UNIVERSITY OF CALIFORNIA
RIVERSIDE

Molecular Characterization of *LATERAL ORGAN BOUNDARIES (LOB)* in *Arabidopsis thaliana*

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

Doctor of Philosophy

in

Plant Biology

by

Robert Aaron Koble

August 2015

Dissertation Committee:
Dr. Patricia Springer, Chairperson
Dr. Linda Walling
Dr. Thomas Eulgem

The Dissertation of Robert Aaron Koble is approved:

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Committee Chairperson

University of California, Riverside

Acknowledgements

It is with my upmost gratitude to Dr. Patricia Springer for allowing me to work in her laboratory for the past 6 years. She has been a great mentor providing guidance and expertise in all aspects of my scientific career. I also thank my Dissertation Committee, Dr. Linda Walling and Dr. Thomas Eulgem for invaluable help in completing my dissertation. Also, I want to thank Dr. John Harada for giving me the opportunity to perform undergraduate research in his laboratory and inspiring me to pursue a PhD in Plant Biology. Like many undergraduate students in the sciences, I was pre-med, but after experiencing the awesome research on plants, I decided to switch to study Plant Biology.

Thank you to Dr. Cris Kuhlemeier for allowing me to intern in his lab for two months at the University of Berne, Switzerland. This was an invaluable experience to learn micro-application of chemicals and to see how science is conducted in another part of the world.

Thank you to Dr. Christian Fankhauser for the *pks2-2* and *phot1-5* seed. Thank you to Dr. Sean Cutler for use of the LATCA library used in the chemical genetics study. Thank you to Dr. Yunde Zhao for the *npy1-1, npy1-2,* and *nph3-1* mutant seeds. Also, thank you to Dr. Zhenbiao Yang for teaching me the chemical genetics screening protocol. Thank you to Dr. Paul Larsen for assistance with the ethylene treatments.

Lastly, heartfelt thanks to all Springer Lab members past and present. Your work has been key for the experiments in this dissertation. From creating the constructs I used to laying down the foundation for avenues of exploration, I am truly grateful.

Dedication

For my friends and family.

Above all else: my rock, my partner in crime - my wife.

ABSTRACT OF THE DISSERTATION

Molecular Characterization of *LATERAL ORGAN BOUNDARIES (LOB)* in *Arabidopsis thaliana*

by

Robert Aaron Koble

Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, August 2015
Dr. Patricia S Springer, Chairperson

Leaves form at the periphery of the shoot apical meristem (SAM) and are separated from the SAM by a boundary region. Boundary cells are smaller and divide less frequently than the surrounding cells. The *Arabidopsis LATERAL ORGAN BOUNDARIES (LOB)* gene is expressed in all organ boundaries and functions to separate lateral organs. *LOB* encodes a transcription factor that differentially regulates the expression of target genes. This dissertation focuses on characterizing the molecular function of LOB in *Arabidopsis* and its role in the separation of lateral organs and responses to blue-light.

In Chapter 1, I demonstrate that LOB directly regulates the expression of genes involved in the blue-light hyponastic response, including *PHOTOTROPIN1 (PHOT1), PHYTOCHROME KINASE SUBSTRATE 2 (PKS2),* and *NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3)*. LOB directly binds to the DNA regions of *PHOT1* and *PKS2* in yeast and *in planta*. *lob* mutants have altered responses to blue-light illumination and *LOB* transcript is regulated by blue-light, likely through *PHOT1* and *PKS2.* This chapter suggests that the boundary region is an important player in the blue-light hyponastic response in *Arabidopsis.*

In Chapter 2, I show that LOB directly regulates the expression of *NAKED PINS IN YUC MUTANTS (NPY1)*. *NPY1* transcript is altered in plants with varying levels of LOB activity and LOB binds to the promoter region of *NPY1*. However, *npy1-1* mutants do not have altered responses to blue-light nor do they exhibit fusions of lateral organs. Further experiments are required to investigate the biological relevance of the regulation of *NPY1* by LOB.

Work in Chapter 3 shows that LOB interacts with HISTONE DEACETYLASE (HDT3) in yeast and in onion epidermal cells. Furthermore, *hdt3-2* mutants exhibit fusions of the axillary stem and cauline leaf and correlates with the decreased transcript level of *BAS1* compared to wild-type plants. A second allele, *hdt3-1* causes a more sensitive hyponastic response to short-term blue-light exposure. These data suggest that LOB interacts with HDT3 for the separation of lateral organs and for proper blue-light responses.

In Chapter 4, I report the results of a chemical genetics screen to identify chemicals that inhibit the *LOB* over-expression phenotype. One chemical, LAT24D02, was identified in this screen and selected for further analysis. Although LAT24D02 inhibited the *LOB* over-expression phenotype, it did not suppress the induction of a direct target of LOB, suggesting that it does not directly inhibit LOB function. Therefore, LAT24D02 affects another unidentified LOB-dependent pathway and further experiments are required to understand the mode of action of LAT24D02.

