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Characterization of Leaflet Inclination and LOB-Domain Genes in *Vigna unguiculata* (L.) Walp (Cowpea)

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by

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Dedication

This dissertation is dedicated to the village it took to raise me.

ABSTRACT OF THE DISSERTATION

Characterization of leaflet inclination and LOB-domain genes in *Vigna unguiculata* (L.) Walp (Cowpea)

by

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Plants undergo organogenesis throughout their lifetime by maintaining populations of pluripotent cells at their apices. The shoot apical meristem (SAM) produces lateral organs, such as leaves, at the periphery of the SAM. The SAM and the lateral organs are separated by the boundary region, an area where growth is restricted. The boundary region plays an important role in regulating shoot architecture and influences leaf inclination. The *Arabidopsis* transcription factor *LATERAL ORGAN BOUNDARIES* (*LOB*) is expressed in organ boundaries and functions to limit growth and separate organs. This dissertation focuses on the molecular control of leaflet angle and boundary formation in cowpea and *Arabidopsis.*

In chapter 1, I identify the cowpea *LOB* ortholog, termed *VuLOB* (Vigun09g091300) and characterize expression. *VuLOB* transcripts accumulation in the pulvini and are positively regulated by the phytohormone brassinosteroid (BR). Treatment with the BR biosynthesis inhibitor propiconazole inhibits cowpea leaflet inclination in response to water deficit, demonstrating that BRs are required to promote leaflet inclination in cowpea.

In chapter 2, I use genome-wide association studies (GWAS) to identify regions of the cowpea genome correlated with variation in pulvinus size and leaflet inclination after water-deficit. Significant peaks correlated to the variation in pulvinus size, but no peaks correlated with leaflet angle were identified. A candidate gene, *VuLBD41* (Vigun05g006100), was selected for additional characterization. Transcripts of *VuLBD41* accumulate in the pulvini of cowpea. However, functional analysis using *Arabidopsis* did not reveal any function in regard to development. Transgenic plants overexpressing *VuLBD41* appear phenotypically wild-type and its function remains unknown.

In chapter 3, I characterize the genetic relationship between *LOB* and putative targets, *TREHALOSE-6-PHOSPHATE PHOSPHATASE I/J* (*TPPI* and *TPPJ*). LOB directly regulates *TPPJ*, but not *TPPI*. Whereas higher order mutants did not have an enhancement of the organ fusions found in *lob* mutants, they exhibited a significant increase in the number of branches. This suggests that these genes have some role in the boundary. Further, I show that ectopic *TPPJ* accumulation in the boundary is sufficient to rescue the *lob* mutant phenotype. This suggests that LOB requires *TPPJ* to restrict growth at the boundary.

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Introduction

1. Functions of the shoot apical meristem (SAM)

Plants produce organs throughout their lives. In contrast, animals form all of their organs during embryogenesis. Plants have the ability to continuously produce organs due to the formation and maintenance of meristems. Meristems are structures containing populations of pluripotent cells that exist at both apices of the plant. In the shoot, the shoot apical meristem (SAM) produces lateral organs such as leaves and floral organs, as well as the stems (Gallois et al. 2002). In the root, the root apical meristem (RAM) produces the cell files of the root (Jiang and Feldman 2005). In the SAM, cells that maintain pluripotency occupy the central zone, while cells in the peripheral zone become incorporated into lateral organ primordia, switching fate (Somssich, et al 2016). As lateral organs form, cells in the peripheral zone are incorporated into newly formed organs and divisions of the central-zone cells replenish the population of meristematic cells. The pluripotent cells of the SAM are maintained through a negative-feedback loop controlled by *WUSCHEL* (*WUS*), a gene that encodes a non-cell autonomous transcription factor and *CLAVATA 1/3* (*CLV1/3*), which encode a receptor kinase and ligand, respectively (Yadav, Tavakkoli, and Reddy 2010). In the negative feedback loop, WUS positively regulates *CLV3* transcription, CLV3 protein is perceived by CLV1, and through a signaling cascade *WUS* is negatively regulated (Somssich, et al 2016). This feedback loop ensures that the meristem is maintained in both size and number of

pluripotent cells. In *Arabidopsis*, plants that carry a mutation in *WUS* fail to maintain their meristems and the meristem is rapidly consumed after the formation of the first true leaves (Mayer et al. 1998). In contrast, plants that carry a mutation in *CLV3* have an abnormally large meristem, caused by an expansion of the WUS expression domain, and produce more lateral organs than their wild-type counterparts (Fletcher et al. 1999). This feedback loop ensures that the plants properly maintain their indeterminacy.

SAM identity and maintenance is regulated by the *KNOX* family of homeodomain transcription factors (Jackson, Veit, and Hake 1994). Class-1 *KNOX* genes are expressed in the SAM and are repressed in initiating organs by the *MYB* transcription factor ASYMMETRIC LEAVES1 (AS1) and the LBD transcription factor ASYMMETRIC LEAVES2 (AS2) (Byrne, Simorowski, and Martienssen 2002). Loss-of-function mutations in the *KNOX* gene *SHOOTMERISTEMLESS* (*STM*) result in failure to produce a SAM and seedling lethality (Barton and Poethig, 1993). This phenotype is attributed to *AS1*/*AS2* ectopic expression in the meristem causing the meristem cells to differentiate and meristematic identity to be lost (Byrne et al. 2002). In contrast, mutants of *AS1* and *AS2* have ectopic KNOX accumulation in the differentiated leaves, causing aberrant leaf shapes (Semiarti et al. 2001). The first gain-of-function mutation described for *KNOX* genes was in maize where plants that were over expressing *KNOTTED1* had patches of cells with meristem identity on their leaves (Jackson, Veit, and Hake 1994; Vollbrecht et al. 1991). After leaf primordia have initiated, AS1 and AS2 interact to form a complex with the DNA of several *KNOX* genes to repress their expression in the initiating organs (Lodha, Marco, and Timmermans 2013).

2. Functions of the boundary region and genes involved in boundary function

In the shoot apical meristem, cells in the central zone slowly divide and their daughter cells populate the peripheral zone. Cells in the peripheral zone will be specified to form a primordium, which will continue to divide and ultimately form a determinate structure, such as a leaf or floral organ. In between the cells in the peripheral zone of the meristem and the cells that have begun to differentiate are the cells of the boundary region (Hussey 1971). The boundary region is a unique population of cells that have their own defining characteristics. Boundary cells divide infrequently, as shown by Hussey 1971, where fewer cells in the boundary are arrested in mitosis compared to the neighboring populations of cells. The cells in the boundary are also smaller relative to the cells in adjacent regions, thus the boundary is a region of restricted growth between the pluripotent cells of the SAM and the differentiating cells of the lateral organs (Hussey 1971). The boundary region has several functions. Boundaries can not only influence leaf shape and complexity, but also affect the architecture, or shape, of the entire plant body.

A subset of the boundary cells will grow out and acquire meristem identity to become axillary meristems (Mitsuhiro, Aida, and Tasaka 2006). Axillary meristems play a role in patterning the architecture of the plant by producing numerous reproductive branches (Cline 1991). However, branching is often suppressed by the primary shoot through the phenomenon known as apical dominance (Cline 1991). Apical dominance is, in part, controlled by auxin and cytokinin. Auxin is synthesized in the SAM and is

transported basipetally where it represses the outgrowth of axillary meristems (Bartrina et al 2011). Axillary meristems remain dormant in the leaf axil and will break dormancy after the concentration of auxin within it is lowered. This allows for cytokinin to accumulate and promote the outgrowth of the branch (Bartrina et al. 2011). Branching is highly advantageous for light capture and reproductive success in wild species but can reduce yields in crop plants. Thus, the regulation of shoot architecture through shoot branching can have a high impact on productivity.

As the plant matures, cells at the base of the floral organs and petioles are fated to be sites of abscission. Abscission is partially required for reproductive success of a plant and requires that a distinct population of cells form an abscission zone (AZ) (Y. Lee et al. 2018). In *Arabidopsis*, the patterning of the AZ is regulated by two boundary-expressed transcription factors, *BOP1* and *BOP2* (McKim et al. 2008). In order to abscise, cells of the AZ must communicate with the neighboring cells. Interplay between the hormones auxin and ethylene are required to regulate abscission (Abeles and Rubinstein 1964). High concentrations of auxin will prevent abscission while high concentrations of ethylene will promote abscission. In the presence of ethylene, various *KNOX* transcription factors are up regulated to promote cell wall remodeling and degrading enzymes for cell separation and organ abscission (McKim et al. 2008).

The boundary plays a crucial role in establishing leaf angle (Kong et al. 2017). This has been most apparent in maize where the ligular region between the blade and sheath establish the angle of the leaf (Lewis et al. 2014). The ligular region consists of an epidermal fringe called the ligule that is surrounded by an outgrowth of tissue called the

auricle (Sylvester, Cande, and Freeling 1990). This region of the maize leaf is of interest to breeders since it impacts leaf angle, which can influence planting density in the field. In maize, several genes involved in the proximal-distal patterning of the leaf are required for proper formation of the ligular region. For example, *liguleless1* (*lg1*) encodes a SQUAMOSA PROMOTER BINDING PROTEIN (SBP) transcription factor and mutants lacking a functional *lg1* gene fail to produce a ligule or auricles and the resulting leaf angles are narrower compared to wild-type leaves (Moreno et al. 1997). *liguleless2* (*lg2*) encodes a bZIP transcription factor that is also required for patterning the ligular region (Walsh, Waters, and Freeling 1997). The mutant phenotype, at the ligular region, of *lg2* is similar to that of *lg1*, but the ligule and auricles are only missing from the midrib region while still forming in the margins of the leaf (Walsh, Waters, and Freeling 1997). Both *lg1* and *lg2* have been identified among the 30 quantitative trait loci (QTL) that contribute to leaf angle, putting them as two of the major players involved in regulating shoot architecture (Mantilla-Perez and Salas Fernandez 2017).

2A. *LATERAL ORGAN BOUNDARIES* (*LOB*)

In *Arabidopsis*, the transcription factor LATERAL ORGAN BOUNDARIES (LOB) is expressed in plant organ boundaries such as the base of lateral roots and floral organs, the base of the pedicels, and at the junction between the primary stem, axillary branch, and cauline leaf (called the paraclade junction) (Shuai, Reynaga-Peña, and Springer 2002). *LOB* was identified in an enhancer trap screen where it was shown to be

expressed in all organ boundaries (Shuai, Reynaga-Peña, and Springer 2002). However, plants that carry a mutation in *lob* only show a slight defect at the paraclade junction where the adaxial side of the cauline leaf fails to separate from the branch it subtends (Bell et al. 2012). This phenotype suggests that *LOB* is required for organ separation at the boundary, but some redundancy exists given *LOB*'s wider expression pattern. *Arabidopsis* plants over-expressing *LOB* are dwarf in comparison to wild-type plants (Shuai, Reynaga-Peña, and Springer 2002). Their rosette leaves are smaller and more compact, and they produce fewer flowers that are both male and female sterile (Shuai, Reynaga-Peña, and Springer 2002). Etiolated seedlings also fail to form an apical hook compared to wild type. This suggests that *LOB* may function in the boundary to limit growth, either by repressing cell division and/or cell expansion (Bell et al. 2012).

LOB encodes a transcription factor and is the founding member of the LOB-domain family (Shuai, Reynaga-Peña, and Springer 2002; Husbands et al. 2007). In *Arabidopsis*, this family has 43 family members, categorized into 2 classes based on the presence of a conserved DNA-binding domain (the LOB domain) at the N-terminus (Shuai, Reynaga-Peña, and Springer 2002). The LOB domain varies slightly between the two classes. In class 1 proteins, the LOB domain contains a zinc-finger motif, $CX_2CX_6CX_3C$, with 4 conserved Cys residues, called the C-block that functions in DNA binding, a predicted coiled-coil domain, $LX_6LX_3LX_6L$ involved in protein-protein interactions, and a conserved GAS block (Shuai, Reynaga-Peña, and Springer 2002). The class II LBDs only contain the C-block. The LOB domain has been shown to bind a conserved DNA motif, 5'-(G)CGGC(G)-3', where the 4 nt core is highly conserved and

the 2 flanking nucleotides are variable, though G is the most common (Husbands et al. 2007).

LOB has been shown to regulate numerous biological processes including responses to brassinosteroids (BRs), blue light, and trehalose biosynthesis (Bell et al. 2012). To regulate separation at the paraclade junction, LOB limits growth through direct upregulation of *PHYB ACTIVATION TAGGED SUPRESSOR1* (*BAS1*), which encodes a P450 brassinosteroid catabolism enzyme (Bell et al. 2012). Thus, LOB activity limits BR from accumulating in the boundary. *lob* mutants have hyperaccumulation of BR at the paraclade junction, causing an overgrowth of the boundary. When *BAS1* is under the regulation of the *LOB* promoter, this mutant phenotype is suppressed (Bell et al. 2012). In an opposite manner, *Arabidopsis* plants over-expressing LOB have increased *BAS1* transcript and are BR-deficient (Bell et al. 2012).

2B. *LATERAL ORGAN FUSION1* (*LOF1*)

LATERAL ORGAN FUSION1 (*LOF1*) was identified through an enhancer trap screen and was shown to be active in organ boundaries, which include the region between the SAM and developing leaves on the adaxial side, the paraclade junction, and at the base of the floral organs and pedicels (Lee, Geisler, and Springer 2009). Cloning of *LOF1* has revealed that it encodes a MYB-transcription factor (Lee, Geisler, and Springer 2009). *Arabidopsis* plants carrying a mutation in *lof1* have cauline leaves that are not fully separated from the axillary branch, a phenotype that is highly variable, as well as

the loss of accessory buds at the paraclade junction (Lee, Geisler, and Springer 2009). This suggests that *LOF1* is required for organ separation and meristem maintenance. A homolog of *LOF1, LOF2*, functions with *LOF1* as *lof1; lof2* double mutants have an enhanced phenotype (Lee, Geisler, and Springer 2009). *lof1 lof2* mutants have additional organ fusions at the pedicels and inflorescence stem and cauline leaves forming decurrent strands down the main shoot (Lee, Geisler, and Springer 2009). Additionally, *LOF1* functions in concert with other boundary and meristem maintenance genes. *lof1* mutants fail to make accessory buds (Lee, Geisler, and Springer 2009). These plants have reduced *STM* transcripts in leaf axils. In addition, when *lof1* mutant plants are crossed with a weak *stm* allele, the resulting double mutants have an enhanced defect in meristem maintenance and show organ fusions in vegetative growth, a phenotype not found in either single mutant (Lee, Geisler, and Springer 2009). Transcripts of *CUP-SHAPED COTYLEDON 2*/*3* (*CUC2* and *CUC3*) were reduced in *lof1,* which suggests that there is a positive genetic relationship between *CUC2/CUC3* and *LOF1* (D. K. Lee, Geisler, and Springer 2009). While higher order mutants between *lof1* and *cuc2/cuc3* did not display vegetative phenotypes, these mutant plants do have severe fusion defects during the reproductive phase (Lee, Geisler, and Springer 2009). Both *lof1 cuc2* and *lof1 cuc3* double mutants display pedicel-stem fusions, and more severe fusions of cauline leaf to the axillary branch (Lee, Geisler, and Springer 2009). These double mutants also had meristem maintenance defects where the leaf axils sometimes contained either a solitary flower or lacked a meristem (Lee, Geisler, and Springer 2009). This further suggests that *LOF1* functions in organ separation and meristem maintenance.

In tomato, the *LOF1* ortholog *TRIFOLIATE* (*TF*) functions not only in meristem maintenance, but also compound leaf development (Ahmad et al. 2013). *tf* mutants have simpler leaves compared to wild type, they have lost many of their leaflets and have reduced axillary buds, producing fewer branches (Ahmad et al. 2013). This phenotype is interesting because it shows a conserved role of *LOF1*'s role in meristem maintenance and provides some evidence that there is a connection between the boundary region and leaf complexity.

2C.*CUP-SHAPED COTYLEDON* (*CUC*)

Arabidopsis contains three *CUP-SHAPED COTYLEDON* homologs (*CUC1*, *CUC2*, *CUC3*), which encode transcription factors that function in the boundary between the lateral organs and SAM (Aida, et al. 1997; Aida, et al.1999). These genes are part of the *NAC* (*NO APICAL MERISTEM*/*ATAF*/*CUP-SHAPED COTYLEDON*) gene family (Ooka et al. 2003). The *CUC* genes function redundantly to maintain the meristem and separate the lateral organs. The *cuc1* and *cuc2* single mutants do not have any detectable mutant phenotype, however, a *cuc1 cuc2* double mutant has fused cotyledons and doesn't produce any shoots (Aida, Ishida, and Tasaka 1999). These mutants fail to initiate a meristem during embryogenesis, as in wild type plants CUC1 and CUC2 are required to activate *STM* (M. Aida, Ishida, and Tasaka 1999). *CUC3* has a stronger role in organ separation rather than meristem maintenance. *cuc3* single mutants have more defects in floral organ fusions and axillary shoot formation compared to either *cuc1* or *cuc2* single

mutants (Hibara et al. 2006). The phenotypes observed in *cuc3* single mutants are strongly enhanced by *cuc2*, but not *cuc1,* which suggests partial redundancy within this family.

In other plant species, the function of the *CUC*s is partially conserved. In *Petunia, NO APICAL MERISTEM* is required for meristem initiation in the embryo and proper flower formation (Souer et al. 1996). In *nam* mutants, no meristem is formed during embryogenesis and the resulting seedling contains two cotyledons that are fused at the petioles (Souer et al. 1996). Occasionally *nam* mutants develop shoots and produce flowers that contain extra petals and anthers, which fuse to the extra petals and aberrant gynoecium (Souer et al. 1996). This suggests a partially conserved role with the *Arabidopsis CUC*s.

In tomato, *GOBLET* encodes a *CUC2* ortholog and its transcript accumulates at the boundary between the leaf and the SAM, as well as at the base of the leaflets of the compound leaf (Berger et al. 2009). *gob* mutants have fused cotyledons and meristems that terminate prematurely suggesting that the function of *GOB* and *CUC2* is conserved (Berger et al. 2009).

2D. *LATERAL SUPPRESSOR* (*LAS*)

LATERAL SUPPRESSOR (*LAS*) encodes a transcription factor in the *GRAS* (*GIBBERELLIC ACID INSENSITIVE*/ *REPRESSOR OF GA1*/ *SCARECROW*) family of transcriptional regulators (Greb et al. 2003). *LAS* was first described as the tomato mutant

lateral suppressor (*ls*). *ls* mutants have defects in axillary meristem formation during the vegetative phase of development (Schumacher et al. 1999). Additionally, *ls* mutants have defects in flower development where they fail to produce petals and have reduced fertility. In *Arabidopsis*, *las* mutants fail to produce axillary meristems during vegetative development (Greb et al. 2003). However, the *las* mutant differs from the *ls* mutant in that the former produces petals in flowers albeit these mutant plants have delayed petal abscission (Greb et al. 2003).

The formation of axillary meristems is dependent on *STM*. During development *STM* transcripts accumulate in the meristem and the leaf axils where axillary meristems will form but are down regulated in all developing lateral organs (Long and Barton 2000). In *las* mutants, *STM* transcripts fail to accumulate in the axils of older leaf primordia, which suggests that *STM* accumulation in the leaf axils is dependent on LAS function and further that LAS functions to promote axillary meristem formation through *STM* (Greb et al. 2003).

2E. *BLADE-ON-PETIOLE* (*BOP*)

BLADE-ON-PETIOLE 1&2 (*BOP1/2*) are homologs that encode BTB/POZ domain proteins. The Arabidopsis *bop1* mutant was first isolated from an EMS screen. *bop1* mutants exhibited ectopic blade tissue growing from their petioles (Chan, et al. 2003). Additionally, *bop1* mutant leaves have an altered shape and flowers have a variable number of organs (Chan, et al. 2003). The mutant phenotype observed in *bop1*

can be explained by the ectopic accumulation of *KNOX* transcripts (Chan Ma Ha et al. 2003). These transcripts, which are normally restricted to the shoot apical meristem, cause the fate of the petiole to switch and produce ectopic blade tissue. This role of *BOP1* is similar to the roles of AS1 and AS2, which form a complex and repress *KNOX* transcripts from the initiating lateral organs (Chan, et al. 2003). Plants carrying mutations in *bop1* together with either *as1* or *as2* have a higher complexity of leaflets compared to either single mutant, suggesting that these genes interact to prevent *KNOX* transcripts from accumulating ectopically (Chan, et al. 2003). *BOP2* has an overlapping expression pattern with *BOP1* and the *bop1 bop2* mutant phenotype is more severe than either of the single mutants (Chan Man Ha et al. 2007). As expected, both *BOP1* and *BOP2* negatively regulate *KNOX* transcript accumulation in the leaves (Chan Man Ha et al. 2007). The *BOP*s are expressed in organ boundaries and regulate shoot architecture through the regulation of abscission and morphogenesis of the lateral organs, further providing evidence of their global role in plant development.

3. Brassinosteroids and their role in development

Plant hormones (phytohormones) participate in nearly all of the growth and development that occurs during a plant's life. They influence the patterning of various organs, control cell division and expansion, regulate flowering, and play a role in immunity and pathogen defense. Brassinosteroids (BRs) are a class of phytohormones that were first isolated in *Brassica napus* pollen in the 1970s (Clouse 2011). These

hormones are found throughout the plant kingdom and influence a number of traits involved in development and morphogenesis, as well as various physiological responses (Clouse 2011).

BRs are synthesized in the endoplasmic reticulum (ER) and primarily play a role in cell division and elongation. Several genes involved in BR biosynthesis and perception have been characterized in several plant species, primarily *Arabidopsis.* Plants deficient in BR biosynthesis or perception have the classic BR-deficient phenotype – they are dwarf and sterile (Clouse, Langford, and McMorris 1996). The most severe BR-deficient phenotype can be seen in plants that have a loss-of-function mutation in *BRASSINOSTEROID INSENSITIVE1* (*BRI1*) (Clouse, Langford, and Mcmorris 1996). *BRI1* encodes a membrane-spanning leucine-rich-repeat receptor-like kinase that physically interacts with the active BR, brassinolide (BL) (Friedrichsen et al., 2000.). Plants with *bri1* mutations are severely dwarf, dark green with downward curling leaves, have delayed flowering, male sterility, altered vascular morphology, and reduced apical dominance (Clouse, Langford, and Mcmorris 1996). The wide range of phenotypes demonstrates that BR perception is required for a plethora of process throughout development.

During development, BRs have a role in specifying the fate of meristems. In *Setaria viridis*, a panicoid grass, the inflorescence meristem differentiates into either a reproductive spikelet or a sterile bristle. A mutant screen for *Setaria* plants that produced few to no bristles revealed a mutation in a Cytochrome P450 724B, termed *Bristleless1* (*Bsl1*), which is involved in BR biosynthesis (Yang et al. 2017). Transcripts of *Bsl1*

accumulate in young spikelet meristems and at the base of the developing bristles, likely to influence cell elongation of the bristle (Yang et al. 2017). Mutations in *bsl1* cause a homeotic transformation of the bristle meristem into a spikelet meristem producing two spikelets (Yang et al. 2017). A similar phenotype is observed when wild type plants are treated with the BR biosynthesis inhibitor propiconazole (Yang et al. 2017). This suggests that BRs are required for the meristem to adopt bristle identity and shows a role for it in lateral organ initiation in *Setaria.*

BRs have a role in influencing agricultural traits. In grasses such as maize and rice, leaf erectness is a desirable trait as it affects both photosynthesis and planting density (Zhang, Bai, and Chong 2014). Erect leaves allow the plant to capture more light for photosynthesis since the lower leaves are not shaded by the upper leaves (Truong et al. 2015). Research on BRs have shown that they contribute to the control of leaf angle in rice. In the early 80's, experiments were carried out on the lamina joint, a boundary between the blade and sheath of the rice leaf, which includes a fringe called the ligule and two auricles that aid in connecting the blade to the sheath (Wada and Marumo 1981). BRs were applied to the lamina joint and the resulting change of angle was calculated. BRs alter lamina joint angle in a concentration-dependent manner, where high concentrations increased the leaf angle and the removal of BRs decreases the leaf angle (Wada and Marumo 1981). This is also apparent in rice plants that are mutants deficient in BR biosynthesis or signaling, which have the more erect leaves compared to wild-type plants.

4. Formation and function of the pulvinus

Legumes are able to modify the angle of their leaves and leaflets by utilizing a structure at the base called the pulvinus (Allen 1969). The pulvinus is a thickened joint and in legumes is able to incline the leaves under a plethora of stimuli. Legumes have been shown to fold their leaves in response to high temperature, water-deficit, various light conditions, and as a circadian response in which they fold leaves up at night in a so-called sleeping behavior (Yu and Berg 1994; Shackel and Hall 1979; Donahue, Berg, and Vogelmann 1990; Kawaguchi 2003). The pulvinus has two groups of cells that differentially contract and expand, the extensor and flexor cells. During inclination, the extensor cells on the adaxial side of the pulvinus undergo an efflux of K+ ions, which are transported to the flexor cells on the abaxial side of the pulvinus (Cote 1995). This causes the water potential in the flexor cells to become more negative, allowing water to be transported out of the extensor cells, causing them to shrink, and into the flexor cells causing them to swell (Cote 1995). This ultimately drives the pulvinus to incline the leaves.

