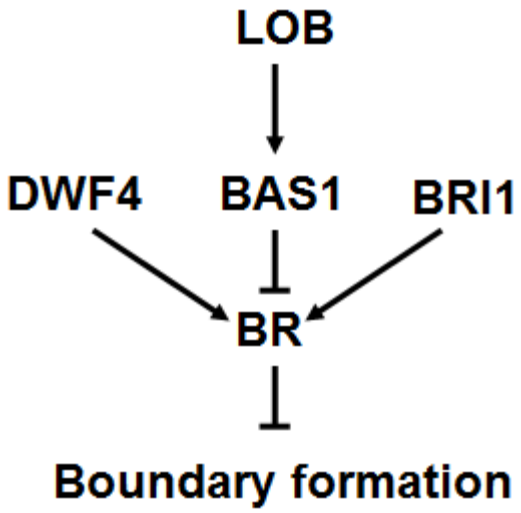

LOB
DWF4
BAS1
BRI1
BR
Boundary formation

Table1.1 Oligonucleotide Sequences

Primer Name	Sequence
lob-3-LP	GCATCCACGCAAACTACAATC
lob-3-RP	CACAAAATCTTTGGAGCAAGC
bas1-2F	CCGACAAGAGGGAATTTGAA
bas1-2R	TCTTCCAAGTCCGGAACAAA
SALK_LBa1	TGGTTCACGTAGTGGGCCATCG
DS3-4N	CCGTCCCGCAAGTTAAATATG
SET223'	GCTCTAGACTCACATGTTACCTCCTTGC
ET22-1	TTCTTCGCCGGAAATGCA
PBAS1-F	GGATCCGGCCTCTCTATTTGTCTGA
PBAS1-R	CCCGGGATCTTTCTTGGTCTCCAC
BAS1 1 Mut1 Top	GTCCTCCAATGGCAACCCCTACGCCGTT
BAS1 1 Mut1 Bottom	AACGGCGTAGGGGTTGCCATTGGAGGAC
BAS1 ChIP 1 F	CCCGGGAAACCTCAATTCGTTGACCTTCAC
BAS1 ChIP 1 R	CCCGGGTTTGCTTGCTGGACTATTTGAGC
QACT-F	GCACCCAGTTCTACTCACAG
QACT-R	CAACATACATGGCAGGGAC
BAS1F	GGCGGAGACAAAACGCTAT
BAS1R	CGGTAGGTGCATGCTGATAA

Primer Name	Sequence
QBAS1-F	CAATCATAGCGGTCCATCAT
QBAS1-R	GGAGCCAAGTGAAAGGTGAA
DWF4-F	CACCATGTTCGAAACAGAGCATCA
DWF4-R	TTACAGAATACGAGAAACCCTA
BRI1-F	CACCATGAAGCTTTTTCAAGCTTC
BRI1-R	TCATAATTTTCCTTCAGGAAC
BAS1 Y1H1-attB4	GGGGACAACTTTGTATAGAAAAGTTGTATTACTAGAAAATGATGTG
BAS1 Y1H1-attB1r	GGGGACTGCTTTTTTGTACAAACTTGCACCAACAAAGAAAAGAGAG
BAS1 F RE probe	CCCGGGAGTCGTTGTTGAAGCTGATAGAGC
BAS1 cds RP	GGATCCTCAATCCTCATGATTGGTCA
LOB gateway F2	CACCATGGCGTCGTCATCAAACTC
LOB-R	GGATCCTCACATGTTACCTCCTTGCT
QLOB-F	GGCGTCGTCATCAAACTCAT
QLOB-R	CGTTGCTTGCTCCAAAGATT
ACT2-F	AAAATGGCCGATGGTGAGG
ACT2-R	ACTCACCACCACGAACCAG
GUS plus-F	CGCGTCAGTACCTGGGAGA
GUS plus-R	GTGATGGTGATGGTGATGGC

Chapter 2 *Arabidopsis BRI1-LIKE 3* (*BRL3*) is a downstream effector of LATERAL ORGAN BOUNDARIES in the boundary region

Abstract

The Meristem-Organ (M-O) boundary is essential for separating the shoot apical meristem and lateral organs. Boundary specification is controlled by boundary-specific genes, including an important LBD family transcription factor, *LATERAL ORGAN BOUNDARIES (LOB).* Previous research revealed that in *Arabidopsis*, *LOB* activates transcription of the brassinosteroid (BR) suppressor *BAS1* to suppress BR signaling in the boundary region. Local suppression of BR signaling is required to limit cell growth in the boundary. In this chapter, we studied another *LOB* downstream pathway mediated by a BR receptor, *BRL3*. In the M-O boundary, *LOB* activates *BRL3* transcription by binding to the *BRL3* promoter. Promoter-reporter fusion experiments revealed that *BRL3* and *LOB* have partially overlapping expression patterns in the M-O boundary region. Moreover, mis-expression of *BRL3* in the boundary caused an organ fusion phenotype and correspondingly altered cellular structure and boundary gene expression in the boundary. This research identified a novel *LOB* downstream regulatory mechanism mediated by *BRL3* and provided a better understanding of the complex *LOB* genetic pathway in M-O boundary specification.

Introduction

The boundary region is an essential structure for normal morphogenesis of both plants and animals by separating two organs with different identities (Aida and Tasaka, 2006a). In plant development, all above ground organs are generated from a group of undifferentiated cells at the shoot apex, called the shoot apical meristem (SAM) (Bowman and Floyd, 2008). At the periphery of the SAM, lateral organ primordia emerge and give rise to leaves and flowers. A special type of boundary tissue named the Meristem-Organ (M-O) boundary is located between the SAM and primordia (Aida and Tasaka, 2006b). The plant M-O boundary has two general functions (Aida and Tasaka, 2006b). First, the M-O boundary functions as a physical barrier to prevent cell mixing between the SAM and lateral organs. Second, the M-O boundary is also required for maintaining SAM identity. Morphologically, the M-O boundary also has special properties. Compared to cells in either the SAM or lateral organ primordia, cells in the M-O boundary are slow growing and dividing, forming a concave valley-like geometry, which can be easily recognized under microscope (Reddy et al., 2004; Aida and Tasaka, 2006a; Rast and Simon, 2008).

The specification and maintenance of the M-O boundary are controlled by boundary-specific genes. Mutants defective in function in one or more boundary specific genes exhibit defects in lateral organ separation and meristem maintenance (Aida et al., 1997; Aida et al., 1999; Schumacher et al., 1999; Greb et al., 2003; Vroemen et al., 2003; Dean et al., 2004; Aida and Tasaka, 2006a; Belles-Boix et al., 2006; Borghi et al., 2007; Rast and Simon, 2008; Bureau et al., 2010; Rast and Simon, 2012). The detailed functions of boundary genes were introduced in the Introduction Chapter.

LATERAL ORGAN BOUNDARIES (LOB) encodes a LBD family transcription factor and gains its name due to its specific expression pattern at lateral organ boundaries (Shuai et al., 2002; Husbands et al., 2007). LOB controls downstream gene expression via binding to a conserved motif in the promoters of target genes (Husbands et al., 2007). *LOB* is important for boundary maintenance since its loss-of-function mutants show a fusion phenotype between the axillary stem and lateral cauline leaf (Bell et al., 2012). To search for possible targets of *LOB* function, a microarray experiment using a Dexamethasone-inducible *35S:LOB-GR* line was carried out (Bell et al., 2012). This microarray experiment revealed that after LOB activation, a negative regulator of brassinosteroid (BR) hormone signaling, *BAS1*, was up-regulated rapidly. BR is a group of poly-hydroxylated steroids functioning as an important growth-promoting hormone (Wang et al., 2004). *BAS1* encodes a cytochrome P450 protein that catalyzes C26 hydroxylation to inactivate BR (Neff et al., 1999). Previous work in the Springer lab and the work reported in Chapter 1 of this dissertation showed that LOB indeed binds the *BAS1* promoter and activates *BAS1* transcription in the boundary region. Expression of *BAS1* in the boundary region, under the *LOB* promoter, can rescue the boundary fusion phenotype in *LOB* loss-of-function mutants. Moreover, mis-activation of BR signaling in the boundary region enhanced the boundary fusion phenotype of a *LOB* loss-of-function mutant, which further supports the importance of LOB-mediated BR suppression in regulation of boundary specification and/or growth.

In the microarray experiment conducted to identify LOB downstream target genes, another BR signaling related gene, *BRL3*, was also identified (Bell et al., 2012). 4 hours after DEX induction, *BRL3* mRNA was up-regulated 4 fold. Moreover, putative LOB binding DNA motifs can also be found in the promoter DNA sequences of the *BRL3*

gene. This interesting finding suggests that in addition to *BAS1*, *BRL3* might be another target downstream of LOB in the boundary region. *BRL3* encodes a BR receptor in *Arabidopsis*, which is structurally similar to BRI1, the primary BR receptor (Cano-Delgado et al., 2004). *BRI1* encodes a leucine-rich repeat receptor-like kinase that functions on the plasma membrane as a BR receptor (He et al., 2000; Wang et al., 2001). The extracellular receptor domain of BRI1 binds BR with high affinity, and once it binds BR, the intracellular effector domain changes conformation and triggers the downstream signaling events (He et al., 2000; Wang et al., 2001; Hothorn et al., 2011; She et al., 2011). BR is a growth-promoting hormone in plants, thus *BRI1* loss-of-function mutant show a severe dwarf plant phenotype (He et al., 2000; Gudesblat and Russinova, 2011). *BRL1, BRL2,* and *BRL3* are *BRI1* homologues in the *Arabidopsis* genome (Cano-Delgado et al., 2004). *BRL1* and *BRL3* have 43% sequence identity with *BRI1* (Cano-Delgado et al., 2004). They contain four conserved domains: a N-terminal signal peptide, an extracellular LRR motif containing domain, a transmembrane domain and an intracellular Ser/Thr kinase domain. *BRL1* and *BRL3* can bind with BR with high affinity. Accordingly, they can complement *BRI1* loss-of-function phenotype when expressed under the control of the *BRI1* promoter (Cano-Delgado et al., 2004). Previous studies also revealed that *BRI1* is expressed more uniformly in multiple tissues, while *BRL1* and *BRL3* expression patterns are more restricted in vascular tissue (Cano-Delgado et al., 2004).

In this chapter, I confirmed that up-regulation of LOB activity indeed causes an increase in *BRL3* expression level using semi-quantitative and quantitative PCR methods and native promoter GUS fusion analysis. Both ChIP and yeast-one-hybrid analysis *revealed* that LOB protein binds to the *BRL3* promoter region. Promoter:GUS

fusion experiments revealed that mutation in the LOB binding motif in the *BRL3* promoter slightly reduced *BRL3* expression in the boundary region, consistent with the hypothesis that LOB up-regulates *BRL3* expression in the boundary region. Moreover, mis-expression of *BRL3* in the boundary region caused a boundary fusion phenotype and correspondingly changed cellular structure in boundary and boundary-specific gene expression. Finally, through studying the genetic interactions between *BRLs* and *LOB* loss-of-function mutants, I found that *lob brl1 brl3* triple mutant demonstrates an enhanced boundary fusion phenotype.

Results

BRL3 expression is activated by LOB in the boundary region

According to a previous microarray study in our lab using a dexamethasone-inducible *35S:LOB-GR* line (Bell et al., 2012), *BRL3* transcripts were increased in response to LOB activation. Furthermore, *BRL3* transcripts were also increased in the *35S:LOB-GR* treated with DEX plus cycloheximide (CHX), a protein synthesis inhibitor, compared to that treated only with CHX. This result indicated that *BRL3* could be directly activated by LOB in the absence of new protein synthesis, suggesting that it is a direct target of LOB regulation.

To confirm that *BRL3* transcripts were indeed up-regulated by LOB, I performed semi-quantitative and quantitative RT-PCR analysis with the boundary tissue of plants in various backgrounds with different *LOB* expression levels. Semi-quantitative RT-PCR results indicated that *BRL3* transcripts were increased in paraclade junction tissues of *35S:LOB-GR* plants after DEX treatment compared to that of mock-treated *35S:LOB-GR* plants (Figure 2.1A). On the other hand, *BRL3* transcripts were decreased in the

paraclade junction of *LOB* loss-of-function mutant *lob-3* plants compared to that of *Col* wild-type control plants (Figure 2.1B). Consistently, quantitative RT-PCR showed that *BRL3* mRNA level was increased by 4-fold in paraclade junction tissues of DEX-treated *35S:LOB-GR* plants compared to that in mock-treated plants (Figure 2.1C). The quantitative RT-PCR experiment also indicated that *BRL3* mRNA level was decreased by 40% and 20% in the boundary tissues of *LOB* loss-of-function mutants *lob::DsE* and *lob-3* plants compared to that of *Ler* wild-type and *Col* wild-type control plants, respectively. In summary, both semi-quantitative and quantitative PCR results indicate that LOB positively controls expression of *BRL3* in the organ boundaries.

LOB binds to the promoter of *BRL3*

As a typical transcription factor, the LOB protein is located in the nucleus and functions in controlling expression of downstream genes via binding to their promoters (Husbands et al., 2007). To test whether LOB activates *BRL3* expression by binding to the promoter of *BRL3 in vivo*, Chromatin Immunoprecipitation (ChIP) was carried out. We used *35S:LOB-GR* plants that were either treated with mock or DEX, and the *BRL3* promoter sequence including the putative LOB binding site (G)CGGC(G), was tested for enrichment in chromatin that co-purified with either the LOB antibody or control GST antibody. Enriched DNA fragments were examined by semi-quantitative PCR with several primer pairs spanning different regions of the *BRL3* promoter. One fragment approximately 3 kb upstream of the *BRL3* coding region was enriched following ChIP with the LOB antibody in the DEX-treated *35S:LOB-GR* plants, whereas this fragment was not enriched in the *35S:LOB-GR* plants treated with mock (Figure 2.2 A, B). Consistently, this fragment around 3 kb upstream of *BRL3* coding sequence was not

enriched by GST antibody in the *35S:LOB-GR* plants treated with mock or DEX (Figure 2.2 B). Further, quantitative PCR on DNA isolated after ChIP with the LOB antibody revealed that the enrichment of this fragment in DEX-treated *35S:LOB-GR* plants was approximately 4-fold (Figure 2.2 C). Fragments in other regions of the *BRL3* promoter that included (G)CGGC(G) putative binding sites were not enriched by the LOB antibody when LOB activity is increased (Data not shown).

To further confirm the binding affinity between LOB and the *BRL3* promoter, a yeast one-hybrid experiment was carried out. The fragment in the *BRL3* promoter that was enriched following pull-down with the LOB antibody in the ChIP experiment, was integrated into the genome of yeast strain YM4271 using two constructs with HIS and URA selectable markers, respectively. A third plasmid including DNA sequences encoding a fusion between LOB and the Gal 4 activation domain (LOB-AD) was transformed into the yeast strain YM4271 containing the *BRL3* promoter fragment integrations (Figure 2.3 A). The yeast one hybrid results showed that yeast cells with the *BRL3* promoter driving the HIS selectable marker and LOB-AD fusion protein can grow on yeast minimal medium lacking histidine, uracil, and tryptophan (SD-His/-Ura/-Trp) plus varying concentrations of 3-aminotriazole (3-AT) up to 10 mM (Figure 2.3 B), while the yeast clones with *BRL3* promoter:HIS integration and the AD protein control were only able to grow on SD/-His /-Ura/-Trp but not on SD/-His /-Ura/-Trp plus concentrations of 3-AT low as 5 mM (Figure 2.3 B), suggesting that LOB binds with *BRL3* promoter in yeast.

In summary, based on the ChIP experiment and yeast-one-hybrid assay, the LOB protein can bind to a fragment of the *BRL3* promoter approximately 3-kb upstream

from the coding region *in vivo* and in yeast, suggesting that *BRL3* functions as a downstream target of LOB.

BRL3 **expression pattern is partially overlapping with** ***LOB***

Since LOB binds with *BRL3* promoter and activates *BRL3* transcription, we would expect that both genes have overlapping expression patterns. We next compared the native gene expression patterns of *BRL3* and *LOB*. According to a previous study (Cano-Delgado et al., 2004), a *GUS* reporter line driven by the *BRL3* promoter is mainly expressed in the vascular tissues of organs such as the leaf and roots. No *pBRL3:GUS* expression in organ boundary was reported previously. However, the *BRL3* promoter generated in that study only includes a promoter fragment around 700 bp upstream of the *BRL3* coding region (Cano-Delgado et al., 2004), which does not include the putative LOB binding sites, thus it is possible that this short form of the *BRL3* promoter does not contain all necessary regulatory elements. To test this possibility, a new *GUS* reporter line including a longer *BRL3* promoter fragment of 3641 bp upstream of the *BRL3* coding region was generated. In this new *pBRL3:GUS* line, GUS activity was detected in both seedling and flowering stages. In the seedlings, GUS activity was observed in all veins of the leaves, and phloem and xylem in roots, with the highest intensity at the site of lateral root initiation, and near the SAM where the mid vein of leaves merged (Figure 2.4 D, E). In the flower of *pBRL3:GUS* plants, GUS activity was detected in all floral organs: sepals, petals, stamens and carpels, with the strongest activity concentrated at the boundary between siliques and pedicels (Figure 2.4 H). In the inflorescence of *pBRL3:GUS* plants, GUS activity was clearly detected in the vascular tissues of cauline leaves and the boundary between stem and lateral organs, but not in the boundary

between stem and pedicels (Figure 2.4 F, I). For comparison, in *pLOB:GUS* seedlings, *GUS* is expressed in the boundary between leaves and hypocotyls, and the boundary between the primary root and lateral roots (Figure 2.4 A, B) (Shuai et al., 2002). In *pLOB:GUS* flowering plants *GUS* was specifically expressed in the junction parts between two different organs, such as the boundary between pedicels and flowers or lateral organs and stem (Figure 2.4 C, G) (Shuai et al., 2002). By comparing the GUS staining patterns in these two different lines, I found that *BRL3* and *LOB* share partially overlapping expression in the organ boundary in both seedlings and flowers, consistent with the conclusion that *BRL3* expression is activated by LOB in boundaries.

LOB up-regulates *BRL3* promoter activity in plants

Microarray analysis and RT-PCR results suggested that *BRL3* transcripts are upregulated by LOB activation. To further confirm that LOB indeed upregulates *BRL3* promoter activity in plants, I examined *pBRL3:GUS* expression pattern in various plant backgrounds with different LOB levels.

I examined the effect of LOB activation on *BRL3* promoter activity by crossing *pBRL3:GUS* and *35S:LOB-GR* plants. In seedlings of *pBRL3:GUS* in the *Col* control background, GUS activity was moderately detected in stipules and leaf veins after both mock and DEX treatment (Figure 2.5 A, B). In flowering *pBRL3:GUS* plants treated with mock or DEX, GUS activity was detected in the floral organs including the junction between the flower and pedicel (Figure 2.5 E, F). Therefore, the GUS activity in *pBRL3:GUS* plants was not affected by DEX treatment. In seedlings of *pBRL3:GUS 35S:LOB-GR* plants treated with mock, the pattern of GUS activity was similar to that in *pBRL3:GUS Col* control plants treated with mock (Figure 2.5 C). However, in

pBRL3:GUS 35S:LOB-GR seedlings treated with DEX for 24 h, the GUS signal was dramatically increased and was detected throughout the seedling including cotyledons, young true leaves and SAM (Figure 2.5 D). GUS intensity was significantly increased in the most organs of flowering *pBRL3:GUS 35S:LOB-GR* plants treated with DEX for 4 h (Figure 2.5 H). Young tissues such as flower buds, cauline leaves, axillary meristems and even the boundary between stem and secondary shoots displayed a higher intensity of *GUS* activity following DEX treatment. As a control, GUS expression in mock-treated *pBRL3:GUS 35S:LOB-GR* flowering plants (Figure 2.5 G) was comparable to that in the *Col* background treated with mock (Figure 2.5 E) or DEX (Figure 2.5 F). RT-PCR was used to examine transcript levels of both *GUS* and native *BRL3* in *pBRL3:GUS 35S:LOB-GR* plants and showed that transcripts of both genes were higher in the paraclade junctions in DEX-treated plants compared to that treated with mock (Figure 2.5 I).