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Introduction

1. Structures and functions of the shoot apical meristem

Unlike animals, plants exhibit indeterminate growth, which allows for the continual generation of organs throughout their life. All post-embryonic organs originate from apical meristems. Plants have two apical meristems, the shoot apical meristem (SAM) and the root apical meristem (RAM) (Weigel and Jurgens 2002). The SAM is located at the aerial tip of the plant and functions to create lateral organs such as leaves (Braybrook and Kuhlemeier 2010). The SAM maintains a reservoir of pluripotent stem cells, which are located in a small region called the central zone. In the peripheral zone of the SAM, lateral organs emerge from founder cells. Daughter cells produced from the division of stem cells in the central zone replenish the cells in the peripheral zone that are lost to organ formation (Szymkowiak and Sussex 1996). The stem cell population is maintained by a negative feedback loop controlled by the *WUSCHEL (WUS)* gene, which encodes a non-cell autonomous transcription factor, and the CLAVATA1/3 (CLV1/3) receptor kinase-ligand complex (Clark et al. 1993; Clark et al. 1995; Clark et al. 1997; Fletcher et al. 1999; Brand et al. 2000; Perales and Reddy 2012). *Arabidopsis* plants carrying a mutated *WUS* gene fail to maintain stem cells. Conversely, *clv3* mutants have an enlarged meristem (Clark et al. 1995; Laux et al. 1996). This feedback model is explained as *WUS* positively regulating stem cell identity by activating *CLV3* expression, which in turn represses *WUS* activity. This mechanism allows for an equilibrium in the stem cell population throughout development, resulting in indeterminacy (Schuster et al.

2014). Plants that fail to maintain this feedback loop have abnormal meristems (Laux et al. 1996).

The *KNOX* family of homeodomain transcription factors functions to regulate SAM identity and maintenance (Barton and Poethig 1993; Jackson et al. 1994; Long et al. 1996; Kerstetter et al. 1997). KNOX proteins interact with BELL1-like transcription factors, which fine-tunes their binding affinity and cellular localization (Byrne et al. 2003; Smith and Hake 2003; Smith et al. 2004). The first evidence that the *KNOX* family of genes play a role in meristem identity came from mutants that over-express *KNOTTED1* in maize leaves, which have 'knots' of meristem-like tissue in the leaf (Bryan and Sass 1941; Freeling and Hake 1985; Hake et al. 1989; Vollbrecht et al. 1991; Smith et al. 1992; Greene et al. 1994). Plants with a non-functional copy of the *Arabidopsis thaliana* ortholog of *KNOTTED1, SHOOT MERISTEMLESS (STM),* fail to produce lateral organs due to consumption of the stem cells in the SAM (Endrizzi et al. 1996). Furthermore, *STM* is expressed specifically in the meristem and is down-regulated in the initiating leaf primordia (Jackson et al. 1994; Long et al. 1996). At a mechanistic level, *KNOX* genes repress the expression of leaf-promoting genes in the SAM (Hay and Tsiantis 2010).

In the initiating lateral organ, ASYMMETRIC LEAVES1 (AS1) and AS2 physically interact to form a repressive complex that binds to the DNA of several *KNOTTED-LIKE HOMEOBOX* family genes to negatively regulate their expression (Byrne et al. 2000; Ori et al. 2000; Semiarti et al. 2001; Xu et al. 2003; Phelps-Durr et al. 2005). Furthermore, *Arabidopsis as1* and *as2* single mutants resemble plants over-

expressing *STM* (Tsukaya and Uchimiya 1997; Byrne et al. 2000; Ori et al. 2000; Semiarti et al. 2001; Sun et al. 2002). However, in *as1* and *as2* mutants, *KNOX* gene expression is down-regulated in the initiating primordia, therefore AS1/AS2 function to maintain repression of *KNOX* genes in the leaf, but are not responsible for the initial down-regulation.

All aerial lateral organs, such as leaves, branches, and flowers, are produced at the flanks of the SAM (Braybrook and Kuhlemeier 2010). A major contributor to the initiation of lateral organs is auxin, which is necessary and sufficient for lateral organ growth (Reinhardt et al. 2000; Vanneste and Friml 2009). *Arabidopsis* plants deficient in auxin transport caused either by chemical application of 1-N-naphthylphthalamic acid (NPA), an inhibitor of auxin transport, or by mutations in the auxin transport protein PINFORMED1 (PIN1), fail to form inflorescences (Okada et al. 1991; Serrano-Cartagena et al. 1999; Scanlon 2003). However, lateral organs initiate from the flank of the meristem when exogenous auxin is applied to these plants (Reinhardt et al. 2000).