In model legumes, such as *Medicago truncatula* and *Lotus japonicus,* little is known about the genetic regulation of pulvinus formation. Previous work has shown that an ortholog of *LOB* is required for the pulvinus formation in both species (Chen et al. 2012; Kawaguchi 2003). In *Medicago,* the gene *ELONGATED PETIOLULE1* (*ELP1*) encodes an LBD transcription factor that is expressed in the boundary region (Chen et al. 2012). Loss-of-function mutants fail to make pulvini and instead make petiolules at the

base of the leaflets. The lack of a pulvinus results in leaves being unable to fold up at night into the sleeping position (Chen et al. 2012; Zhou et al. 2012). The pulvinus is a radially symmetric structure with a large, centralized vascular bundle surrounded by motor cells. Epidermal cells of the pulvinus have cell walls with convolutions that run horizontally along the structure and allow for the cells to change in surface area during inclination. In *elp1* mutants, the pulvinus is replaced by a structure that has bilateral symmetry with a large vascular bundle in the abaxial domain and two smaller vascular bundles in the abaxial domain (Chen et al. 2012). Additionally, the epidermal cells of this structure lack the cell wall convolutions typically found on the surface of the pulvinus, instead resembling epidermal cells in a petiole or petiolule (Chen et al. 2012). Additionally, cloning of the gene *SLEEPLESS* in *Lotus japonicus* and *Apulvinic* in *Pisum sativum* has revealed that these are the *LOB* orthologs in their respective species and that their function overlaps with *ELP1* (Chen et al. 2012). However, the regulatory mechanism by which *ELP1* functions to form the pulvinus remains elusive. Boundary genes, including *LOB*, restrict growth to properly form the boundary. Ectopic expression of *LOB* produces dwarf plants and, interestingly, when *ELP1* is ectopically expressed, the resulting transgenic plants are also dwarf (Shuai, Reynaga-Peña, and Springer 2002; Chen et al. 2012). This suggests that both *LOB* and *ELP1* have an overlapping function in restricting growth to form the boundary.

5. Importance of leaf angle

Plant architecture is, in part, influenced by the angle of the leaves relative to the stem. The boundary is where leaf angle is established. Leaf angle is both adaptive and selective in that plants can alter their leaf angle as a means of survival and breeders have selected for upright leaves in crops, particularly grass species, to increase planting density and contribute to overall plant health (Zhang, Bai, and Chong 2014; van Zanten et al. 2010).

Plants alter their leaf angle to maximize carbon gain. As light availability changes throughout the day, plants need to adapt and alter the incidence of light to maximize photosynthesis (van Zanten et al. 2010). This requires that the plant differentially alter the angles of young and old leaves. To maximize carbon gain, the younger leaves at the apical region of the plant have an upright leaf angle relative to the older leaves at the base, which remain horizontal (van Zanten et al. 2010; Truong et al. 2015). This allows reduced shading of the older leaves at the base of the plant, maximizing photosynthesis throughout the plant (Truong et al. 2015). The regulation of leaf angles plays an important role in stress avoidance and contributes to the survival of most plant species under several circumstances.

Leaf hyponastic movement, or the upward bending of the leaves, is a major change in leaf angle that occurs under a variety of stimuli. Hyponastic movements are induced by an environmental stimulus but are independent of the direction of the stimulus. Examples of this include responses to the circadian clock where some plants,

including many legumes, fold up at night into a sleeping position and in response to several stresses (Kawaguchi 2003; Yu and Berg 1994). In the semi-aquatic plant *Rumex palustris*, the rosette undergoes hyponasty when the plant is submerged under water. This is a survival mechanism so that the plant can reach the surface of the water and reestablish gas exchange (Cox et al. 2003).

In *Phaseolus*, high air temperature drives the hyponastic response (Yu and Berg 1994). When grown outdoors, *Phaseolus* plants orient themselves away from the sun during the middle of the day but will face the sun towards the end of the day (Yu and Berg 1994). However, when grown in growth chambers with the light remaining constant, the plants will alter their leaf angle away from the incident light source as the air temperature increases (Yu and Berg 1994). During *Arabidopsis* thermomorphogenesis, the petioles elongate to incline the leaves (Quint et al. 2016). This process is thought to reduce heat captured from the sun and opens up the rosette to increase the rate of transpiration (Quint et al. 2016). Plants with increased transpiration reduce the overall leaf temperature.

In the legume *Mimosa pudica,* leaves undergo nastic movement when they perceive touch. The leaves and leaflets both contain a pulvinus at the base that drives the leaves and leaflets to fold (Song, Yeom, and Lee 2014). This rapid movement is useful for deterring potential predators. While the physiological mechanism that drives the motor cells of the pulvinus to differentially expand and contract is relatively conserved across legumes, the mechanism by which *Mimosa* perceives touch and is able to rapidly fold its leaves remains elusive.

Leaf orientation can have major consequences on light capture and, therefore, carbon gain. Leaf angle is one component of the shade avoidance syndrome (SAS) (Ku et al. 2011; Nozue et al. 2015). SAS encompasses a number of traits that a plant exhibits when low light is perceived by a plant, particularly when a neighbor interferes with its light capture. Some plants exhibit rapid internode elongation as a response to SAS, but rosette plants, such as *Arabidopsis*, do not have this luxury (van Zanten et al. 2010). Instead, leaves elongate their petioles, causing the leaf blade to be elevated. Shade can be partially defined as low blue-light fluence rates or a low red:far-red ratio and plants generally respond to both. In *Arabidopsis*, the phytochrome photoreceptors perceive the red:far-red ratio and are required for the resulting hyponastic growth response (Hohm, Preuten, and Fankhauser 2013). A mutation in the *phytochromes* can cause these plants to be insensitive to shade or be constitutively hyponastic, even in ambient light (Honggui Wang et al. 2013). Blue light is sensed by the cryptochromes and phototropins (Hohm, Preuten, and Fankhauser 2013). Both of these families are positive regulators of leaf hyponasty under low-light conditions.

Ethylene is a phytohormone that is a contributor to hyponastic growth in plants. Ethylene plays a role in hyponastic growth in plants that are tolerant of submergence (van Zanten et al. 2010). Submergence-tolerant plants rapidly accumulate ethylene upon submergence, causing the leaves to undergo hyponasty (Van Zanten et al. 2010). Further, treatment with the ethylene receptor antagonist 1-methylcyclopropene (1-MCP) reduces leaf hyponasty after submergence, which suggests that ethylene has a role in the submergence induced hyponastic response (Cox et al. 2003). Additionally, plants which

are insensitive to ethylene have reduced hyponastic growth during treatments of low blue light (van Zanten et al. 2010). This suggests that ethylene plays an important role in leaf hyponasty induced by multiple environmental factors.

In some species, leaf hyponasty is regulated by the circadian clock. This is particularly common in legumes where the folding up into the sleeping position at night is found throughout the family (Ueda and Yamamura 2000). This movement is brought on by the onset of darkness and is rapidly induced by changes in cellular turgor pressure (Donahue, Berg, and Vogelmann 1990). The sleeping movement is driven by the pulvinus, a structure at the base of the leaves and leaflets that contains specialized parenchyma cells, called motor cells, which differentially expand and contract to drive inclination (Rodrigues and Machado 2007). These movements are heavily influenced by various wavelengths of light; both blue light and red:far-red ratios are sensed by the cryptochromes and phytochromes, respectively, to alter the volume of the motor cells (Donahue, Berg, and Vogelmann 1990). These photoreceptors have been shown to regulate cytosolic calcium (Ca^{2+}), which directly regulates the expansion and contraction of the motor cells (Cote 1995). The folding of the leaves and leaflets into the sleeping position has been a plant phenomenon observed for more than a century (Darwin 1897). However, the function of this sleeping behavior remains unclear.

6. Contributions of the dissertation

This dissertation focuses primarily on the further classification of LOB's role in organ boundary formation in *Arabidopsis* and its role as a boundary gene in cowpea. In chapter I, I identified one *LOB* ortholog in cowpea, *VuLOB* (Vigun09g091300) and show that this gene is transcribed in the pulvini, but also in shoot apices, floral buds, and lateral root junctions, giving it a partial overlapping expression pattern with *AtLOB* and some evidence that it is a boundary gene. This partially contrasts the expression data found using the gene expression browser on Legume Information System (LIS) which suggests that transcripts of *VuLOB* are enriched only in the roots, but it's likely that these data are not at a high enough resolution to detect transcripts elsewhere. Similarly, to *AtLOB*, *VuLOB* is positively regulated by brassinosteroids suggesting at least a partially conserved function between the two genes. However, functional analysis characterized by driving *VuLOB* by the constitutive *35S* promoter reveals that this does not phenocopy *35S:AtLOB*. Instead of producing a dark green, dwarf, and sterile plant, like *35S:AtLOB* plants, *35S:VuLOB* produce plants that are about equal to wild type in size, but produce asymmetric rosettes, have altered leaf shape, and are reproductive, which suggests that these two genes have diverged in function. Furthermore, I characterize some of the requirements for leaf inclination in cowpea. Using split-root assays, I show that the signal to incline is perceived by the roots and transmitted to the leaves. The ability to incline is dependent upon brassinosteroid accumulation. When cowpea plants are treated with the BR biosynthesis inhibitor propiconazole, they fail to incline, and this is correlated with an

altered cellular morphology of the epidermal cells of the pulvinus. Given these data, BR is required to regulate leaf inclination in cowpea.

In chapter II, I utilize a genotyped population of the UCR cowpea germplasm to conduct genome-wide association studies (GWAS) on pulvinus dimensions and leaf angle. I show that *VuLBD41* (Vigun05g006100) is a potential candidate for the control of pulvinus dimensions. The gene expression browser from LIS suggests that transcripts of *VuLBD41* are not highly expressed in the leaf, but with RT-PCR I show that *VuLBD41* is highly expressed in the pulvinus. However, given some functional characterization in *Arabidopsis,* its role as a boundary gene remains elusive. Furthermore, no candidates were obtained with a GWAS conducted on leaf angle suggesting that a rare variant was not detectable in our population.

In chapter III, I show that LOB regulates *TREHALOSE-6-PHOSPHATE PHOSPHATASEJ* (*TPPJ*), but not *TPPI* to form a proper boundary. *TPPJ* and *TPPI* are closely related homologs with an overlapping expression pattern with *LOB* in the boundary of the vegetative shoot. A mutant allele of *TPPJ*, *tppj-1*, does not enhance the *lob-3* mutant phenotype nor does a mutant allele of *TPPI*, but the triple mutant *lob-3; tppi-1; tppj-1* has more branches compared to any of the single or double mutant combinations. The *lob-3* mutant phenotype is rescued by expressing *TPPJ* under the *LOB* promoter, but this phenotype is not rescued by *TPPI*. These data suggest that LOB regulates trehalose accumulation through the regulation of *TPPJ* to properly form the boundary.

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Chapter 1:

Characterization of leaflet inclination in *Vigna unguiculata*

Abstract

Plant organ boundaries influence the angle at which lateral organs emerge from the shoot apical meristem. Leaf angle is an aspect of plant architecture that can impact productivity, as erect leaves allow for planting at a higher density and reduce self-shading to increase photosynthetic efficiency. In some species, leaf angle changes in response to environmental stimuli. *Vigna unguiculata* (cowpea) changes its leaf angle under water-deficit stress, inclining leaflets to reduce exposure to sunlight and minimize water loss. Leaflet inclination is driven by the pulvinus, a structure at the base of the leaves and leaflets. In *Medicago truncatula*, pulvinus formation requires the transcription factor *ELONGATED PETIOLULE1* (*ELP1*). ELP1 is orthologous to *LATERAL ORGAN BOUNDARIES* (*LOB*) in *Arabidopsis thaliana*, a transcription factor that functions to limit growth in organ boundaries, in part by modulating the accumulation of brassinosteroids (BRs). The function of LOB in cowpea and its potential role in modulating BRs to control leaflet angle remains to be understood. This works shows that BR biosynthesis is required for water-deficit induced cowpea leaflet inclination, as BR-deficient plants are unable to incline their leaves. The cowpea *LOB* ortholog, *VuLOB*, has an overlapping expression pattern with *AtLOB* and is regulated by BRs in a similar manner. However, *Arabidopsis* plants overexpressing *VuLOB* do not phenocopy plants

overexpressing *LOB,* nor do they appear to regulate the same genes in a similar manner. These data suggest that *VuLOB* has diverged in function from *AtLOB*.

Introduction

The shoot apical meristem (SAM) is a mass of pluripotent cells from which lateral organs arise. A population of stem cells reside in the central zone at the top of the meristem. These cells divide and their flanking daughter cells comprise the peripheral zone, from which lateral organs form. The boundary region forms between the SAM and initiating primordia and is composed of cells that are smaller than those in the neighboring SAM and developing lateral organ and divide infrequently (Hussey 1971). The boundary region specifies the angle of lateral organs, which can alter plant architecture and can ultimately impact crop yield. The angle of leaves relative to the ground is an important characteristic because it can contribute to both plant survival and productivity (Cox et al. 2003; Truong et al. 2015). Plants can alter leaf angle to maximize or minimize light exposure or to withstand stresses such as heat or flooding (Cox et al. 2003; Koini et al. 2009). Plant breeders have selected for erect leaves in species such as maize and rice because erect leaves allow for planting at a higher density and prevent the shading of lower leaves. This allows for prolonged photosynthesis and provides more energy to the plant for flowering and seed development (Truong et al. 2015).

Restriction of growth in the boundary in the model plant *Arabidopsis thaliana* can, in part, be attributed to the gene *LATERAL ORGAN BOUNDARIES* (*LOB*) (Bell et

al. 2012). *LOB* was identified through an enhancer trap screen where promoter activity was detected at the base of the floral organs, pedicels, lateral roots, and at nodes (Shuai, Reynaga-Peña, and Springer 2002). *LOB* encodes a transcription factor that recognizes a conserved 5'-(G)CGGC(G)-3' motif (Husbands et al. 2007). *LOB* regulates organ separation at the boundaries through a negative feedback loop wherein the phytohormone brassinosteroid (BR) positively regulates *LOB* expression and LOB positively regulates expression of *BAS1,* a cytochrome P450 enzyme that catabolizes brassinosteroids (Bell et al. 2012). Through this negative feedback loop, LOB represses growth at the organ boundaries through the catabolism of BR, a known positive regulator of cell division and cell expansion (Clouse 2011). Loss-of-function *lob* mutants exhibit organ fusion of the cauline leaf to the axillary branch at the paraclade junction, the region on the shoot where the cauline leaf, axillary branch, and primary stem meet (Bell et al. 2012). Further, the *lob* mutant phenotype can be rescued by driving *BAS1* expression under the *LOB* promoter, suggesting that *BAS1* accumulation in the boundary is sufficient to restrict growth (Bell et al. 2012).

Cowpea (*Vigna unguiculata* L. Walp.) is a warm-season legume grown for grain and fodder (Sprent, Odee, and Dakora 2010). Cowpea is cultivated primarily in the arid environments of Sub-Saharan Africa, India, China, Brazil, and America due to its ability to withstand drought and heat (Agbicodo et al. 2009). Cowpea is one of the few legumes that can maintain a high yield after experiencing high temperature and/or drought stress. As a legume it has the added benefit of housing nitrogen fixing bacteria and serves as an important intercrop for many other plant species (Ehlers and Hall 1997). One of the

mechanisms that is thought to contribute to cowpea's tolerance of water-deficit is its ability to incline its leaves (Shackel and Hall 1979). When soil-water availability is low, the roots transmit a signal to the leaves causing them to incline. A structure at the base of the leaves and leaflets called the pulvinus drives leaf inclination in cowpea. During leaf inclination, the cells on the adaxial (upper) side of the pulvinus undergo an efflux of K^+ ions, which causes water to exit the cells and the cells to shrink (Cote 1995). The cells on the abaxial (lower) side undergo an influx of K^+ ions, which causes water to enter the cells allowing them to expand (Cote 1995). The movement of water into and out of the pulvinus causes simultaneous contraction and flexion, which results in the movement of the leaves and leaflets (Cote 1995). The decrease in leaf angle alters plant architecture and reduces the amount of sunlight captured by each leaflet (Shackel and Hall 1979). In turn, this reduces the temperature of the leaves causing a reduction in water loss, which is thought to allow the plant to retain water and maintain water status to survive drought (Shackel and Hall 1979).

Previous research has investigated the formation and function of the pulvinus in several species. In the model legume *Medicago truncatula,* the pulvinus controls the nighttime folding of leaves into a "sleeping" position (Chen et al. 2012). The pulvinus has a unique anatomy and morphology that has been well characterized. The epidermal cells have many convolutions, similar to an accordion, which gives a large surface area allowing for the large changes in volume to occur. Anatomically, the pulvinus is radially symmetric with a large central vascular bundle surrounded by motor cells (Chen et al. 2012). The *Medicago* gene *ELONGATED PETIOLULE1* (*ELP1*) is required for pulvinus

formation. Loss-of-function *elp1* mutants lack pulvini and produce a structure similar to a petiolule (Chen et al. 2012; Zhou et al. 2012). These structures contain epidermal cells that are larger and wider than wild-type pulvini epidermal cells and have lost their radial symmetry (Chen et al. 2012). In the *elp1* mutant, the structure at the base of the leaflets has clear adaxial-abaxial polarity with a central vascular bundle and two smaller vascular bundles exhibiting bilateral symmetry (Chen et al. 2012). Further, these plants are unable to alter their leaf angle (Chen et al. 2012; Zhou et al. 2012). *ELP1* contains the conserved LOB domain and is the ortholog of *LOB* (Chen et al. 2012). The function of the *LOB* ortholog is conserved in both *Lotus japonicus* and *Pisum sativum*. The *Lotus* gene *SLEEPLESS* (*SLP*) is required for pulvinus formation and *Lotus* use pulvini to fold their leaves at night in a similar manner to *Medicago (Chen et al. 2012; Kawaguchi 2003)*. Loss-of-function *slp* mutants lack pulvini and are unable to alter their leaf angle at night (Kawaguchi 2003). In *Pisum sativum, Apulvinic* (*APU*) is required for pulvinus formation. Naturally occurring varieties of *Pisum* have reduced expression of *APU* and have petiolules in place of pulvini (Chen et al. 2012; Marx 1984). Although *Arabidopsis* doesn't have a pulvinus, the boundary region specifies the angle of leaves in a manner similar to the pulvinus in legumes. Thus, the function of these genes is conserved in regulating leaf angle in diverse species.

Currently, the molecular and hormonal regulation of pulvinus formation and function remains elusive. In this study, we show that cowpea encodes a *LOB* ortholog, *VuLOB*, and address its expression pattern and functionality. Further, we show how brassinosteroids are involved in leaflet inclination and their relationship with *VuLOB*.

Results

Cowpeas perceive water-deficit in the roots

In cowpea, water-deficit is thought to be sensed by the roots. A mobile signal, that is likely ABA, is proposed to be transmitted from the roots to the shoot to cause the leaflets to incline (Shackel and Hall, 1979). To determine if roots are the source of a signal to incline under water-deficit, we grew cowpea seedlings in split-root pots where the root system of one plant was divided between two pots of soil. After 3 weeks of acclimation, one pot was continually watered while the other was subjected to drying. Leaves of control plants, where both pots were watered, remained flat while leaves of plants in which water was withheld from both pots inclined. Plants with root systems divided between one watered pot and one dry pot inclined their leaves at the same time as the leaves of plants with roots divided between two dry pots inclined, while the leaves of control plants remained flat (Figure 1.1). Since overall plant turgor was not impacted by withholding water from one pot, our data suggest that the roots sense water-deficit and transmit a signal that stimulates the pulvinus to initiate inclination.

BR deficiency prevents cowpea leaf inclination after water-deficit treatment

BRs are involved in regulating leaf angle in cereal crops. The application of exogenous brassinolide to the rice lamina joint, a boundary within the rice leaf, causes an increase in cell expansion and a dramatic increase in leaf angle (Wada et al. 1984). We asked if perturbing the amount of BR within the plant may affect the formation or the function of the pulvinus in cowpea. There are no mutant populations of cowpea available

and cowpea is recalcitrant to transformation, so we used propiconazole, a BR-biosynthesis inhibitor, to create BR-deficient plants (Hartwig et al. 2012). Continuous treatment of cowpea seedlings with propiconazole (PCZ) in concentrations ranging from 1μM to 8μM resulted in dwarf plants regardless of the concentration used (Figure 1.2). We did not use concentrations smaller than 1μM because a sufficient response was observed at the concentrations used. We measured the length and width of the terminal pulvini and found that formation of the pulvini were unaffected compared to untreated controls (Figure 1.2). To assess whether PCZ treatment affects the function of the pulvinus, we withheld water for several days and calculated the change in leaf angle after control plants had inclined leaves (Figure 1.2). Surprisingly, PCZ-treated plants failed to incline their leaves after several days of water deficit (Figure 1.3). Leaves of PCZ-treated plants neither inclined nor showed signs of wilting, even after untreated plants had wilted (data not shown). To eliminate the possibility that the compact structure of PCZ-treated plants prevented leaflet inclination, we grew seedlings until the unifoliate leaves had fully expanded and then treated them with 16 μM PCZ. Upon emergence of the first pair of trifoliate leaves, which were characteristically small as a result of BR deficiency, half of the plants were subjected to water-deficit stress. After several days of stress, the unifoliate leaves of untreated control plants that had been subjected to water deficit were inclined, but PCZ-treated plants failed to incline (Figure 1.4). This suggests that BR is required for leaf inclination.

Time-lapse imaging revealed that untreated plants had dynamic leaf angles throughout the day. These plants solar tracked throughout the day and inclined to “sleep”

at night, a phenomenon observed in many other legume species. Untreated plants under water-deficit remained inclined throughout the day and had reduced solar tracking, likely to maintain a lower leaf temperature, and inclined to "sleep" upon sunset. However, when plants were treated with propiconazole they are unable to move their leaves, either to solar track, "sleep" at night, or incline under water-deficit. This further suggests that the leaf inclination response requires BR biosynthesis.

PCZ treatment affects pulvinus epidermal cell morphology

Leaf inclination is dependent on multiple factors and therefore there could be multiple explanations as to why cowpeas treated with PCZ fail to incline their leaves. The epidermal cells of the pulvinus have convoluted cell walls, which allow their volume to change when expanding or contracting. Additionally, the motor cells within the pulvinus acquire and lose water in order to expand and contract (Cote 1995). This requires the proper localization of aquaporins to transport water, the vacuole to store water, and the necessary ion channels to move K^+ in the correct direction to lower water potential (Cote 1995). To determine if the PCZ treatment impacted the cellular morphology of the pulvinus, we observed the morphology of the epidermal cells using scanning electron microscopy (SEM). Pulvini of untreated plants have convolutions in the cell wall, which account for the change in volume upon inclination and declination (Figure 1.5). However, in plants treated with PCZ, the pulvinus epidermal cells lacked these convolutions, which may, in part, account for their failure to incline (Figure 1.5). This

demonstrates that BR signaling is needed for development of epidermal cell morphology in the pulvinus.

To examine the impact of PCZ on pulvini that were produced prior to the treatment, which had normal convolutions in their epidermal cells, we grew seedlings, untreated, until the unifoliate leaves had fully expanded. This was followed by a treatment of 16 μM PCZ and water-deficit stress following the emergence of the trifoliate leaves. The fully expanded unifoliate leaves failed to incline after water-deficit, suggesting that, in addition to being required for formation of a functional pulvinus, BR signaling may be needed for some other aspect of leaf inclination. We examined the morphology of pulvini from the unifoliate leaves using SEM. Pulvini on unifoliate leaves formed prior to PCZ treatment had stereotypical cell wall convolutions, indicating that PCZ does not affect the cellular morphology post-development (Figure 1.5). Further, these plants exhibited an obvious BR-deficient phenotype in the newly forming trifoliate leaves with a compact nature and lack of convolutions in their pulvini (Figure 1.5), demonstrating that they responded to the PCZ treatment. These data suggest that BR signaling may be required for the proper function of the motor cells within the pulvinus. In the motor cells, BR signaling may be required for the activation of the ion channels or aquaporins. Another possibility is that the mobile signal required to induce leaflet inclination requires BR signaling and that the removal of BR prevents this signal from being perceived or active.

Plants treated with PCZ do not have a decrease in survival after water-deficit

Leaf inclination is an early and reversible response to water-deficit that happens several days before wilting (Shackel and Hall, 1979). We therefore asked if leaf inclination had an impact on the plants' ability to survive long-term water-deficit. To test this, we grew plants with and without a PCZ treatment. PCZ treatment prevented the leaflets from inclining. After two trifoliate leaves had formed, water was withheld from half of the plants until the untreated plants wilted, as propiconazole treated plants do not exhibit an obvious wilting phenotype. After wilting, all plants were watered without propiconazole and survival was assessed by the formation of new leaves. All plants, regardless of their treatment, were able to form new leaves (data not shown). This suggests that leaflet inclination is not necessary for the plant's ability to survive water-deficit under these greenhouse conditions.