To determine whether any aspect of *BRL3* expression requires LOB activity, I examined *pBRL3:GUS* expression in the *lob-3* background. *pBRL3:GUS* is mainly expressed in young flower buds in the inflorescences of both *Col* (Figure 2.6 A) and *lob-3* plants (Figure 2.6 B). In the boundary cells between axillary meristem and stem, *pBRL3:GUS* signal is significantly reduced in *lob-3* background (Figure 2.6 D, F) compared to that in *Col* background (Figure 2.6 C, E). Since *LOB* is specifically expressed in the paraclade junction region, the local reduction of *pBRL3:GUS* signal in the *lob-3* mutant is consistent with the *LOB* loss-of-function there.

The above experiments using *pBRL3:GUS* in various backgrounds with different levels of LOB activity confirmed that LOB indeed upregulates *BRL3* transcription *in vivo*.

Mutation of the LOB binding site in the *BRL3* promoter slightly reduced *BRL3* expression level

Since LOB can bind with the *BRL3* promoter via a conserved LOB binding site, I determined the effect of mutating this binding site on the *BRL3* expression level. Through PCR-mediated mutagenesis, the two repeat LOB binding sites "CGGCC" centered 3020bp upstream of the *BRL3* CDS (Figure 2.7 A) were mutated to "CAACC" (Figure 2.7 B). Based on our understanding of how LOB binds DNA (Husbands et al., 2007), this mutation is predicted to reduce the binding affinity of LOB for the *BRL3* promoter. The mutated *BRL3* promoter driven *GUS* construct (*pBRL3m:GUS*) was introduced into the *Ler* background. GUS activity in *pBRL3m:GUS* was slightly reduced (Figure 2.7 F-H) compared to the native *pBRL3* promoter driven GUS (*pBRL3:GUS*) expression (Figure 2.7 C-E). I classified the *GUS* expression level into 3 types: weak, moderate, and high level of expression. In native *pBRL3:GUS* plants, the percentages of weak, moderate and high expression level were 15%, 33% and 52%, respectively. In mutated *pBRL3m:GUS* plants, the percentages of weak, moderate and high expression level were 25%, 39% and 36%, respectively. The incomplete reduction of GUS activity by mutating the LOB binding sites indicated that there might be additional LOB binding regions in the promoter region of *BRL3*.

***BRL3* overexpression causes organ fusion**

To understand the function of BRL3 in boundary specification, I generated a *35S:BRL3* overexpression line in the *Ler* background. *35S:BRL3* plants showed some growth defects, such as twisted stems (Figure 2.8 B), which is consistent with previous finding that *BRL3* functions in the vascular tissues (Cano-Delgado et al., 2004). In the

Ler wild-type background, the boundary region clearly separates the stem and cauline leaf, where axillary meristem forms (Figure 2.8 A). When *BRL3* was ectopically expressed throughout the whole plant using the *35S* promoter, the cauline leaf was fused to the axillary stem at the base of these two organs and sometimes the base of cauline leaf extended below the leaf to fuse with the primary stem (Figure 2.8 B, D). This fusion phenotype is similar to that of the *LOB* loss-of-function mutant *lob::DsE* (Figure 2.8 C).

To confirm that the boundary fusion phenotype in *35S:BRL3* overexpression plants was due to ectopic *BRL3* expression locally at the boundary region, I generated boundary specific *pLOB:BRL3* transgenic plants in *Col, Ler* and *lob::DsE* backgrounds. Similar to *35S:BRL3* transgenic plants, *pLOB:BRL3* transgenic plants in both *Col* and *Ler* backgrounds also resulted in fusion between cauline leaf and axillary shoot (Figure 2.9 B, D). Due to the fusion of the cauline leaves and secondary meristem in these plants, the accessory meristem emerges at a position far away from the stem. The length of boundary fusion in *pLOB:BRL3* transformants (Figure 2.9 B, D) is similar to that in *35S:BRL3* transformants (Figure 2.8 B, D) and to that in *lob::DsE* mutant plants (Figure 2.9 E). Furthermore, increased *BRL3* levels in *35S:BRL3* transformants significantly enhanced the boundary fusion phenotype in the loss-of-function mutant *lob::DsE* (Figure 2.9 E, F). Interestingly, the large fusion in the boundary resulted in a change of angles between the axillary and primary shoots. Normally, the axillary shoot in *lob::DsE* is straight and has an approximate 45 degree angle from the stem (Figure 2.9 E). However, the axillary shoot in the *pLOB:BRL3 lob::DsE* transformants was bent at the base of the shoot, then pointed upwards and parallel to the stem (Figure 2.9 F). To confirm that *BRL3* transcript levels were elevated in *pLOB:BRL3 lob::DsE* plants, *in situ*

hybridization was carried out to examine the *BRL3* mRNA level. I used a *BRL3* probe in the C-terminus of the *BRL3* coding region to detect *BRL3* mRNA in the inflorescence tissues (Figure 2.9 G, H). Due to the low transcript level, *BRL3* can't be detected in the inflorescences of *Ler* plants (Figure 2.9 G). However, *BRL3* mRNA was detected in the boundaries between the stem and lateral organs, at the base of the pedicel and base of floral organs in the *pLOB:BRL3* plants (Figure 2.9 H), similar to the pattern of *GUS* expression in *pLOB:GUS* (Figure 2.4 G) (Shuai et al, 2002). RT-PCR analysis also confirmed *BRL3* transcripts were elevated in both inflorescences tissue and dissected paraclade junction tissue of *pLOB:BRL3* mature plants (Figure 2.9 I) compared to wild-type control.

Taken together, mis-expression of *BRL3* in whole plants or specifically in boundary tissue is expected to increase BR signaling, leading to defective boundary formation.

Cellular structure of the boundary region in *pLOB:BRL3* plants is altered

To further understand the cellular changes in *pLOB:BRL3*-induced boundary fusion phenotype, I observed the cellular organization of the boundary region in sections stained with toluidine blue. The cell size in the plant is related to the different development stage. In general, in the *Ler* wild-type background, the shape of boundary cells was close to round and the sizes of boundary cells were similar (Figure 2.10 A), which might be a result of regular cell divisions. In *lob::DsE* backgrounds, the boundary cell shape was more irregular and boundary cell size was bigger than that of *Ler* control plant (Figure 2. C), suggesting irregular cell expansion at the boundary. Furthermore, in boundary of *pLOB:BRL3* and *pLOB:BRL3 lob::DsE,* this cellular structure is disorganized,

with the highest cell intensity and smallest cell size (Figure 2.10 B. D). These observations of boundary cell structure suggested that *BRL3* misexpression in the boundary region activated BR signaling, leading to an increase of cell division rate in the boundary. BR may increase cell expansion in the boundary, but the extent of cell expansion is much lower than cell division, which resulted in smaller cell size of boundary cells in *pLOB:BRL3 lob::DsE* plants.

Altered boundary gene expression in *BRL3* mis-expressing plants

To further study the mechanism by which *BRL3* mis-expression induced organ fusion, I examined expression of boundary genes in the *pLOB:BRL3* plants using promoter:*GUS* fusion analysis. I crossed *lob::DsE* (*LOB* enhancer trap line), *GT185* (*OBO* gene trap line) (Sundaresan et al., 1995), *ET4016* (*LOF1* enhancer trap line) (Lee et al., 2009) and *pLOF2:GUS* (Lee et al., 2009) to *pLOB:BRL3* plants. The pattern of GUS activity of *ET4016* and *GT185* in the *pLOB:BRL3* were similar to those in the *Ler* wild-type background (data not shown). However the levels of *GUS* expression in the *LOB* and *LOF2* lines were significantly decreased in *pLOB:BRL3* plants compared to those in wild-type control (Figure 2.11). In *lob:: DsE* plants, GUS activity driven by the *LOB* promoter resides at the boundary between cauline leaf and axillary shoot, and the boundary region in the inflorescences such as base of flowers and pedicels (Figure 2.11 A, C) (Shuai et al., 2002). The GUS pattern of *lob:: DsE* in *pLOB:BRL3* plants was the same as that in *Ler* wild-type background, but the level of GUS in the junction between fused boundary organs in the *pLOB:BRL3* was significantly reduced compared to that in *Ler* wild-type background (Figure 2.11 B), and the GUS signal in the inflorescences was moderately decreased compared to that in *Ler* wild-type background (Figure 2.11 D). In

pLOF2:GUS plants, GUS activity was clearly restricted at the base of cauline leaves and at the base of flowers (Figure 2.11 E, G). In *pLOF2:GUS pLOB:BRL3* plants, GUS activity was dramatically reduced at the base of flowers and at the base of cauline leaves (Figure 2.11 F, H). In brief summary, mis-expressing of *BRL3* in the boundary region suppressed both *LOB* and *LOF2* expression at the boundary regions, contributing to the defects of boundary separation.

Loss-of-function mutants of *BRLs* enhanced the organ fusion phenotype in *lob* loss-of-function mutant

I examined the genetic interaction between loss-of-function mutants of *BRLs* and *LOB*. Based on the fact that *pLOB:BRL3* mis-expression enhanced the boundary fusion phenotype of *lob-3* mutant, I expected that loss-of-function mutations in *BRL3* and its homologue might suppress the boundary fusion phenotype of the *lob-3* mutant. *brl1-1* and *brl3-1* are knock out mutants that have T-DNA insertions in exons of *BRL1* and *BRL3* (Figure 2.12 A, H); *brl2-1* is a knock-down mutant that has a T-DNA insertion in the 5'-UTR of *BRL2* (Figure 2.12 A, H). Surprisingly, a decrease of *BRL* activity enhanced the boundary fusion phenotype in the *lob-3* mutant (Figure 2.12 B-G, H). In the *lob-3 brl3-1* double mutant (Figure 2.12 F), *lob-3 brl3-1 brl2-1* triple mutant (Figure 2.12 D), and *lob-3 brl3-1 brl1-1* triple mutant (Figure 2.12 G), the boundary fusion phenotype was stronger than that in single mutant of *LOB*, *lob-3* (Figure 2.12 C). Statistical analysis of fusion length at first, second and third nodes from 26-35 plants from each individual genotype confirmed that *lob-3* mutant had the weakest boundary fusion phenotype and *lob-3 brl3-1* mutant slightly enhanced *lob-3* boundary fusion phenotype. Two triple mutants *lob-3 brl3-1 brl1-1* and *lob-3 brl3-1 brl2-1* enhanced the

double mutant *lob-3 brl3-1* boundary fusion phenotype, suggesting a redundancy among *BRL* genes. This observation revealed a complicated genetic interaction between *BRLs* and *LOB*. To explain this interaction, we proposed that in *BRL* loss-of-function mutants, other BR receptor *BRI1* might be up-regulated to compensate and even overcompensate the loss of *BRLs*, thus BR signaling was slightly activated in *BRLs* loss-of-function mutants.

Discussion

Cells at the M-O boundary of plants are specialized for slow growth and division, properties that are important for separation of the SAM and lateral organs (Dumais and Kwiatkowska, 2002; Reddy et al., 2004). Boundary cells also have distinct hormonal activity compared to the SAM and lateral organs (Bowman and Floyd, 2008). In the center of the SAM, cytokinin signaling is active and involved in positive feedback regulation of the SAM identity gene *WUS* (Leibfried et al., 2005; Gordon et al., 2009). In the lateral organ primordia, auxin signaling is active and sustains the rapid growth of lateral organs (Heisler et al., 2005). Besides auxin, BR is a growth promoting plant hormone and BR activity in the L1 layer of plants determines plant organ sizes (Savaldi-Goldstein et al., 2007). In the M-O boundary cells, both auxin reporter and cytokinin reporter are turned off (Heisler et al., 2005; Gordon et al., 2009), indicating suppressed auxin and cytokinin hormonal signaling at the boundary region.

Boundary cell specification is controlled by several boundary specific genes, including a *LBD* family transcription factor LOB. Previous work revealed that LOB locally suppresses BR signaling in the boundary region via up-regulation of a BR inactivating gene *BAS1*. It has been clearly shown that up-regulation of BR signaling in the boundary

region enhanced the boundary fusion phenotype in the *LOB* loss-of-function mutant and down-regulation of BR signaling in the boundary region via *pLOB:BAS1* mis-expression rescued the boundary fusion phenotype of a *LOB* loss-of-function mutant (Bell et al., 2012). Based on previous work in our lab and my work in Chapter 1, a linear model between LOB and BR signaling regulation in boundary has been proposed, according to which LOB negatively regulates BR signaling in the boundary via direct activation of *BAS1* transcription. In other words, local suppression of growth promoting BR signaling in M-O boundary of plants is essential for normal boundary specification and separation of SAM and lateral organs. Similarly, in the valve region of the *Arabidopsis* silique, another growth promoting hormone, auxin, is suppressed to allow specification of valve boundary (Sorefan et al., 2009).

Considering these previous observations, it is a very interesting but seemingly contradictory finding that BRL3, a BR receptor, is also up-regulated by LOB protein activity at the boundary region. Studies in this Chapter clearly showed that on one hand, LOB directly binds to the *BRL3* promoter and activates its expression. On the other hand, mis-expression of *BRL3* in the boundary region led to boundary fusion phenotype and suppressed *LOB* expression at the boundary region. Considering both sides, it is reasonable to conclude that *BRL3* might function in the negative feedback regulation of *LOB* expression in the boundary when *BRL3* expression level is high. This negative feedback regulation might secure the control of boundary specification.

However, the study in this Chapter also revealed that the genetic relationship between *BRL3* and BR signaling is not simple and linear. While mis-expression of *BRL3* caused boundary fusion phenotype, the loss-of-function mutant of *BRL3* also enhanced the boundary fusion phenotype of *LOB* loss-of-function mutant. These observations

suggested that both very high and very low levels of *BRL3* caused increased BR signaling strength at the boundary region. To understand this complex relation between BRL3 and BR signaling strength, we proposed following hypothesis: (1) In *BRL3* loss-of-function mutant, other BR receptors, such as BRI1, might be up-regulated to compensate and even overcompensate the loss of *BRL3*. Under this situation, BR signaling is slightly activated by *BRL3* loss-of-function mutant. (2) In *pLOB:BRL3* mis-expression line, the *BRL3* expression level was dramatically increased in the boundary region, which led to a significant increase in BR signaling strength. This hypothesis not only can explain the genetic interactions between *BRL3* and *LOB*, but also revealed a possible overcompensating mechanism between BRL3 and other BR receptors. Future experiments on expression pattern of BR receptors in boundary of *BRL3* loss-of-function mutant will be helpful to validate our current hypothesis.

Materials and methods

Plant Materials

T-DNA insertional mutants *brl1-1* (SALK_005982), *brl2-1* (SALK_142625) and *brl3-1* (SALK_800036) were isolated from the SALK Institute Genomic Analysis Laboratory collection (Caño-Delgado et al., 2004). *brl1-1* homozygous mutants were identified by PCR-based genotyping with specific primers SALK_005982-LP, SALK_005982-RP and SALK_LBa1 (Table 2.1). *brl2-1* homozygous mutants were identified by PCR-based genotyping with specific primers SALK_142625-LP, SALK_142625-RP and SALK_LBa1 (Table 2.1). *brl3-1* homozygous mutants were identified by PCR-based genotyping with specific primers BRL3-OX-F, BRL3-R and SALK_LBa1 (Table 2.1). The *lob brl3* double mutant was generated by crossing *lob-3*

with *brl3-1* and the homozygous double mutant was confirmed by genotyping with related primers. *lob brl1 brl3* and *lob brl2 brl3* triple mutants were generated by crossing *brl1-1* or *brl2-1* with *lob brl3* double mutant and confirmed by genotyping with related primers.

Growth Conditions

Arabidopsis seeds were sterilized with 95% ethanol for 5 min, then sprayed into the soil and grown under the long-day growth conditions (16 hours light/8 hours dark). For DEX induction, *35S:LOB-GR* seeds were sterilized with 20% bleach containing 0.1% Tween-20 for 5 min, washed with sterile water, and sowed on MS plates (Murashige et al., 1962). After 10 days growth, *35S:LOB-GR* seedlings were incubated with 30 ml 15 μM Dexamethasone (DEX) for 4 hours with shaking, then harvested for ChIP experiment.

Plasmid constructs

To generate the *pBRL3:GUS* construct, a genomic fragment 3641 bp upstream of the *BRL3* coding region was first amplified from *Col* genomic DNA with primers pBRL3-F and pBRL3-S-R (Table 2.1). The amplified fragment was then cloned into the TOPO II vector (Invitrogen), sequenced to confirm integrity, and then subcloned into the binary vector pCB308 (Xiang et al, 2009) using the introduced *Xba*I and *Xma*I restriction sites. To generate the mutated *pBRL3m:GUS* construct, the *BRL3* promoter was first amplified from *pBRL3:GUS* plasmid using primers pBRL3-GATE-F and pBRL3-S-R (Table 2.1), then cloned into the pENTR TOPO vector. The two LOB binding sites in the *pBRL3* promoter in *pBRL3:pENTR* were mutated by amplification with primers pBRL3-muta-F and pBRL3-muta-R (Table 2.1) following site-directed mutagenesis protocol

(Stratagene). The mutated *BRL3* promoter sequence was then subcloned into *pBRL3:GUS* by replacing the native *BRL3* promoter sequence using the *Xba* I and *Xma* I restriction sites. The *35S:BRL3* and *pLOB:BRL3* constructs were made using the GATEWAY cloning system. The *BRL3* full length CDS was amplified with primers BRL3-GATE-F and BRL3 OX-R (Table 2.1), cloned into pENTR TOPO vector. *35S:BRL3* was generated by LR reaction with BRL3-pENTR and pGWB2, while *pLOB:BRL3* was made by LR reaction with BRL3-pENTR and pLOB:Frame A-3'UTR (Described in Chapter 1). The generated constructs were introduced to *Col* and *Ler* wild-type *Arabidopsis* plants using the floral-dip method (Clough et al., 1998).

ChIP assay

The protocol for extraction of DNA samples following ChIP from *35S:LOB-GR* seedlings treated with Mock or DEX was described in Chapter 1. The *BRL3* promoter fragment was amplified with primers BRL3 ChIP-F and BRL3 ChIP-R. *LOB* amplified with QLOB-F and QLOB-R was used for the normalization control for semi-quantitative PCR. *ACTIN2* (*ACT2*) amplified with QACT2-F and QACT2-R was used for the normalization control for quantitative PCR. Primers were shown in Table 2.1.

Gene Expression Analysis

One-month old *35S:LOB-GR* plants were sprayed 0.05% EtOH or 0.05% EtOH+15 μM DEX in 0.02% [v/v] Silwet. Paraclade junction tissue of *Arabidopsis* plants was harvested after 4 h Mock or DEX treatment. *pBRL3:GUS 35S:LOB-GR* seedlings were flooded with 0.05% EtOH or 0.05% EtOH+15 μM DEX for 24h before harvesting. Total RNA was isolated using Trizol extraction buffer and quantified by the NanoDrop

spectrophotometer. The RNA was treated with DNase at 37°C for 1 h before cDNA synthesis. cDNA was synthesized from 3 μg of total RNA using Superscript III following the manufacturer's proto*col* (Invitrogen). For semi-quantitative RT-PCR analysis, *BRL3* was amplified with primers BRL3-F and BRL3-R (Table 2.1), GUS was amplified with primers GUS-F and GUS-R, while *ACT2* amplified with primers ACT2-F and ACT2-R (Table 2.1) was used for equal loading control. For quantitative RT-PCR, BRL3 was amplified with primers QBRL3-F and QBRL3-R (Table 2.1), while *ACT2* amplified with primers QACT2-F and QACT2-R (Table 2.1) was used as a normalization control.