Auxin is transported to the site of organogenesis by a trans-membrane protein called PIN1. PIN1 is polarly localized to the plasma membrane towards the direction of auxin flow to the site of leaf primordia. Auxin response is higher at the site of initiating primordia compared to the surrounding cells (Reinhardt et al. 2003; de Reuille et al. 2006; Jonsson et al. 2006; Smith and Bayer 2009). Once a lateral organ primordium develops, a boundary region forms that physically separates the lateral organ from the SAM (Callos and Medford 1994).

2. Characteristics and function of the boundary region

The cells of the boundary region have three unique characteristics. They grow much slower, divide less frequently, and are smaller than the surrounding cells (Callos et al. 1994; Gaudin et al. 2000). Tomato plants treated with the mitosis inhibitor colchicine exhibit fewer cells arrested in mitosis compared to the cells in the SAM and primordia, suggesting that the rate of cell division is lower in the boundary compared to the surrounding cells (Hussey, 1971). Due to its morphological location and function, the boundary region has a distinct and important role during lateral organ development. The primary function of the meristem-organ boundary is to physically separate the meristem from lateral organs. Plants defective in boundary formation fail to separate the lateral organs from the SAM. Furthermore, the boundary is important for maintaining meristem identity (Rast and Simon 2008). Axillary buds are formed at the boundary between leaves and the SAM. Plants that harbor mutations in boundary expressed genes have fewer axillary stems demonstrating that the boundary is important for axillary stem formation (Greb et al. 2003).

Although a boundary separates lateral organs from the SAM, communication between these two organs persists. This communication is mediated by the boundary as meristem-derived signals are passed through the boundary region to organ primordia to control adaxial cell fate (Sussex 1954; McConnell and Barton 1998; Bowman et al. 2002). A second example is the mechanism of the florigen FLOWERING LOCUS T (FT). FT is produced in the leaf under long day conditions and is transported through the boundary region to the meristem where it interacts with a transcription factor to activate

transcription of meristem-identify genes (Abe et al. 2005; Notaguchi et al. 2008; Wigge et al. 2005).

3. Genes involved in boundary function

Much research in the past several decades has attempted to understand the mechanisms involved in boundary function including gene identification. Several genes are expressed in the boundary and function to separate lateral organs from the SAM and/or to maintain the meristem.

3a. *CUP-SHAPED COTYLEDON (CUC)*

The *CUC* family is composed of three members, *CUC1, CUC2,* and *CUC3* that are expressed in the lateral organ-SAM boundary throughout development (Aida et al. 1999; Aida and Tasaka 2006). These genes encode transcription factors (TFs) and are a part of the *NAC* (*NO APICAL MERISTEM/ATAF/CUP SHAPED COTYLEDON)* gene family. CUC TFs activate *STM* expression in the embryo (Aida et al. 1997). The CUC TFs act redundantly as *cuc1* and *cuc2* single mutants have very subtle phenotypes, however *cuc1cuc2* double mutants do not produce shoots. Furthermore, CUC activity is required for cotyledon boundary formation because *cuc1cuc2* double mutants have fusions of the cotyledons suggesting a lack of separation at this boundary (Aida et al. 1997). *CUC3* is also involved in cotyledon boundary formation (Vroemen et al. 2003; Hibara et al. 2006) as introduction of a *cuc3* mutation into a *cuc1, cuc2,* or *cuc1 cuc2* background resulted in higher rates of plants with fused cotyledons (Vroemen et al. 2003;

Hibara et al. 2006). These observations suggest redundancy in the CUC family of transcription factors and indicate that they function in SAM maintenance and boundary formation.

CUC gene expression is post-transcriptionally regulated by *microRNA 164* (*miR164*). *miR164* is encoded by three gene members *miR164a-c* (Laufs et al. 2004; Sieber et al. 2007). Over-expression of *miR164* results in lower levels of *CUC1/2* transcript but does not affect *CUC3.* Furthermore, *miR164* over-expression plants resemble *cuc1cuc2* mutants, having fused cotyledons, fused sepal margins, and fused petioles (Laufs et al. 2004). Lastly, plants with a version of *CUC2* resistant to *miR164* regulation have larger boundaries than wild-type plants (Laufs et al. 2004). These results show that *CUC* genes are controlled by *miR164* for proper boundary function throughout development.

The *CUC* gene family is highly conserved in several plants including petunia, *Medicago,* and tomato. In petunia, *NO APICAL MERISTEM (NAM)* is required for proper meristem and floral organ formation. *nam* plants have pleiotropic defects including arrested growth of the SAM, fused cotyledons, fused anthers to petals, and extra-antheroid tissue in-between the stamens and carpels. *NAM* is expressed at the boundary of the SAM and cotyledons as well as the base of the stamen primordia (Souer et al. 1996).