Cowpea has one LOB ortholog

In model legumes, *LOB* orthologs are required for the formation of the pulvinus and the pulvinus is required for leaf inclination. We sought to identify and characterize a putative *LOB* ortholog in cowpea. A BLAST search was done using the nucleotide and translated amino acid sequences of LOB orthologs from 3 closely related species, *PsAPU*, *MtELP1,* and *LjSLP* against the cowpea BAC database first and then again once the annotated genome was made available. We identified one putative ortholog in cowpea, which we termed *VuLOB* (Figure 1.7) from both the BAC database and annotated genome. The N-terminal region of LOB-domain proteins contains the

conserved LOB domain, which contains a C block for protein-DNA interactions, a GAS block and a coiled-coil domain for protein-protein interaction. Aligning the N-terminal region of VuLOB with the LOB domains of PsAPU, MtELP1, and LjSLP revealed strong conservation of the LOB domain, indicating that VuLOB is a LOB-domain protein. We also identified 48 total *LATERAL ORGAN BOUNDARIES DOMAIN*(*LBD*) genes in the available cowpea genome and 71 putative LBDs in soybean, a closely related species (Figure 1.6) (Lonardi et al. 2019). A phylogenetic analysis was carried out. The LOB domain of VuLOB was aligned with the 48 LBDs in the cowpea genome, other LOB orthologs in legumes, grasses, and *Arabidopsis*, the LBDs in the same clade as LOB, and 71 putative LBDs in the soybean genome (Figure 1.7). The analysis was inferred with maximum likelihood and 1000 bootstrap replications. VuLOB forms a clade with the previously published LOB orthologs from legumes and LOB from *Arabidopsis*. This suggests that *VuLOB* is likely the *LOB* ortholog in cowpea.

We observed that the original nucleotide sequence obtained from the cowpea BAC sequence contained a start codon and the entire LOB domain but was still incomplete due to the lack of a stop codon. We carried out 3'RACE to obtain full-length transcripts of *VuLOB*. The *VuLOB* transcribed region is 591 bp long and when we aligned it to the cowpea BAC sequence and the sequence from the annotated genome, we identified only 1 exon within the gene (Figure 1.7) (Lonardi et al. 2019)

VuLOB transcript accumulation has a similar pattern to *LOB*

In *Arabidopsis*, *LOB* transcript accumulates at the bases of lateral organs (Shuai, Reynaga-Peña, and Springer 2002). These include the base of the lateral roots, floral buds, pedicels, and nodes. To determine if *VuLOB* has a similar expression pattern as *LOB*, we carried out RT-PCR on RNA isolated from different regions of the plant. *VuLOB* did not accumulate in the terminal or lateral blade of leaflets on trifoliate leaves, the blade of unifoliate leaves, or root tissue lacking lateral root junctions. This expression pattern is consistent with *LOB* in *Arabidopsis* since *LOB* does not accumulate in any blade tissue or root tissue outside of the lateral root junction. We detected *VuLOB* transcripts in the pulvini at the base of the leaves and leaflets, apices, floral buds, and roots with lateral root junctions (Figure 1.8). This consistent expression pattern suggests that *VuLOB* may share a conserved function with *LOB* in the boundary and provides a higher resolution than what is found in the gene expression atlas from LIS

VuLOB is positively regulated by brassinosteroids

Previous work from our lab has shown that in *Arabidopsis LOB* is positively regulated by BRs (Bell et al. 2012). *LOB* transcripts rapidly accumulate following a short-term treatment with brassinolide. To examine the relationship between BRs and *VuLOB*, we excised terminal pulvini from 8-week-old plants and floated them in a solution containing either 1 μM BL or a mock solution. Samples were taken at 2 and 4 hours and transcripts were quantified using qPCR. To determine if pulvini responded to the BL treatment, we examined transcripts of a putative *DWF4* homolog, *VuDWF4*.

DWF4 is the rate limiting enzyme in BR biosynthesis and since BR biosynthesis is under negative feedback regulation, *VuDWF4* transcripts were expected to be down regulated in the presence of BL compared to mock. As expected, *VuDWF4* had decreased transcript accumulation after 2 hours of BL treatment and had begun to level off by 4 hours (Figure 1.9). *VuLOB* had a slight increase in transcript levels following 2 hours of BL treatment and were significantly increased following 4 hours of BL treatment, which suggests that *VuLOB* is rapidly induced by BL in a similar manner to *LOB* (Figure 1.9). *VuLOB* transcript levels were also examined in pulvini that had been incubated in 100 μM PCZ for 48 hours. Unexpectedly, *VuLOB* transcripts were not significantly altered in the response to PCZ treatment, compared to mock treated (Figure 1.9). Further, *VuDWF4* accumulation was not significantly changed in response to PCZ treatment compared to a mock treatment, which could imply that either the samples are not BR-deficient, or the cells had reached homeostasis after 48 hours. It is possible that while BR biosynthesis was blocked, endogenous BL was still present within the tissue, preventing a detectable change in *VuLOB* transcripts.

Ectopic expression of *VuLOB* in *Arabidopsis* does not phenocopy the *LOB* overexpression phenotype

In *Arabidopsis,* LOB functions to restrict growth through the modulation of BR catabolism (Bell et al. 2012). Plants with constitutively active LOB are severely dwarfed compared to wild-type and fail to produce an apical hook when grown in darkness (Shuai, Reynaga-Peña, and Springer 2002; Bell et al. 2012). During the vegetative phase

of development, these plants are compact with highly reduced leaves and during the reproductive phase, have reduced perianth and sterile reproductive organs (Shuai, Reynaga-Peña, and Springer 2002). To test the functionality of *VuLOB*, we cloned the coding sequence of *VuLOB* and expressed it in *Arabidopsis* under both the *35S* promoter and an estradiol inducible promoter. Plants carrying the *XVE:VuLOB* construct in a Col-0 background had no change in phenotype on media containing the mock treatment, compared to wild-type. When *XVE:VuLOB* plants were grown on 100 μM estradiol they resembled wild-type plants, indicating no change in phenotype was observed when *VuLOB* is overexpressed. Transcripts of *VuLOB* were detected at high levels to confirm that the phenotype observed was not due to transgene silencing in the plant. Further, etiolated *XVE:VuLOB* plants grown on media containing a mock estradiol treatment produced an apical hook after 5 days in the dark, similar to wild-type plants. Etiolated *XVE:VuLOB* seedlings grown on media containing 100 μM estradiol also formed an apical hook in a similar manner to wild-type. The transgenic plants resembled wild-type plants, which produce an apical hook during skotomorphogenesis on both mock and 100 μM estradiol plates (Figure 1.10). This suggests that *VuLOB* may only partially restrict growth or restrict growth redundantly and is the first line of evidence that *VuLOB* and *LOB* do not share a common function.

We also analyzed the impact of constitutive overexpression of *VuLOB* by creating the *35S:VuLOB* construct and transforming it into Col-0. We wanted to observe phenotypes of plants overexpressing *VuLOB* at later stages in development since we were unable to reproducibly induce *XVE:VuLOB* on soil (Figure 1.10). *35S:VuLOB* plants

produced a rosette that appeared to lack the spiral phyllotaxy found in wild-type plants (Figure 1.10). After bolting, *35S:VuLOB* plants produced branches with wide angles, reproductive flowers, and set viable seed (Figure 1.10). *35S:VuLOB* plants were not dwarfed compared to untransformed Col-0 (data not shown). Comparatively, *35S:LOB* plants produce a reduced rosette and remain dwarf upon the transition to flowering where the flowers are both male and female sterile (Shuai, Reynaga-Peña, and Springer 2002). This suggests that the function of *VuLOB* may have diverged from *LOB*. To test this, we analyzed transcript levels of several known targets of *LOB* in 14-day-old seedlings, paraclade junctions, and inflorescences of 4-week-old plants. First, we observed transcripts relating to BR biosynthesis and signaling. *LOB* has been shown to positively regulate *BAS1, BRL3*, and *DWF4* (Bell et al. 2012). In *35S:VuLOB* transgenic plants, we found that *BAS1* transcripts increased in seedlings and inflorescences compared to wild-type plants. *BRL3* and *DWF4* had an increase in transcript accumulation in all tissue types compared to wild-type plants. Next, we checked genes that regulate plant development, specifically genes that influence cell size and/or cell proliferation. We examined transcript accumulation for *BOP1*, *HEC1*, and *LNG2,* genes which *LOB* has been shown to positively regulate (Bell et al. 2012). We detected an increase in transcript accumulation in both *BOP1* and *HEC1* in all tissue types in *35S:VuLOB* plants compared to wild-type. *LNG2* appeared to be slightly upregulated in seedlings and inflorescences, but not in paraclade junctions of *35S:VuLOB* plants when compared to wild-type. Finally, we looked at the transcript accumulation pattern of *XTH16*, *PME41*, and *TCH4,* genes which encode proteins involved in modifying the cell wall. *LOB* negatively regulates

these genes in *Arabidopsis* (Bell et al. 2012). However, we found that when compared to wild-type, *VuLOB* regulates these genes in a different manner. While *XTH16* was downregulated in *35S:VuLOB* paraclade junctions, transcript levels were unchanged in seedlings and inflorescences (Figure 1.10). *PME41* transcripts were also down regulated in *35S:VuLOB* paraclade junctions, but transcripts appeared to be upregulated in seedlings and inflorescences (Figure 1.10). In a similar manner to *PME41*, *TCH4* was upregulated in *35S:VuLOB* seedlings and inflorescences, but unchanged in paraclade junctions. These data suggest that *VuLOB* has partially diverged in function from *LOB* given that it only regulates a subset of the genes we tested in a similar manner.

The transgenic plants partially phenocopy an *as2* mutant phenotype (Semiarti et al. 2001). The leaves are similar in size to Col-0, but have larger lobes at the leaf base and appear more irregular in their symmetry. Further, compared to Col-0, which makes a spiral rosette, *35S:VuLOB* plants produce a rosette that fails to produce an obvious spiral. We examined *AS2* transcripts in *35S:VuLOB* transgenic plants. In seedlings and paraclade junctions, *AS2* transcripts were reduced compared to wild-type, but transcript accumulation was unchanged in inflorescences (data not shown). This suggests that the *35S:VuLOB* may negatively regulate *AS2*.

In *Arabidopsis*, LOB has been shown to negatively regulate leaf inclination upon blue-light illumination (unpublished). To assess *VuLOB*'s role in leaf inclination in cowpea, we quantified transcripts of *VuLOB* in pulvini of inclined leaves and compared them to transcripts on pulvini in flat leaves. However, transcript levels of *VuLOB* are not

significantly changed upon leaf inclination (Figure 1.11). This suggests that changes in *VuLOB* transcripts are not required for water-deficit induced leaf inclination.

Discussion

Legumes utilize a pulvinus to alter their leaflet angle under an array of stimuli (Chen et al. 2012; Yu and Berg 1994; Kawaguchi 2003). Many legumes fold their leaves up at night in a "sleeping" position while others utilize this mechanism to regulate their physiology (Kawaguchi 2003; Yu and Berg 1994). Cowpea folds its leaves in response to water-deficit stress (Shackel and Hall 1979). When soil water availability is low the leaves incline. This inclination reduces the amount of sunlight the leaves receive, preventing the leaves from increasing in temperature, thus preventing water loss through transpiration (Shackel and Hall 1979). In model legumes, such as *Lotus japonicus* and *Medicago truncatula*, the *LOB* orthologs *SLEEPLESS* and *ELONGATED PETIOLULE1* are required for the formation of the pulvinus, however, their mode of action remains elusive. Here we characterize the *LOB* ortholog and leaf inclination in cowpea, an economically important legume.

Leaf angle is a complex trait that is controlled by many factors. Many plants have fixed leaf angles, but some are able to change their leaf angle under numerous conditions. Many plants change their leaf angle in response to stress, either inclining during submergence to reach oxygen or under heat stress as a sun-avoidance mechanism (Cox et al. 2003; Yu and Berg 1994). Many legumes are able to incline their leaves under stress as well as solar track to maximize carbon gain throughout the day (Shackel and Hall

1979). The leaf movement found in legumes is unique because it is reversible, setting them apart from other plants. In cowpea, the leaflets are inclined during water-deficit. Using a split-root assay we determined that cowpea roots sense water deficit. A mobile signal, likely abscisic acid (ABA), is synthesized in the roots undergoing water-deficit and mobilized to the leaves, causing the pulvinus to incline the leaves. It has been shown that ABA contracts the motor cells of the pulvinus and auxin expands them, but the signaling cascade has yet to be elucidated (Iino, Long, and Wang 2001).

Since the 1980s, it has been known that BR signaling is able to control leaf angle (Wada et al. 1984). Experiments carried out on the lamina joint in rice have shown that the application of BRs increases leaf angle, resulting from an increase in cell expansion at the lamina joint. In contrast, rice mutants that are BR deficient, either reduced in biosynthesis or signaling, have erect leaves compared to WT (Yamamuro et al. 2000). We hypothesized that BRs control leaflet angle in cowpea and used PCZ to inhibit BR biosynthesis in the plant. PCZ treatment produced a characteristic BR-deficient phenotype, resulting in compact, dwarf plants. Upon water-deficit, PCZ treated plants were unable to incline their leaves and, further, were unable to fold into the "sleeping" position at night. PCZ treatment resulted in alteration of cell shape in the epidermis. Untreated plants produced epidermal cells that have convolutions that presumably allow for the adjustment in cell volume during expansion and contraction. However, pulvini formed during PCZ treatment lacked convolutions, which presumably impacted their ability to incline. Further, when cowpea seedlings were treated with PCZ after their leaves were fully developed, pulvini were unable to incline. Epidermal cells were

morphologically normal in these leaves, suggesting that either the motor cells were impaired and required BR signaling to function or that the signal to incline is somehow blocked or inactive. Vacuole size is dependent on BR signaling and treatment with BR biosynthesis inhibitors can cause the vacuole to be decreased in size and more fragmented (Yamagami et al. 2018). In the pulvinus, a smaller and more fragmented vacuole could prevent the motor cells from functioning properly to incline the leaves.

Cowpea contains a single *LOB* ortholog within its genome. This gene, *VuLOB* (Vigun09g09300), is orthologous to other *LOB* genes in related legumes and forms a clade with the *Arabidopsis LOB* gene. In legumes, the pulvinus specifies the angle of leaves and leaflets and because of this we sought to understand the similarities that *VuLOB* has with *LOB*. Functional analysis has shown that *LOB* and *VuLOB* have diverged functions. While *LOB* has been shown to restrict growth via BR modulation at the boundary, *VuLOB* likely functions in an alternative pathway or in concert with other players to form the boundary. It does not appear that *VuLOB* restricts growth since mature plants aren't dwarf in comparison to wild-type plants and etiolated seedlings produced an apical hook. This presents the possibility that *LOB* has diverged in function throughout evolution and that it does not restrict growth in some species. It would be interesting to see if other *LOB* orthologs in distantly related species restrict growth in some manner or not.

While *VuLOB* does not have a conserved function with *LOB*, it still overlaps with *LOB* in regard to its regulation. Both *VuLOB* and *LOB* are positively regulated by BR suggesting that BR modulation is likely required for pulvinus formation and/or function.

It will be interesting to address possible interactors of *VuLOB,* which may bias it towards different modes of regulation. LOB preferentially regulates BR accumulation at the boundary through regulation of *BAS1*, and also regulates cell wall modification enzymes and trehalose accumulation. It is possible that *VuLOB* has a stronger affinity for a different downstream target. Further analysis of *VuLOB*'s downstream targets will need to be assessed.

BRs are required for pulvinus function and *LOB*, which is regulated by BR, is required for pulvinus formation in the cool-season legumes *Medicago*, *Lotus,* and *Pisum*, but little is known about the role of *LOB* and pulvinus formation in the warm-season legumes. *VuLOB*'s transcripts are not altered when the leaflets are inclined which suggests that changes in *VuLOB* transcripts may not be required for pulvinus function. This also suggests that the formation and function of the pulvinus is regulated independently and that *VuLOB*'s putative role is solely for the formation. These data also support the notion that BRs regulate *VuLOB* and leaf inclination independently. This is unsurprising given that BRs regulate several independent aspects of development. Since *VuLOB* regulates cell-wall modifying genes, it would be interesting to see if these genes are differentially expressed during leaf inclination.

Materials/Methods

Phylogenetic analysis:

The cowpea VuLOB sequence was obtained by BLAST search using the available BAC sequence database first and then the annotated genome with the amino acid

sequences of AtLOB (**Q9FML4)**, PsAPU (I6QB37), MtELP1 (**H2D439**), and LjSLP **(I6QJ63)** (http://138.23.178.42/blast/blast.html). These sequences were later confirmed once the available genome was available (*Vigna unguiculata v1.1*) in the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html#%21info?alias=Org_Vunguiculata_er). The *Arabidopsis* LBD amino acid sequences were downloaded from DATF database (http://atrm.cbi.pku.edu.cn/). Using BLASTP, all of the *Arabidopsis* LBDs were used as query against the cowpea and soybean genome, respectively. The hits that contained a significant E-value of <1E-3, contained the conserved C-block, and the conserved G in the GAS block were used as candidate sequences. All putative splice variants were retained in the analysis. The LOB domain sequences were aligned using MUSCLE. Phylogenetic analysis was conducted using the maximum-likelihood algorithm from the MEGA 7.0 software with 1,000 bootstraps (https://www.megasoftware.net/).

3' RACE:

3'RACE was carried out using RNA from cowpea CB46 floral buds as described in Frohman, et al. 1988. First-strand cDNA synthesis was carried out following the manufacturer's instructions using SuperScript IV (Invitrogen) and the primer Qt (Table 1.1). First-round PCR amplification used the primers APU-like 3' RACE 1 and Qo followed by a second round of amplification using the primers APU-like 3' RACE 2.3 and Qi. 3'RACE products were cloned into the pMiniT plasmid (New England

Biosciences) following the manufacturer's instructions and sequenced with Sanger sequencing.

Plant growth conditions and propiconazole treatment:

For propiconazole treatments, cowpea (*Vigna unguiculata* L. Walp) accession CB46 plants were grown in trays of vermiculite (Therm-O-Rock). Vermiculite was washed in water before planting. Seeds were directly sown to trays and given 1 liter of water every other day with or without 4 μM propiconazole. A pinch of fertilizer (MiracleGro) was added weekly. For tissue collection, seeds were directly sown in 2-gallon pots with UCR mix and watered every other day. 8-week-old plants were used in all assays unless otherwise stated. For SEMs, pulvini of 3-week-old plants treated with propiconazole or untreated were dissected and imaged live in the SEM (Hitachi TM-1000).

To grow *Arabidopsis* plants on media, seeds were surface sterilized in 70% ethanol for 2 minutes, 10 minutes in 50% bleach, and rinsed 3 times with sterile water. Seeds were stratified in sterile water for 3 days at 4°C in the dark. The seeds were then plated onto Murashige and Skoog (MS) media (pH 5.7) and placed in a growth chamber at 23°C and illuminated with white light at ~100 $\mu M/m^2s$ in a 16-hour light/ 8-hour dark cycle. For plants grown on soil, seedlings were sterilized and sown to MS plates as described above and transplanted 7 days after germination to UCR Sunshine Mix supplemented with Osmocote (14-14-14) and Marathon. The plants were illuminated with white light at ~100 $\mu M/m^2s$ in a 16-hour light/ 8-hour dark cycle. *Arabidopsis* plants

were transformed via floral dip (Clough and Bent 1998) to create transgenic plants. All transformants were selected on MS media supplemented with 100 μM hygromycin B (Invitrogen).

Split-root assay:

Cowpea CB46 seeds were germinated in soil and grown in a glasshouse until 2-weeks-old. 2-week-old plants were removed and placed in a 50 ml conical vial with 2 windows cut out of the bottom and a slit, cut to fit in between two 4-inch pots. Root systems were divided between the 2 pots and plants were given several weeks to recover. Water was withheld from one of the pots and inclination was imaged (Samsung Galaxy S7).

Transcript analysis:

Total RNA from cowpea tissue was extracted using the CTAB buffer described in Untergasser 2008. Total RNA from *Arabidopsis* tissue was isolated using Trizol reagent. All pulvini tissue was ground fresh in the buffer and all other tissues were flash-frozen in liquid nitrogen before RNA was extracted. For RT-PCR, 1 μg of RNA was treated with DNase for 30 minutes at 37°C and cDNA synthesis was carried out using SuperScript IV following the manufacturer's instructions. Cowpea cDNA was equalized using the *VuEF1a* primers and the *VuLOB* primers were used to amplify the transcript of *VuLOB* in various tissues with 35 cycles. *Arabidpsis* cDNA was equalized with the *ACT2* primers and all primers used for functional analysis are in Table 1.1.

Excised pulvini assay:

For the excised pulvini assay, terminal pulvini were excised from 8-week-old plants and incubated in 1 µM BL, 100 µM PCZ, or a mock solution, in petri dishes on the lab bench in light. After each time point, samples were immediately put in a CTAB buffer containing β-mercaptoethanol and RNA extraction was carried out as described in Untergasser 2008. 1 µg of RNA was treated with DNase 1 for 30 minutes at 37°C and cDNA synthesis was carried out using SuperScript IV (Thermo Fisher) following the manufacturer's instructions. qPCR assays were carried out on a Bio-Rad CFX Connect and relative transcripts were calculated based on the Pfaffal method. Significance was calculated using an ANOVA.

Cloning:

The coding sequence of *VuLOB* was obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#%21info?alias=Org_Vunguiculata_er), amplified using the primers APU-like entry clone F and R (Table 1.1) and cloned into pENTR/D/TOPO (Thermo Fischer) following the manufacturer's instructions to create *pENTR:VuLOB*. *pENTR:VuLOB* was recombined into *pMDC7* using LR clonase II (Thermo Fischer) to create *XVE:VuLOB*. To create *35S:VuLOB*, *pENTR:VuLOB* was recombined into *pGWB2* using LR clonase II (Thermo Fischer).

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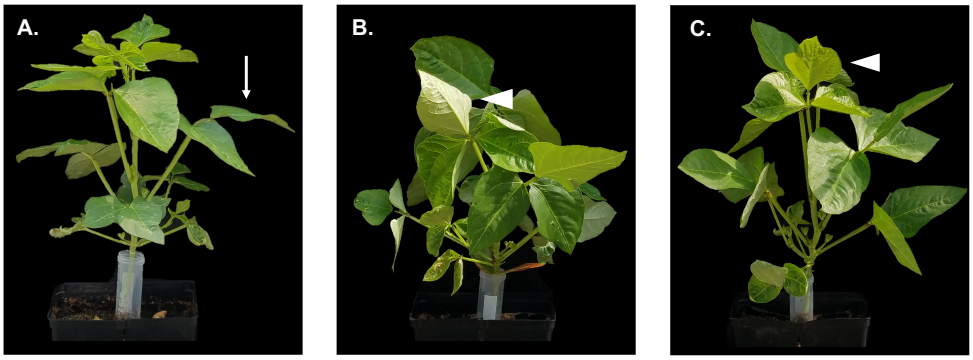
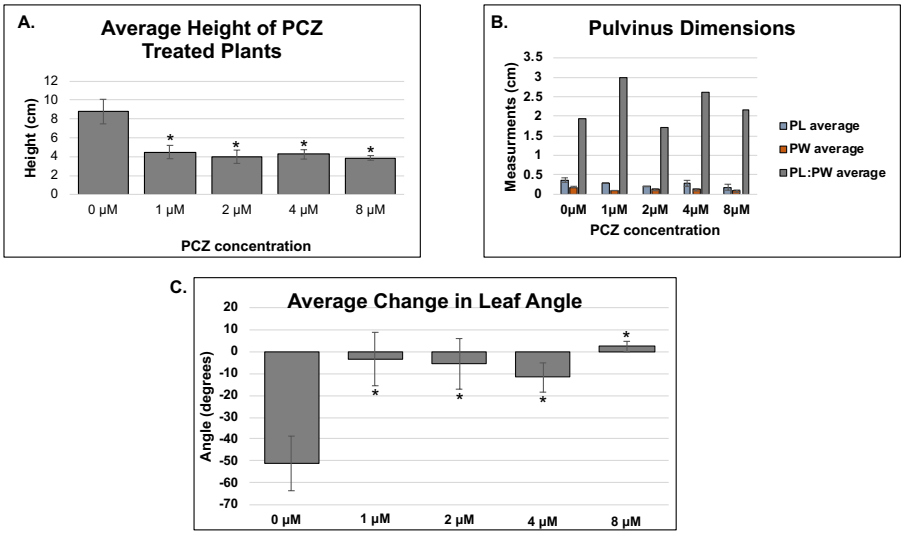


Figure 1.1 Split root assay. Cowpea leaves incline when water-deficit is perceived by the roots. Plants were grown with roots divided between two pots. When both pots were watered (A), leaves remain uninclined; when water was withheld from both pots the leaves inclined (B). Leaves inclined when 1 pot was watered and the other was allowed to dry out (C). The white arrow indicates an uninclined leaflet. The white arrowheads indicates an inclined leaflet. The plus indicates a pot that was watered, and the minus is a pot that had water withheld. The pots are aligned side-by-side.



ANOVA, $p<0.01$

Figure 1.2 Quantification of phenotypes of plants treated with propiconazole.
(A) Height of 3-week-old cowpea seedlings treated with various concentrations of propiconazole. Measurements were taken from the base of the hypocotyl to the shoot apical meristem. (B) Dimensions of the terminal pulvinus. Length, width, and the ratio between length and width are shown. (C) Difference of terminal leaflet angle after water-deficit. N= 10, *= $p<0.01$. Error bars represent SD. Significance was calculated with ANOVA.