Yeast one hybrid assay

BRL3p:pMW#2 and *BRL3p:pMW#3* constructs were generated with GATEWAY cloning method (Invitrogen). The *BRL3* promoter fragment (-3099bp to -2688bp) was amplified from *Col* genomic DNA using primers BRL3 Y1H1-attB4 together with BRL3 Y1H1-attB1R (Table 2.1), which contained introduced attB4 and attB1R sites, and subsequently cloned into pDONR4R-P1R. *BRL3p:pMW#2* and *BRL3p:pMW#3* constructs were generated using the LR recombination reactions between *BRL3-pDONR4R-P1R* and *pMW#2* or *pMW#3*, respectively. The plasmids *BRL3p:pMW#2* and *BRL3p:pMW#3* were transformed into yeast strain YM4271 sequentially as previously described (Deplancke et al., 2006). Autoactivation was detected by patching the positive clones on SD-His-Ura + 0, 5, 10, 20, or 40 mM 3-AT and beta-Gal Assay (Clontech). The clones with the lowest autoactivation were used for LOB-pDEST^{AD} (Chapter I) transformation. The yeast clones that were most sensitive to 3-AT and did not show X-gal activity were used as recipients for introduction of *LOB-pDEST^{AD}* constructs, and

selected on SD-His-Ura-Trp with appropriate concentration of 3-AT. All primers are shown in Table 2.1).

In Situ Hybridization

Inflorescence tissues were fixed in 4% paraformaldehyde (with 1xPBS) and embedded in paraffin. A truncated *BRL3* cDNA was amplified from BRL3-pENTR with primers Sp6-BRL3-F and T7-BRL3-R (Table 2.1). The *BRL3* anti-sense probe was synthesized using T7 RNA polymerase, while the *BRL3* sense probe was synthesized using Sp6 RNA polymerase. The *in situ* hybridization protocol to label *BRL3* transcripts in *pLOB:BRL3* plants was performed as previously described (Yu et al., 2009).

GUS staining, histology and microscopy

GUS staining and sectioning of boundary tissue were carried out as previously described (Lee et al., 2009). Imaging and microscopy procedures were described previously (Lin et al., 2003).

References:

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Figure 2.1 LOB upregulates *BRL3* transcript accumulation in boundary tissues.

(A, B) RT-PCR analysis of *BRL3* transcript levels in dissected paraclade junctions of *35S:LOB-GR* plants that were mock-treated or induced with DEX (A) and *Col* wild-type and *lob-3* mutants (B). *BRL3* transcript levels were increased in *35S:LOB-GR* in response to DEX treatment and decreased in *lob-3* relative to *Col*. 28 cycles and 30 cycles of amplification of *BRL3* were performed in *35S:LOB-GR* samples and *lob-3* mutant, respectively. 20 cycles of amplification of *ACTIN2 (ACT2)* served as a loading control.

(C) Quantitative RT-PCR analyses of *BRL3* transcripts in dissected paraclade junctions in *Ler* and *Col* wild-type, *lob::DsE* and *lob-3* mutants, and mock- and DEX-treated *35S:LOB-GR* plants. *ACT2* transcripts were amplified as a normalization control. Compared with that in wild-type control plants, the level of *BRL3* transcripts was decreased in *lob* mutants. Compared with that in mock-treated *35S:LOB-GR* plants, the level of *BRL3* transcripts was increased 4-fold in Dex-treated plants. n means biological replicates, n=3. Error bar indicates S.D.

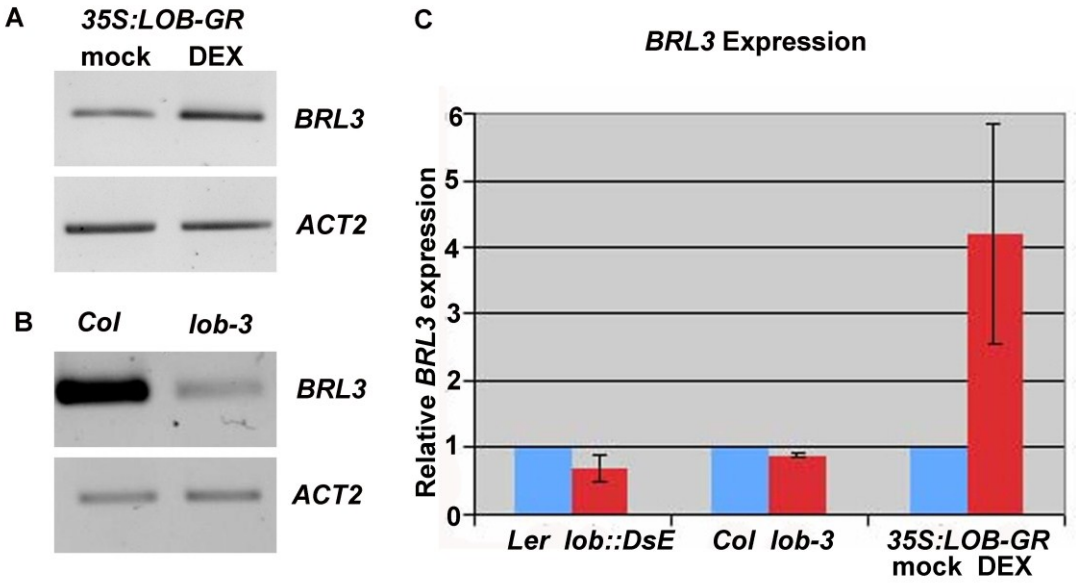

A
35S:LOB-GR
mock
DEX
BRL3
ACT2
B
Col
lob-3
BRL3
ACT2
C
BRL3 Expression
Relative BRL3 expression
0
1
2
3
4
5
6
Ler
lob::DsE
Col
lob-3
35S:LOB-GR
mock
DEX

Figure 2.2 LOB binds the *BRL3* promoter *in vivo*.

(A) Cartoon representing the structure of the *BRL3* gene, composed of 1 exon. The two putative *LOB* binding sites CGGCC (marked with red) are located approximately 3 kb upstream of the *BRL3* coding region.

(B, C) ChIP assay demonstrating that LOB binds with the *BRL3* promoter. PCR products amplified from DNA obtained before (input) or after ChIP with anti-LOB or anti-GST antibodies. Ten-day old *35S:LOB-GR* seedlings treated with mock or 15 μM DEX for 4 hours were used to extract crude chromatin for ChIP assay. The LOB protein and associated DNA were pulled down in ChIP samples using anti-LOB antibody. Samples using the GST antibody served as a negative control. Primers spanning the putative LOB-binding sites were used to amplify the *BRL3* promoter fragment. (B) PCR products amplified using semi-quantitative PCR. Primers amplifying the *LOB* genomic sequence were used for normalization. 40 cycles and 34 cycles of amplification of *BRL3* promoter fragment and LOB were performed, respectively. (C) PCR products amplified using quantitative PCR. Primers amplifying the *ACT2* genomic sequence were used for normalization The *BRL3* fragment was enriched by 4-fold in the ChIP sample with the LOB antibody from DEX-treated *35S:LOB-GR* plants relative to mock-treated. *BRL3* DNA was not enriched in the ChIP sample using the GST antibody. n means biological replicates, n=3. Error bar indicates S.D.

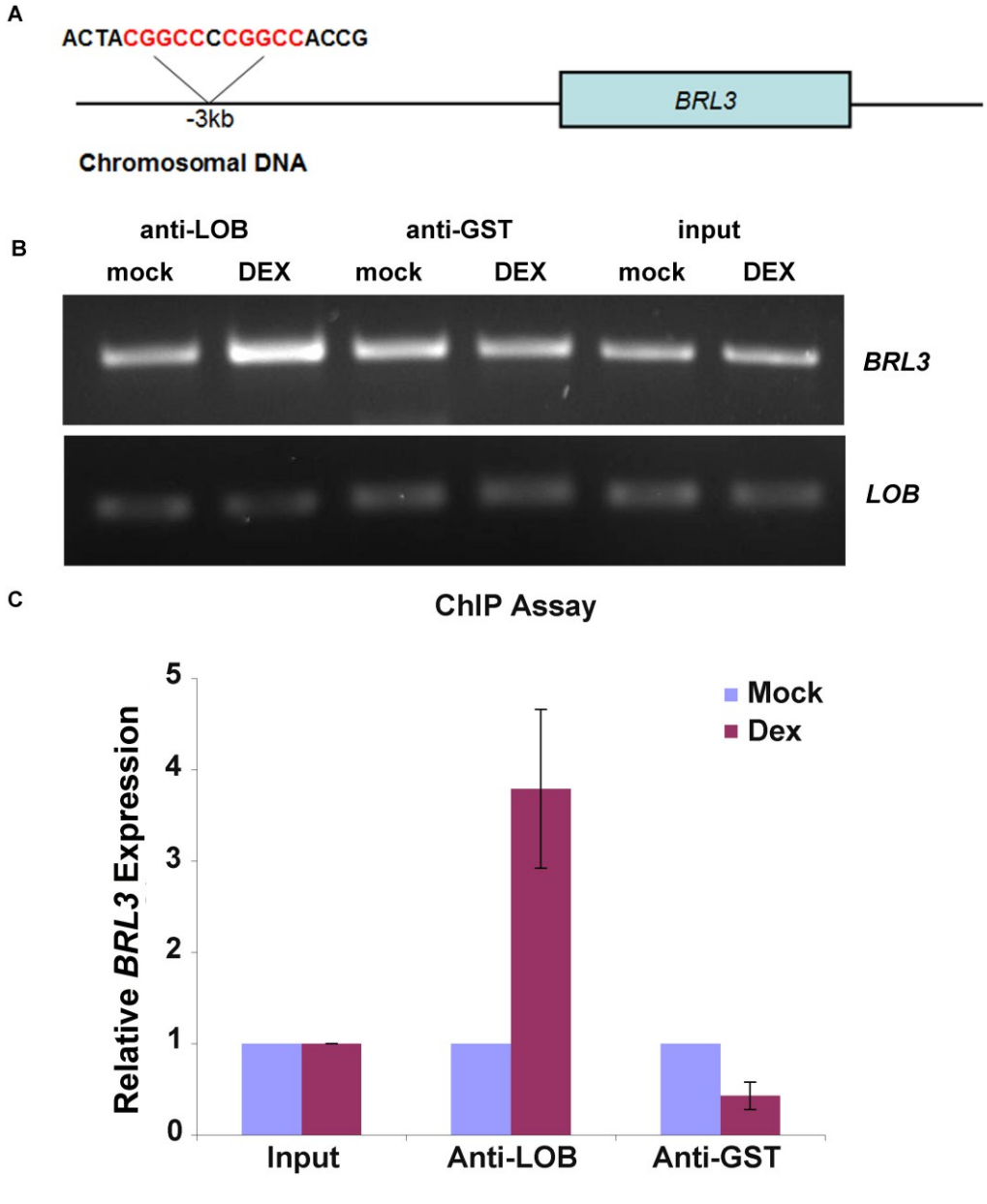
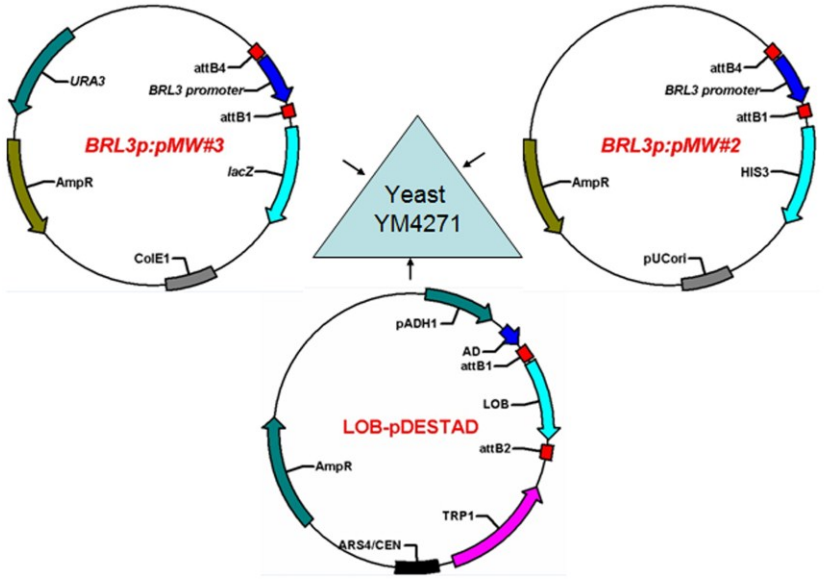

A
ACTACGGCCCCGGCCACCG
-3kb
BRL3
Chromosomal DNA
B
anti-LOB
anti-GST
input
mock
DEX
mock
DEX
mock
DEX
BRL3
LOB
C
ChIP Assay
Relative BRL3 Expression
5
4
3
2
1
0
Mock
Dex
Input
Anti-LOB
Anti-GST

Figure 2.3 LOB binds with *BRL3* promoter in a yeast one-hybrid assay.

(A) The *BRL3* promoter fragment was integrated into *pMW#2* and *pMW#3* constructs, and transformed into Yeast strain YM4271. LOB was fused to the Gal4 DNA-binding domain in the pDEST^{AD} construct. The yeast clones with *BRL3p:pMW#2* and *BRL3p:pMW#3* constructs that were most sensitive to 3-AT and did not show X-gal activity were used as recipients for introduction of *LOB-pDEST*^{AD} constructs.

(B). Yeast YM4271 cells carrying the *BRL3* promoter driving the *HIS3* reporter gene and an empty AD control show minimal growth on selective growth SD/-His/-Ura/-Trp medium but cannot grow on SD/-His/-Ura/-Trp containing various concentrations of 3-AT (upper lane). Yeast cells carrying the *BRL3* promoter-reporter construct and *LOB-AD* fusion constructs grow well on 3-AT selective growth medium up to 10 mM 3-AT level (lower lane).

A


URA3
attB4
BRL3 promoter
attB1
BRL3p:pMW#3
lacZ
AmpR
ColE1
Yeast
YM4271
attB4
BRL3 promoter
attB1
BRL3p:pMW#2
HIS3
AmpR
pUCori
pADH1
AD
attB1
LOB
LOB-pDESTAD
attB2
AmpR
TRP1
ARS4/CEN

B

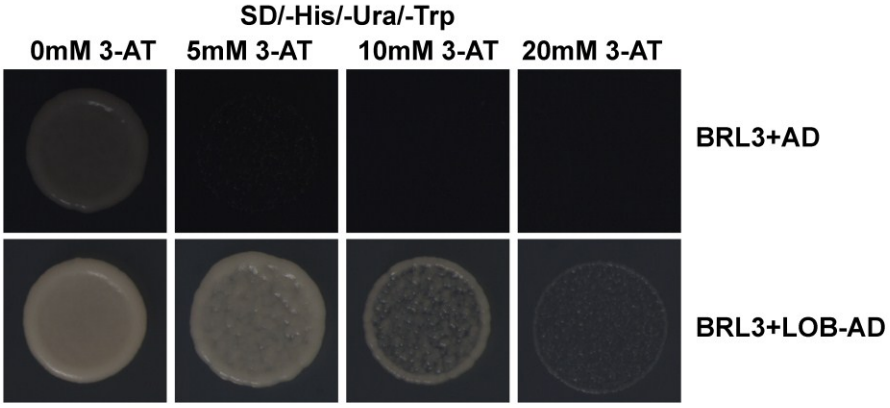

SD/-His/-Ura/-Trp
0mM 3-AT
5mM 3-AT
10mM 3-AT
20mM 3-AT
BRL3+AD
BRL3+LOB-AD

Figure 2.4 *LOB* and *BRL3* expression pattern in *Arabidopsis* plants.

(A-C,G) GUS activity in *pLOB:GUS* plants. Whole mount of young seedling (A), root (B), and inflorescence (G). Longitudinal section of paraclade junction (C).

(D-F, H, I) GUS activity in *pBRL3:GUS* plants. Whole mount of young seedling (D), root (E) and inflorescence (H,I). Longitudinal section of paraclade junction (F).

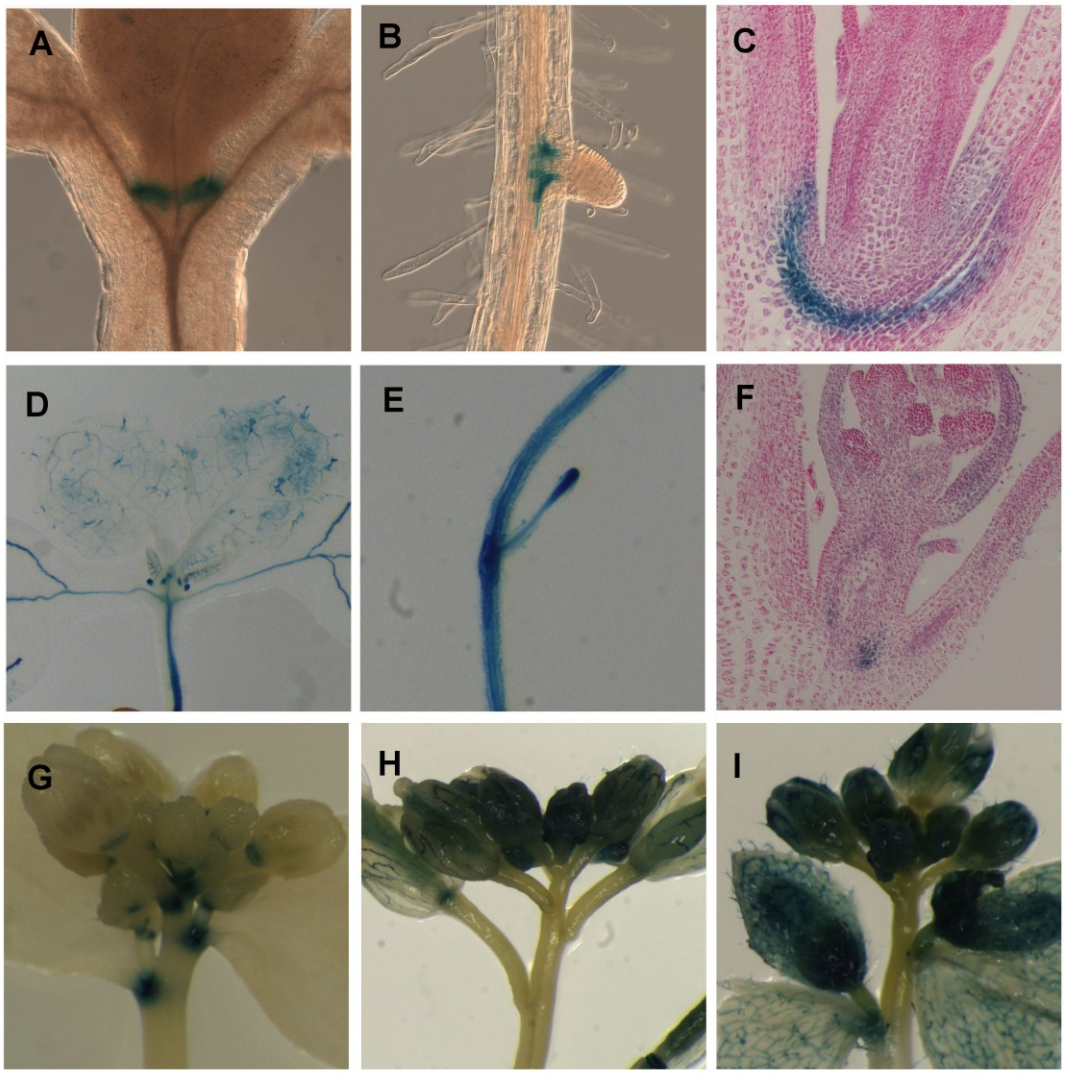
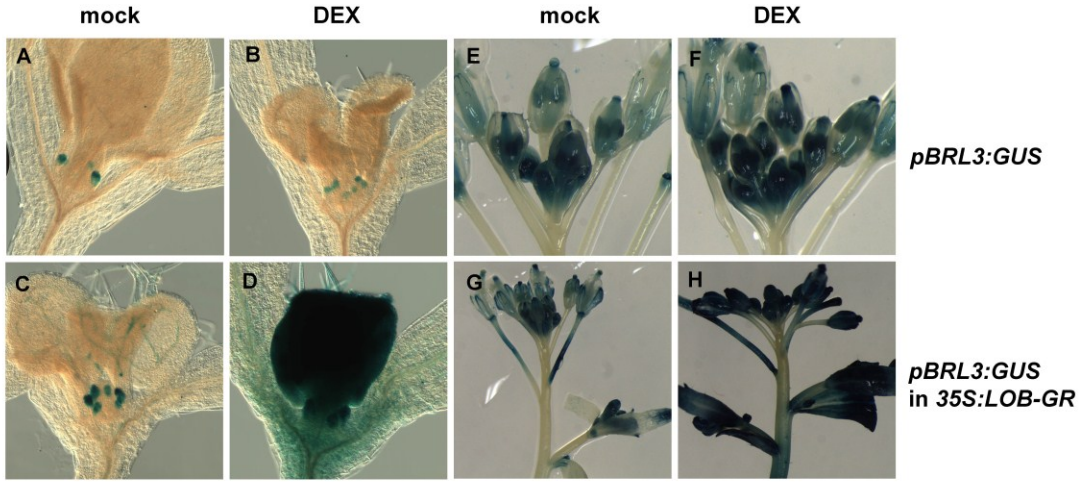

A
B
C
D
E
F
G
H
I

Figure 2.5 LOB activates *BRL3* transcription.

GUS expression pattern in *pBRL3:GUS* (A, B, E, F) and *pBRL3:GUS 35S:LOB-GR* (C, D, G, H) plants mock treated (A, C, E, G) or treated with DEX (B, D, F, H). *pBRL3:GUS* expression was detected in stipules and leaf veins of young seedlings (A, B) and in the young flower buds in the inflorescences (E, F). No significant difference was detected between *pBRL3:GUS* plants treated with mock (A, E) and those treated with DEX (B, F). GUS activity was significantly increased in *pBRL3:GUS 35S:LOB-GR* plants treated with DEX (D, H) compared to those mock-treated (C, G). (I). RT-PCR analysis revealed that *BRL3* transcripts are up-regulated by LOB. Both *GUS* and native *BRL3* transcripts were increased in *pBRL3:GUS 35S:LOB-GR* plants treated with DEX compared to that treated with mock. RT-PCR products were detected after 28 cycles of amplification for *BRL3* transcripts or 26 cycles of amplification for *GUS* transcripts. 20 cycles of amplification for *ACT2* served as a loading control.


mock
DEX
mock
DEX
A
B
E
F
pBRL3:GUS
C
D
G
H
pBRL3:GUS
in 35S:LOB-GR

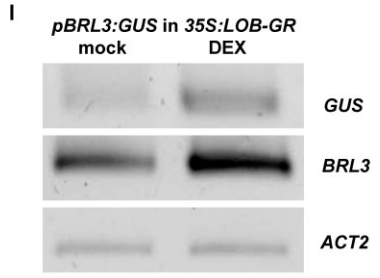

I
pBRL3:GUS in 35S:LOB-GR
mock
DEX
GUS
BRL3
ACT2

Figure 2.6 *BRL3* transcription is reduced in the *lob-3* loss-of-function mutant.

GUS expression pattern in *pBRL3:GUS* (A, C, E) and *pBRL3:GUS lob-3* (B, D, F) inflorescences (A, B) and paraclade junctions (C-F). (E-F) show longitudinal sections of paraclade junctions.

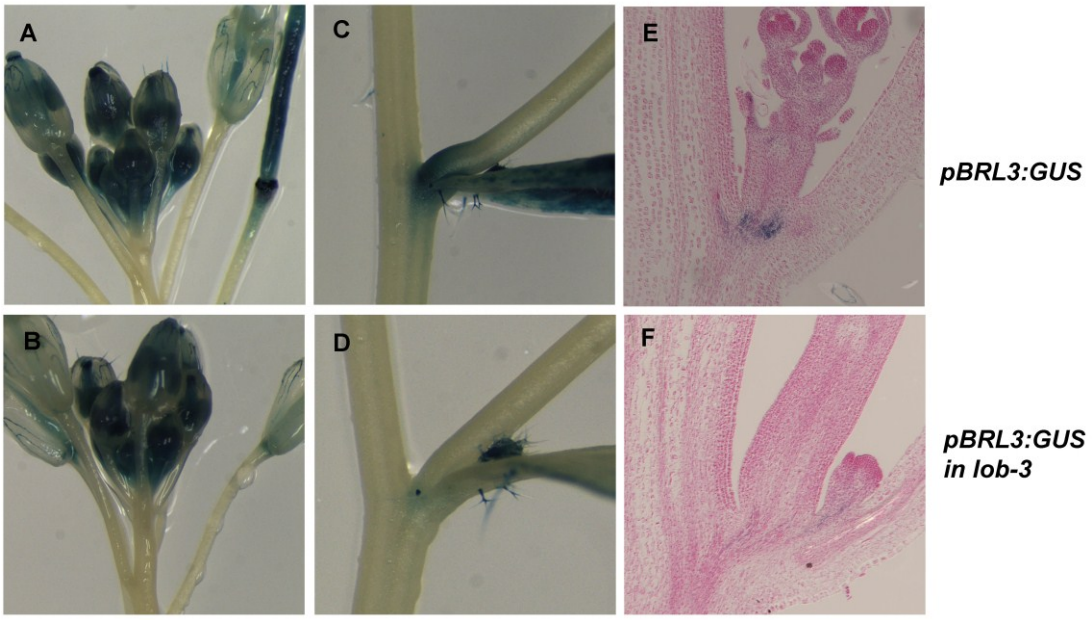

A
C
E
B
D
F
pBRL3:GUS
pBRL3:GUS
in lob-3

Figure 2.7 Mutagenesis of LOB-binding site in *BRL3* promoter.

(A, B) Cartoon representing structure of *pBRL3:GUS* constructs. The putative LOB binding sites in the 3 kb upstream of the *BRL3* ORF (A, shown in red) and mutated putative *LOB* binding sites (B, shown in red).

(B) GUS activity of *pBRL3:GUS* (C, D, E) and *pBRL3:GUS* (F, G, H). GUS signal in the *pBRL3m:GUS* is slightly decreased compared to that in *pBRL3:GUS*. For both *pBRL3:GUS* and *pBRL3m:GUS* plants, the *GUS* expression level was classified into 3 types: low (left); moderate (middle); high (right).

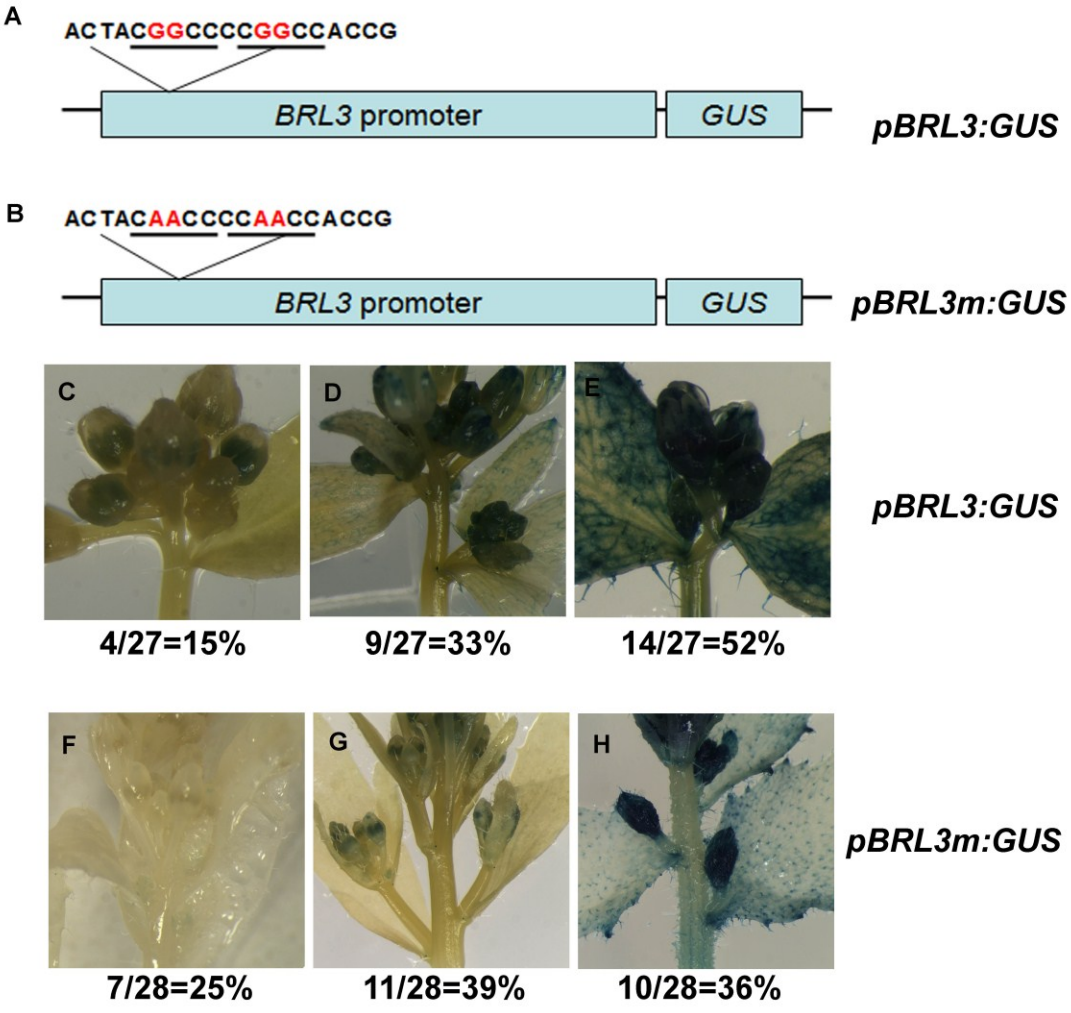

A
ACTACGGCCCCGGCCACCG
BRL3 promoter
GUS
pBRL3:GUS
B
ACTACAACCCCAACCACCG
BRL3 promoter
GUS
pBRL3m:GUS
C
D
E
pBRL3:GUS
4/27=15%
9/27=33%
14/27=52%
F
G
H
pBRL3m:GUS
7/28=25%
11/28=39%
10/28=36%

Figure 2.8 Overexpression of *BRL3* causes fusion in the boundary region of mature plants.

Paraclade junction of *Ler* (A), *35S:BRL3* (B, D), and *lob::DsE* (C).

A
Ler
B
35S:BRL3 in Ler
C
lob::DsE
D
35S:BRL3 in Ler

Figure 2.9 Mis-expression of *BRL3* in boundary enhanced boundary fusion phenotype of *lob::DsE*.

(A-D) Paraclade junction of *Col* (A); *pLOB:BRL3* in *Col* (B); *Ler* (C); *pLOB:BRL3* in *Ler* (D); *lob::DsE* (E); and *pLOB:BRL3 lob::DsE*. In wild-type *Col* and *Ler*, the axillary shoot and cauline leaf are well separated (A, C). *pLOB:BRL3* mis-expression lines in *Col* and *Ler* caused moderate fusion between axillary meristem and leaf (B, D). *pLOB:BRL3* enhances the *lob::DsE* phenotype (E,F).

(G, H) *In situ* hybridization detecting *BRL3* transcripts in *Ler* wild-type plants (G) and *pLOB:BRL3* transformants (H). (I) RT-PCR analysis of *BRL3* transcript accumulation in *pLOB:BRL3* plants. Total RNA was extracted from inflorescence tissues of *Ler* (Lane 1) and *pLOB:BRL3* plants (Lane 2) or from dissected paraclade junctions of *Ler* (Lane 3) and *pLOB:BRL3* (Lane 4) plants. *ACT2* was used as a loading control. 27 cycles of amplification for *BRL3* and 20 cycles of amplification for *ACT2* were performed.

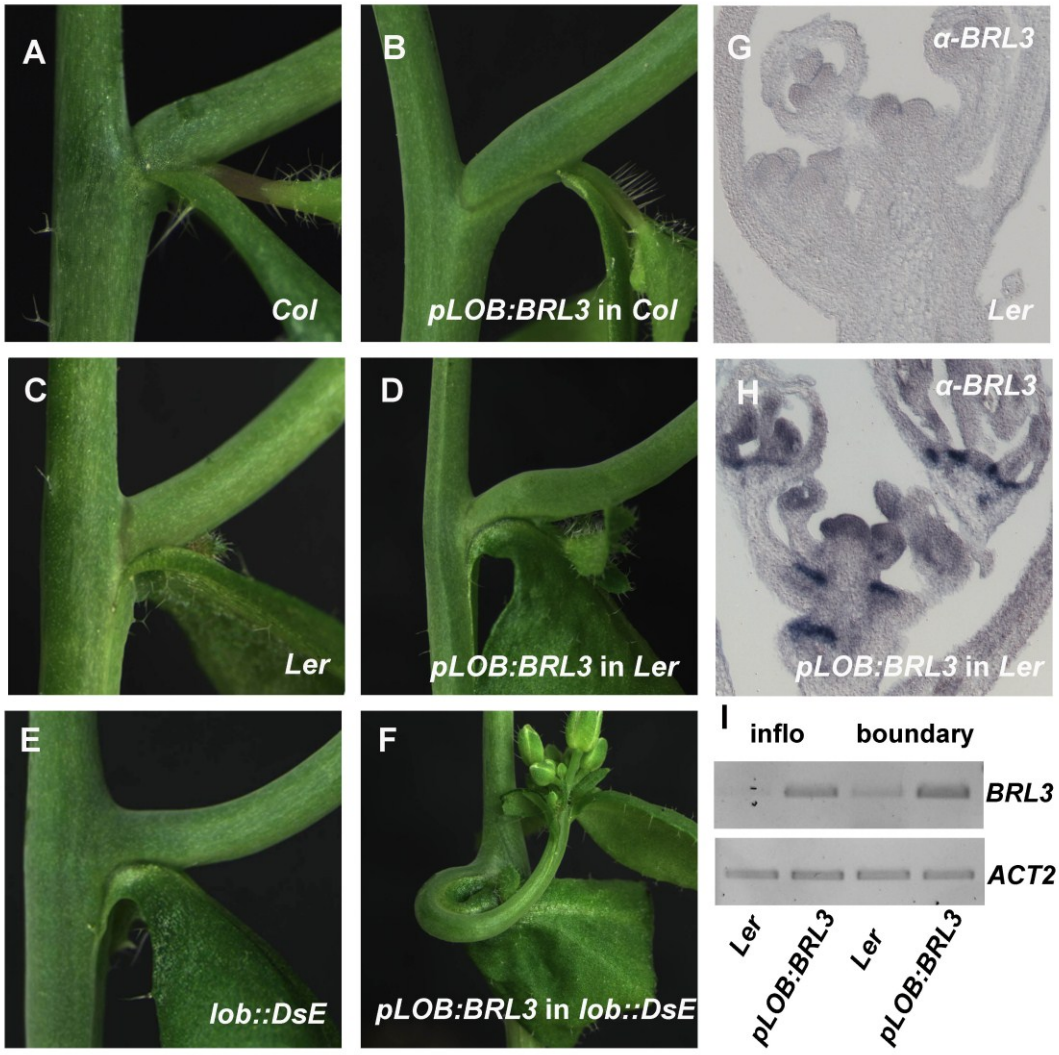

A
Col
B
pLOB:BRL3 in Col
G
α-BRL3
Ler
C
Ler
D
pLOB:BRL3 in Ler
H
α-BRL3
pLOB:BRL3 in Ler
E
lob::DsE
F
pLOB:BRL3 in lob::DsE
I
inflo
boundary
BRL3
ACT2
Ler
pLOB:BRL3
Ler
pLOB:BRL3

Figure 2.10 Characteristics of boundary cells in *pLOB:BRL3* plants.

Toludine blue staining of sections showing cell shape in the paraclade junction between the cauline leaf and axillary shoot of *Ler* (A), *pLOB:BRL3* (B), *lob::DsE* (C) and *pLOB:BRL3 lob:DsE* (D).

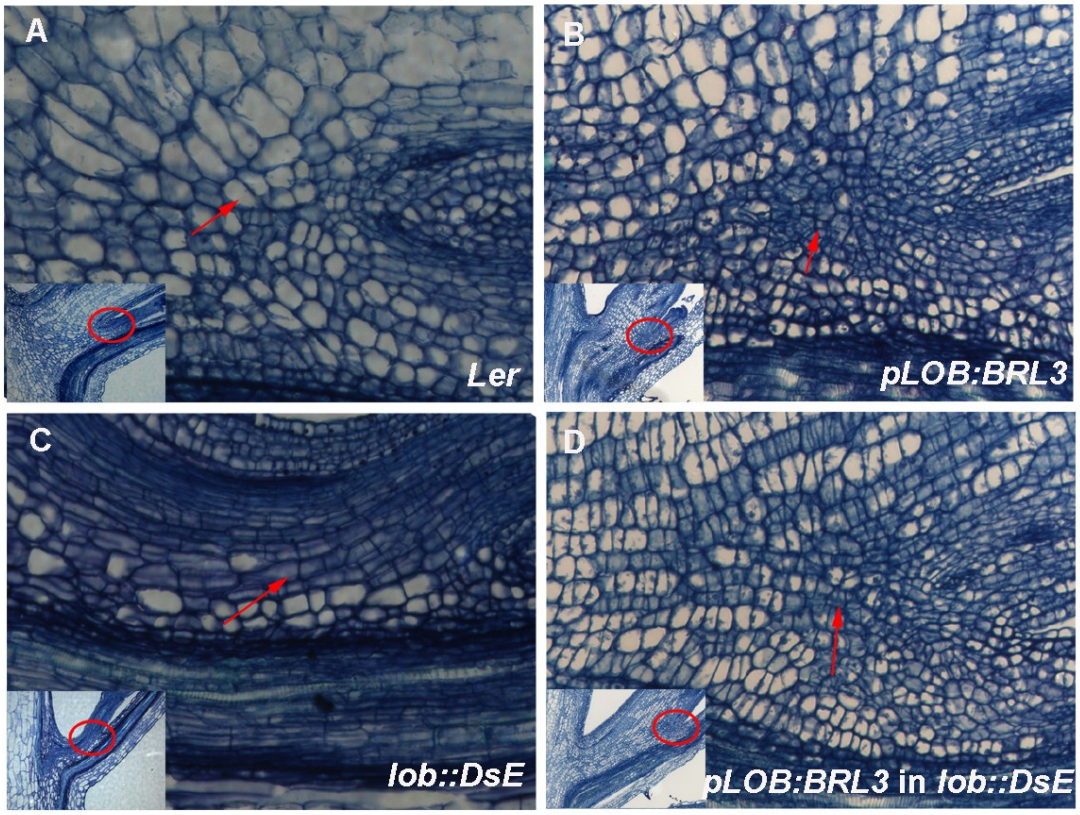

A
Ler
B
pLOB:BRL3
C
lob::DsE
D
pLOB:BRL3 in lob::DsE

Figure 2.11 Expression of boundary genes in *pLOB:BRL3* plants.

(A-D) *GUS* expression in *lob::DsE* (A, C) and *lob::DsE pLOB:BRL3* (B, D) paraclade junctions (A, B) and inflorescences (C, D). (E-H) *GUS* expression in the *pLOF2:GUS* (E, G) and *pLOF2:GUS pLOB:BRL3* (F, H) paraclade junctions (E, F) and inflorescences (G, H).