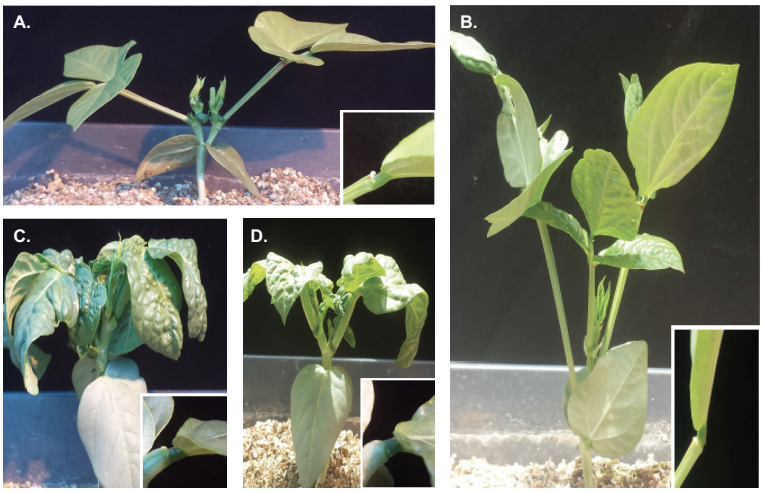


Figure 1.3 Leaf inclination phenotypes of cowpea seedlings.
(A) Cowpea seedling under well-watered conditions. (B) Cowpea seedling after water-deficit treatment. (C) Propiconazole treated seedling under well-watered conditions. (D) Propiconazole treated seedling after water-deficit treatment. Insets show the terminal pulvinus.

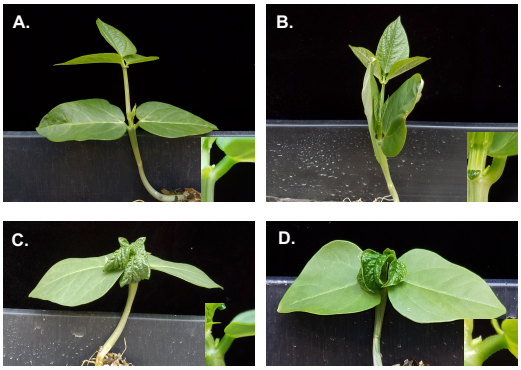


Figure 1.4 Cowpea seedling phenotypes after propiconazole treatments post-leaf expansion.
(A) Cowpea seedling with fully expanded unifoliate leaves under well-watered conditions. (B) Inclined unifoliate leaves after water-deficit. (C) Seedling treated with propiconazole under well-watered conditions. (D) Seedling treated with propiconazole after water-deficit.

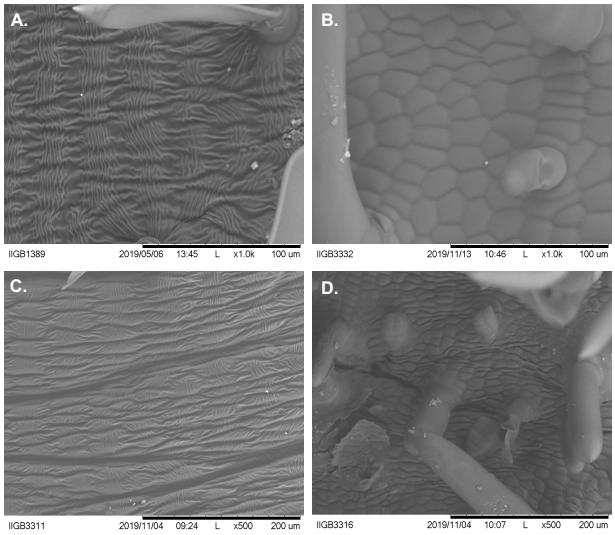
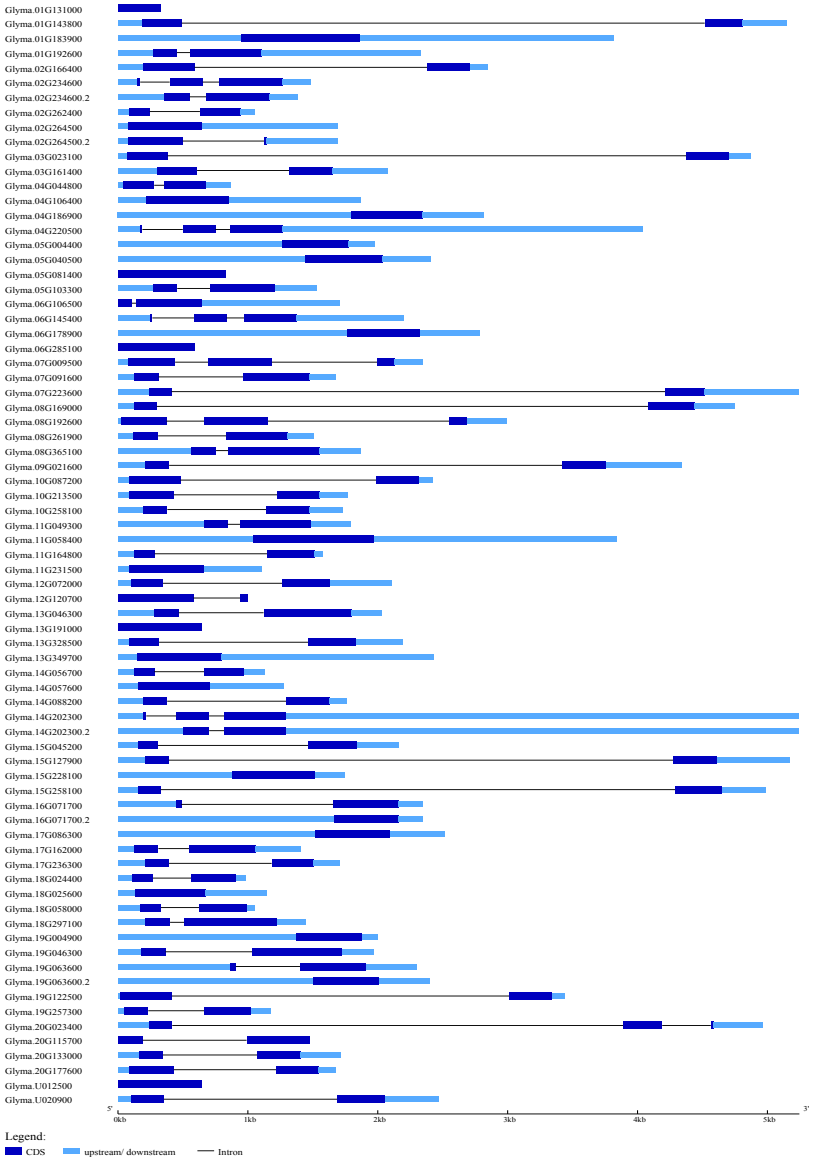


Figure 1.5 Pulvinus epidermal cell morphology.
Scanning electron microscope (SEM) images showing morphology of the epidermal cells of the terminal pulvinus in 3-week old plants and the unifoliate pulvinus. Seedlings grown (A) without propiconazole treatment or (B) treated with 4 μM PCZ. Seedlings grown until full leaf expansions and then treated with propiconazole show(C) the convolutions of the unifoliate pulvinus or (D) the loss of the convolutions in the developing terminal pulvinus.

A.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Legend:
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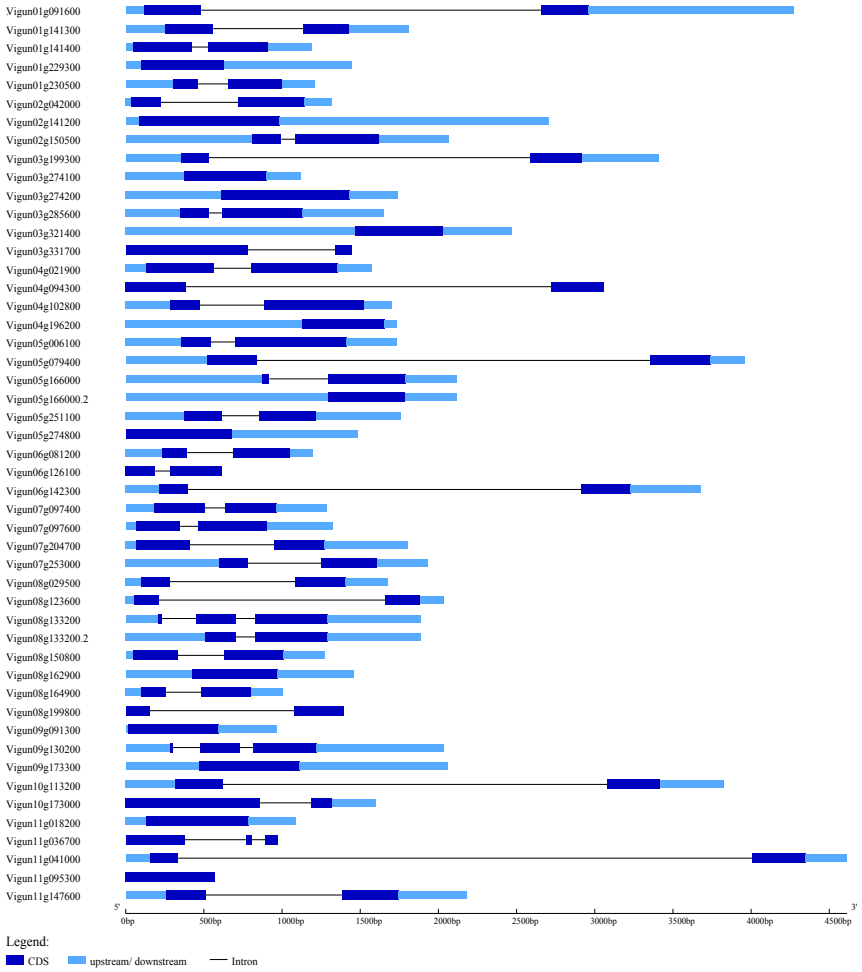
B.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Vigun11g041000
Vigun11g095300
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5'
3'
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500bp
1000bp
1500bp
2000bp
2500bp
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3500bp
4000bp
4500bp
Legend:
CDS
upstream/ downstream
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Figure 1.6 Putative LOB-domain genes in *Glycine max* and *Vigna unguiculata.*
Gene structures of all putative LOB-domain genes and their splice variants in soybean (A) and cowpea (B).

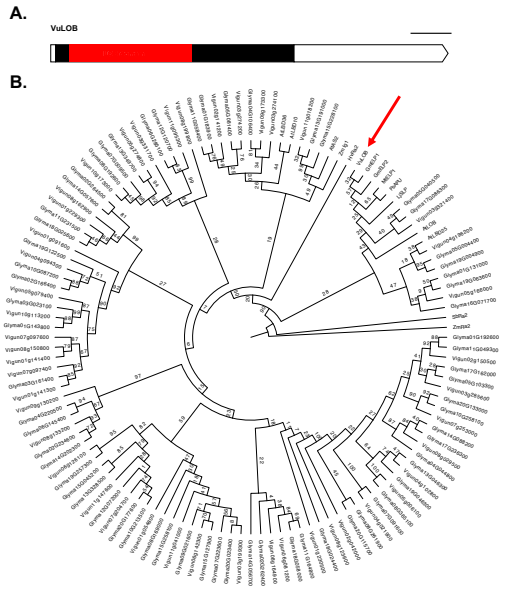

Figure 1.7 Phylogenetic analysis of LOB-domains in legumes.
(A) Cartoon representative of VuLOB. The filled box represents the exon, the LOB domain is showed in red, and the white boxes are the 5' and 3' untranslated regions. The black line represents 100 bp. (B) Phylogenetic analysis from presumptive LOB orthologs in *Medicago, Lotus, Pisum, Glycine, Maize, Sorghum, Hordeum, Arabidopsis*, and LOB-domains from proteins in *Vigna* and *Glycine*. The analysis was inferred using maximum likelihood and the tree was evaluated with 1000 bootstrap replications.

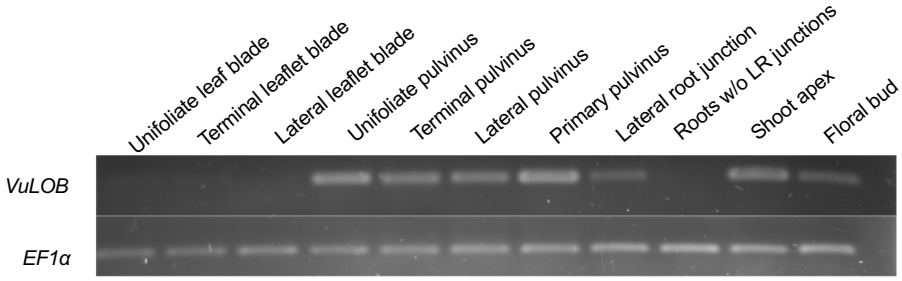

Figure 1.8 Expression pattern of *VuLOB*.
RT-PCR showing transcript accumulation of *VuLOB*. RNA was isolated from various above ground regions from 8-week old plants or roots from 4-week old plants. Transcripts accumulate in all pulvini, lateral root junctions, apices, and floral buds. RT-PCR was carried out with 35 cycles for *VuLOB*. *EF1α* was used as a control with 25 cycles.

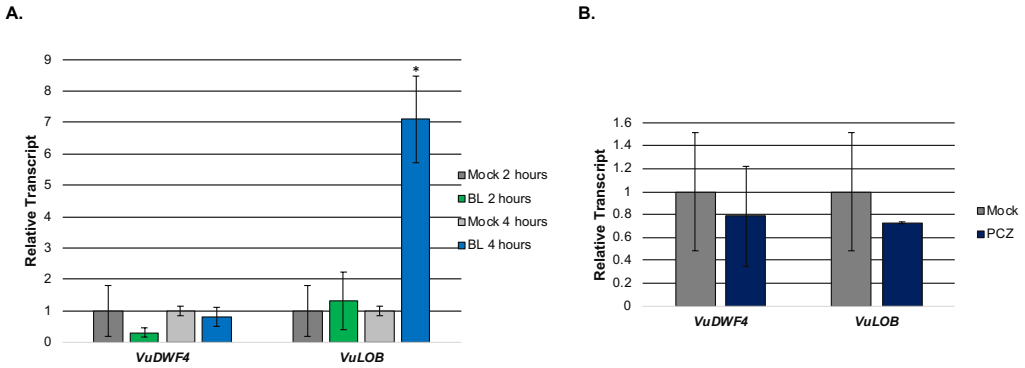

Figure 1.9 *VuLOB* transcript levels respond to brassinosteroid treatment.
(A) Relative transcript levels of *VuDWF4* and *VuLOB* in isolated pulvini of 8-week old plants treated with BL for 2 hours or 4 hours compared to mock treated n=4. (B) Relative transcript levels of *VuDWF4* and *VuLOB* in isolated pulvini of 8-week old plants treated with PCZ for 48 hours compared to mock treated, n=3. Error bars represent standard error, * = $p<0.05$. Significance was determined by Student's t-test.

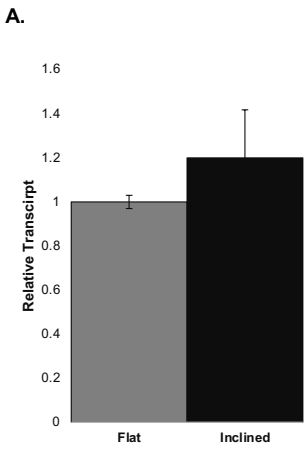

Figure 1.10 Transcripts of *VuLOB* in response to leaf inclination.
Relative transcript levels of *VuLOB* in the terminal pulvini of inclined leaves after water-deficit compared to terminal pulvini of flat leaves under well-watered conditions. N=3. No significant difference was detected between the two treatments. Significance was calculated with Student's t-test.

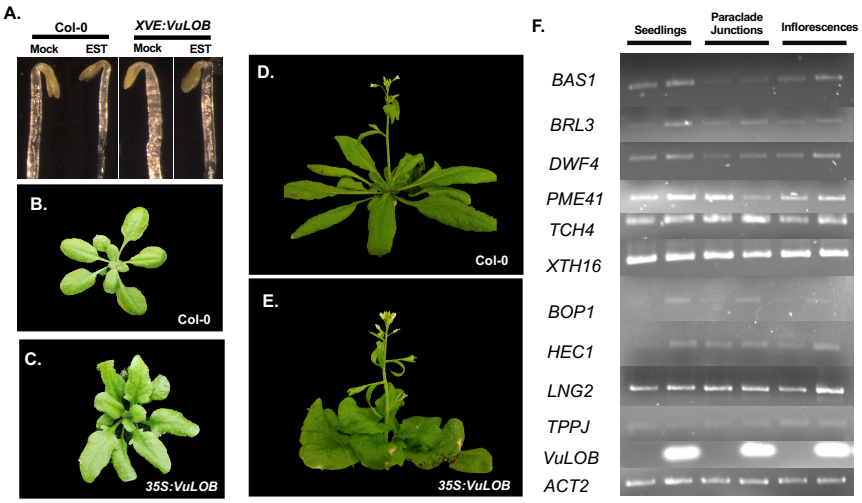

Figure 1.11 Plant and molecular phenotypes of *35S:VuLOB*.
(A) Dark-grown 5-day-old seedlings of Col-0 and *XVE:VuLOB* grown on media with 100 μM β-EST or mock treatment. (B-C) Phenotypes of 17-day old adult vegetative plants. (D-E) Phenotypes of 4-week old plants after flowering. (F) RT-PCR analysis showing transcript accumulation of known *AtLOB* targets in various tissues of Col-0 and *35S:VuLOB*. *ACT2* was used as a control, with 22 cycles; all other PCRs were carried out with 28 cycles.

Table 1.1

Primer Name	Sequence 5' -> 3'	Tm (°C)
VuAPU-like F	AAGATATTCGGAGCGAGCAA	54
VuAPU-like R	TGGCAGAATCAAGCTCCTTT	54.5
VuEf1a F	GGAGCAAAAGTCACCACCAT	55.6
VuEF1a R	AGGTCCACCAACCTTGACTG	57.1
APU-like entry clone F	CACCATGGCATCATCGAGCTCCTA	60.6
APU-like entry clone R	TCACAAGTTGTTATTACCTCCTCCTTCT	57.3
attB2 R	CCACTTTGTACAAGAAAGCTGGGT	57.5
ELP1-like CDS F	AAGTGGGTTTTATAGGCCCTC	54.5
qVuEF1a F	TCAACAGCCTGAGCCTGAGC	59.9
qVuEF1a R	GGAAAAGTATCTGGCCTCCAGTTG	57.6
qVuELP1-like.2 F	TCCAACCATCACTCACAACTCTTAC	56.6
qVuELP1-like.2 R	TTATTACCTCCTCCTTCTCCTCCC	57.1
qVuDWF4.2 F	TGACCAACCTCAACACTTCAATCC	57.3
qVuDWF4.2 R	GAAAGTTATTGTTTGCCGCGTTCTT	56.8
APU-like 3'RACE 1	CCACCAGAAGAGCCTCAGAA	56.5
APU-like 3'RACE 2.3	GAGGAGAAGGAGGAGGTAATAACA	55.3
Qt	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTT	83.22
Qo	CCAGTGAGCAGAGTGACG	60.84
Qi	GAGGACTCGAGCTCAAGC	60.42
ACT2-N	AAAATGGCCGATGGTGAGG	66.9
ACT2-C	ACTCACCACCACGAACCAG	63.8
BAS1F	GGCGGAGACAAAACGCTAT	55.5
BAS1R	CGGTAGGTGCATGCTGATAA	59.72
BRL3F	TTACGACGTTCCCACTCACA	60.16
BRL3R	CAGCCTTGCCATACCAAAAT	59.96
DWF4(F)2	CTCAAGACGAGGCCAAAAAG	56.0
DWF4(R)2	TCTTTGAGTGCTTTGCGATG	55.0
PME41 RT F	CTTTCAACTCCGCCACTTTG	54.2
PME41 RT R	ACACCACTCTGAACCATCCAAC	57.2
TCH4 RT F	ATGGCGATCACTTACTTGCTTC	55.4
TCH4 RT R	ACATTTGTGTGAAGTGTGTAAGG	53.5
XTH16 RT F	CCCCCAATGGGTCGAATCTTG	58.2
XTH16 RT R	GATGTGTTGTGGTCTCCAGAC	55.5
HEC1 RT F	CATCAGATGGAGAAGCTTCCTGAG	57
HEC1 RT R	CTAAGAATCTGTGCATTGCCC	54
LNG2 RT F	GTCCAGACTTTGGAATCAAAC	51.7

LNG2 RT R	GCAGAAGCAAACTTCATTAACC	52.7
MS TPPJ RT F	TCATAACGGTCTCTACAG	47.6
MS TPPJ RT R	GATGATTCGTTCGAACATGTTA	51
2190548-F	TCTAAGTACCAAAGCCTCG	60.8
2190548-R	AAGACGGATCAACAGTACGG	61.5

Chapter 2:
Genome-wide Association Studies Reveal Candidate Gene for Pulvinus Size in *Vigna unguiculata* (Cowpea L. Walp)

Abstract

Leaf angle is an important agronomic trait that has implications in high density planting and plant health. Legumes utilize the pulvinus, a structure at the base of their leaves and leaflets, to alter leaf angle in response to various stimuli. Cowpea plants incline their leaves under periods of water-deficit as a mechanism of sun-avoidance. Genome-wide association studies (GWAS) were carried out to identify regions of the genome that associate with the variation in water-deficit induced leaflet angle and pulvinus dimensions. While no significant single nucleotide polymorphisms (SNPs) associated with change in leaf angle were identified, several significant peaks were identified that are associated with pulvinus dimensions. A candidate gene, *LATERAL ORGAN BOUNDARIES DOMAIN41* Vigun05g006100 (*VuLBD41*), is highly enriched in the cowpea pulvini. *LBD41* is orthologous to *Arabidopsis LBD40* and *LBD41*. Functional analysis in *Arabidopsis* does not predict how *VuLBD41* might function in the boundary. *Arabidopsis* plants overexpressing *VuLBD41* have a larger variation in branch number but no other discernible boundary phenotype. Further experiments need to be carried out to explore *VuLBD41* function in the pulvinus.

Introduction

Cowpea is a warm-season grain legume primarily grown in Sub-Saharan Africa. Due to cowpeas' resilience to drought and high temperature, it is advantageous to grow as a high-protein grain crop in this region (Hall 2012). One of the mechanisms thought to contribute to cowpea drought-tolerance is the ability to incline its leaves under water-deficit stress (Shackel and Hall 1979). When soil-water availability is low, the roots transmit a signal to the leaves causing them to incline (Shackel and Hall 1979). Leaf inclination in cowpea is driven by a structure at the base of leaves and leaflets called the pulvinus. During leaf inclination, the adaxial motor cells undergo an efflux of K^+ that causes water to exit and the cells to shrink (Cote 1995). In contrast, the abaxial motor cells undergo an influx of K^+, which causes water to enter and the cells to expand (Cote 1995). The differential flexion and expansion of the pulvinus motor cells drives the leaves and leaflets up, decreasing their exposure to the sun, and decreasing the rate of water loss (Shackel and Hall 1979).

Little is known about the regulation of leaf angle and pulvinus size in cowpea. Leaf angle is established by the boundary, which is defined early in development. The boundary region forms between the shoot apical meristem (SAM) and the initiating primordia and is composed of cells that are smaller than cells in the neighboring regions that divide infrequently (Hussey 1971). In *Arabidopsis*, boundary formation can in part be attributed to the function of *LATERAL ORGAN BOUNDARIES* (*LOB*) (Shuai, Reynaga-Peña, and Springer 2002; Bell et al. 2012). *LOB* encodes a transcription factor that is functional in plant organ boundaries and modulates brassinosteroid (BR)

accumulation through the regulation of a BR catabolism enzyme BAS1 (Bell et al. 2012; Husbands et al. 2007). BRs have long been known to regulate leaf angle (Abe 1991). Classic experiments done with rice show that the application of BR to the lamina joint, a boundary between the blade and the sheath of the leaf, causes an increase in the angle of the blade in relation to the shoot (Zhang, Bai, and Chong 2014). BRs promote cell expansion at the lamina joint, which increases leaf angle. In contrast, BR-deficient rice plants have erect leaf angles. In cereal crops, erect leaf angles have two advantages for increased yield (Truong et al. 2015). Firstly, erect leaves drastically affect the architecture of the shoot. This allows for high density planting of rice. Secondly, erect leaves can increase photosynthetic efficiency of the plant (Truong et al. 2015). Young leaves with erect leaf angles at the top of the plant won't shade the older leaves at the base. This allows for sunlight to be evenly distributed across the plant instead of being captured by the young leaves (Truong et al. 2015). With an even distribution of sunlight, leaf temperature is maintained to allow for more efficient photosynthesis and the plant can use this energy for flowering and seed set (Truong et al. 2015). In contrast, plants with flat leaf angles can have an increased leaf temperature, which reduces photosynthetic efficiency. The older leaves also prematurely senesce, and the plant may divert energy into leaf production, rather than flowering.