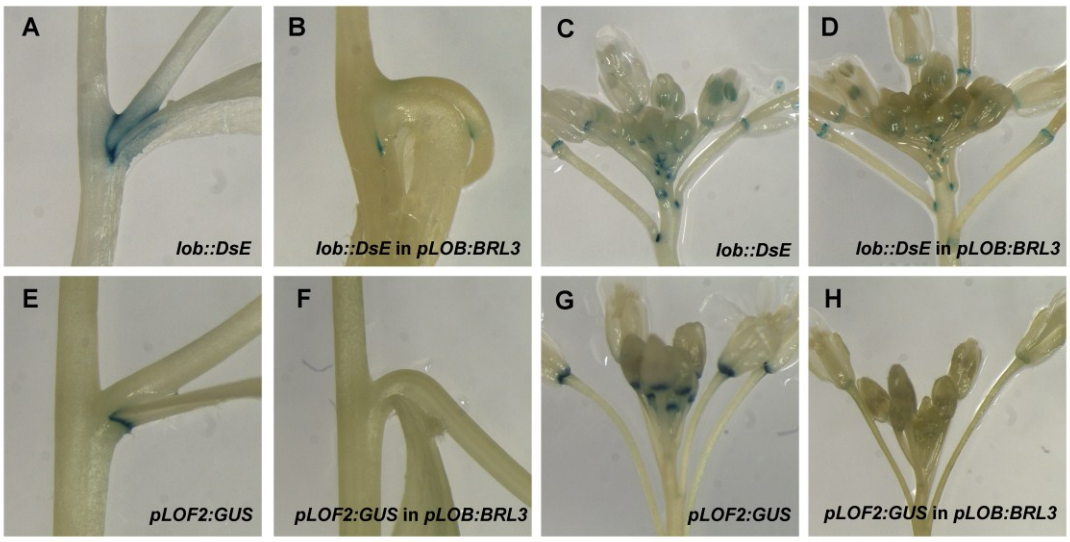
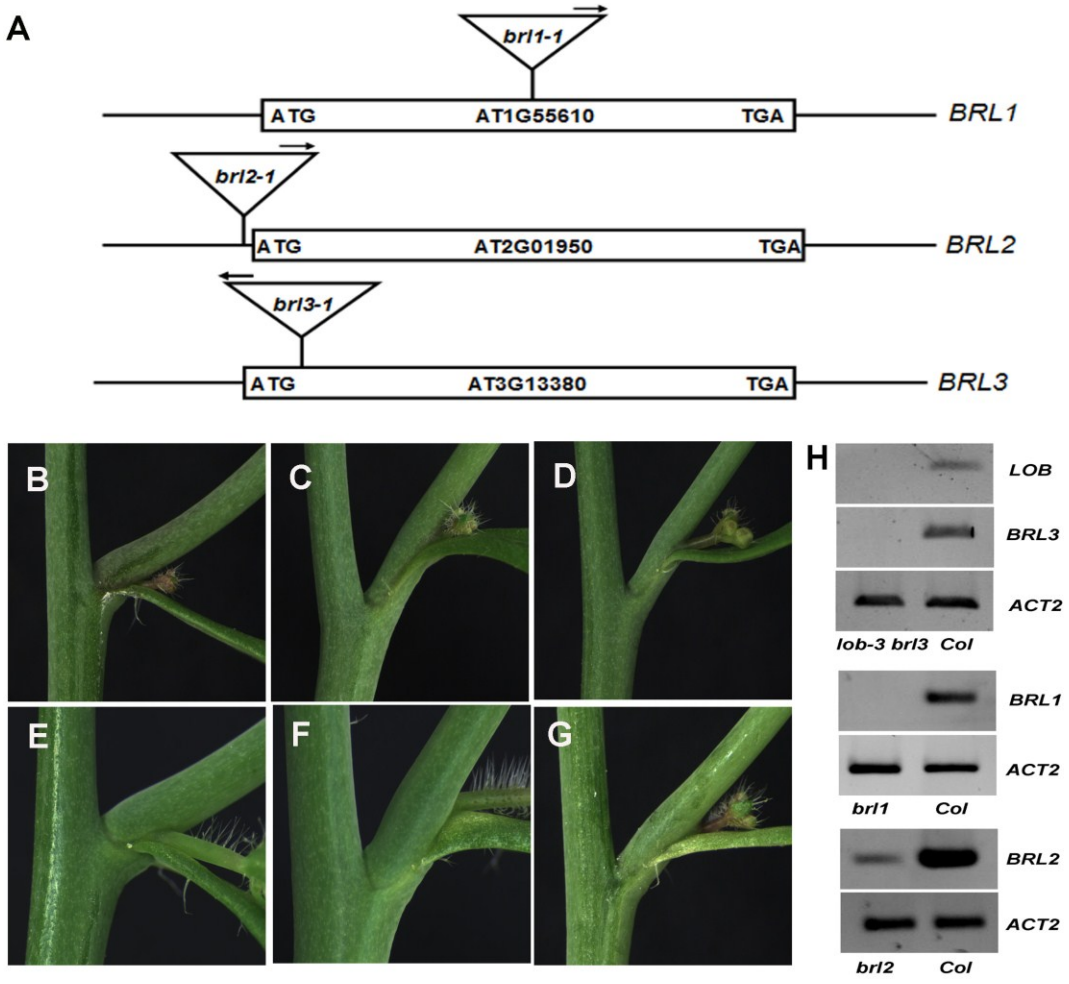

A
lob::DsE
B
lob::DsE in pLOB:BRL3
C
lob::DsE
D
lob::DsE in pLOB:BRL3
E
pLOF2:GUS
F
pLOF2:GUS in pLOB:BRL3
G
pLOF2:GUS
H
pLOF2:GUS in pLOB:BRL3

Figure 2.12 Genetic interactions between *BRL* loss-of-function mutants and *lob-3* mutants.

(A) Cartoon showing T-DNA insertion sites in *brl1-1*; *brl2-1;* and *brl3-1* mutants. T-DNA was inserted in the exon of *BRL1* in *brl1-1*, in the 5'-UTR of *BRL2* in *brl2-1,* and in the exon of *BRL3* in *brl3-1*.

(B-G) Phenotype of paraclade junction in *Col* wild-type (B), *lob-3* (C), *lob-3 brl3-1 brl1-1* triple mutant (D), and *brl3-1* (E), *lob-3 brl3-1* double mutant (F), and *lob-3 brl3-1 brl2-1* triple mutant (G). (H) RT-PCR analysis showed *lob-3*, *brl1-1* and *brl3-1* are knock-out mutants, while *brl2-1* are knock-down mutants. RNA was extracted from paraclade junctions. 26 cycles of amplification for *LOB* and 30 cycles of amplification for *BRLs* were performed. 20 cycles of amplification for *ACT2* was served as a loading control.

(I) Length of fusion in *lob brl* mutants. The boundary of first three nodes was measured in *Col*, *lob-3*, *lob-3 brl3-1* double, *lob-3 brl3-1 brl1-1* triple, *lob-3 brl3-1 brl2-1* triple mutants. n means the number of plants, n>30. Error bar indicates S.D.


A
brl1-1
ATG
AT1G55610
TGA
BRL1
brl2-1
ATG
AT2G01950
TGA
BRL2
brl3-1
ATG
AT3G13380
TGA
BRL3
B
C
D
E
F
G
H
LOB
BRL3
ACT2
lob-3 brl3
Col
BRL1
ACT2
brl1
Col
BRL2
ACT2
brl2
Col

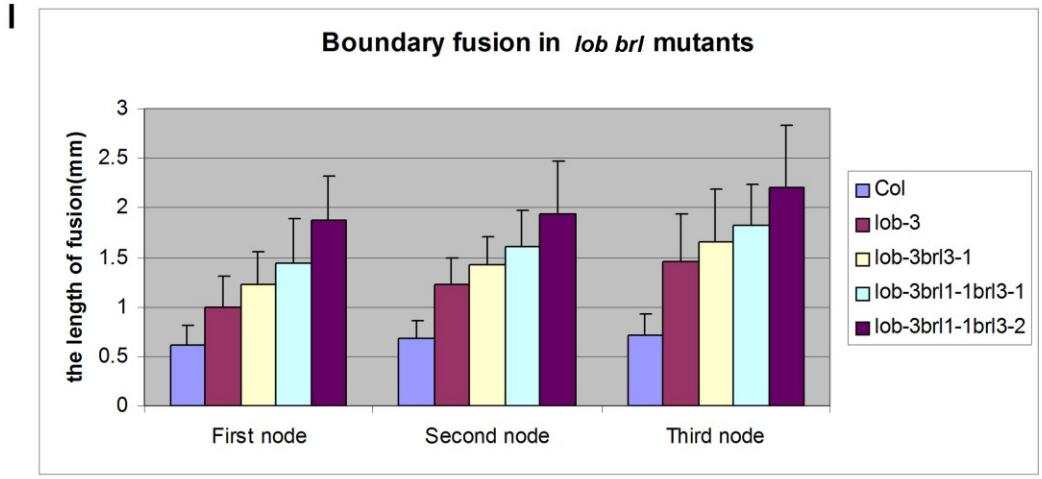

I
Boundary fusion in lob brl mutants
the length of fusion(mm)
3
2.5
2
1.5
1
0.5
0
First node
Second node
Third node
Col
lob-3
lob-3brl3-1
lob-3brl1-1brl3-1
lob-3brl1-1brl3-2

Figure 2.13 Model for BRL3 mediated BR signaling in boundary region.

In the boundary region, suppressed BR signaling is required boundary integrity. Increased LOB activity up-regulates both *BAS1* and *BRL3* expression. On one hand, BAS1 decreases BR signaling and ensures the boundary specification process. On the other hand, BRL3 activates BR signaling and represses the expression of boundary genes such as *LOB* and *LOF2*, forming a negative feedback to prevent over-activation of *LOB* gene expression and boundary specification.

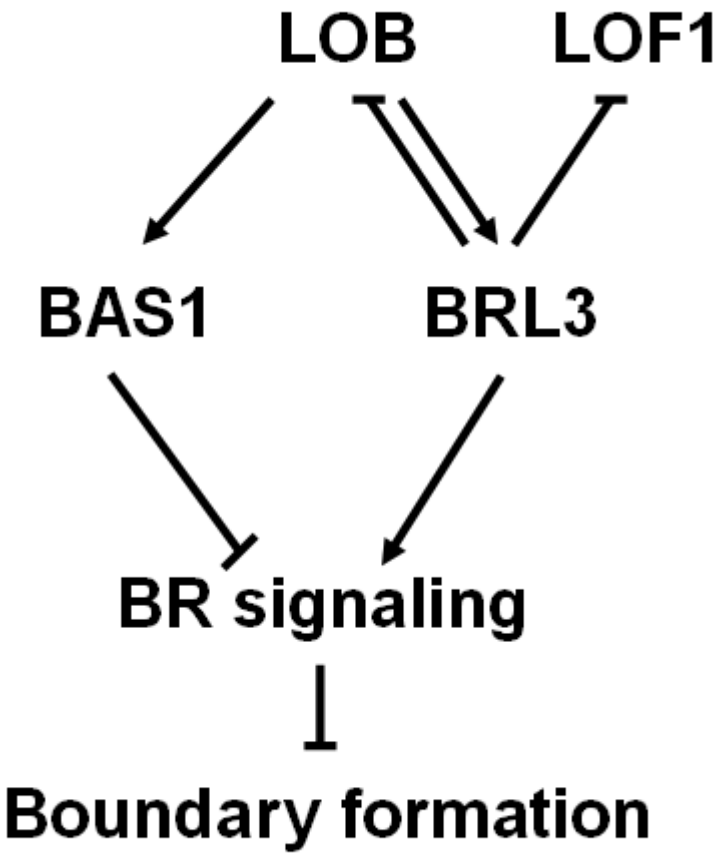

LOB
LOF1
BAS1
BRL3
BR signaling
Boundary formation

Table 2.1 oligonucleotides sequence

Primer Name	Sequence
pBRL3-F	TCTAGATAGTCACGAGTTTGATGG
PBRL3-S-R	CCCGGGGTTATTAGCCCACAAAGTG
pBRL3-muta-F	CAACCCTACAACCCCAACCACCGAGCC
pBRL3-muta-R	GGCTCGGTGGTTGGGGTTGTAGTAGGGTTG
pBRL3-GATE-F	CACCTAGTCACGAGTTTGATGG
BRL3-GATE-F	CACCATGAAACAACAATGGCAGTT
BRL3 OX-R	GTCGACTTAAGGCTCCTTATCTCGTGAT
BRL3 CHIP-F	GGCCGAAGAAACTGACAAA
BRL3 CHIP-R	CTCGAAGTCCTCCTTTCACG
BRL3-F	TTACGACGTTCCCACTCACA
BRL3-R	CAGCCTTGCCATACCAAAAT
QBRL3-F	TTGAGAGCCTTCCGACATCT
QBRL3-R	CATCAGCCAGTTTAGCCTTGT
Sp6-BRL3-F	ATTTAGGTGACACTATAGCAACAATGGCAGTTCTTGA
T7-BRL3-R	TAATACGACTCACTATAGGGCAATAGTCATCTCCGGGGA
BRL3 Y1H1-attB4	GGGGACAACTTTGTATAGAAAAGTTGTGGACACGAGAGA ATCTTTG
BRL3Y1H1-attB1R	GGGGACTGCTTTTTTGTACAAACTTGGATGCCGTTGATT TCATTTG
ACT2-F	AAAATGGCCGATGGTGAGG
ACT2-R	ACTCACCACCACGAACCAG
QACT-F	GCACCCAGTTCTACTCACAG
QACT-R	CAACATACATGGCAGGGAC

Primer Name	Sequence
QLOB-F	GGCGTCGTCATCAAACTCAT
QLOB-R	CGTTGCTTGCTCCAAAGATT
GUS-F	GGGTCAATAATCAGGAAGTG
GUS-R	AGCCAGTAAAGTAGAACGG
SALK_005982-LP	ATATGGATGTTGCCGAATCTG
SALK_005982-RP	CTGTAAAGCGCCATGACTAGC
SALK_070890-LP	TCTACAACGGACAATTCCTTCC
SALK_070890-RP	GAAATTTTCAGGGAGGGTTCC
BRL3 ChIP 2 F	GGGGGCAAGCTCGCCGGGAAATTGAAG
BRL3 OX-R	GTCGACTTAAGGCTCCTTATCTCGTGAT

Chapter 3 Genetic interaction between *LATERAL ORGAN BOUNDARIES* (*LOB*) and *LATERAL SUPPRESSOR* (*LAS*) at the boundary region

Abstract

The plant meristem-organ (M-O) boundary functions to provide a barrier between the SAM and emerging lateral organ primordia. Boundary cell specification and maintenance are tightly controlled by various boundary genes, including the LBD family transcription factor *LOB*. In previous chapters, it was shown that LOB regulates organ boundary formation by limiting growth, in part by negatively regulating brassinosteroid accumulation and response. To identify genes that function with *LOB*, a mutant screen was conducted in the *lob* loss-of-function mutant background. One identified mutant revealed a novel interaction between *LATERAL SUPPRESSOR* (*LAS*) and *LATERAL ORGAN BOUNDARIES (LOB)*. Loss of function in the GRAS transcription factor *LAS* dramatically enhances the boundary fusion phenotype of the *lob* loss-of-function mutant. *lob las* double mutants exhibit disorganized cells in the fused boundary in a background dependent manner. In addition to the synergistic effect on boundary integrity, *LAS* and *LOB* also have synergistic interactions to control axillary meristem formation. Moreover, gene expression analysis revealed that *LAS* is required for *LOB* expression in the boundary region, but not sufficient to up-regulate *LOB* expression. Finally, this study revealed that *LAS* overexpression inhibits growth, leading to dwarf plants, possibly via a novel *LOB* independent pathway. Overall, LAS and LOB functions partially overlap to maintain the boundary and promote axillary meristem formation.

Introduction

The plant meristem-organ (M-O) boundary is essential for normal separation of organs and functions in many aspects of plant development (Aida and Tasaka, 2006). Cells in the boundary region have unique features, such as highly polarized shape, slow division and limited expansion, which leads to the "valley" like shape of boundary and ensures the boundary tissue as a stable physical barrier to separate different organs (Dumais and Kwiatkowska, 2002; Reddy et al., 2004; Aida and Tasaka, 2006). Boundary cell specification is a process controlled by several key boundary-specific genes (Bowman and Floyd, 2008; Rast and Simon, 2008). This dissertation focuses on the boundary-specific gene *LATERAL ORGAN BOUNDARIES* (*LOB*), which encodes a LBD family transcription factor (Shuai et al., 2002; Husbands et al., 2007). LOB is important for normal boundary specification, since *lob* loss-of-function mutants exhibit organ fusion between the stem and cauline leaf (See Chapter 1 and Bell et al, 2012). Overexpression of *LOB* suppresses cell growth and leads to dwarf plants, very similar to brassinosteroid (BR) defective mutants (Shuai et al., 2002; Gudesblat and Russinova, 2011). As a DNA-binding transcription factor, LOB is proposed to function by binding to the promoters of downstream target genes to control their expression level (Husbands et al., 2007). Previous work in the Springer Lab and work in Chapter 1 and 2 identified two *LOB* downstream targets *BAS1* and *BRL3*, linking *LOB* to the BR signaling pathway. On one hand, LOB activates the transcription of the BR negative regulator *BAS1* (Neff et al., 1999), thus inactivating BR signaling locally at boundary cells. Local suppression of BR signaling is essential for boundary integrity since mis-expression of positive regulators of BR signaling in boundary tissues, such as *DWF4*, *BRI1* and *BRL3* all lead to an organ fusion phenotype (See Chapter 1 and Chapter 2). On the other hand, LOB directly up-

regulates expression of a BR receptor, *BRL3* (Cano-Delgado et al., 2004), to form a negative feedback loop, possibly maintaining some basal level of BR signaling strength in the boundary region (See Chapter 2). Although downstream pathways of LOB have been identified and studied in our lab, how *LOB* expression level in the boundary is controlled and how *LOB* interacts with other boundary genes to maintain boundary specification is not well understood.

LATERAL SUPPRESSOR (LAS) encodes an *Arabidopsis* transcription factor in the GRAS family (Bolle, 2004). GRAS-family proteins are plant specific and feature a conserved VHIID motif (Bolle, 2004). GRAS proteins are involved in various plant development processes, including root development (Helariutta et al., 2000), shoot apical meristem maintenance (Stuurman et al., 2002), axillary meristem formation (Schumacher et al., 1999; Greb et al., 2003), phytochrome signaling, and gibberellin signal transduction (Bolle, 2004). *LAS* and its orthologues in several different plant species have a conserved role in axillary meristem formation (Schumacher et al., 1999; Greb et al., 2003; Li et al., 2003). In tomato plants, the *lateral suppressor (ls)* loss-of-function mutant blocks axillary meristem formation during vegetative growth, which is very similar to the *Arabidopsis las* mutant (Schumacher et al., 1999; Greb et al., 2003). In rice plants, the *LAS* orthologue gene *MONOCULM1* (*MOC1*) also has a similar function in axillary meristem formation (Li et al., 2003). *MOC1* is specifically expressed in axillary buds and boundary regions. A loss-of-function mutant of *MOC1* has only one main culm and lacks tillers, suggesting that *MOC1* functions in the boundary region to promote initiation and outgrowth of axillary buds. In *Arabidopsis*, *LAS* is specifically expressed at the boundary region and functions as an important regulator of axillary meristem formation. The transcriptional control of *LAS* involves an enhancer element 3.2

kb downstream of the coding region (Raatz et al., 2011). Recent studies based on promoter swap experiments revealed that this enhancer sequence is required and sufficient to specify the boundary specific expression pattern. *LAS* is involved in three processes: (1) Similar to its orthologues in other plants (tomato and rice), a *LAS* loss-of-function mutant has reduced number of axillary meristems during the vegetative stage of development (Schumacher et al., 1999; Greb et al., 2003); (2) a *LAS* loss-of-function mutant sometimes has defects in separation between the axillary shoot and main stem (Greb et al., 2003); (3) *LAS* mediates crosstalk between the SAM and lateral organ primordia. The lateral organ primordia specific gene *YABBY* provides local morphogenic cues for flower development and *YABBY* misexpression in flowers results in numerous immature petals and abolished stamens and carpels. A *LAS* loss-of-function mutant can suppress this *YABBY* misexpression phenotype by an unknown mechanism (Goldshmidt et al., 2008). Although *LAS* and its orthologues in plants clearly play an important role in the boundary region for axillary meristem initiation, the functions of *LAS* in specification of boundary cell identity and the genetic interaction between *LAS* and other boundary genes were not known.