Previous research on model legumes has revealed that *LOB* orthologs are required for pulvinus formation (Chen et al. 2012). In *Medicago truncatula,* the pulvinus controls the nighttime folding of leaflets. The *Medicago* gene *ELONGATED PETIOLULE1* (*ELP1*) is required for pulvinus formation (Chen et al. 2012). Loss-of-function *elp1*

mutants lack pulvini and are unable to fold their leaves (Chen et al. 2012; C. Zhou et al. 2012). This gene has a conserved function in both *Lotus* and *Pisum*, where loss-of-function mutants of the *ELP1* ortholog fail to produce a pulvinus, producing a petiolule in its place (Chen et al. 2012).

In soybean, *GmILPA1* has been shown to regulate pulvinus size (Gao et al. 2017). *GmILPA1* encodes a subunit of the anaphase-promoting complex/cyclosome, which regulates motor cell proliferation within the pulvinus (Gao et al. 2017). Mutants of *Gmilpa1* have fewer motor cells within their pulvini and enlarged vascular bundles (Gao et al. 2017). The morphological change of the pulvinus impacts the change in petiole angle that soybean exhibits throughout the day (Gao et al. 2017). These plants have larger petiole angles compared to wild type plants and have a reduced solar tracking throughout the day (Gao et al. 2017). Thus, pulvinus size affects both the architecture of the plant as well as its nyctinastic movement throughout the day. A BLAST search using *GmILPA1* as query against the annotated cowpea genome, reveals a putative cowpea ortholog to *GmILPA1*, Vigun02g173100, which is expressed in all organs based on LIS (Lonardi et al. 2019).

Arabidopsis encodes 43 *LBD* genes (Shuai, Reynaga-Peña, and Springer 2002) and while several of these genes have been well-characterized with regards to their role in development, many of their roles are poorly understood. Particularly, the class II LBDs have remained the most elusive. In *Arabidopsis*, there are six genes that make up this class (*LBD37-42*). These genes are grouped based on the sequence similarity of their LOB-domain. The conserved N-terminus of these proteins lacks the coiled-coil domain

found in the class I protein, suggesting that they may not bind DNA, however LBD41 was shown to bind DNA *in vitro* (Husbands, et al. 2007; Majer and Hochholdinger 2011). *LBD37*, *LBD38*, and *LBD39* are involved in nitrogen metabolism and have been shown to negatively repress anthocyanin biosynthesis (Rubin et al. 2009). Transcripts of these genes are rapidly (30 minutes) induced upon the treatment of N-depleted seedlings with NO_3 (Rubin et al. 2009). Loss-of-function mutants accumulate anthocyanins under sufficient nitrogen conditions while WT plants do not (Rubin et al. 2009). When constitutively expressed, *35S:LBD37*, *35S:LBD38*, and *35S:LBD39* fail to produce anthocyanins under nitrogen depletion, providing further evidence that these genes function to negatively regulate the accumulation of anthocyanins under nitrogen starvation. *LBD40* is a gibberellin responsive gene (Zentella et al. 2007). When seedlings were treated with GA, transcripts of *LBD40* were down regulated. In transgenic plants carrying a stabilized repressor of GA signaling (DELLA), *LBD40* transcripts were increased, suggesting a negative relationship between GA and *LBD40*. It has been proposed that *LBD40* functions in the negative regulation of GA-mediated development (Zentella et al. 2007). *LBD41* is the most poorly understood class II LBD gene. It has been shown to be a hypoxia-response gene, but its role in development has yet to be addressed (T. A. Lee and Bailey-Serres 2019). In *Arabidopsis*, LBDs regulate many different processes and could be implicated in crop species for beneficial traits relating to stress tolerance and increased yield.

Here, we use genome-wide association studies (GWAS) to identify regions of the cowpea genome that contribute to variation in pulvinus size and leaflet inclination. We

identify one candidate which may contribute to the variation in pulvinus size and begin to explain its function using *Arabidopsis*.

Results

Genome-wide association study reveals candidates for pulvinus size

We carried out a genome-wide association study (GWAS) to identify regions of the cowpea genome that are associated with variation in the size of the pulvinus. The terminal pulvini of plants in a cowpea minicore population were measured when plants reached one month of age. The terminal pulvinus of the youngest, fully-expanded trifoliate leaf was measured and the pulvinus of three plants for each line available was measured. After the measurements were taken, length:width ratios were calculated and QTLs for pulvinus dimensions were identified using the MLM method in TASSEL v5 (https://www.maizegenetics.net/tassel). This method identified several peaks on chromosomes 5, 6, 7, and 8 (Figure 2.1). Upon further evaluation of the genes associated with significant single nucleotide polymorphisms (SNPs), we identified a gene likely orthologous to *AtLBD41*, hereby referred to as *VuLBD41* (Vigun05g006100). In *Arabidopsis*, *LBD* genes have a wide range of functions including leaf development and boundary formation, but the function of *LBD41* is poorly understood. It is possible that *VuLBD41* has a novel function in cowpea. A significant SNP peak associated with a *BRASSINOSTEROID-6-OXIDASE2* (*VuBR6ox2)* encoding gene was also identified. *BR6ox* genes are involved in brassinosteroid biosynthesis and in maize have been shown to accumulate in the boundary between the blade and sheath (Johnston et al. 2014). Other

candidates included genes involved in ABA biosynthesis, flower and fruit patterning, cell shape, cuticle formation, and megaspore development (Table 2.1). However, these genes were not pursued for further analysis. The predicted functions of these genes were based on their functions in *Arabidopsis*; therefore, it is possible that these genes have gained a novel role in pulvinus formation in cowpea. Further experiments are needed to test this.

Analysis of pulvinus length and width measurements separately revealed a single significant SNP on chromosomes 1 and 10 for pulvinus length, while no significant SNPs were identified for pulvinus width (Figure 2.2). Since the ratio of length to width gave us the most significant SNPs, these data were pursued further. More experiments need to be carried out to determine the significance of the genes in the region of the two SNPs found to associate with pulvinus length.

Genome-wide association study for leaflet angle

We also carried out GWAS on the minicore to identify genomic regions associated with the variation in leaflet angle after water-deficit. The difference in leaflet angle was calculated by determining the change in angle of the terminal leaflet of 3-week-old seedlings following water deficit for three days. We attempted to identify QTLs for leaf angle using the MLM method in TASSEL v5, but no significant peaks were identified (Figure 2.3). After adjusting the significance threshold, we were still unable to identify any significant SNPs. It may be that the SNPs associated with the phenotype are undetectable for two possibilities. One possibility is that this trait is controlled by the presence or absence of a gene, which would not be detected with GWAS. The other

possibility could be due to a transposable element (TE) which could explain the variation rather than a SNP. It is estimated that the cowpea genome contains 39.2% TEs (Lonardi et al. 2019), which could account for the variation in the phenotype.

Phylogenetic analysis of VuLBD41

A phylogenetic analysis was performed to determine relationships between *VuLBD41* and the class II LBD genes in *Arabidopsis*. Aligning the amino acid sequence of the LOB domain of VuLBD41 with the *Arabidopsis* class II LBD proteins revealed that VuLBD41 forms a clade with both LBD40 and LBD41 (Figure 2.4). LBD40 is a DELLA target (Josse et al. 2011) and LBD41 is a hypoxia response gene (Liu et al. 2005). LBD41 is a putative interactor with TOPLESS due to the presence of the EAR domain in the C-terminal region of the gene (Causier et al. 2012). This suggests that it acts as a transcriptional repressor, but its role in development has not been characterized. While VuLBD41 is similar in sequence to LBD40 and LBD41, its function in pulvinus formation remains a mystery since LBD40 and LBD41 have no known role in development.

Transcript accumulation pattern of GWAS candidates

Further characterization of the *VuLBD41* expression pattern shows a high accumulation of transcript in the pulvini at the base of the terminal and lateral leaflets as well as in the pulvinus at the base of the leaf (Figure 2.5). Transcripts of *VuLBD41* had strong accumulation in the lateral root junctions and floral buds which is consistent with

the gene data available on LIS (Figure 2.5). A lower level of *VuLBD41* transcript was detected in the roots without lateral root junctions, apices, and blades of the leaves and leaflets (Figure 2.5). These data indicate that *VuLBD41* is expressed in the pulvinus, which is consistent with a putative function in regulating its size.

In *Arabidopsis*, *BR6ox2* is involved in BR-biosynthesis. Knock-out mutants of brassinosteroid-6-oxidases in multiple species exhibit the stereotypical dwarf phenotype resulting from a BR-deficiency (Makarevitch et al. 2012; Shimada et al. 2003). Brassinosteroid accumulation in blade/sheath boundaries in rice has been shown to increase leaf angle (Wada et al. 1984) and BR manipulation has been a target for achieving the ideal plant architecture (Cai et al. 2016; Jin et al. 2008). Characterization of the expression pattern showed that *VuBR6ox2* transcripts accumulated in all tissues at low levels (Figure 2.5). The broad expression makes *VuBR6ox2* a less compelling candidate to have a specific role in regulation of pulvinus size. Further experiments to analyze the effect of brassinosteroids on the size of the pulvinus could be carried out.

Functional analysis of VuLBD41

To explore the function of *VuLBD41,* we created transgenic *Arabidopsis* plants overexpressing *VuLBD41* driven by the *35S* promoter (Figure 2.6). We chose three independent transgenic lines, each with confirmed high levels, using RT-PCR, of *VuLBD41* in their inflorescences, to examine for shoot architecture defects. In three independent transgenic lines, no obvious phenotype was observed compared to wild-type plants. We examined transgenic plants grown side-by-side with Col-0. These transgenic

plants exhibited no organ fusions during vegetative growth or in paraclade junctions, as seen in *lob* or *lof1* mutant plants (Bell et al. 2012; D.K. Lee, Geisler, and Springer 2009). No obvious branch angle defects were observed, but the number of branches formed from the main shoot was more variable (Figure 2.6). Col-0 plants had an average of 3.4 branches while *35S:VuLBD41* plants had an average of 3.7 branches. These averages were not statistically significantly different, however *35S:VuLBD41* plants had more variation, with the number of branches ranging from 1 to 7 in 4-week old plants. In contrast, Col-0 plants showed less variation in branch number, containing between 3 and 5 paraclade junctions. Given that the class II LBD proteins lack a coiled-coil domain, VuLBD41 may require a cofactor *in planta* to bind DNA. In this case, constitutive expression of *VuLBD41* alone would not be sufficient to confer a phenotype.

Discussion

Plant architecture can heavily impact crop yield. Particularly, leaf angle is an important agricultural trait because it can positively affect planting densities and plant health to increase yield (Truong, et al. 2015). In grass species, such as maize and rice, the molecular mechanisms underlying the control of leaf angle have been elucidated. Multiple QTLs have been identified in maize that contribute to the variation in leaf angle (Mantilla-Perez and Salas Fernandez 2017). However, the mechanisms behind the control of leaf angle in legumes is poorly understood and no QTLs have been identified to explain the variation in leaf angle. Here we identify several QTLs that contribute to the

variation in dimensions of the pulvinus and begin to characterize one candidate gene that may contribute to this variation.

In legumes such as cowpea and soybean, leaf angle is regulated by a structure at the base of the leaves and leaflets called the pulvinus. The pulvinus is a joint that can differentially expand and contract to drive leaf inclination (Cote 1995). In soybean plants with a reduced pulvinus, the structure fails to alter leaf angle and the resulting plants have very wide leaf angles, thus implicating pulvinus size as a significant contributor to leaf angle (Gao et al. 2017).

In order to identify regions of the cowpea genome that contribute to variation in pulvinus dimensions and leaf angle under water-deficit, we carried out a genome-wide association study (GWAS) using a diverse sub-population of UCR's cowpea germplasm collection termed the minicore (unpublished). While we were not able to identify QTLs that contribute to the variation in leaflet angle in this population, we were able to identify several QTLs that potentially account for the variation in the dimensions of the pulvinus. Variation in leaflet angle may be undetectable by GWAS due to the fact that it is caused by a transposable element or the presence or absence of a gene. Further, the timing to water-deficit induced leaflet inclination was a trait not analyzed. This phenotype may have more variation across the minicore since different accessions may require a different threshold of water-deficit in order to incline. One QTL identified as a potential contributor to the variation in the dimensions of the pulvinus was on chromosome 5 and had two candidate genes, *VuLBD41* (Vigun05g006100) and *VuBR6ox2* (Vigun05g030100). *VuBR6ox2* showed a low level of transcript accumulation in all tested

tissue types, suggesting that it may not contribute directly to the size of the pulvinus. However, *VuLBD41* had high levels of transcript accumulation in all of the cowpea pulvini as well as in the roots, shoot apex, and floral buds, thus giving us evidence that this is a good candidate gene to pursue further.

Phylogenetic analyses places *VuLBD41* in a subclade with the *Arabidopsis* genes, *LBD40* and *LBD41* making it difficult to tell what gene *VuLBD41* is orthologous to. In *Arabidopsis,* the functions of *LBD40* and *LBD41* are poorly understood. Both of these genes are Class II LBD transcription factors that differ from Class I LBDs in that they lack a coiled-coil domain but contain an EAR domain in the C-terminal region of the protein, which is involved in transcriptional repression. The presence of an EAR domain suggests that these transcription factors interact with TOPLESS to repress transcription of downstream targets (Causier, et al. 2012). Their function in development has not been characterized, although *LBD40* is likely a target of DELLA proteins and *LBD41* is a hypoxia-responsive gene, but no mutant phenotype involved in boundary formation has been reported. Further, overexpression of either of these genes in *Arabidopsis* did not result in detectable abnormal phenotypes. This suggests that a cofactor, presumably *TOPLESS*, is required to increase *LBD40* and/or *LBD41* activity *in planta*. In a similar manner, overexpression of *VuLBD41* in *Arabidopsis* yields no detectable abnormal phenotype which could suggest that it requires a cofactor to increase its activity. It would be interesting to test the relationship between *TPL*/*VuTPL* and these LBDs.

Materials and Methods

Plant Materials:

The UCR cowpea minicore population (unpublished) contained 384 accessions, of which 382 were grown in the field at UCR's Agricultural Operations in the summer of 2017 for determination of pulvinus dimensions. For the leaflet inclination GWAS experiment, 295 accessions were grown in trays of vermiculite in a glasshouse with a daytime temperature of 31°C with no supplemental light.

All transgenic *Arabidopsis* lines were in a Col-0 background. For selection of the transgene, seeds were surface sterilized with 70% ethanol for 5 minutes, 50% bleach and 0.02% Tween-20, and rinsed 5 times with sterile water. Seeds were stratified in the dark for 3 days at 4 ℃ and plated on 1X Murashige and Skoog (MS) media (pH 5.7) with 1 % sucrose and supplemented with 100 μM hygromycin B (Invitrogen). The plates were placed in an incubator at 23°C and illuminated with white light at ~100 $\mu M/m^2s$ in a 16-hour light/ 8-hour dark cycle for 1 day followed by a 5 day dark germination to select for transformants. Seedlings with an elongated hypocotyl were transplanted to UCR Sunshine Mix supplemented with Osmocote 14-14-14 and Marathon and incubated in light as described above.

Phenotyping:

When the field-grown plants were one-month old, the length and the width of the terminal pulvinus of the youngest, fully expanded leaf was measured with a digital

caliper (Mitutoyo). Three plants from each accession were measured and then averaged before calculating the ratio between the length and the width. Glasshouse grown plants were grown with 3-5 plants per accession in 2 trays. After 3 weeks of normal growth, 1 tray was continually watered while water was withheld from the other. The terminal leaflet of the oldest leaf of the control plants was imaged immediately. After 3 days without water, the terminal leaflet of the oldest leaf was imaged after checking that the vermiculite was dry. Each accession had 3 to 5 replicates per tray. Leaflet angle was measured from the terminal pulvinus to the midrib of the terminal leaflet. Leaflet angles were measured in ImageJ (Rueden et al. 2017). An average of flat angles and inclined angles were calculated and the difference between the two averages was taken and used for genome-wide association study (GWAS).

GWAS analysis:

GWAS was performed using the available SNP dataset from the iSelect array on a subset of the minicore population using the mixed-linear model (MLM) function in Tassel version 5.0 (https://www.maizegenetics.net/tassel) to account for population structure (Zhou et al. 2010; Muñoz et al. 2017). The $-\log_{10}(p)$ values were plotted against the physical location of each single nucleotide polymorphism (SNP) across the 11 chromosomes available from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Vunguiculata_er) (Lonardi et al. 2019). A Bonferroni correction was applied to correct for multiple

comparisons with a significance cutoff at 0.05 and the total number of tested SNPs equaling 51,128.

RT-PCR/ RNA extraction:

For cowpea, total RNA was extracted using the CTAB method as described in Untergasser 2008 and *Arabidopsis* total RNA was isolated with Trizol reagent following the manufacturer's instructions. All pulvini tissues were ground fresh in buffer and all other tissues were flash frozen in liquid nitrogen prior to RNA extraction. For RT-PCR, 1 µg of RNA was treated with DNase for 30 minutes at 37℃ and cDNA synthesis was carried out using SuperScript following the manufacturer's instructions. cDNA was equalized using the *VuEF1a* primers and the *VuLBD41* and *VuBR6ox2* primers were used to check the transcript of *VuLBD41* and *VuBR6ox2* in various tissues, respectively. ACT2 primers were used to equalize *Arabidopsis* cDNA.

Phylogeny:

The cowpea *VuLBD41* sequences was obtained through a BLAST search in the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html#%21info?alias=Org_Vunguiculata_er). Both the *Arabidopsis LBD40* and *LBD41* nucleotide sequences were used as query. The sequences of *VuLBD41*, *AtLOB*, *ZmRA2*, and all of the *Arabidopsis* Class II LBDs were aligned using MUSCLE. Phylogenetic analysis was conducted using the maximum-

likelihood algorithm using MEGA 7.0 software with 1,000 bootstraps (https://www.megasoftware.net/).

Cloning:

VuLBD41 was amplified from pulvinus cDNA using the primers found in Table 2.1 and cloned into pENTR/D/TOPO (Thermo Fischer) following the manufacturer's instructions to create *pENTR:VuLBD41*. *pENTR:VuLBD41* was recombined into *pGWB2* using LR Clonase II (Thermo Fischer) to create *35S:VuLBD41*. *35S:VuLBD41* was introduced into *Agrobacterium* strain GV3101 using electroporation and transgenic plants were created using the floral dip method (Clough and Bent 1998).

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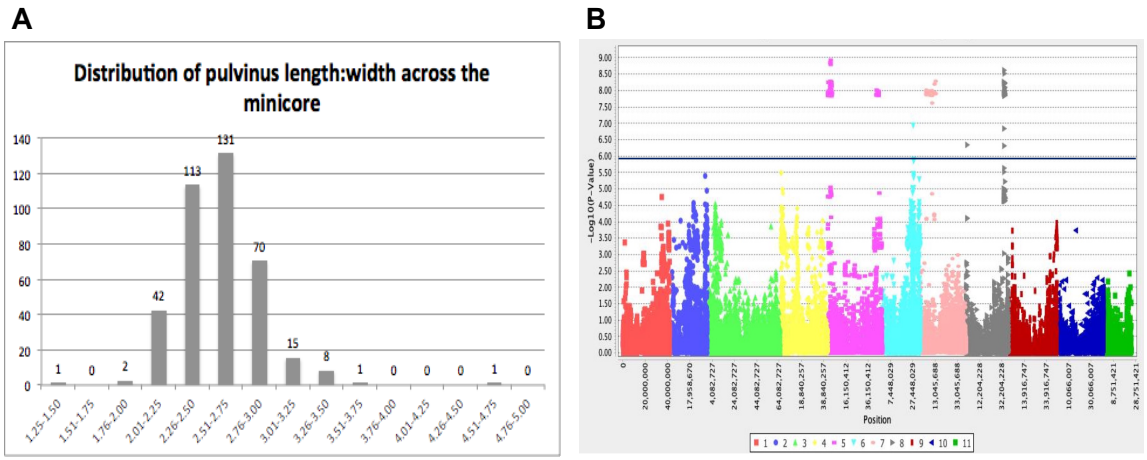

Figure 2.1 Genome-wide association study (GWAS) for the pulvinus dimensions trait.
(A) Distribution of the length to width ratio of the terminal pulvinus in the cowpea families in a minicore population.
(B) GWAS for pulvinus dimensions. Blue line denotes Bonferroni correction at 5.93. *VuLBD41* is on chromosome 5.

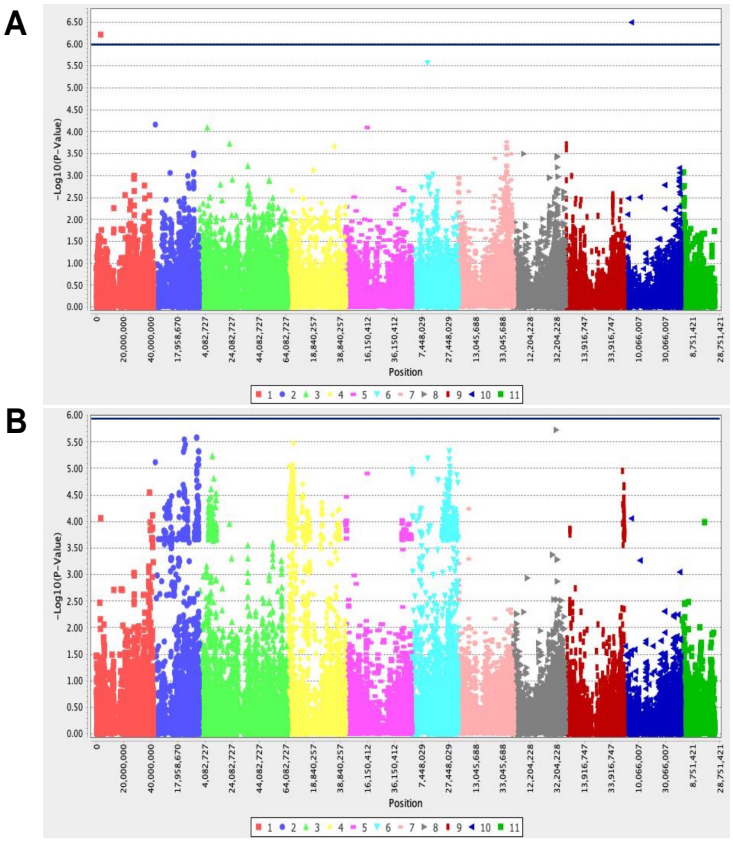

Figure 2.2 GWAS for individual pulvinus length and width dimensions.
GWAS for (A) pulvinus length and (B) pulvinus width.
Blue line denotes Bonferroni correction at 5.93. A cowpea minicore was used for the population.

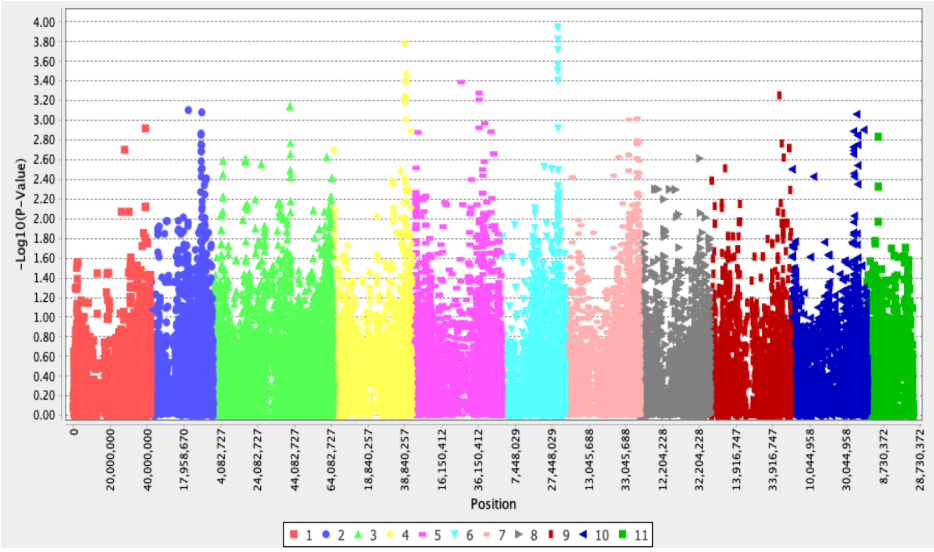

Figure 2.3 GWAS for the change in leaflet angle after water-deficit. GWAS for leaflet angle within the minicore. The Bonferroni correction is at 5.93. A cowpea minicore was used for the population.

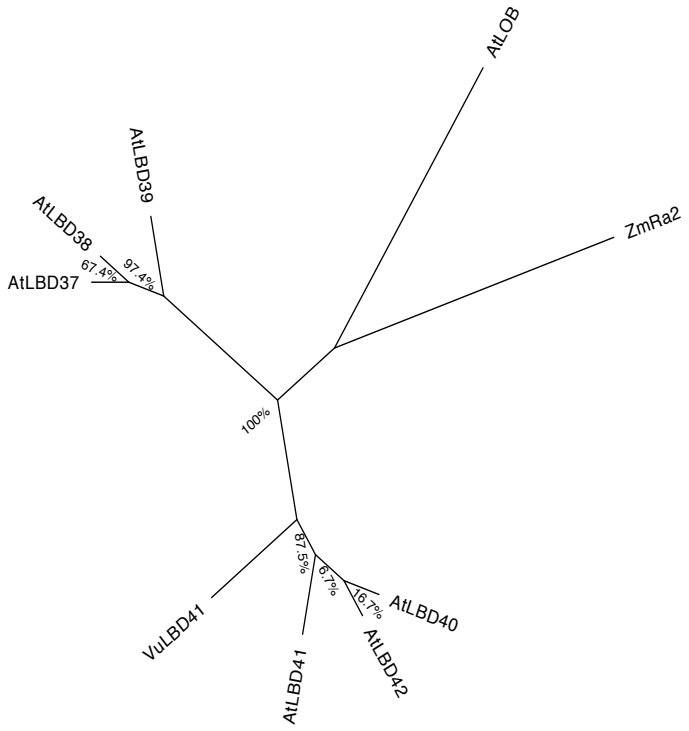

Figure 2.4 Phylogenetic analysis of *VuLBD41*.
Phylogenetic analysis of the Class II LBD genes in *Arabidopsis*, *VuLBD41*, *LOB*, and *ZmRa2*. The analysis was inferred using maximum likelihood and the tree was evaluated with 1000 bootstrap replications.