In addition to *LAS* and *LOB*, several other boundary genes also control boundary cell identity or integrity. *CUP-SHAPED COTYLEDON (CUC)* genes are members of the NAC-domain containing transcription factor family. *CUC* genes are required for boundary formation throughout plant development, SAM formation and axillary meristem initiation. Interestingly, CUC activity positively regulates *LAS* expression, since *LAS* mRNA levels are decreased in *cuc3 35S:MIR164A* plants, in which CUC activity is decreased, and increased in the *mir164abc* mutant, in which CUC activity was increased (Raman et al., 2008). *ORGAN BOUNDARY1 (OBO1)* encodes a small protein of 133 amino acids

expressed specifically in the boundary region between the SAM and lateral organs (Cho and Zambryski, 2011; Takeda et al., 2011). *OBO1* is a direct target of CUC1 (Takeda et al., 2011), indicating a genetic hierarchy between some boundary genes. *LATERAL ORGAN FUSION1 (LOF1) and LATERAL ORGAN FUSION2 (LOF2)* encode two boundary-specific transcription factors of the MYB family. *LOF1* and *LOF2* loss-of-function mutants have fusion between the axillary stem and cauline leaf and defects in accessory meristem formation (Lee et al., 2009). The *las-4 lof1-1* double mutant exhibits enhanced fusion relative to the *lof1* single mutant and a decrease in the number of axillary meristems compared to *las-4*, suggesting that different boundary genes have partially redundant functions (Lee et al., unpublished).

In Chapter 3 of this dissertation, I first identified a loss-of-function allele of *las* that enhances the *lob* loss-of-function organ fusion phenotype. In *lob las* double mutants, cauline leaves and axillary meristems are dramatically fused together and cell organization in the fused region is abnormal. The *lob las* double mutant also has stronger defects in axillary meristem formation compared to the *las* single mutant, suggesting that *LOB* also contributes to axillary meristem formation. Moreover, this study revealed that *LAS* is required for normal expression of *LOB* and other boundary genes, including *CUC1, CUC2, LOF1, LOF2* and *OBO1*. Finally, I found that *LAS* overexpression inhibits plant growth, which is similar to the effect of *LOB* overexpression. However, *LAS* overexpression does not up-regulate *LOB* transcription, suggesting a possible novel LAS-dependent pathway for boundary cell growth inhibition and identity specification.

Result

EMS screen and map-based cloning identified a *las* mutation as *lob* enhancer

In *Ler* wild-type plants, the cauline leaf and axillary shoot boundary are clearly separated (Figure 3.1 A). In contrast, mutations in *LOB* cause moderate fusion in the boundary between the cauline leaf and axillary shoot (Figure 3.1 B). In order to identify additional genes in the *LOB* genetic network, *lob::DsE* plants were mutagenized using ethyl methane sulfonate (EMS) to identify mutants that enhance the *lob* phenotype (Shuai et al, unpublished). Loss-of-function mutations in downstream targets, cofactors, or genes that function redundantly with *LOB* might lead to an enhanced organ fusion phenotype in the *lob* mutant background. One mutant, from the screen, identified in the M2 generation and named Line *7735*, caused a dramatic fusion in the boundary between cauline leaf and axillary shoot (Figure 3.1 C). The severe fusion in *7735* sometimes led to bending of the leaves and axillary shoots. Even more, accessory meristems, which form in the junction between cauline leaves and axillary shoots in wild type, were not formed in *7735* mutants.

To identify the gene responsible for the enhanced phenotype in the *7735* mutant, the M2 mutant, which was in the *Ler* ecotype, was crossed to *Col* wild-type plants and F2 plants with the mixed ecotype were used for map-based cloning using SSLP markers (Lukowitz et al., 2000). Using this approach, the mutation site in *7735* was identified, located at position 649 bp of the *LAS* coding sequence. This mutation is within the conserved VHIID domain, leading to an amino acid change of Glycine to Arginine (Figure 3.2 A, B). Based on the map-based cloning result, the mutation in *LAS* gene was renamed as *las-13* mutation.

To isolate the *las-13* mutation from *the lob::DsE* background, the *7735* M2 mutant was backcrossed with *Ler* wild-type and *las-13* homozygotes were identified in the F2 generation. According to previous work, *LAS* loss-of-function reduces the number of vegetative axillary meristems (Schumacher et al, 1999; Greb et al., 2003). In the *las-13* mutant (Figure 3.3 B), only 0-1 meristem was produced in the rosette, while *Ler* wild-type plants generate around 4 rosette meristems (Figure 3.3 A, Figure 3.4 J). The reduction in rosette meristem formation indicates that the *las-13* mutation disrupts the native LAS function in the *7735* plant. To verify that the *las-13* mutation was the only mutation responsible for the reduced rosette meristem phenotype, we generated the *pLAS:LAS* plasmid, in which the *LAS* coding region is expressed under the control of the native *LAS* promoter. The *pLAS:LAS* construct was transformed into the *las-13* single mutant. *pLAS:LAS las-13* plants produced multiple axillary meristems in rosette leaf axils (Figure 3.3 C). The number of axillary meristems in *pLAS:LAS las-13* plants was similar to the number in *Ler* wild-type plants (Figure 3.3 A), confirming that the *las-13* mutation indeed causes the reduced meristem phenotype.

To further analyze the function of *LOB* and *LAS* in boundary maintenance and organ separation, we measured the length of the fusion between cauline leaves and axillary shoots at paraclade junctions (Figure 3.4 K). In the *Ler* wild-type plants, cauline leaves and axillary shoots were almost completely separated (Figure 3.4 A, K). In the *lob::DsE* single mutant, the base of cauline leaves was attached to the base of axillary shoots and the length of fusion was approximately 2 mm (Figure 3.4 B, K). As a result, the accessory meristem emerged further away from the stem but the orientation of the lateral organs was normal. In the *las-13* single mutant, the cauline leaf and axillary shoot were separated normally, however accessory meristem production was suppressed. In

some cases, the base of cauline leaves extended down the stem, producing a decurrent strand in the *las-13* mutant (Figure 3.4 D, J, K). In the *lob::DsE las-13* double mutant, the length of the fusion between cauline leaves and axillary shoots was approximately 5 mm (Figure 3.4 G, K), which is significantly longer than that in *lob::DsE* single mutants. To further confirm the genetic interaction between *LOB* and *LAS*, we generated another *las lob* double mutant combination by crossing *lob::DsE* with *las-11* in the *Ler* background (Goldshmidt et al., 2008). The *las-11* single mutant did not cause a fusion phenotype (Figure 3.4 E), while the *lob::DsE las-11* double mutant exhibited severe boundary fusion like that in the *lob::DsE las-13* double mutant (Figure 3.4 G, H). These data support the conclusion that *LOB* and *LAS* have a synergistic effect on boundary specification or maintenance. However, the synergistic interaction between *LOB* and *LAS* genes is not clear in the *Col* ecotype background. In *Col*, the *lob-3* mutant has a moderate fusion between the cauline leaf and axillary shoot (Figure 3.4 C) and the *las-4* mutant in *Col* does not show fusion (Figure 3.4 F). The *lob-3 las-4* double mutant has a moderate fusion phenotype (Figure 3.4 I), which is similar to *lob-3* single mutant. There are at least two possible reasons for the weak boundary fusion phenotype of *lob-3 las-4* double mutant in *Col* background. One possibility is that *lob-3* and/or *las-4* mutants could be relatively weaker alleles compared with *lob::DsE* and *las-11* mutants, such that the amount of LOB and LAS activity in *lob-3 las-4* double mutant is still above the threshold to show synergistic interaction. The other possible reason is that the synergistic effect between *LOB* and *LAS* requires some factors which are only present in *Ler* ecotype, but not in *Col* ecotype.

LOB contributes to axillary meristem formation

las mutants produce fewer axillary meristems and produce no accessory meristems (Greb et al., 2003), whereas *lob* mutants do not exhibit defects in axillary meristem formation (Figure 3.4 J). To examine whether *LOB* plays a role in axillary meristem formation, we counted the number of axillary meristems in the different backgrounds in long-day grown plants. The rosette axillary meristems were produced in the axils of rosette leaves, which form during vegetative development, whereas axillary meristems and accessory meristems were generated in the axils of cauline leaves, which are produced during reproductive development. In 4-week-old *Ler* wild-type and *lob::DsE* plants, approximately four rosette meristems, three axillary meristems, and one accessory meristem were produced on average. In the *las-13* single mutant, the number of axillary meristems was the same as that in the *Ler* or *lob::DsE* plants. However, *las-13* plants produced fewer rosette meristems (0~2) and never generated accessory meristems in the junction between cauline leaves and axillary shoots (Figure 3.4 J). In the *lob::DsE las-13* double mutant, the number of accessory meristems and axillary meristems was the same as the number produced in the *las-13* single mutant. However, the number of rosette meristems was decreased in *lob::DsE las-13* double mutants compared to that in the *las-13* single mutant (Figure 3.4 J). Taken together, we concluded that *LOB* has a function in the control of axillary meristem generation via synergistic interactions with *LAS*.

Boundary cell organization was altered in *lob las* mutants

To further understand the cellular changes in enlarged boundary fusion in *lob::DsE las-13* double mutant, I observed the cellular organization of the boundary

region in sections stained with toluidine blue. In wild-type plants, cells in the boundary region of the paraclade junction are smaller compared to the adjacent cells in the stem, due to the slower cell expansion and cell division in the boundary region (Dumais and Kwiatkowska, 2002; Reddy et al., 2004) (Figure 3.5 A). The boundary cells were quite uniform in size. Similarly, the boundary cells in *las-13* were also well organized even the cell size was smaller than that in the *Ler* wild-type background (Figure 3.5 B). This is maybe due to they are different development stage. In the *lob::DsE* mutant, cells in the fused region were longer than in wild-type, due to elongation along the axis parallel to the boundary gap. The rate of cell division in different boundary cells appears to be non-uniform (Figure 3.5 C). In the *lob::DsE las-13* double mutant, the cells in the fused region were much longer along the horizontal axis than those in the wild-type, *lob::DsE,* or *las-13* plants (Figure 3.5 D), probably due to the abnormal cell division and cell expansion activities. Overall, the presence of mildly-disorganized and severely-disorganized cells in boundary region of *lob::DsE* and *lob::DsE las-13* plants is consistent with the severity of the fusion phenotype in *lob::DsE* and *lob::DsE las-13* double mutant, respectively.

LAS is required for expression of _LOB_ and other boundary genes

To investigate the genetic relationship between *LOB* and *LAS*, I examined the *LOB* expression pattern in the *LAS* loss-of-function mutant, using both *GUS* reporter and RT-PCR analysis. In *lob::DsE* seedlings, GUS activity was specifically detected in the boundary between leaves and hypocotyls (Figure 3.6 A), while in *lob::DsE las-11* and *lob::DsE las-13* seedlings, the *GUS* expression pattern was similar to that in the *lob::DsE Ler* plants but the level of GUS activity was significantly decreased (Figure 3.6 B, C). In *lob::DsE* adult plants, GUS activity was very strong in the boundary regions including the

paraclade junctions, the base of pedicels and base of floral organs (Figure 3.6 D). In *lob::DsE las-11* and *lob::DsE las-13* adult plants, weak GUS activity was detected in some boundary tissues, such as the junction between stem and cauline leaves, or the base of floral organs. In the boundary between the shoots and pedicels, GUS activity was decreased to a level too low to be detected (Figure 3.6 E, F). RT-PCR analysis with boundary tissues of *Ler* and *las-13* mutant plants also showed that *LOB* transcripts were decreased in the *las-13* mutant compared to that in *Ler* control plants (Figure 3.6 K). These two experiments both suggested that *LAS* is required for *LOB* expression in boundaries. However, the dependency of *LOB* expression on normal *LAS* expression is not absolute, because in the *Col* ecotype, I failed to detect a significant difference in *LOB* expression in *Col* wild-type and *las-4* mutant plants (Figure 3.6 G-J), indicating the diversity of boundary gene interaction network among different ecotypes.

I further carried out quantitative RT-PCR analysis on several boundary-specific genes in *lob* and *las* mutants. Quantitative RT-PCR results indicate that the transcript levels of five boundary specific genes, *CUC1, CUC2, LOF1, LOF2* and *OBO1*, were all significantly reduced in dissected paraclade junctions of *las* and *lob::DsE las-13* mutant plants (Figure 3.7). These results further suggested that *LAS* is important for normal boundary gene expression. Interestingly, in the *lob::DsE* single mutant, expression of these boundary genes is not altered (Figure 3.7), suggesting that decreased activity of these boundary genes together with reduced activity of *LOB* in *lob::DsE las-13* mutant contributes to enhanced boundary fusion.

LAS overexpression suppressed plant growth

Cells at the boundary region are usually slow growing and slow dividing, making the boundary tissue a very stable physical barrier to separate different organs (Hussey, 1971; Dumais and Kwiatkowska, 2002; Reddy et al., 2004). The boundary-specific gene *LOB* has been shown to inhibit growth when overexpressed throughout the plant (Shuai et al., 2002). To further investigate the function of *LAS*, I generated *35S:LAS* overexpression plants and DEX-inducible *35S:LAS-GR* plants. *35S:LAS* plants were smaller than *Col* wild-type plants (Figure 3.8 A,B). Compared to *Col* wild-type, the leaves in *35S:LAS* plants were rounder with a short petiole. Consistently, *35S:LAS-GR* seedlings treated with 10 μM DEX were dwarfed and produced fewer rosette leaves (Figure 3.8 E), while *35S:LAS-GR* seedlings treated with mock were much larger (Figure 3.8 D). These results indicate that overexpression of *LAS* suppressed growth in both seedling and adult stages. Semi-quantitative RT-PCR analysis with whole seedlings showed that *LOB* transcript levels were not affected by increased *LAS* transcripts level (Figure 3.8 C), indicating that *LAS* alone is not sufficient to activate *LOB* transcription. These data suggest that *LAS* overexpression suppressed plant growth in a LOB-independent manner.

Discussion

The plant SAM is responsible for generation of lateral organs and renewal of itself. The meristem is composed of different functional zones (Bowman and Floyd, 2008). Gene expression patterns and hormone signaling pathways in different zones of the SAM are also unique and give rise to the different cellular identity in various zones. In the central zone of the SAM, *WUS* and *CLV3* are expressed and together with high

cytokinin signaling activity form a feedback loop to maintain the undifferentiated cell status (Leibfried et al., 2005; Gordon et al., 2009). In lateral organ primordia, active auxin response promotes rapid cell growth and division to form new leaves and flowers (Reinhardt et al., 2003; Heisler et al., 2005). Several boundary-specific genes are expressed in a band of cells between the meristem and lateral organ primordia to ensure the limited expansion and slow division characteristic of this cell identity (Aida and Tasaka, 2006). The boundary region serves not only as a barrier in the shoot apex, but also actively communicates between the shoot apical meristem and emerging lateral organ primordia (Goldshmidt et al., 2008).

Previously, it has been shown that the boundary specific transcription factor LOB controls boundary integrity between axillary stems and lateral cauline leaves by local suppression of BR signaling (See Chapter 1 and 2). The moderate boundary fusion phenotype of *lob* loss-of-function mutant provided a good starting point for an EMS mutagenesis to identify additional factors involved in boundary specification or maintenance. Through this study, mutation in another boundary specific gene *LAS* has been found to enhance the moderate boundary fusion phenotype of the *LOB* loss-of-function mutant. Previously, *LAS* has been revealed to be important for axillary meristem formation, but did not have a clear role in boundary specification between lateral cauline leaf and axillary shoots (Greb et al., 2003). This study revealed that the *lob las* double mutant has dramatic fusion between axillary stems and lateral cauline leaves, demonstrating the synergistic genetic interaction between *LOB* and *LAS* gene for boundary specification and/or maintenance. Consistent with the finding that *LAS* has a function in boundary specification, this study also found that overexpressing *LAS* inhibits growth and reduced plant size. It is possible that specific expression of *LAS* in the

boundary region locally suppresses cell growth and maintains the boundary cell identity. In general, it is a common feature that boundary genes control boundary cell identity or integrity via suppression of cell division, since *LOB*, *CUC* and *LAS* overexpressing plants all exhibited smaller leaf size or indented leaf shape (Shuai et al., 2002; Laufs et al., 2004).

On the other hand, previous studies found that LOB controls boundary cell specification, but not axillary meristem formation (Bell et al., 2012,). The *lob las* double mutant also has stronger reduction of axillary meristem formation than the *las* single mutant, revealing a novel role for LOB in axillary meristem formation. Taken together, this study and previous studies about LOB and LAS functions provide clear evidence that both genes contribute to two processes in plant development: integrity of the boundary region and axillary meristem formation. It is not coincidental that the boundary region and axillary meristem formation are co-controlled by boundary genes. In plant developmental processes, the axillary meristem must form after the existing organ boundary, which provides a landmark for axillary meristem. Thus, boundary genes *LOB* and *LAS* have partially overlapping function in both boundary specification or integrity and axillary meristem formation. Interestingly, the control of boundary specification and meristem formation by boundary genes is not unique for later adult stages of plant. For example, in early plant seedling development, CUCs are required for both boundary specification between cotyledons and SAM formation (Long and Barton, 1998; Takada et al., 2001). One explanation would be that the boundary region between cotyledons provides the landmark required for SAM formation during embryogenesis.

Through the study of *LOB* gene expression in *LAS* loss-of-function mutants, I found that LAS is required for *LOB* expression in the boundary region. Moreover, not

only *LOB*, several other boundary genes such as *LOF1*, *LOF2* (Lee et al., 2009), *CUC1*, *CUC2* (Long and Barton, 1998; Takada et al., 2001) and *OBO1* (Cho and Zambryski, 2011) also require *LAS* for their normal expression levels at the boundary region, suggesting multiple cross talking interactions between different boundary genes. Future studies using different mutant combinations of boundary genes may reveal more details of the complex interactive genetic network for boundary specification.

Finally, overexpressing *LAS* alone cannot increase *LOB* transcript level, suggesting that LAS does not directly up-regulate *LOB* expression and other factors are required. As a result, although both LAS and LOB inhibit growth when overexpressed, LAS suppression of growth is not directly dependent on LOB. Currently, a detailed mechanism of the *LAS* genetic pathway leading to boundary specification and axillary meristem formation is not well understood. To further understand this *LAS*-dependent genetic network, similar to the approach used in LOB functional study, a microarray analysis comparison between transient LAS overexpressing plant and control plant would provide new information of putative LAS downstream targets.

Materials and methods

Plant growth conditions

Plants were grown in soil at 23°C under long-day conditions (16h light/8h dark). *las-4* was in the *Col* background (Greb et al., 2003) and was genotyped by dCAPS primers LAS-F and LAS-R (Table 3.1). The amplified fragment from the *las-4* mutant was then digested using *Spe*I. *las-11* and *las-13* were in *Ler* background. *las-11* was genotyped by primers *las-11* SAP-WT or *las-11* SAP-Mut together with *las-11* SAP-R.

las-13 was genotyped by CAPS primers LAS-CAPS-F together with LAS-CAPS-R. The amplified fragment from the *las-13* allele can be digested by *Hpa*II. *35S:LAS-GR* seeds were sterilized with 20% bleach+0.01% Tween-20 and sowed on MS plate plus 0.03% EtOH (mock) or MS plate plus +10μM DEX in 0.03% EtOH.

Mapping-based cloning

The *lob::DsE* plants were mutagenized with ethyl methanesulfonate (EMS) as described (Robinson-Beers et al., 1992). M1 plants were grown on soil in pools of ~20 M1 plants per pool. M2 seeds were harvested and planted in soil to screen for enhanced fusion. One plant with enhanced fusion was identified for further study and nominated Line *7735*. The mapping population was generated from the F2 plants of *7735* mutant crossed to *Col* wild-type plant. First, 5 pools of 10 mutants with mixed background and enhanced boundary fusion were tested by SSLP markers nga280, nga111, ciw2, ciw3, nga1126, nga168, nga162, ciw11, ciw4, nga6, ciw5, ciw6, nga1107, CTR1, ciw9 and ciw10 (Lukowitz et al., 2000). Second, the SSLP marker nga280 was used to test 500 individual mutants from the mapping population that showed enhanced boundary fusion, which revealed that the distance from the mutation site to the marker nga280 was 0.1 cM. *LAS* is located in this region according to the annotation of BAC clones and *LAS* coding sequence was amplified and cloned from *7735* mutant and the *Ler* parental line and sent to sequence with M13 primers.

Morphological analysis

The *Ler*, *lob::DsE*, *las-13*, *lob::DsE las-13* plants were grown in long-day conditions and one-month old plants (30-36 plants) were used for quantitative analyses.

The number of rosette meristems, accessory meristems and axillary meristems were determined. The boundary in the second node from rosette was used to measure the length of boundary fusion from the end of cauline leaf attached to the stem to where the axillary stem separated from the cauline leaf.

Plasmid constructs

The *pLAS:LAS* construct was made using the GATEWAY cloning system. The *LAS* coding sequence was amplified with primers LAS-GATE-F and LAS-GATE-R (Table 3.1) from *Col* wild-type inflorescence cDNA and cloned into pENTR vector (Invitrogen), generating *LAS:pENTR* construct. The *LAS* promoter (from 3967 bp upstream of the CDS to the start codon) was amplified from genomic DNA of *Col* wild-type plants and inserted into *PCB302-5* (Springer lab) using introduced *Sac*I restriction sites to make the destination vector *pLAS:PCB302-5*. *pLAS:LAS* construct was generated from an LR recombination reaction (Invitrogen) between *LAS:pENTR* and *pLAS:PCB302-5*. *35S:LAS* was generated using the GATEWAY system with *LAS:pENTR* and the destination vector pGWB2 (Nakagawa et al. 2007). To make *35S:LAS-GR*, the *LAS* CDS, without a stop codon, was amplified from *Col* wild-type inflorescences cDNA with primers LAS-GATE-F and LAS-SAAB-R (Table 3.1), and cloned into *pENTR* vector to generate *LAS-pENTR-1* vector. *35S:LAS-GR* was constructed using the GATEWAY system with *pLAS:pENTR-1* and modified destination vector *pGR(B)* (Mangeon et al). The *pLAS:LAS*, *35S:LAS* and *35S:LAS-GR* constructs were introduced to *las-13* mutant or *Col* wild-type using the floral-dip method (Clough et al., 1998).

Histological analysis

Dissected paraclade junctions (around 1 cm in length) including stems, axillary shoots, and cauline leaves were cut from 4-week old plants and fixed with 4% paraformadehyde in 1xPBS for 24 h. The fixed tissues were embedded with paraplast and sectioned in 10 µm thick sections with a microtome. The sections were dewaxed with citrosolv twice and stained with 1% toluidine blue for 2 min. After rinsing two times with water and dehydration through a series of ethanol dilutions, the samples were mounted with permount (Fisher Scientific, USA) and finally observed with DIC microscope.

GUS Staining

GUS staining was done as described (Sundaresan et al., 1995). The inflorescence tissues or 7-d seedlings were soaked in GUS solution (0.1% 5-bromo-4-chloro-3-indolyl-beta-glucuronic acid) and vacuum infiltrated for 10 min. The vials were covered with foil and incubated in 37°C for 16h, followed by destaining with 70% ethanol. Finally, images were taken using stereomicroscope (LEICA MZ12) or compound microscope with DIC optics (LEICA) and a SPOT camera (Diagnostic Instruments).

Gene expression analysis

For the *las-13* mutant, the RNA was extracted from dissected paraclade junction tissue. For *35S:LAS* plants, total RNA was extracted from 10-d-old seedlings. The cDNA was synthesized from 3 µg of total RNA with Superscript III following the manufacturer's proto*col*s (Invitrogen), and followed by semi-quantitative RT-PCR or quantitative RT-PCR. For semi-quantitative RT-PCR, gene-specific primers were designed for *LAS*

(LAS-CAPS-F and LAS-CAPS-R), *LOB* (EMB28 and EMB29). ACTIN (ACT2) amplified with primers ACT-F and ACT-R was served as a loading control. For quantitative RT-PCR, gene-specific primers were designed for *LOB* (QLOB-F and QLOB-R), *BAS1* (QBAS1-F and QBAS1-R), *BRL3* (QBRL3-F and QBRL3-R), *CUC1* (QCUC1-F and QCUC1-R), *CUC2* (QCUC2-F and QCUC2-R), *LOF1* (QLOF1-F and QLOF1-R), *LOF2* (QLOF2-F and QLOF2-R), *OBO1* (QOBO1-F and QOBO-R) (Table 1.1 in Chapter 1, Table 3.1).

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Figure 3.1 *7735* mutant enhances fusion in the *lob::DsE* mutant.

Paraclade junctions of *Ler* wild-type (A), *lob::DsE* (B), and *7735* (C). In wild-type, the cauline leaf and axillary shoot are completely separated, *lob::DsE* exhibits moderate fusion, which is enhanced in *7735*.

A
Ler
B
lob::DsE
C
7735

Figure 3.2 *7735* contains a lesion in *LATERAL SUPPRESSOR* (*LAS*).

(A). Sequence of *LAS* coding region spanning the identified point mutation (G to A), located 649 bp downstream of the *LAS* ATG in the *7735* mutant and the corresponding sequence in *Ler.*

(B). Alignment between the LAS protein and other members of the *Arabidopsis* GRAS family. GRAS proteins have the conserved VHIID domain (labeled with underline) and LXXLL-motif (labeled with double underline) at the C-terminus. The altered amino acid in the 7735 mutant (labeled with asterisk) resides in the VHIID region.

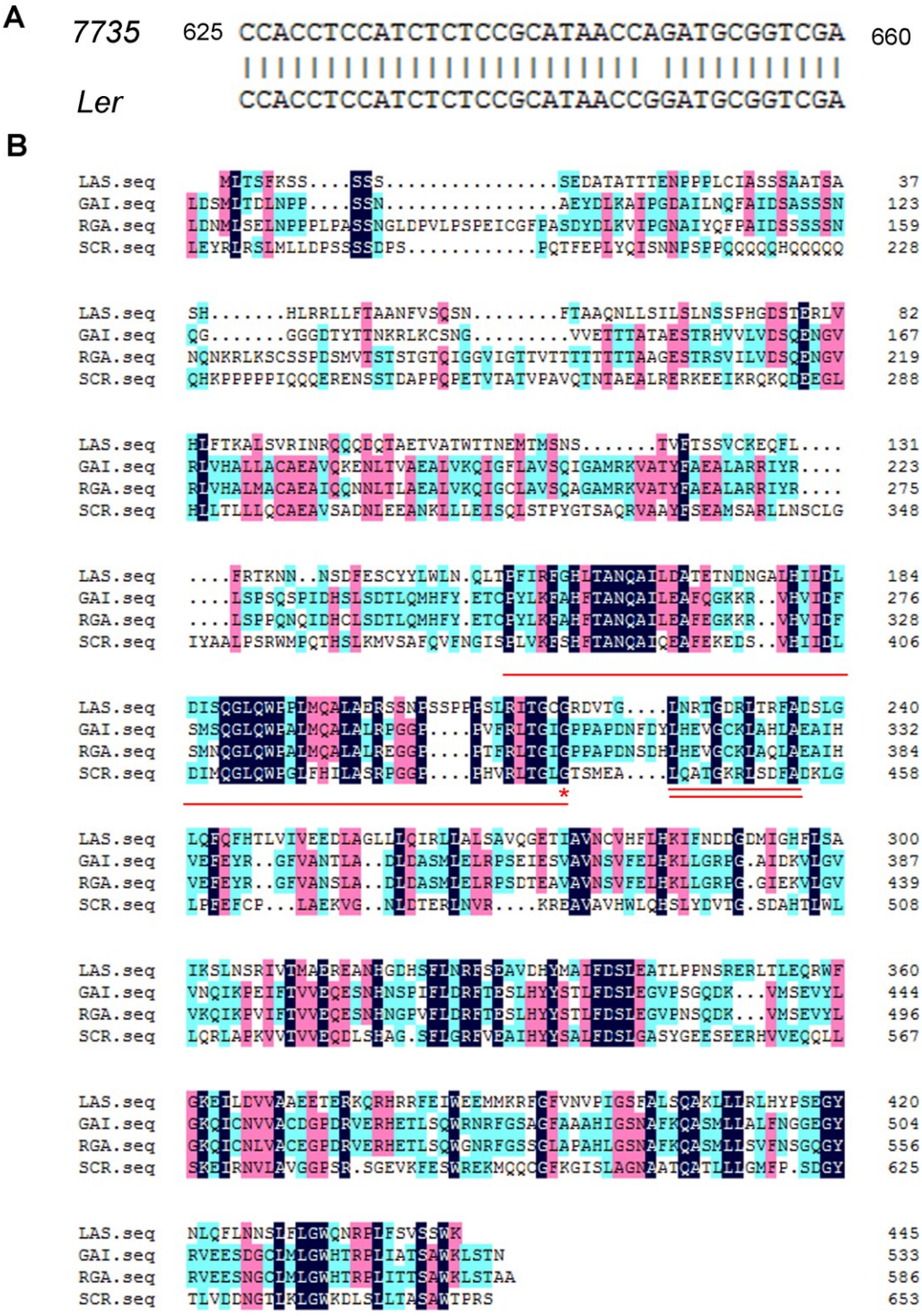

A
7735 625 CCACCTCCATCTCTCCGCATAACCAGATGCGGTCGA 660
Ler CCACCTCCATCTCTCCGCATAACCGGATGCGGTCGA
B
LAS.seq
GAI.seq
RGA.seq
SCR.seq

Figure 3.3 *las-13* mutant is complemented by *pLAS:LAS* transgene.

Rosette of *Ler* wild-type plant showing initiating axillary meristems (A), *las-13* with reduced axillary meristems (B), and *pLAS:LAS las-13*, in which axillary meristems are restored (C). Arrows show the position of axillary meristem.

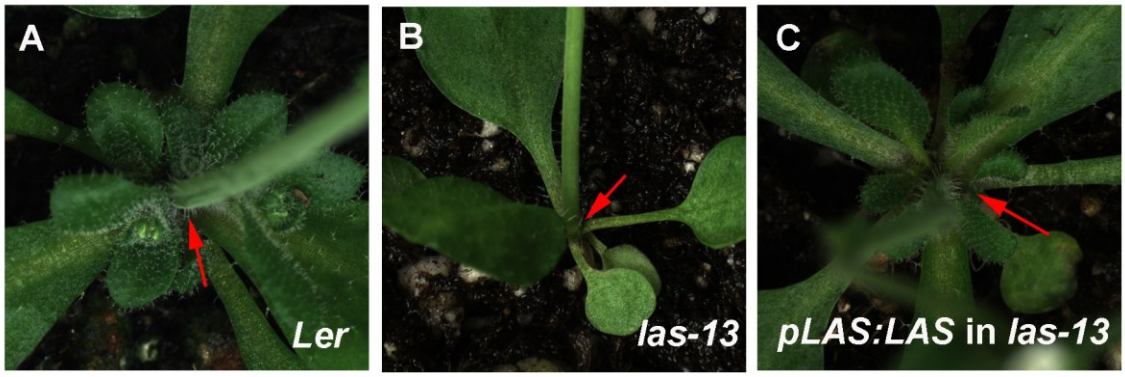
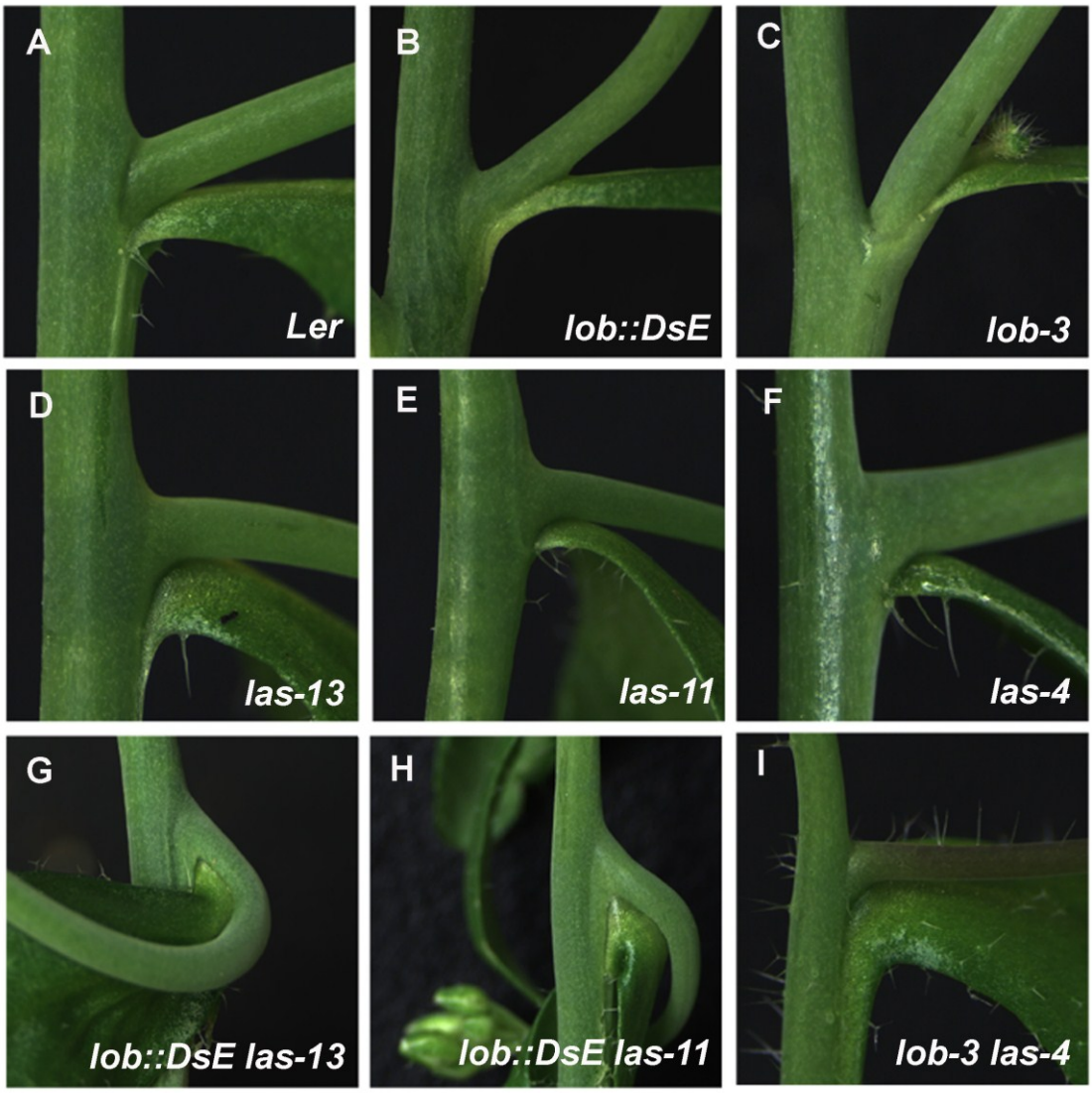

A
Ler
B
las-13
C
pLAS:LAS in las-13

Figure 3.4 *las* mutations in *Ler* background enhance boundary fusion phenotype of *lob* mutant.

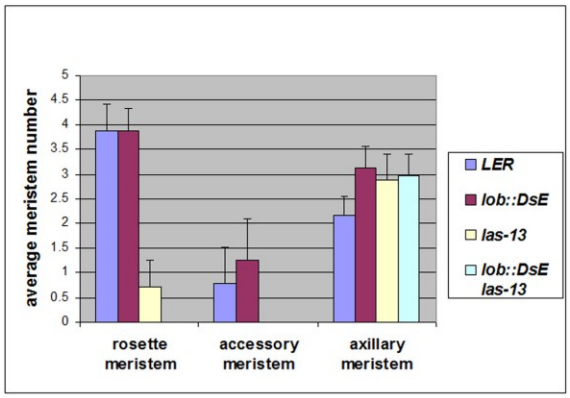
(A-I) Paraclade junction in *Ler* (A); *lob::DsE* in *Ler* background (B); *lob-3* in *Col* background (C); *las-13* in *Ler* (D); *las-11* in *Ler* (E); *las-4* in *Col* (F); *lob::DsE las-13* (G); *lob::DsE las-11* (H); and *lob-3 las-4* (I).

(J) The number of rosette axillary meristems (rosette meristem), accessory meristems, and axillary meristems on inflorescence stem in *Ler*, *lob::DsE*, *las-13*, and *lob::DsE las-13*. (K) The length of fusion in *Ler*, *lob::DsE*, *las-13*, and *lob::DsE las-13*.

n means the number of plants, n>30. Error bar indicates S.D.


A
Ler
B
lob::DsE
C
lob-3
D
las-13
E
las-11
F
las-4
G
lob::DsE las-13
H
lob::DsE las-11
I
lob-3 las-4

J


average meristem number
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
rosette meristem
accessory meristem
axillary meristem
LER
lob::DsE
las-13
lob::DsE las-13

K

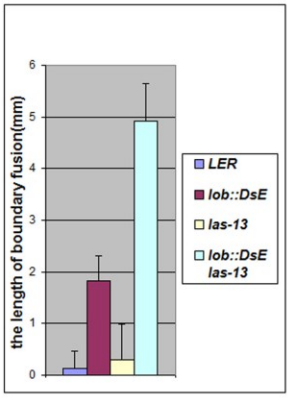

the length of boundary fusion(mm)
6
5
4
3
2
1
0
LER
lob::DsE
las-13
lob::DsE las-13

Figure 3.5 Characteristics of boundary cells in the *lob, las,* and double mutants.

Toludine blue stained cross sections of paraclade junctions of *Ler* (A); *las-13* (B); *lob::DsE* (C); and *las-13 lob::DsE* (D) plants. The red box highlights the boundary cells. The inset in left corner of each panel shows a low magnification view for (A-D). Arrow shows the boundary region.

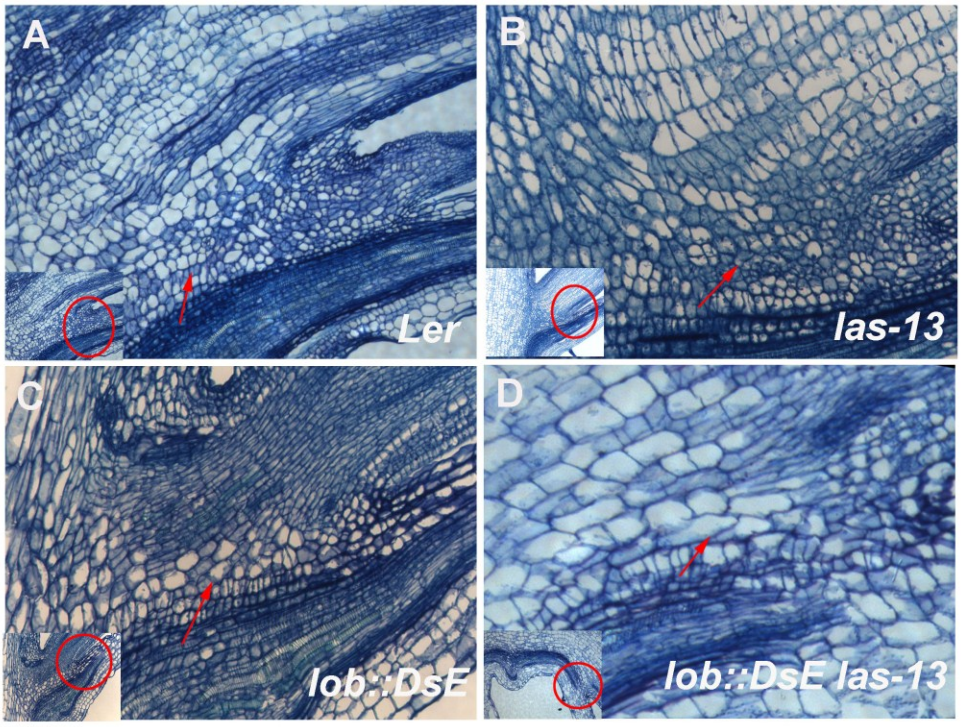

A
Ler
B
las-13
C
lob::DsE
D
lob::DsE las-13

Figure 3.6 *LOB* expression level is reduced in *las* mutants in the *Ler* background.