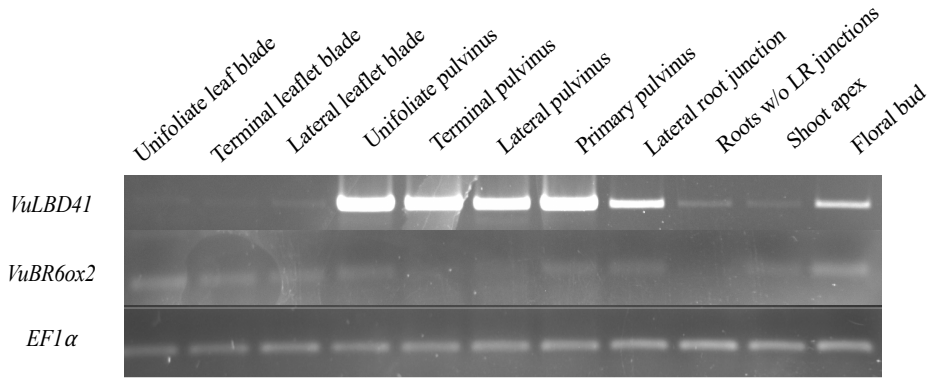

Figure 2.5 Transcript accumulation pattern GWAS candidates.
RT-PCR showing transcript accumulation of *VuLBD41* and *VuBR6ox2.* RNA was isolated from various above ground regions from 8-week old plants or roots from 4-week old plants. *VuLBD41* transcripts accumulate in all pulvini, roots, apices, and floral buds. *VuBR6ox2* transcripts accumulate in all samples tested, except for roots without lateral root junctions. RT-PCR reactions were carried out with 35 cycles for *VuLBD41* and 30 cycles for *VuBR6ox2*. *EF1α* was used as a control with 25 cycles.

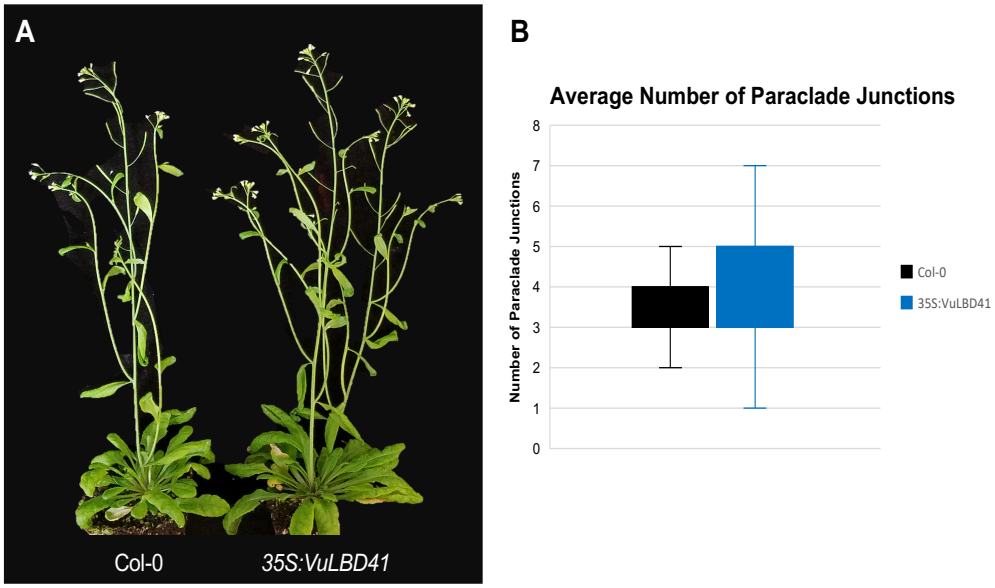

Figure 2.6 Phenotypes of *35S:VuLBD41*.
(A) Phenotypes of 5-week old *Arabidopsis* Col-0 wild-type and *35S:VuLBD41* plants after the transition to flowering. (B) Quantification of the average number of paraclades produced on the primary shoot. N= 16. Error bars represent SD. Significance was calculated Student's t-test. No significant difference was detected between the average paraclade junction numbers.

Table 2.1

Cowpea Genome I.D	***Arabidopsis* gene name**	**Function**
Vigun05g006100	LBD40	GA/ DELLA response
Vigun05g006500.1	ABA4	ABA biosynthesis
Vigun05g029200.1	FUL	Fruit patterning/ Floral evocation
Vigun05g029300.1	SEP2	Flower development
Vigun05g030100.1	BR6OX2	BR signalling
Vigun05g039000.1	SPL1	Thermotolerance
Vigun05g040100.1	MYB16	MIXTA-like, cuticle formation
Vigun08g178900	AGO4	Megaspore development

Table 2.2

Primer Name	Sequence 5' -> 3'	Tm (°C)
VuEf1a F	GGAGCAAAAGTCACCACCAT	55.6
VuEF1a R	AGGTCCACCAACCTTGACTG	57.1
VuLBD40 F	CTTCGTCCAGCGATCTTTCG	55.9
VuLBD40 R	TCAAGCCGAGCATTCAAGCC	58.4
VuBR6ox R	CTCCTCCTACCTCTTCCCAC	56.1
VuLBD41 EC F	CACCATGCGGATGAG	49.9
VuLBD41 EC R	TCAAGCCGAGCATTCAAGCC	52.9
attB2 R	CCACTTTGTACAAGAAAGCTGGGT	57.5
ACT2-N	AAAATGGCCGATGGTGAGG	66.9
ACT2-C	ACTCACCACCACGAACCAG	63.8

Chapter 3

LOB regulates *TPPJ* for proper organ boundary formation in *Arabidopsis thaliana*

Abstract

Lateral organs, including leaves and floral organs, are derived from pluripotent cells in the shoot apical meristem (SAM). Cells in the center of the SAM slowly divide and populate the peripheral zone, where a subset of founder cells will be specified to form organ primordia. These cells will continue to divide to form outgrowths that eventually become mature organs. The boundary region occupies the space between the pluripotent cells of the SAM and initiating organ primordia and is a region of reduced growth that is necessary for proper organ outgrowth. The transcription factor LATERAL ORGAN BOUNDARIES (LOB) functions in the boundary to restrict growth and has been shown to directly regulate *TREHALOSE-6-PHOSPHATE PHOSPHATASEJ* (*TPPJ*), but not its paralog *TPPI*. Mutations in *LOB* result in an overgrowth of the boundary, which leads to the failure of the cauline leaf to separate from the axillary branch. This mutant phenotype is rescued by ectopically expressing *TPPJ* in the boundary, providing evidence that the *lob* mutant phenotype can, in part, be explained by the mis-regulation of *TPPJ* transcripts. Furthermore, *lob*; *tppj*; *tppi* triple mutants have an increased number of paraclade junctions compared to wild type, suggesting that this mutant has a partially impaired boundary region. However, these triple mutant plants do not exhibit any enhancement in the *lob* mutant phenotype and the relationship between LOB and the TPPs remains unclear.

Introduction

All plant lateral organs, such as leaves and floral organs, arise from the shoot apical meristem (SAM). While organ development is completed during animal embryogenesis, plants continue to form organs throughout their lifetime. The SAM consists of pluripotent cells in the central zone that divide to maintain themselves as well as populate the peripheral zone. Maintenance of the SAM requires a balance of producing lateral organs and replenishing the meristematic cells (Fletcher et al. 1999). Some of the cells in the peripheral zone will ultimately switch fate and produce lateral organs (Gallois et al. 2002). A boundary region lies between the central zone and the initiating primordia (Hussey 1971). Plant organ boundaries have unique characteristics, with cells are smaller than their neighbors and divide infrequently (Hussey 1971).

Proper boundary formation is necessary during lateral organ development as the boundary has several roles during this process. After leaves initiate, a subset of the boundary cells will produce an axillary meristem at the base of the leaf (Long and Barton 2000). Mutations in the boundary gene *LATERAL SUPPRESSOR* (*LAS)* in *Arabidopsis* and *LS* in tomato produce plants that have reduced numbers of axillary meristems (Schumacher et al. 1999; Greb et al. 2003). The boundary is also necessary for organ separation and this is dependent on several genes. *LATERAL ORGAN FUSION 1/2* (*LOF1/2*) encode MYB- transcription factors that are functional in organ boundaries (Lee, Geisler, and Springer 2009). While *lof2* mutants don't display any abnormal phenotype, *lof1* mutants fail to separate the axillary branches from their subtending

cauline leaves (Lee, Geisler, and Springer 2009). This region between the primary stem, axillary branch, and cauline leaf is called the paraclade junction. Examination of *lof1; lof2* double mutants revealed that the failure in organ separation was enhanced, demonstrated by the failure of the pedicels to separate from the inflorescence stem and more severe fusions of the cauline leaf to the primary stem (Lee, Geisler, and Springer 2009). These data suggest that *LOF1* and *LOF2* have a functional overlap in organ separation.

Additionally, the three *CUP-SHAPED COTYLEDON* (*CUC*) genes act redundantly and are required for the separation of the cotyledons and maintenance of the meristem (Aida et al. 1997; Hibara et al. 2006). Plants carrying loss-of-function mutations in any combination of two of these genes have fused cotyledons and fail to maintain a meristem (Aida et al. 1997). This phenotype is caused by the failure of the *CUCs* to activate *SHOOTMERISTEMLESS* (*STM*), a gene required for meristem identity (Aida, Ishida, and Tasaka 1999). These observations place the *CUC* genes in an interesting role regulating both organ separation and meristem maintenance.

In *Arabidopsis*, *LATERAL ORGAN BOUNDARIES* (*LOB*) encodes a transcription factor that is also required for organ separation at the paraclade junction (Husbands et al. 2007; Bell et al. 2012). *LOB* was first discovered in an enhancer trap screen where it was shown to be expressed at the base of the floral organs, pedicels, leaves, cotyledons, and lateral roots (Shuai, Reynaga-Peña, and Springer 2002). The *lob* mutants have a subtle phenotype at the paraclade junction where the adaxial side of the cauline leaf fails to separate from the abaxial side of the axillary branch (Bell et al. 2012). However,

overexpression of *LOB* produces a dwarf, sterile plant, suggesting that *LOB* functions to limit growth, either by regulating cell expansion or division in the boundary to control lateral organ development (Shuai, Reynaga-Peña, and Springer 2002; Bell et al. 2012).

To identify downstream targets of *LOB*, a microarray was carried out after *LOB* expression was induced. Interestingly, LOB was shown to regulate a suite of genes involved in the BR response, cell wall modifications, the blue light response, and trehalose biosynthesis (Bell et al. 2012). So far, the most widely characterized LOB target is *PHYB ACTIVATION TAGGED SUPPRESSOR 1* (*BAS1*), which encodes a cytochrome P450 enzyme that functions in the catabolism of BRs (Bell et al. 2012). LOB was shown to have a direct relationship with *BAS1* through its increase in transcript accumulation independent of protein synthesis and binding to the promoter and 3rd exon of *BAS1* shown both with ChIP-PCR and EMSA (Bell et al. 2012).

TREHALOSE-6-PHOSPHATE PHOSPHATASE J (*TPPJ*) was also identified as a potential direct target of LOB in the microarray experiments (Bell et al. 2012). *Arabidopsis* contains 11 functional TPPs, which are active throughout the plant and function in trehalose biosynthesis by converting trehalose-6-phosphate (T6P) into trehalose (Vandesteene et al. 2012). Trehalose is a non-reducing sugar that is formed by the linkage of two glucose molecules (Paul 2007; Lunn et al. 2014). There are five known trehalose biosynthesis pathways and in plants and other eukaryotes, the most common pathway is the OtsA-OtsB pathway (Paul et al. 2010). This pathway is distinct in that an intermediate metabolite, trehalose-6-phosphate (T6P), is formed. The enzyme trehalose-6-phosphate synthase (TPS) transfers a glucose molecule from UDP-glucose to glucose-

6-phosphate to make T6P and UDP (Paul et al. 2010; Ponnu, Wahl, and Schmid 2011). T6P is dephosphorylated by trehalose-6-phosphate phosphatase (TPP) producing trehalose and inorganic phosphate (Paul et al. 2010; Ponnu, Wahl, and Schmid 2011). Trehalose is found in high abundance in bacteria, fungi, and invertebrates, where it is synthesized via alternative pathways than to the pathway found in plants (Paul 2007). In these organisms, trehalose acts as an osmoprotectant and prevents proteins from aggregating under stress (Paul 2007). However, trehalose is found in trace amounts in plants and, with few exceptions, it's role in stress and development remain unclear (Ponnu, Wahl, and Schmid 2011).*TPPJ* has promoter activity in the shoot apex, root, leaf hydathodes, and filaments in the flower (Vandesteene et al. 2012). Activity in the shoot apex overlaps with *LOB* (Shuai, Reynaga-Peña , and Springer 2002; Vandesteene et al. 2012). *TPPI*, a closely related homolog of *TPPJ*, has an overlapping expression pattern with *TPPJ* (Vandesteene et al. 2012) and while presumably not a direct target of LOB, has a mutant phenotype of organ fusion at the paraclade junction (unpublished data). Due to apparent redundancy, mutations in most *TPP* genes do not result in observable mutant phenotypes in *Arabidopsis* (Vandesteene et al. 2012). However, in maize, mutants in the *TPP* gene *Ramosa3* (*RA3*) have branching defects in the tassel (Satoh-Nagasawa et al. 2006). *ra3* mutants have meristem determinacy defects in both the tassels and ears causing an increase in branch number in the tassels and ectopic branching in the ears (Satoh-Nagasawa et al. 2006; Claeys et al. 2019). Mutated versions of *RA3* and a closely related homolog, *TPP4*, that disrupt the phosphatase domain and are unable to catalyze trehalose synthesis, rescue the *ra3* mutant phenotypes suggesting that

the enzymatic activity of these proteins is not required for the control of branching in maize (Claeys et al. 2019). This suggests that TPP enzymes may play a regulatory role in plant development and that the protein has a moonlighting function.

The precursor to trehalose, trehalose-6-phosphate (T6P), has a role in plant development and response to stress (Paul et al. 2010; Fichtner et al. 2017; Yadav et al. 2014). T6P is indispensable during plant development (Schluepmann et al. 2003). *Arabidopsis* plants carrying a mutation in the *TPS1* gene, and thus are unable to synthesize T6P, are arrested in embryo development at the torpedo stage (Wahl et al. 2013). Further, *tps1* mutant plants carrying an inducible *TPS1* transgene, providing TPS1 activity during embryo development, show developmental abnormalities at later developmental stages (Wahl et al. 2013). These plants have a slower germination rate, are short in stature during vegetative development, and are delayed in flowering (Wahl et al. 2013). During the reproductive phase, these plants have reduced apical dominance and aerial rosettes (Wahl et al. 2013). This demonstrates that T6P has a clear role in multiple aspects of plant development. Plants containing weak alleles of the *tps1* mutation fail to flower, but this can be restored through the application of exogenous sucrose, which suggests that T6P plays a role as a signaling molecule for sucrose during the transition to flowering (Wahl et al. 2013).

Data from a microarray that identified putative targets of LOB provides evidence that LOB directly regulates *TPPJ* and, potentially, its homolog *TPPI* given their overlapping expression patterns. Data from this chapter suggests that *TPPJ* is directly regulated by LOB and that TPPJ accumulation in the boundary is sufficient to rescue the

lob mutant phenotype at the paraclade junction. However, the *tppj* mutants have no detectable phenotype suggesting redundancy within the family and higher order mutants with *lob* and *tppi* only have an enhanced number of branches, but no enhancement of organ fusions is detected. These results suggest that *LOB* regulates multiple pathways to separate organs at the paraclade junction.

Results

Induction of ectopic LOB activity alters *TPPJ* transcripts

In a previous microarray experiment, *TPPJ* was identified as a downstream target of LOB (Bell et al, 2012). To further investigate this interaction between LOB and *TPPJ*, we confirmed this regulation independently of the microarray using the *35S:LOB-GR* dexamethasone (DEX)-inducible construct. After a 4-hour DEX treatment, *TPPJ* transcript levels had increased >2.0 fold in 9-day-old seedlings, compared to mock-treated seedlings (Figure 3.1). However, *TPPI* transcripts were not significantly differentially expressed. These transcripts had decreases <2.0 fold when treated with DEX, compared to the mock treated seedlings (Figure 3.1). We then asked if the increase in *TPPJ* transcript after LOB induction was independent of protein synthesis. 9-day-old seedlings containing the *35S:LOB-GR* construct were treated with both DEX and the translational inhibitor cyclohexamide (CHX). *TPPJ* transcripts were increased >2.0 fold in DEX + CHX treated seedlings compared to CHX alone and *TPPI* transcripts were unchanged between the two treatments. These data suggest that LOB directly regulates *TPPJ* expression in seedlings.

lob; tppi; tppj triple mutants have an increased number of branches

Confirmation of the microarray experiment described above led us to test the genetic relationship between LOB and the *TPP* genes. We crossed *lob-3* to *tppi-1; tppj-1* to examine the phenotypes of single mutants and all higher order mutant combinations. We were specifically interested in traits that affect shoot architecture. After the plants had one open flower on their oldest branch we measured organ fusions at the paraclade junction, the length of the base of the leaf as it extends down the primary stem, also known as the decurrent strand, the angle of the branch relative to the primary stem, and branch number off of the primary shoot. *lob-3* mutants exhibited more contact between their cauline leaves and axillary branches (0.485 mm) compared to Col-0 (0.16 mm). Additionally, *tppj-1* and *tppi-1* single mutant plants resembled Col-0 when examined for contact between the cauline leaf and axillary branch, having 0.15 and 0.18 mm of contact, respectively. Higher order mutants carrying the *lob-3* allele exhibited organ fusion at the paraclade junction, but the length of contact between the cauline leaf to the axillary branch was not statistically significant from *lob-3* (Figure 3.2). *TPPI* mutant plants contained decurrent strands at the base of the cauline leaf, which cause the leaf base to extend down the primary shoot. We measured decurrent strands at the same developmental stage for all the mutants available. Col-0, *lob-3*, *tppj-1*, and *lob-3; tppj-1* plants did not produce decurrent strands at the paraclade junction. All higher order mutants carrying the *tppi-1* allele had decurrent strands of varying lengths, but this was not changed with the addition of the *lob-3* allele (Figure 3.3). The angle of the first

branch was measured for each of the genotypes. *lob-3* plants had a smaller branch angle when compared to wild-type plants (Figure 3.4). However, *tppi-1*, *tppj-1*, and *tppi-1; tppj-1* plants had a slightly increased branch angle when compared to wild-type (Figure 3.3). The branch angle of *lob-3; tppj-1* closely resembled the *lob-3* single mutants, while *lob-3; tppi-1* and *lob-3; tppi-1; tppj-1* are partially restored to a wild-type phenotype (Figure 3.4). Plants were examined for the number of branches along the primary shoot. Single mutants typically resembled the wild-type control, with the exception of *tppi-1,* which had a statistically significant increase in branch number (Figure 3.4). The *lob-3; tppi-1; tppj-1* triple mutant had a statistically significant increase in the number of branches, with an average of 6 branches (Figure 3.4). These data suggest that *LOB* and the *TPP* genes have a genetic relationship.

Ectopic *TPPI* and/or *TPPJ* accumulation are not responsible for the *LOB* overexpression phenotype

LOB functions in restricting growth through the modulation of brassinosteroids. Transgenic lines that constitutively express *LOB* are dwarf and sterile. The *LOB* over-expression phenotypes, both light-grown and dark-grown are partially rescued when crossed to a BR-signaling mutant but are not rescued by the exongenous application of brassinolide. We wanted to further test the genetic relationship between *LOB* and the *TPP* genes. We crossed plants carrying the *35S:LOB-GR* construct into the *tppi-1; tppj-1* double mutant. Siblings from this cross were plated on 5 μM dexamethasone and mock plates without sucrose and grown under white light for 10 days (Figure 3.5). There was

no detectable change in phenotype among the genotypes. Since LOB restricts growth, ectopic expression prevents the plants from forming an apical hook when seedlings are germinated in the dark. We hypothesized that ectopic *LOB* activity acts through the *TPP* genes to restrict growth. We asked if mutations in the TPP genes could restore apical hook formation in *35S:LOB-GR* plants. In mock-treated *35S:LOB-GR* plants, an apical hook formed after the plants were germinated in the dark (Figure 3.4). In contrast, no apical hook was formed in *35S:LOB-GR* seedlings grown in the dark on media containing 5 μM dexamethasone, (Figure 3.5). In a similar manner, *tppi* or *tppj* single mutants carrying the *35S:LOB-GR* transgene produced an apical hook when mock treated and lacked an apical hook when the transgene was induced with DEX (Figure 3.5) Further, *tppi; tppj* double mutants carrying the *35S:LOB-GR* transgene formed an apical hook when mock treated and were unable to form the apical hook upon DEX induction (Figure 3.5). These data suggest that the *TPPs* are not required for the *LOB* over-expression phenotype.

Accumulation of TPPJ in the boundary is sufficient to rescue the *lob* mutant phenotype

Mis-regulation of *TPPJ* or *TPPI* may cause the *lob-3* mutant phenotype at the paraclade junction. We hypothesized that the misregulation of *TPPJ* contributes to the *lob* phenotype and tested this by generating *pLOB:TPPJ* and *pLOB:TPPI* expression constructs to target *TPPJ* and *TPPI* to the boundary and introduced them into both wild type (Col-0) and *lob-3* backgrounds. Wild-type plants containing either transgene

appeared morphologically normal with an average contact length between the cauline leaf and axillary branch of 0.140 mm for *pLOB:TPPI* Col-0 and 0.138 mm for *pLOB:TPPJ* Col-0, similar to wild-type plants (Figure 3.6). In *lob-3* mutant plants, the contact between the cauline leaf and the axillary branch was measured at 0.620 mm and when compared to *pLOB:TPPJ lob-3*, which had an average contact length of 0.240 mm, shows that the introduction of the transgene rescues the *lob-3* mutant phenotype. However, *pLOB:TPPI lob-3* plants had an average contact length of 0.570 mm, and that difference from *lob-3* s not statistically significant (Figure 3.6). This suggests that misregulation of *TPPJ*, and not *TPPI*, in the boundary contributes to the *lob* phenotype.

TPS1 accumulation in the boundary is not sufficient to induce organ fusions

LOB modulates BR accumulation at the boundary through the activation of *BAS1*. When BR biosynthesis or signaling genes are expressed in the boundary under the *LOB* promoter, organ fusions are increased at the paraclade junction. These fusions are more severe in *lob-3* mutant plants. If LOB regulates *TPPJ* in the boundary, then a *lob* mutant is expected to have reduced TPP activity and possibly hyperaccumulation of T6P in the boundary. We therefore wanted to test whether the *lob-3* fusion phenotype results from a hyperaccumulation of T6P in the boundary. To test this, we introduced a construct expressing TPS1 under the LOB promoter (*pLOB:TPS1)* to drive synthesis of T6P in the boundary. *TPS1* encodes a trehalose-6-phosphate synthase enzyme, which is active in the first step in trehalose biosynthesis. In Col-0 plants carrying the *pLOB:TPS1* transgene no abnormal phenotype was observed (data not shown). Cauline leaves and axillary branches

were separated at the paraclade junction. Further, the *lob-3* mutant phenotype was not enhanced with the introduction of *TPS1* at the boundary (data not shown). This suggests that ectopic T6P accumulation in the boundary may not cause overgrowth of the boundary.

CyclinB1;1 transcript is not altered in *lob; tppi; tppj* mutants

The presence of organ fusions and increased branching in higher-order mutants of *lob*, *tppi*, and *tppj* suggest that the cell cycle may be mis-regulated in the meristems and/or boundaries of these plants since an overactive cell cycle might potentially result in overgrowth of tissues. To address this, we measured *CyclinB1;1* transcript accumulation in both seedlings and inflorescences of all genotypes. *CyclinB1;1* is a G2/M regulator in *Arabidopsis* that is active in both shoot and root tissues and transcript accumulation of *CyclinB1;1* serves as a good proxy for cells that are about to divide. Five-day-old seedlings and inflorescences with unopened flowers and less than 1 cm of internode elongation were harvested and transcripts were quantified with qRT-PCR. While *CyclinB1;1* was differently regulated, though not significantly, no discernable trend was detected (Figure 3.7). This suggests that *CyclinB1;1* is not misregulated in the boundary region of these plants.

Complementation of the *tppi* and *tppj* phenotype

In *Arabidopsis, tppi-1*mutant plants make decurrent strands at the base of the cauline leaves and *tppj-1* mutant plants have no detectable phenotype. To confirm that the

phenotypes observed corresponded to mutations in *tppi* and/or *tppj* we generated constructs placing the coding sequence of both genes under the control of the estradiol-inducible synthetic transcription factor XVE (Zuo et, al. 2000). *XVE:TPPI* was transformed into Col-0 and *tppi-1* and *XVE:TPPJ* was transformed into Col-0 and *tppj-1*. Seeds were placed on media containing 5, 10, 25, 50, and 100 μM β-estradiol or mock treated. While *tppj* has no detectable mutant phenotype at 10 days post germination, *tppi* mutants appeared smaller due to a developmental delay (Figure 3.7). Compared to wild-type plants of the same age, *tppi-1* mutant seedlings have smaller leaves. Transgenic plants carrying the *XVE:TPPI* construct in a *tppi* mutant background have a slight recovery of growth compared to mock treated plants and phenotypically resemble wild-type plants (Figure 3.8). This phenotype was not concentration dependent for the range of concentrations tested and only one representative concentration is shown. No change in phenotype was observed for *tppj* plants carrying the *XVE:TPPJ* transgene and grown on estradiol, compared to mock, which suggests that ectopic *TPPJ* transcript accumulation doesn't have any effect on the development of the plant.

TPPJ can compensate for TPPI

TPPJ and *TPPI* have partial overlapping expression patterns and encode proteins which are 75% identical in sequence (Vandesteene et al. 2012). To test whether the TPP genes can compensate for one another, we introduced the *pLOB:TPPJ* and *pLOB:TPPI* constructs into *tppi* and *tppj* mutants, respectively. No change in phenotype was observed in *pLOB:TPPI tppj* plants compared to *pLOB:TPPI* Col-0 (Figure 3.10). However,

compared to *tppi* mutants, plants containing either the *pLOB:TPPJ* or *pLOB:TPPI* constructs made fewer decurrent strands (Figure 3.9). These plants resembled wild-type plants with the exception of the occasional decurrent strand in *pLOB:TPPJ tppi* plants. These data suggest that *TPPJ* accumulation in the boundary is sufficient to compensate the *tppi-1* mutant phenotype.

LOB binds to the promoter region of *TPPJ* in yeast

LOB has been shown to bind the DNA motif (G)CGGC(G), where the Gs flanking the motif are variable (Husbands et al. 2007). The promoter regions of both *TPPI* and *TPPJ* contain three putative LOB binding sites. To test if LOB directly binds to either *TPPI* or *TPPJ* promoters, we carried out yeast-1-hybrid assay using 918 base pairs (bp) and 980 bp upstream of the ATG of *TPPI* and *TPPJ*, respectively. These DNA fragments were cloned upstream of a *HIS* reporter gene and integrated into the yeast genome to create both *pTPPI:HIS* and *pTPPJ:HIS*. A clone containing the LOB coding sequence fused to the GAL4 activation domain was transformed into yeast cells containing the promoter:HIS integration. Yeast cells containing the *pTPPI:HIS* construct grew on media lacking HIS, but were unable to grow in media lacking HIS and containing 3-Amino 1, 2, 4-Triazole (3-AT) (Figure 3.11). This suggests that LOB does not bind to the promoter of *TPPI* in yeast. In contrast, yeast cells containing the promoter region of *TPPJ* and LOB grew on media lacking HIS with and without 3-AT, whereas yeast cells containing the empty vector did not grow on 3-AT containing media (Figure 3.11). These results suggest that LOB is able to bind to its putative binding site in the

promoter of *TPPJ*, but not at any of the putative binding sites in the promoter region of *TPPI*.

Discussion

Plants require proper organ boundary formation to establish an optimal architecture. In order to form proper boundaries, plants have evolved multiple independent pathways to regulate this process. Boundaries are a region of reduced growth and improper boundary formation can result in organ fusions, reduced branching, and lethality. Growth is regulated in multiple ways, including through the modulation of brassinosteroid (BR) accumulation. BRs promote cell division and expansion, and in the boundary, the transcription factor LOB prevents their accumulation by regulating expression of *BAS1*, which encodes a BR catabolic enzyme. *lob* mutants have a hyperaccumulation of BR in organ boundaries, resulting in failure of the organs to separate at the paraclade junction. From a previous microarray dataset, LOB was shown to regulate *TPPJ*, an enzyme involved in trehalose biosynthesis. A precursor to trehalose and a substrate for the TPPs, trehalose-6-phosphate is a signaling molecule that promotes cell division in some contexts (Ponnu et al. 2011). Since LOB activity limits growth, we hypothesized that LOB regulation of *TPPJ* expression functions to restrict growth in the boundary. We characterized the relationship between LOB, *TPPJ*, and its homolog *TPPI*, and their role in boundary formation. We chose to look at *TPPI* because of its close homology to *TPPJ*. *LOB* and *TPPJ* have a positive relationship given that transcripts of *TPPJ* are upregulated when *LOB* is overexpressed. However, *TPPI* transcripts were not

significantly altered in response to *LOB* overexpression suggesting that these two do not have a genetic relationship. We tested whether LOB directly binds to the promoters of *TPPI* or *TPPJ* using a yeast-1-hybrid assay. This experiment demonstrated that LOB directly binds to the promoter region *TPPJ,* in a region containing two putative LOB binding sites in a tandem arrangement. No interaction was observed between LOB and the promoter region of *TPPI*, despite the presence of canonical binding site motifs. This provides further evidence that LOB has a direct and specific relationship with *TPPJ*.

In *Arabidopsis*, *tppj* mutants display no detectable mutant phenotype. Further, when combined with a *lob* mutation, the resulting double mutant plants had no enhancement of the *lob* mutant phenotype. *Arabidopsis* contains 10 *TPP* genes which could suggest that there is redundancy among these genes in the boundary. We tested whether the *TPP* genes exhibit redundancy in the boundary by examining *lob; tppi; tppj* triple mutants. Enhancement of organ fusions relative to single mutants was not observed, but these plants had a significant increase in the number of paraclade junctions. These data could suggest that the boundary is functioning improperly due to an increase in T6P accumulation or the TPP genes have a separate role from their enzymatic activity as suggested by Claeys et al 2019. Further experiments would need to be carried out to provide evidence for this.

Driving *TPPJ* expression under the *LOB* promoter is sufficient to rescue the *lob* mutant phenotype. However, *TPPI* accumulation in the boundary doesn't rescue the mutant phenotype. This provides further evidence that LOB regulates *TPPJ* to restrict growth at the boundary and that mis-regulation of *TPPJ* contributes to the *lob* mutant

phenotype. Because LOB regulates BR accumulation through the direct regulation of *BAS1* and BAS1 accumulation at the boundary is sufficient to rescue the *lob* mutant phenotype, we hypothesize that the regulation of *TPPJ* by LOB is an independent pathway to restrict growth in boundary cells. This is pathway is likely redundant with the BR pathway and serves as a backup. We did not examine the genetic interactions between *BAS1* and *TPPJ*. It would be interesting to see if a *lob; tppj; bas1* mutant has any enhanced mutant phenotype as this would further tease apart the hypothesis these pathways are separate, but redundant.

TPPJ and TPPI share ~75% amino acid identity. We examined their ability to compensate for each other by introducing a transgene carrying the coding sequence of either gene driven by the *LOB* promoter into mutants of both genes. *tppj* mutants have no detectable mutant phenotype and the introduction of *pLOB:TPPI* or *pLOB:TPPJ* did not change the phenotype. However, the phenotype of *tppi* mutants, which are smaller than wild-type plants and have decurrant strands, was suppressed by both the *pLOB:TPPI* and *pLOB:TPPJ* transgenes. These data suggest that TPPJ and TPPI can compensate for each other, at least in this context.

T6P is a signaling molecule that can be used to allocate carbon sources, such as sucrose, to accumulate in various cells. These carbon sources can be metabolized, and the resulting energy used for cell division. Thus, T6P is an important molecule for growth and hyperaccumulation of T6P at the boundary could cause an overgrowth, leading to organ fusions. Given that *Arabidopsis* plants carrying the *tppi* mutation produced organs that failed to separate from one another we, hypothesized that boundary cells have an

increased rate in cell division. Transcripts of *CyclinB1;1*, a G2/M marker, were not significantly altered in any mutants in both seedlings and inflorescences. This suggests that the potential misregulation of the cell cycle in the boundary cells is not dependent on *CyclinB1;1* or that the cell-cycle is not mis-regulated in these mutants. The relationship between T6P accumulation and boundary cell division remains elusive.

This chapter suggests that LOB regulates trehalose accumulation in the boundary as a secondary mechanism to restrict growth. Proper boundary formation requires that the cells of the boundary slow down or cease their rate of division. We hypothesize that LOB may positively regulate *TPPJ* to synthesize trehalose from T6P, a known sucrose signal, to stop the cells of the boundary from dividing. However, the TPP enzymes in this pathway may act redundantly, since we could not detect any mutant phenotypes in *tppj*. Further, double mutants of *lob; tppj* did not exhibit enhanced organ fusions. Interestingly, the *pLOB:TPPJ* transgene is able to fully rescue the *lob* mutant phenotype suggesting that TPPJ, and subsequently, trehalose accumulation are sufficient to restrict growth at the boundary. No evidence provided suggests that *TPPI* is under the same regulation by LOB. The *tppi* mutant does display organ fusion phenotypes, but they are different than the *lob* mutant phenotype. Decurrent strands form in *tppi* plants but are not seen in *lob* mutant plants. However, previous data from our lab has shown that *lof1; lof2* double mutants produce decurrent strands at the paraclade junction, similarly to *tppi* (Lee et, al. 2009). Both *LOF1* and *LOF2* are boundary expressed genes and do not interact with *LOB*. We do not yet know the targets of LOF1 and LOF2 and it would be interesting to

see if they regulate *TPP* genes at the boundary. Further experiments to show the detailed relationship between these genes are required.

Materials and Methods

Plant materials and growth conditions:

All mutant plants (*lob-3*, *tppi-1*, and *tppj-1*) and all transgenic plants (*35S:LOB:GR*, *XVE:TPPI*, *XVE:TPPJ*, *35S:TPPI*, *35S:TPPJ*, *pLOB:TPPI*, *pLOB:TPPJ,* and *pLOB:TPS1*) were in the Col-0 background. For plants grown on media, seeds were surface sterilized in 70% ethanol for two minutes, treated with 50% bleach for ten minutes, and rinsed three times with sterile water. Seeds were stratified in sterile water for three days at 4°C in the dark. The seeds were then sown onto Murashige and Skoog (MS) media (pH 5.7) without the addition of sucrose and placed in a growth chamber at 23°C, illuminated with white light at ~100 μM/m^2s in a 16-hour light/8-hour dark cycle. For plants grown on soil, seedlings were sterilized and sown to MS plates as described above and transplanted seven days after germination to Sunshine Mix #1 supplemented with Osmocote (14-14-14) and Marathon. The plants were illuminated with white light at ~100 μM/m^2s in a 16-hour light/8-hour dark cycle.

Generation of higher order mutants and transformation:

Crosses between *lob-3* and *tppi-1; tppj-1* parental lines were carried out to generate all double mutant combinations and the *lob-3; tppi-1; tppj-1* triple mutant. Crosses between *pLOB:TPPI* Col-0 and *tppj-1* or *tppi-1* were used for *tppi-1* suppression

and crosses between *pLOB:TPPJ* Col-0 and *tppj-1* or *tppi-1* were used for *tppj-1* suppression.

To create transgenic plants, *Arabidopsis* was grown in standard conditions as described above. Multiple plants were grown in 4-inch pots. All binary vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 using electroporation and *Arabidopsis* plants were transformed via floral dip (Clough and Bent 1998). Primary transformants (T1 generation) were selected with BASTA (Finale, AgrEvo); T2 single-locus transformants (identified by segregation ratios) and T3 homozygous transformants were selected on MS media supplemented with 50 μM phosphinothricin (Sigma). All other transformants were selected on MS media supplemented with 100 μM hygromycin B (Invitrogen).

For the *35S:LOB:GR* induction, dexamethasone was used at 5 μM concentration. For *XVE* seedling induction, β-estradiol (Sigma) was used at concentrations of 5, 10, 25, 50, and 100 μM.

Cloning:

The 1113 bp CDS of *TPPJ*, 1143 bp CDS of *TPPI*, and 2829 bp CDS of *TPS1* were all PCR amplified and introduced into *pENTR-D/TOPO* (Invitrogen) to create entry clones. To create *pLOB:TPPI*, *pLOB:TPPJ*, and *pLOB:TPS1*, all entry clones were linearized and recombined with the *pLOB:3'UTR-IGR* destination vector with the Gateway reaction using LR clonase. To create XVE expression clones, entry clones of *pENTR:TPPI* and *pENTR:TPPJ* were recombined with *pMDC7* with the Gateway

reaction using LR clonase, respectively. To create *35S:TPPI* and *35S:TPPJ*, linearized entry clones of *pENTR:TPPI* and *pENTR:TPPJ* were recombined with *pGWB2* with the Gateway reaction using LR clonase, respectively.

Transcript analysis:

Tissue from the seedling shoots, paraclade junctions, or inflorescences were collected, and flash frozen in liquid nitrogen. Total RNA was isolated using Trizol reagent following the manufacturer's protocol. 1 µg of RNA was used for cDNA synthesis and first-strand cDNA was synthesized using SuperScript IV following the manufacturer's instructions (Thermo Fisher). *ACT2* primers were used as an internal control for both RT-PCR and qRT-PCR. All other gene- and transgene-specific primers are listed in Table 3.1. All qRT-PCR experiments used SYBR green on a BioRad CFX Connect and data were analyzed using BioRad iQ5 software. Relative transcripts were calculated with the Pfaffl method (Pfaffl 2004) and significance was calculated with a one-way ANOVA.

Yeast-1-hybrid:

The 918-bp region upstream of the ATG in the *TPPI* promoter and the 980-bp region upstream of the ATG in the *TPPJ* promoter were PCR amplified with B1R and B4 Gateway adaptors. These products were cloned into pDONR-P4-P1R entry vector

(Invitrogen) using BP clonase per the manufacturer's instructions. These products were then cloned into the pMW #2 vector, placing them upstream of the HIS auxotrophic selectable marker, using the LR reaction. pMW #2-pTPPI and pMW #2-pTPPJ were transformed into yeast as described and checked for auto-activation (Deplancke et al. 2004). The LOB-AD and Empty-AD plasmids were transformed into yeast with a lithium acetate protocol. Independent colonies isolated and checked for self-activation on 20, 40, 60, 80, and 100 μM 3-AT. The colonies which had the lowest amount of self-activation were used for yeast-1-hybrid.

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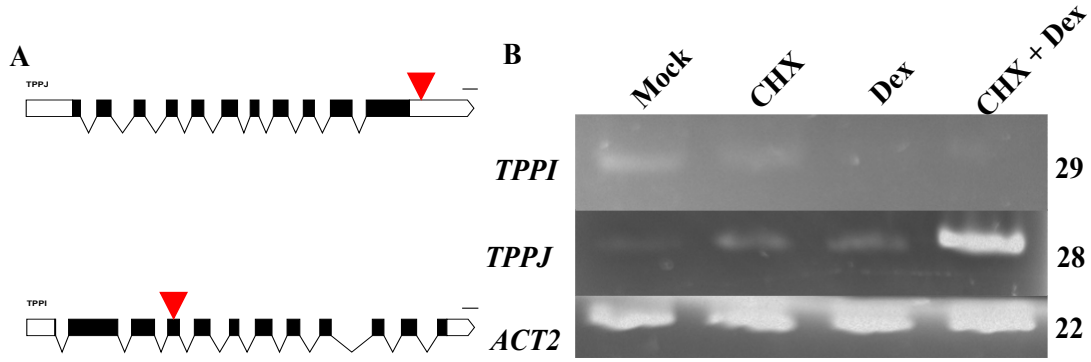


Figure 3.1 Transcript levels of *TPPI* and *TPPJ* after induction of LOB.
(A) Gene structures of *TPPJ* and *TPPI*. Black bars indicate exons, black lines indicate introns, and white boxes indicate UTRs. Red triangles indicate T-DNA insertions. (B) RT-PCRs of *TPPI* and *TPPJ* from *35S:LOB:GR* seedlings treated with mock, cycloheximide (CHX), dexamethasone (Dex), or CHX + Dex for 4 hours. 22 cycles were used for *ACT2* control and 28 cycles were used for *TPPI* and *TPPJ.*

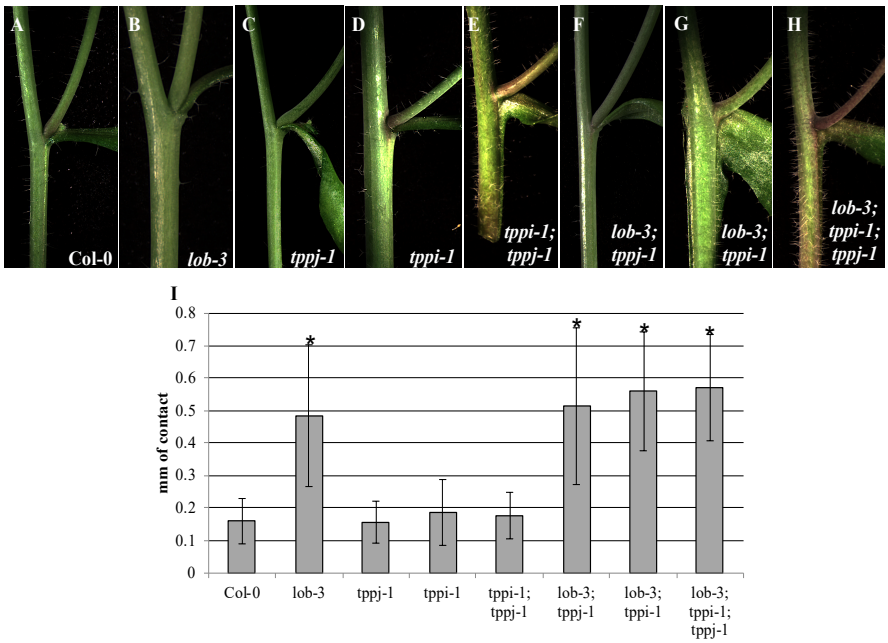
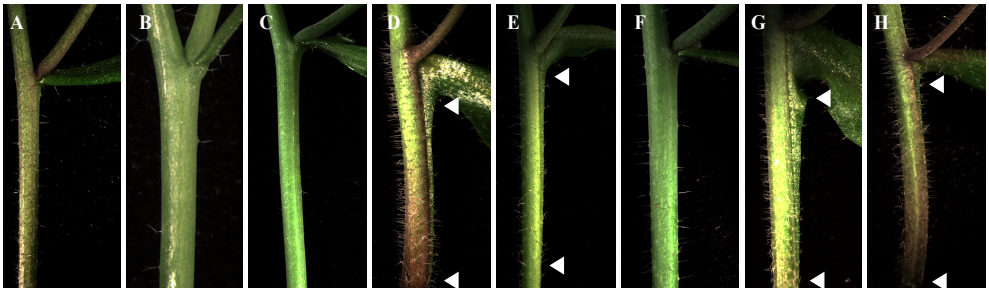


Figure 3.2 Higher order mutants between *LOB*, *TPPI*, and *TPPJ* do not have enhanced organ fusions. (A-H) Paraclade junctions of Col-0 (A), *lob-3* (B), *tppj-1* (C), *tppi-1* (D), *tppi-1; tppj-1* (E), *lob-3; tppj-1* (F), *lob-3; tppi-1* (G), and *lob-3; tppi-1; tppj-1* (H). (I) Quantification of the length of contact between the adaxial side of the cauline leaf to the axillary branch. All measurements were taken on the first paraclade junction after the first flower on the corresponding branch had opened. N= 15, * $p<0.05$, error bars indicate SD. Significance was calculated by ANOVA.



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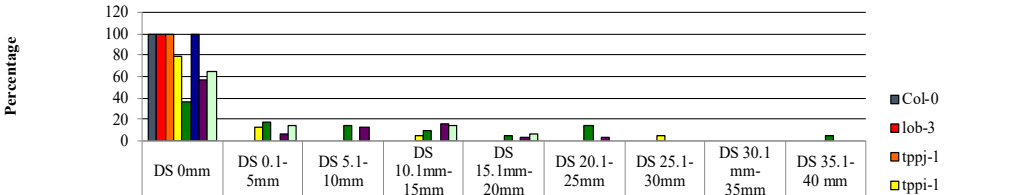

	DS 0mm	DS 0.1-5mm	DS 5.1-10mm	DS 10.1mm-15mm	DS 15.1mm-20mm	DS 20.1-25mm	DS 25.1-30mm	DS 30.1 mm-35mm	DS 35.1-40 mm
Col-0	100	0	0	0	0	0	0	0	0
lob-3	100	0	0	0	0	0	0	0	0
tppj-1	100	0	0	0	0	0	0	0	0
tppi-1	78.26	13.04	0	4.35	0	0	4.35	0	0
tppi-1; tppj-1	36.36	18.18	13.63	9.09	4.54	13.63	0	0	4.54
lob-3; tppj-1	100	0	0	0	0	0	0	0	0
lob-3; tppi-1	56.67	6.67	13.33	16.67	3.33	3.33	0	0	0
lob-3; tppi-1; tppj-1	64.28	14.28	0	14.28	7.142	0	0	0	0

Figure 3.3 Decurrent strands are only present in plants carrying the *tppi-1* mutation.
(A-H) Stems just below the first paraclade junctions of Col-0 (A), *lob-3* (B), *tppj-1* (C), *tppi-1* (D), *tppi-1; tppj-1* (E), *lob-3; tppj-1* (F), *lob-3; tppi-1* (G), and *lob-3; tppi-1; tppj-1* (H). (I) Quantification of the percentage of decurrent strands binned based on length. All measurements were taken on the first paraclade junction after the first flower on the corresponding branch had opened. N= 15.

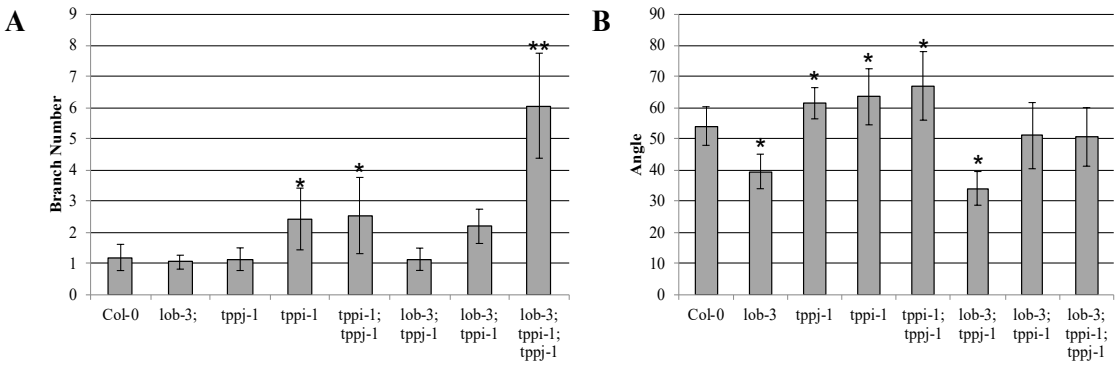

Figure 3.4 *lob-3; tppi-1; tppj-1* triple mutant plants have an increase in branch number but no change in branch angle.

(A) Quantification of the number of branches off the primary shoot. (B) Quantification of the branch angle of the first formed branch. All measurements were taken after the first flower opened on the first formed branch. N = 15, * = $p<0.05$, ** = $p<0.01$. Error bars indicate SD.

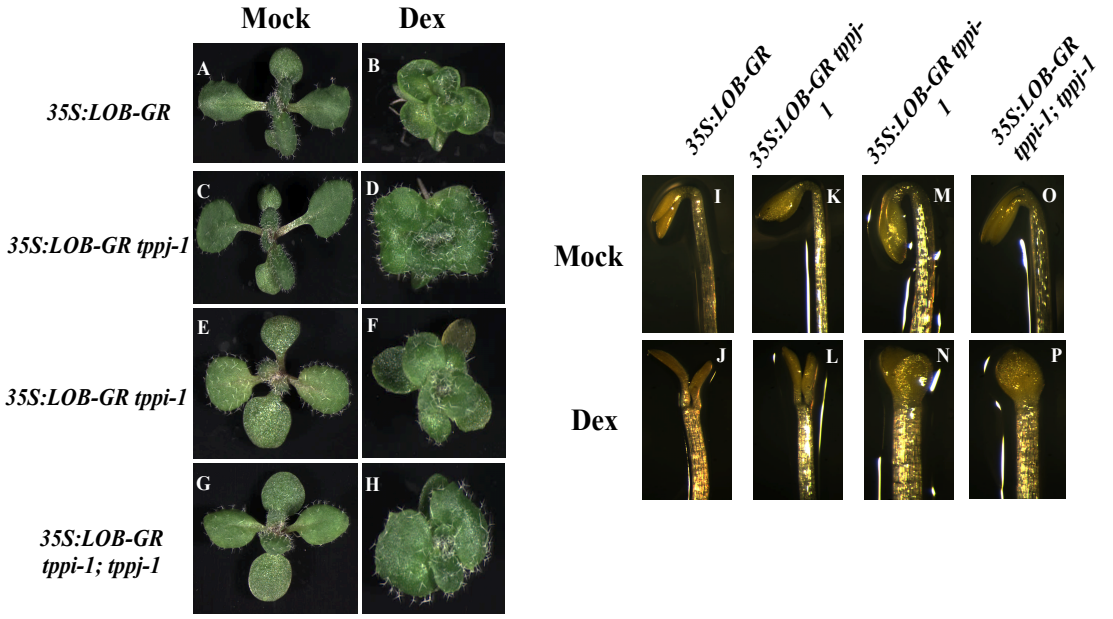

Figure 3.5 *TPPJ* and *TPPI* are not responsible for the LOB over-expression phenotype in light or dark grown seedlings.
(A-H) 10-day old, light grown seedlings on media lacking sucrose in the presence of a mock treatment (A,C,E,G) or 5 µM Dexamethasone (B,D,F,H). (I-O) 5-day old, dark grown seedlings on sucrose lacking MS media in the presence of a mock treatment (I,K,M,O) or 5 µM Dexamethasone (J,L,N,P).