(A-J) GUS activity in seedlings (A-C, G, H) and inflorescences (D-F, I, J) of *lob::DsE* (A, D); *lob::DsE las-13* (B, E); *lob::DsE las-11* (C, F); *pLOB:GUS* in *Col* (G, I); and *pLOB:GUS las-4* (H, J).

(K). RT-PCR analysis shows *LOB* mRNA level is reduced in dissected paraclade junctions of *las-13* mutant compared to that in the boundary of *Ler* control plant. 22 cycles of amplification for *LOB* was performed. 20 cycles of amplification for *ACT2* was served as a loading control.

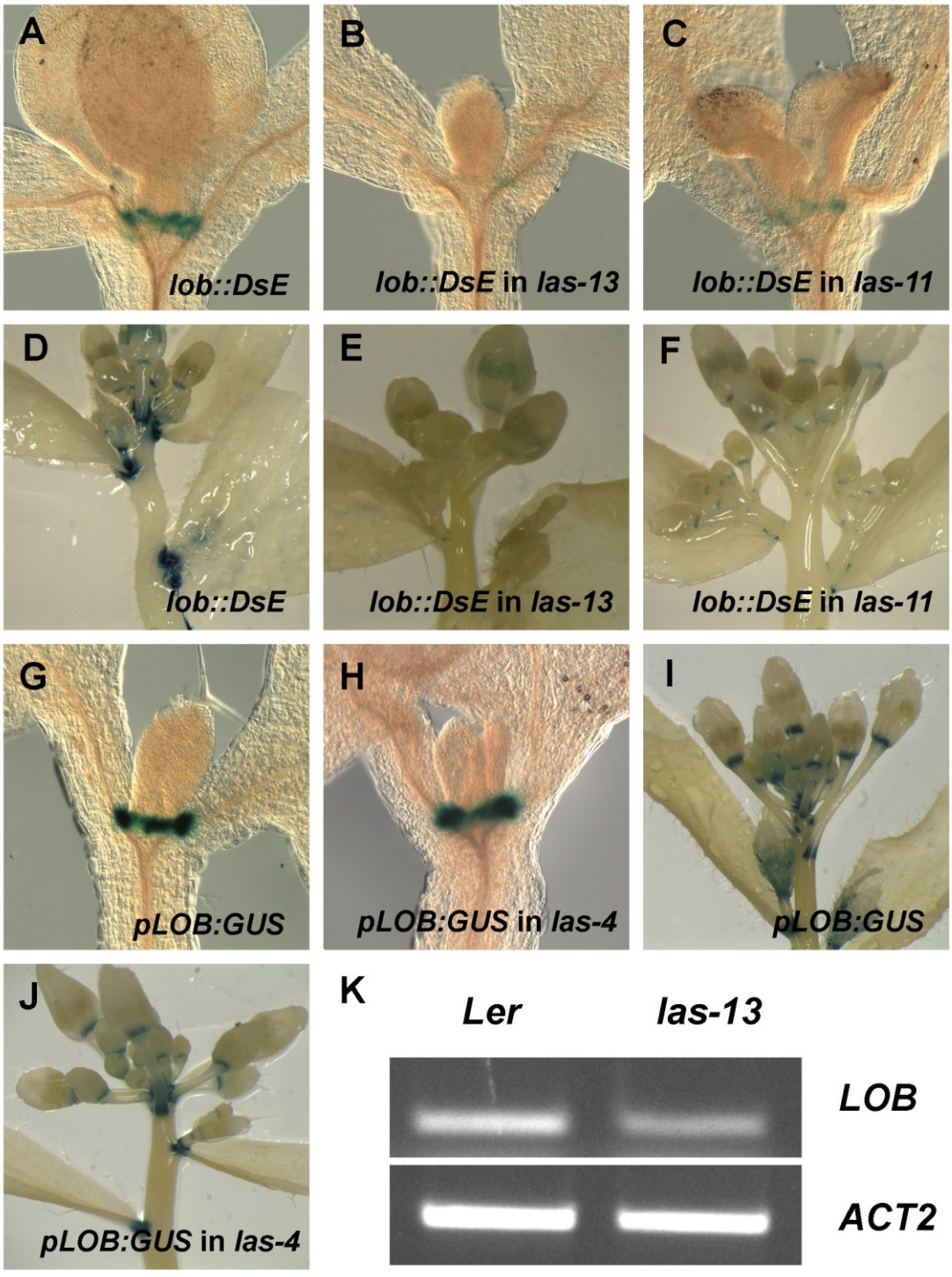

A
lob::DsE
B
lob::DsE in las-13
C
lob::DsE in las-11
D
lob::DsE
E
lob::DsE in las-13
F
lob::DsE in las-11
G
pLOB:GUS
H
pLOB:GUS in las-4
I
pLOB:GUS
J
pLOB:GUS in las-4
K
Ler
las-13
LOB
ACT2

Figure 3.7 Quantitative RT-PCR analysis of boundary gene transcript accumulation in *las* and *lob* mutants.

Transcript levels of *LOB*, *CUC1, CUC2, LOF1, LOF2,* and *OBO1* in dissected paraclade junctions of *Ler*, *lob::DsE*, *las-13*, and *lob::DsE las-13*. *ACT2* was served as a loading control.

n means biological replicates, n=3. Error bar indicates S.D.

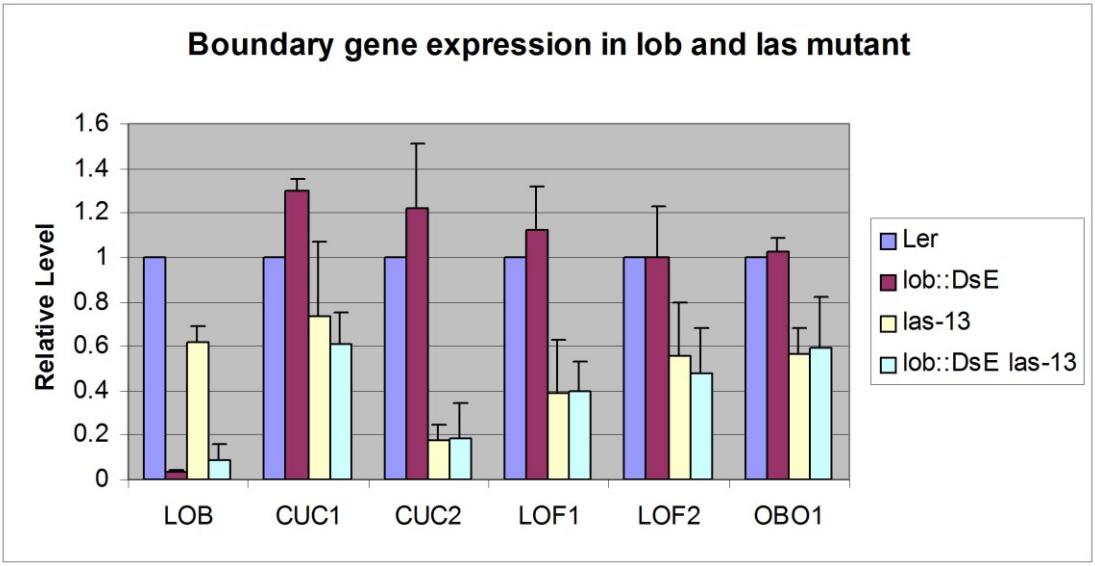

Boundary gene expression in lob and las mutant
Relative Level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LOB
CUC1
CUC2
LOF1
LOF2
OBO1
Ler
lob::DsE
las-13
lob::DsE las-13

Figure 3.8 Overexpression of *LAS* suppresses plant growth.

(A) *Col* plant; (B) *35S:LAS* plant; (C) Mock-treated *35S:LAS-GR* plant; (D) *35S:LAS-GR* plant grown on 10 μM DEX. (E) Semi-quantitative PCR analyses of *LOB* and *LAS* transcript levels in *Col* and *35S:LAS* seedlings. RT-PCR was detected by 30 cycles of amplification for *LAS* and 28 cycles of amplification for *LOB*. 20 cycles of amplification of *ACT2* was served for a loading control.

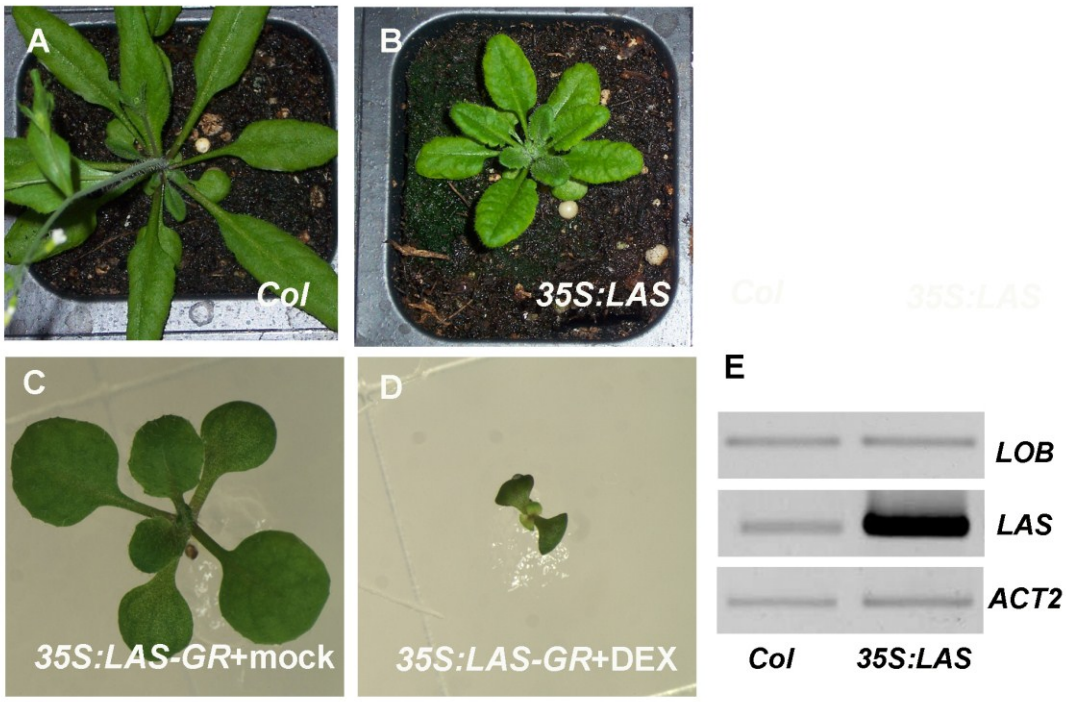

A
Col
B
35S:LAS
C
35S:LAS-GR+mock
D
35S:LAS-GR+DEX
E
LOB
LAS
ACT2
Col
35S:LAS

Table 3.1 Oligonucleotide Sequences

Primer Name	Sequence
LAS-F	GCCGCTCAAAACTTACTCTCAAT
LAS-R	GTGTGAAACTGGAATTGGAGACC
LAS-CAPS-F	AACCAAGCTATCCTCGACGC
LAS-CAPS-R	TCCCAAATCTCAAACCTCCG
Las11-SAP-WT	GAGTCTTGTTACTATCTTGG
Las11-SAP-Mut	GAGTCTTGTTACTATCTTGA
Las11-SAP-R	ACTGCTAGGGTTTGATGA
LAS-GATE-F	CACCATGCTTACTTCCTTCAAATCCT
LAS-GATE-R	TCATTTCCACGACGAAACGG
LAS-SAAB-R	TTTCCACGACGAAACGGAG
pLAS-SacI-F	GAGCTCGCCACCTAATGAAGTGTAC
pLAS-SacI-R	GAGCTCTTGAAAAGATAGAAAAAGATGCTT
QLOB-F	GGCGTCGTCATCAAACTCAT
QLOB-R	CGTTGCTTGCTCCAAAGATT
QCUC1-F	TACCCAACGGGACTGAGAAC
QCUC1-R	GCGGAGGAGGAAATGTAATG
QCUC2-F	GAGGAGGAGGAGCAACTGTG
QCUC2-R	GAAACGAGGAAAAGGGTCAA
QLOF1-F	TCTTGGTCCTCGTCGTCTTC
QLOF1-R	GCATCAGCCTCTCCTCTTCTT
QLOF2-F	GGTAACAAATGGGCGATGATA
QLOF2-R	AACCTGGGAAGCAAGGAACT
QOBO1-F	CGCTCTTATCGGTCGTCTTC
QOBO1-R	CGCTTTCGCTTCTTCTTCTC

CONCLUSIONS

The plant shoot apical meristem (SAM) consists of a group of undifferentiated cells at the shoot apex, which are responsible for both generation of all the lateral organs, such as leaves and flowers, and renewal of the meristem cells themselves (Bowman and Floyd, 2008). Within the SAM, there are several different functional zones with unique gene expression patterns and hormonal activities. The meristem-organ boundary resides at a band-like region between the SAM and lateral organ primordia (Aida and Tasaka, 2006). The boundary region is not only a physical barrier in the shoot apex, but also actively communicates between the SAM and emerging lateral organ primordia (Aida and Tasaka, 2006; Goldshmidt et al., 2008).

The LBD family gene *LOB* has been identified as a boundary-specific gene and is the focus of this dissertation. *LOB* encodes a protein containing a LBD domain, which is conserved throughout the *LBD* family (Shuai et al., 2002). LOB is a transcription factor and controls downstream gene expression by binding to a LOB binding motif (Husbands et al., 2007). Previous studies also revealed that *LOB* is important for boundary maintenance (Shuai et al., 2002; Bell et al., 2012). First, *LOB* was initially identified as a gene specifically expressed in the boundary region, between the SAM and lateral organs, through a gene trap screening of *GUS* reporter gene expression patterns (Shuai et al., 2002). Second, loss-of-function *lob* mutations result in moderate fusion between the axillary stem and cauline leaf (Bell et al., 2012). Finally, *35S:LOB* overexpression greatly reduces growth in various tissues, making plants dwarf (Shuai et al., 2002). Taken together, *LOB* is specifically expressed at the boundary region and locally suppressed growth to ensure normal boundary cell identity. However, the *LOB* downstream genetic

pathway and genetic interaction between *LOB* and other factors controlling boundary specification were not known before this dissertation study.

LOB controls boundary maintenance through two counteracting BR signaling pathways. On one hand, LOB directly activates *BAS1*, a BR repressor, to inhibit BR signaling in the boundary region. *BAS1* is a member of cytochrome P450 gene family and catalyzes C26 hydroxylation to inactivate BR (Neff et al., 1999). BR is one important growth-promoting hormone controlling plant shoot architecture (Gudesblat and Russinova, 2011). Previous studies revealed that active BR signaling in the epidermal cell layer is critical for cell size and plant growth control (Savaldi-Goldstein et al., 2007). Through this dissertation study, it was found that inhibition of BR signaling in the boundary region is required for limiting cell expansion and division, which are required to maintain boundary integrity, since boundary specific overexpression of positive regulators for BR signaling, *DWARF4* and *BRI1*, leads to fused boundary, which is similar to *lob* loss-of-function mutant phenotype. Interestingly, in the valve region of the *Arabidopsis* silique, it has been reported that auxin signaling is suppressed and this local auxin level minimum leads to limited cell growth in the valve region (Sorefan et al., 2009), suggesting that local suppression of growth promoting hormone signaling might be a general mechanism in plant boundary maintenance. On the other hand, LOB directly activates transcription of the BR receptor *BRL3*. BRL3 is a BR receptor in *Arabidopsis*, which is structurally similar to BRI1 (Cano-Delgado et al., 2004). BRL3 is a functional BR receptor, since it complemented the *bri1* loss-of-function phenotype when expressed under the control of the *BRI1* promoter (Cano-Delgado et al., 2004). Consistent with its function as a BR receptor, boundary specific mis-expression of *BRL3* leads to fused boundary and decreased *LOB* expression. Considering LOB activates *BRL3*

transcription and *BRL3* overexpression inhibits *LOB* expression, there is a negative feedback regulation for *LOB* expression domain and BR signaling. Negative feedback regulation is not a unique feature for BR signaling in the boundary, but a common theme for control of plant hormone responses and plant development. For the first example, BR biosynthesis is under negative feedback control. When BR signaling activity is high, expression of the BR biosynthesis gene *CPD* is inhibited by BR, to down-regulate BR level (Wang et al., 2004). A second example involves cytokinin activation of type A ARR targets, which in turn reduce cytokinin responses to prevent over-activation of the cytokinin pathway (Hwang et al., 2012). Another example is the negative feedback regulation of *WUSCHEL (WUS)* and SAM identity. In the SAM, *WUS* up-regulates *CLAVATA3 (CLV3)* expression, which in turn restricts the *WUS* expression domain, forming a negative feedback loop (Bowman and Floyd, 2008). Negative feedback loops provide a mechanism for precise regulation of a signaling pathway.

LOB is not the only important factor for boundary maintenance but also functions synergistically with *LAS* to control both boundary maintenance and axillary meristem formation. *LAS,* a transcription factor in the GRAS family of *Arabidopsis* (Bolle, 2004), has been revealed to be important for axillary meristem formation, but was not known to have a clear role in boundary specification between the lateral cauline leaf and axillary shoot (Greb et al., 2003). *lob las* double mutants have dramatic boundary fusion between the stem and lateral cauline leaves, demonstrating the synergistic genetic interaction between *LOB* and *LAS* for boundary maintenance. Consistent with the finding that *LAS* has a function in boundary specification, overexpressing *LAS* inhibits plant growth and reduced plant size. It is possible that specific expression of *LAS* in the boundary region locally suppresses growth and maintains boundary cell identity or

boundary integrity. In general, it is a common feature that boundary genes control boundary maintenance and/or cell identity via suppression of cell growth, since *LOB*, *CUC* and *LAS* overexpressing plants all showed suppression of cell growth in smaller leaf size or indented leaf shape (Shuai et al., 2002; Laufs et al., 2004). On the other hand, previous studies found that *LOB* controls boundary integrity, but did not find an effect on axillary meristem formation (Bell et al., 2012). The *lob las* double mutant also has stronger reduction of axillary meristem formation than *las* single mutant, revealing a novel role of *LOB* in the process of axillary meristem formation. Interestingly, the synergistic interactions are not only found between *LOB* and *LAS*, but also between other boundary genes. For example, the *las* loss-of-function mutant enhances the *lof1* loss-of-function mutant in both boundary fusion and axillary meristem initiation (Dong-Keun Lee's dissertation). In a broader sense, the control of boundary specification and meristem formation by boundary genes is not unique for adult stages of plant as revealed in this study. For example, in seedling development, *CUCs* are required for both boundary specification between cotyledons and SAM formation (Long and Barton, 1998; Takada et al., 2001). One explanation for these variations of the common theme would be that the boundary region between cotyledons provides the landmark required for SAM formation during embryogenesis as demonstrated by *cuc1 cuc2* mutant, whereas boundary region between stem and lateral leaves provides positional information needed for axillary meristem formation as demonstrated by *lob las* mutant.

In order to further understand the *LOB* dependent boundary maintenance pathway, future studies should be carried out in three directions. First, the microarray screen for *LOB* downstream targets also revealed other putative candidate genes. Further study of these candidate genes will identify new branches of *LOB* genetic

pathways and enhance our understanding of boundary cell identity maintenance. Second, although we know that *LOB* is specifically expressed in lateral organ boundaries, the upstream regulators for *LOB* are not well characterized. A genome-wide yeast-one-hybrid screen using *LOB* promoter fragments would provide clues for the direct regulators of *LOB*. Further study on the upstream genetic pathway of LOB would provide a more complete understanding of how boundary identity is established and maintained. Finally, in addition to *LAS*, other boundary specific genes may also have synergistic genetic interactions with *LOB*. For example, *LAS* is required for the normal boundary expression pattern of *LOB* and several other boundary genes such as *LOF1*, *LOF2*, *CUC1*, *CUC2* and *OBO1* (Aida et al., 1997; Aida et al., 1999; Hibara et al., 2006; Lee et al., 2009; Cho and Zambryski, 2011; Takeda et al., 2011), indicating multiple interactions between different boundary genes. To test this possibility, extensive crossing of different boundary gene loss-of-function mutants will provide valuable information and help to elucidate the complex genetic networks for boundary identity maintenance.

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