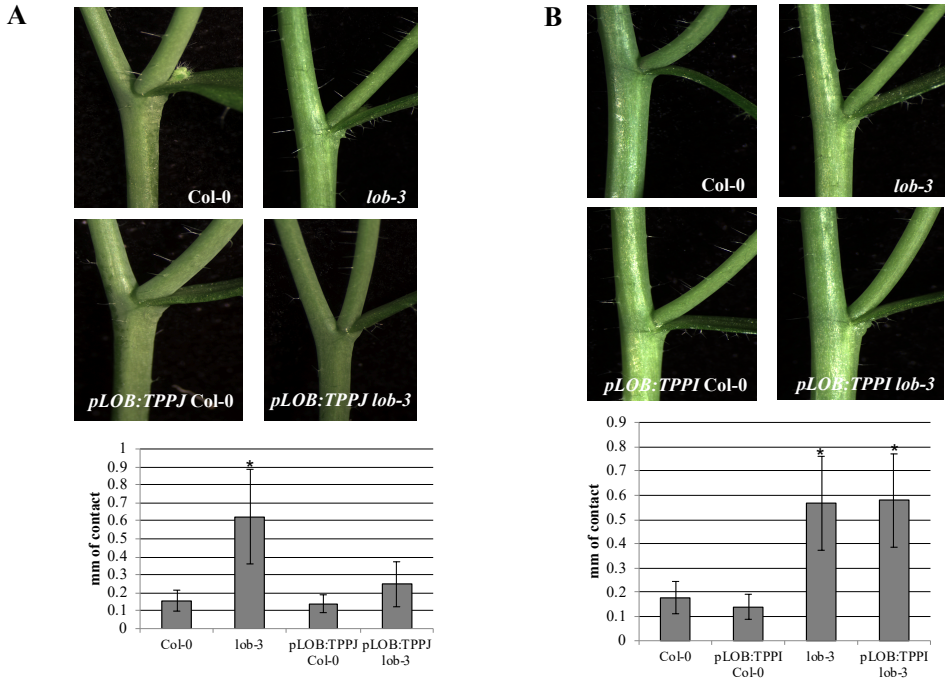

Figure 3.6 Accumulation of TPPJ, but not TPPI, in the boundary is sufficient to rescue the *lob* phenotype. (A) Paraclade junctions of Col-0, *lob-3*, *pLOB:TPPJ* Col-0, *pLOB:TPPJ lob-3* grown side by side. (B) Paraclade junctions of Col-0, *lob-3*, *pLOB:TPPI* Col-0, *pLOB:TPPI lob-3* grown side by side.
(C) Quantification of the millimeters of contact between adaxial side of cauline leaf and abaxial side of the axillary branch. All measurements were taken on the first paraclade junction after the first flower on the corresponding branch had opened. N= 10, * =p<0.05, error bars indicate SD. Significance was calculated by ANOVA.

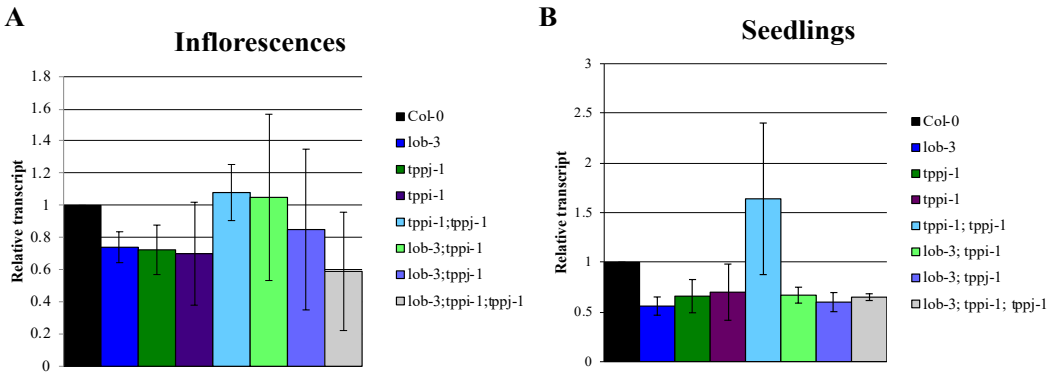

Figure 3.7 *CyclinB1;1* transcripts are not changed in inflorescences or seedlings in higher order mutants.
Relative transcript levels of *CyclinB1; 1* in inflorescences (A) and seedlings (B). Data represents the average of 2 biological replicates. Significance was calculated with ANOVA.

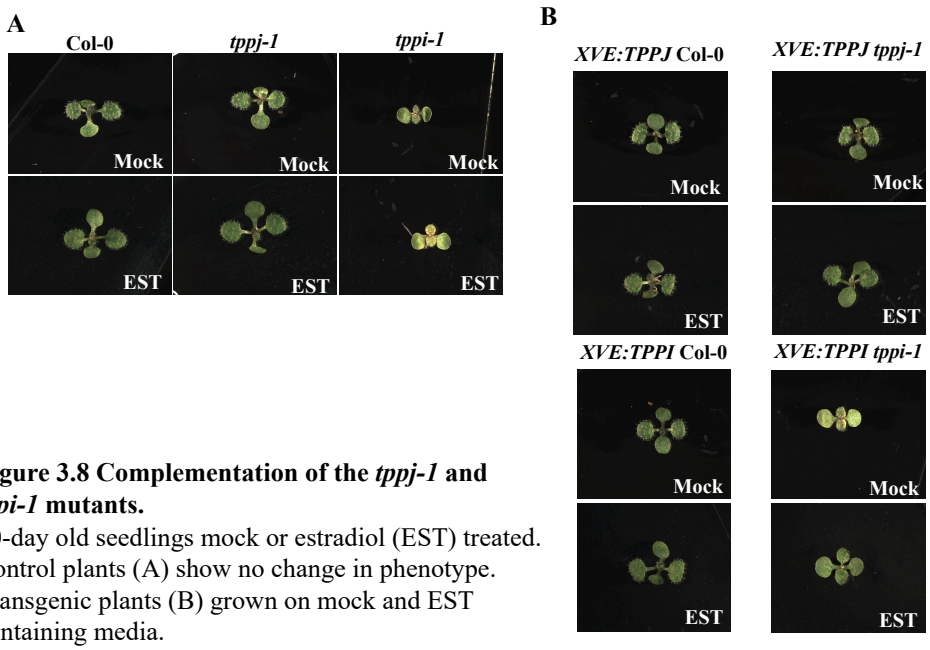

Figure 3.8 Complementation of the *tppj-1* and *tppi-1* mutants.
10-day old seedlings mock or estradiol (EST) treated. Control plants (A) show no change in phenotype. Transgenic plants (B) grown on mock and EST containing media.

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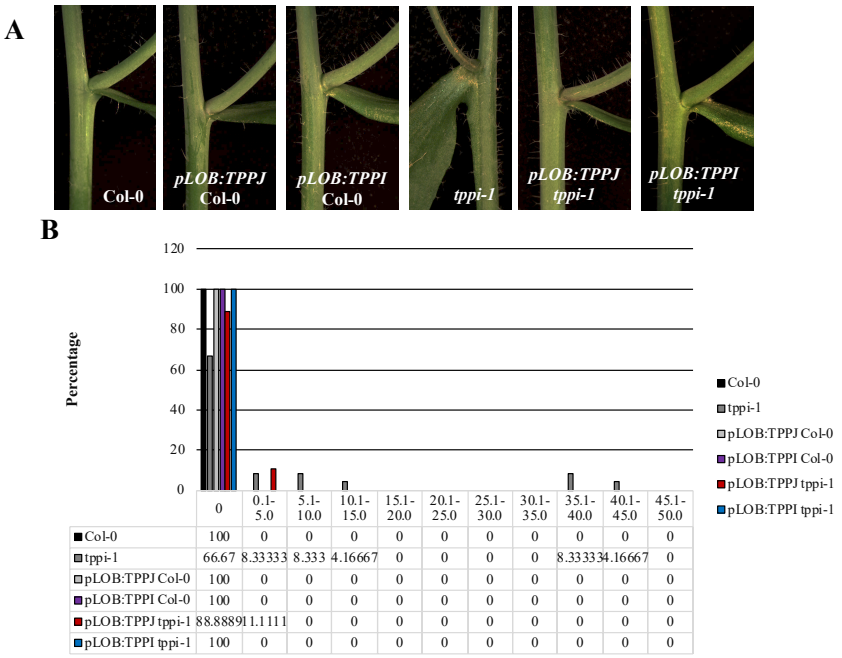

	0	0.1-5.0	5.1-10.0	10.1-15.0	15.1-20.0	20.1-25.0	25.1-30.0	30.1-35.0	35.1-40.0	40.1-45.0	45.1-50.0
Col-0	100	0	0	0	0	0	0	0	0	0	0
tppi-1	66.67	8.33333	8.333	4.16667	0	0	0	0	8.33333	4.16667	0
pLOB:TPPJ Col-0	100	0	0	0	0	0	0	0	0	0	0
pLOB:TPPI Col-0	100	0	0	0	0	0	0	0	0	0	0
pLOB:TPPJ tppi-1	88.8889	11.111	0	0	0	0	0	0	0	0	0
pLOB:TPPI tppi-1	100	0	0	0	0	0	0	0	0	0	0

Figure 3.9 *tppi-1* mutants are rescued by TPPJ or TPPI accumulation in the boundary. (A) Paraclade junction phenotypes of Col-0, *tppi-1*, *pLOB:TPPJ* Col-0, *pLOB:TPPI* Col-0, *pLOB:TPPJ tppi-1*, *pLOB:TPPI tppi-1*. (B) Quantification of the percentage of decurrant strands binned based on length. All measurements were taken on the first paraclade junction after the first flower on the corresponding branch had opened. N= 16.

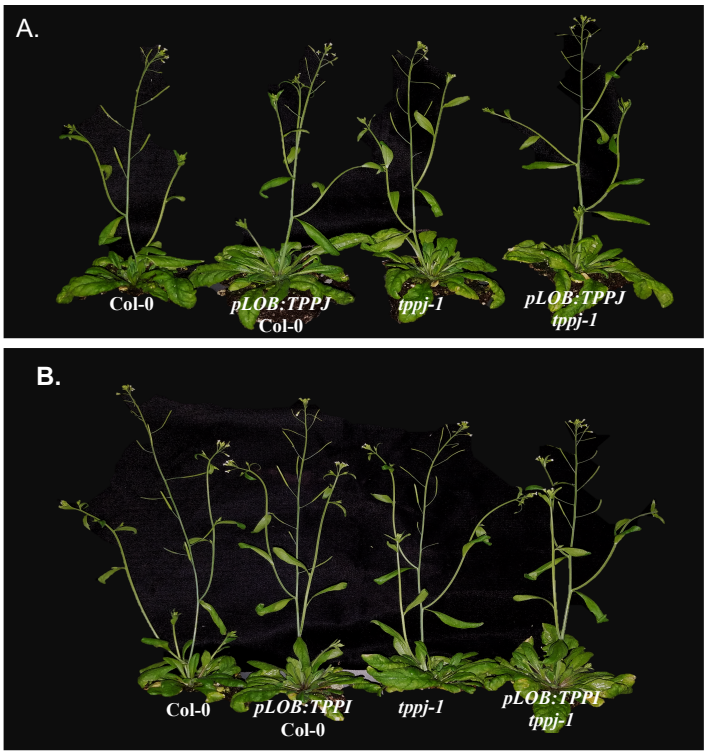

Figure 3.10 *tppj-1* mutants have no change in phenotype when complemented with *pLOB:TPPJ* or *pLOB:TPPI*.
Plants containing *pLOB:TPPJ* (A) or *pLOB:TPPI* (B) and controls at 4-weeks-old.

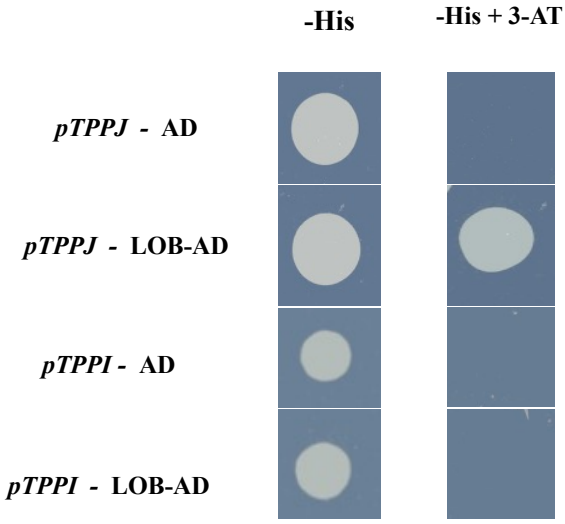

Figure 3.11 LOB binds to the promoter of *TPPJ*, but not *TPPI in vivo*.
Yeast-1-hybrid shows that LOB binds the promoter region of *TPPJ* on selective media in the presence of 20mM 3-AT. LOB does not bind to *TPPI* in the presence of 3-AT.

Table 3.1

Primer name	Sequence 5' -> 3'	Tm (°C)
LOB-RKF	CCACACACAGTCCATGCATTA	55.3
LOB-RKR	GCGTCGTCATCAAACTCATA	52.6
TPPI-1 520 INTRON FW	TTAATCTGTTTTGACTTGATCATATTTTG	51.4
TPPI-1 1101 INTRON RV	AATCAATACTCAACTCATATGTACGACAA	54.5
tppj-1 FW PRIMER 2086	ATAAATTAGCTACTACGAGGGAGAGGA	56.3
tppj-1 RV PRIMER 2572	AATATATCCCCAAATCGATAAAAGTTC	51.7
LBa1	TGGTTCACGTAGTGGGCCATCG	73
LBb1.3 SALK	ATTTTGCCGATTTCGGAAC	51.5
LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC	68
RK TPPI RTF	TGACCCAAGGTCGGAAGGTTT	58.8
RK TPPJ RTF	TTCGGTCGGTGGTAAAGAACTATCC	58.2
attB2 R	CCACTTTGTACAAGAAAGCTGGGT	57.5
TPS1 entry clone F	CACCATGCCTGGAAATAAGTA	52.5
TPS1 entry clone 3'UTR	CACTTGACACTGTCTCGGGATA	56.1
ACT2-N	AAAATGGCCGATGGTGAGG	66.9
ACT2-C	ACTCACCACCACGAACCAG	63.8
MS TPPJ RT F	TCATAACGGTCTCTACAG	47.6
MS TPPJ RT R	GATGATTCGTTCGAACATGTTA	51
MS qTPPI F	GAAGAAATGGAGCGAACTGG	53.8
MS qTPPI R	CGTCACCAATATAAACCGGG	53.3
qCyclinB1;1 Tsu F	CCGGAACTGAATCTGCTTAGG	55.4
qCyclinB1;1 Tsu R	GCGACTCATTAGACTTGTTCA	52.5
TPPI attb4 F	GGGGACAACTTTGTATAGAAAAGGTGGAAGTCGCTTTTAGGTCTAATAG	64.5
TPPI attB1 R	GGGGACTGCTTTTTTGTACAAACTTGTATCTGTGGTTTTTCCACGACAA	66.4
TPPJ attB4 F	GGGGACAACTTTGTATAGAAAAGGTGGAGAGCGTGATGGTTTTATTAC	65
TPPJ attB1 R	GGGGACTGCTTTTTTGTACAAACTTGCTATAAAAACAGGGGATGAGCA	66.4

Conclusion

Leaves are formed at the periphery of the shoot apical meristem (SAM). The undifferentiated cells of the SAM slowly divide within the central zone and eventually populate the peripheral zone. These cells will switch fate and begin dividing to form the primordia of lateral organs (Gallois et al. 2002). Between the central zone of the SAM and the newly forming lateral organ, a small group of cells called the boundary region reside (Hussey 1971). These cells are unique from their neighbors given that they are smaller and divide more infrequently (Hussey 1971). The boundary region has roles in regulating organ separation, forming axillary meristems, and specifying leaf angle (Greb et al. 2003; Lee, Geisler, and Springer 2009; Lewis et al. 2014). Leaf angle is an aspect of plant architecture that can influence plant productivity (Truong et al. 2015). Plants with leaves that are more erect relative to the ground have an equal distribution of light capture, which prevents the younger leaves from becoming too hot and the older leaves from prematurely senescing (Truong et al. 2015). Further, erect leaf angles allow for high-density planting.

In *Arabidopsis,* several genes are expressed in the boundary to regulate the formation of the boundary region and facilitate organ separation (Lee, Geisler, and Springer 2009; Bell et al. 2012). One gene, *LATERAL ORGAN BOUNDARIES* (*LOB*), encodes a transcription factor that is expressed at the base of the floral organs, lateral roots, and at the paraclade junction (Shuai, Reynaga-Peña, and Springer 2002; Husbands et al. 2007). Plants with mutations in *LOB* exhibit a subtle phenotype at the paraclade junction where the cauline leaf fails to separate from the axillary branch (Bell et al.

2012). *LOB* is the founding member of the LOB-domain family of transcription factors (Shuai, Reynaga-Peña, and Springer 2002). This is a 43-member, plant-specific family that has both defined and undefined functions. LOB has been shown to physically interact with DNA sequences carrying the 5'-(G)CGGC(G)-3' motif where the flanking G's are variable (Husbands et al. 2007). Further, LOB directly regulates a brassinosteroid (BR) catabolism enzyme, *PHYB ACTIVATION TAGGED SUPPRESSOR1* (*BAS1*), to modulate the accumulation of BR in the boundary region (Bell et al. 2012).

In this dissertation, I characterized the *LOB* ortholog in cowpea, a warm-season legume that utilizes a pulvinus to incline its leaves under water-deficit conditions (Shackel and Hall 1979). This response is thought to decrease the temperature of the leaves, which ultimately reduces the rate of transpiration. We hypothesize that abscisic acid (ABA) is partially responsible for the movement of the pulvinus, given that ABA is induced in response to drought and has been shown to contract motor cells in protoplasts of *Phaseolus* pulvini (Sobeih et al. 2004; Iino, Long, and Wang 2001). Furthermore, we showed that BR accumulation is required for leaf inclination in cowpea. Treatment with the BR biosynthesis inhibitor propiconazole (PCZ) resulted in cowpea seedlings being unable to incline their leaves after water-deficit. BR-deficient plants formed pulvini that lacked the stereotypical convolutions in the epidermal cells and failed to incline. Additionally, leaves treated with PCZ after normal development failed to incline after water-deficit. What roles do BRs have in the epidermis and motor cells of the pulvinus? We hypothesize that BRs may control microtubule orientation in the epidermis to produce the necessary convolutions for function or that BR signaling is required for

proper vacuole formation. In the *Arabidopsis* hypocotyl brassinosteroids mediate the stability and orientation of microtubules (Wang et al. 2012). BR-deficient plants fail to elongate their hypocotyls during skotomorphogenesis (Wang et al. 2012). BRs have also been shown to regulate microtubule organization in the pavement cells of *Arabidopsis* (Liu et al. 2018). Plants that are unable to synthesize or perceive BR have abnormal pavement cell shapes and altered organization of the microtubules (Liu et al. 2018) The relationship between BRs and microtubule organization may provide evidence for the role BRs play in shaping the epidermal cells of the pulvinus. In the motor cells, BR signaling may be required for the proper size of the vacuole. The root meristematic cells of *Arabidopsis* treated with BL increase the size of the vacuole while the vacuole is smaller when treated with a BR biosynthesis inhibitor (Yamagami et al. 2019). BR signaling at the vacuole membrane may be required for its expansion in the motor cells of the pulvinus. Without this signaling, it is possible that the motor cells won't be able to expand and contract.

Previous reports demonstrate that *LOB* functions to restrict growth in the boundary region (Bell et al. 2012). BR promotes cell division and elongation, and LOB positively regulates *BAS1,* which catabolizes BR in the boundary to restrict growth. Functional analysis of the *LOB* ortholog in cowpea, *VuLOB* (Vigun09g09130), does not provide evidence that it has a conserved function with *LOB*, as *Arabidopsis* plants overexpressing *VuLOB* do not phenocopy plants overexpressing *LOB*. This suggest that these two genes have diverged in function. How might *VuLOB* regulate the formation of the boundary? One possibility is that it may regulate genes involved in cell wall

modifications. From a microarray dataset, *LOB* was shown to regulate several genes that modify the cell wall (Bell et al. 2012). However, these genes have not been further tested for a specific role in boundary formation.

LOB has been shown to restrict growth in the boundary and in some legumes is required for pulvinus formation (Bell et al. 2012; Chen et al. 2012). However, in chapter 2, I show that *VuLOB* does not associate with variation in pulvinus size or leaflet angle after water-deficit. *VuLOB* may only be responsible for the formation of the pulvinus, and not necessarily the variation in the size or function. Using Genome-Wide Association Studies (GWAS) I identified several significant genomic regions that associate with pulvinus shape (length:width ratio). SNPs associated with a LOB-domain transcription factor, *VuLBD41* (Vigun05g006100) were identified in one of these regions. Based on the LIS gene expression data, *VuLBD41* is highly expressed in the roots and flowers and has low levels of transcripts in the leaves. However, I have shown that transcripts of VuLBD41 accumulate at high levels in the pulvini. When overexpressed in *Arabidopsis*, no obvious change in phenotype was observed. One possible reason for the lack of an overexpression phenotype is that VuLBD41 may require a cofactor to regulate target genes. A second possibility is that VuLBD41 has post-translational modifications that either target it for protein degradation when expressed in a heterologous system or prevent it from binding DNA. It would be useful to knock out *VuLBD41* orthologs in model legumes to examine the impact on development of the pulvinus. Furthermore, in *Arabidopsis*, *LBD41* is a hypoxia-response gene (Liu et al. 2005; Lee and Bailey-Serres 2019). It would be interesting to determine if 35S:*VuLBD41* exhibits an altered

phenotype under stress conditions. Plants alter their leaf angle under submergence and heat stress and *VuLBD41* could be the link between leaf angle and stress in cowpea (van Zanten et al. 2010).

How does LOB regulate the accumulation of metabolites to form the boundary? LOB has been shown to regulate a suite of genes involved in hormone biosynthesis and signaling in the boundary (Husbands et al. 2007; Bell et al. 2012). In chapter 3, I show that LOB binds the *TPPJ* promoter in yeast. *TPPJ* encodes a trehalose-6-phosphate phosphatase, which is involved in the last step of trehalose biosynthesis. Trehalose-6-phosphate (T6P) is dephosphorylated by TPP enzymes to produce trehalose. T6P metabolism results in allocation of sucrose and has a role in plant development and stress responses (Paul et al. 2010; Yadav et al. 2014; Fichtner et al. 2017). Given that T6P promotes growth, it is possible that LOB regulates *TPPJ* to prevent T6P from accumulating in the boundary. It is likely that this is one mechanism by which LOB restricts growth in the boundary.

To further understand pulvinus development, leaf angle, and boundary formation, I propose that the following experiments be carried out:

1. Explain the *VuLOB* overexpression phenotype. *LOB* regulates a suite of genes involved in brassinosteroid responses, cell-wall modifying enzymes, blue light signaling, and trehalose biosynthesis (Bell et al. 2012). However, *Arabidopsis* plants overexpressing *VuLOB* do not phenocopy the *35S:LOB* transgenic plants. I propose to use RNA-seq to explore the genes that *VuLOB* regulates in *Arabidopsis*.

2. Explore what genes are differentially expressed in the cowpea pulvinus. The pulvinus requires many ion transporters and aquaporins in order to function (Cote 1995). Often, these genes are regulated in a circadian fashion (Oikawa et al. 2018). It would be interesting to use RNA-seq to look at differentially regulated transcripts in pulvini of inclined leaves vs flat leaves to see which transcription factors regulate these ion channels and aquaporins after water-deficit.

3. Understand how *VuLBD41* functions in pulvinus development. Cowpea has been recalcitrant to transformation using previous methods, but using either a model legume system or an improved method for cowpea transformation could be used to study gene function (Bett et al. 2019; Che et al. 2019). Advancements in genome editing have allowed us to be more precise in engineering plant genomes (Ding et al. 2016). A future approach to assess *LBD41* gene function is to create a knock-out mutant of an ortholog of *LBD41* in a model legume such as *Medicago truncatula* or cowpea.

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