The *Medicago truncatula NO APICAL MERISTEM* (*MtNAM)* gene is involved in SAM maintenance, cotyledon and leaflet separation, and floral organ formation. Mutant *mtnam* plants exhibit fused leaflets as well as a decreased number of floral organs and fusions of the petals and stamens. *MtNAM* is expressed at the boundary of the embryonic

meristem and cotyledon as well as leaflet primordia and the boundaries of leaflets (Cheng et al. 2012). Given that *mtnam* plants have fused leaflets and that *MtNAM* is expressed at the boundary of leaflets, boundary expressed genes play an important role in complex leaf development. For example, *CUC* genes are expressed during leaflet formation in several compound leaf species and plants with lower levels of *CUC3* transcript have smoother leaflet margins as well as fused leaflets compared to control plants. Lastly, no *CUC3* expression was observed in the simple leaves of *S. lycopersicum lanceolate* mutants, suggesting that boundary expressed genes are required for compound leaf development (Blein et al. 2008).

GOBLET (*GOB*) is the tomato *CUC2* ortholog and is expressed at the boundary of the SAM and lateral organ primordia, as well as the flanks of initiating leaflets. Wild-type tomato plants have leaves with primary, secondary, and intercalary leaflets and lobed leaflet margins. However, tomato plants carrying a mutation in the *gob* gene have smooth leaflet margins and fused leaflets (Berger et al. 2009). Interestingly, micro-RNA control of *CUC2* is conserved in tomato. Plants containing a *miRNA* resistant version of *GOB (Gob-4d)* have leaflets with a higher degree of lobing compared to wild-type. Furthermore, tomato plants with strong constitutive over-expression of *miR164* have fewer leaflets and resemble *gob* mutants. Lastly, plants that over-express *miR164* specifically in lateral organs lack secondary leaflets and have smoother margins (Berger et al. 2009). Taken together, *GOBLET* is necessary for proper leaflet formation and compound leaf development.

3b. *LATERAL SUPPRESSOR (LAS/LS)*

LATERAL SUPPRESSOR (LS) was first described in 1964 as a tomato mutant (Malayer and Guard 1964). Tomato plants lacking functional LS protein failed to form axillary meristems (Schumacher et al. 1999). *LS* encodes a GRAS-domain transcription factor. In 2003, it was shown that the *Arabidopsis LS* ortholog (*LAS*) is also is involved in axillary meristem formation (Greb et al. 2003). *Arabidopsis* plants that lack functional LAS protein fail to form rosette axillary shoots and have axillary stem fusions to the primary stem. Unlike tomato plants with defective LS protein, *Arabidopsis las-4* mutants have petals, although abscission of these floral organs is delayed (Greb et al. 2003). *LAS* is expressed on the adaxial side of leaf primordia corresponding with the future site of the axillary meristem. Interestingly, *LAS* has an overlapping role with the *CUC* family of genes. The *las* mutation enhances the *cuc* mutant phenotype as *las cuc2* and *las cuc3* double mutants had fewer tertiary shoots than *las* single mutants and frequently have rosette leaf fusions (Hibara et al. 2006).

Additionally, LAS regulates *STM* expression for proper axillary meristem development. *STM* expression is an early marker for axillary meristem development (Long and Barton 2000). In the axils of wild-type plants, *STM* expression is localized to a small group of cells on the adaxial side of the leaf-meristem boundary. However, in *las-4* plants, the expression of *STM* in the cells is absent (Greb et al. 2003). Taken together, *LAS* functions to promote axillary shoot development through regulation of *STM* expression in the adaxial leaf boundary.

3c. *REGULATOR OF AXILLARY MERISTEM1 (RAX1)*

REGULATOR OF AXILLARY MERISTEM1 (RAX1) is the putative *Arabidopsis* ortholog of the tomato R2R3 MYB transcription factor BLIND, which functions to control axillary meristem formation (Schmitz et al. 2002). There are three members of the *RAX* family in *Arabidopsis* and these genes are involved in regulating axillary meristem formation (Müller et al. 2006). Dominant-negative *rax1-1D* mutants have fewer axillary stems after flowering, suggesting *RAX1* is involved in axillary meristem formation (Keller et al. 2006). *RAX1* is expressed in the axils of young leaf primordia, marking the future site of axillary meristem formation (Keller et al. 2006; Müller et al. 2006). RAX1 acts through *CUC* genes, as *CUC2* transcript is lower in *rax1-1D* mutants and *cuc* mutations enhanced the *rax1* branching phenotype (Keller et al. 2006). More recently, it has been shown that RAX1 functions in concert with a bHLH transcription factor, REGULATOR OF AXILLARY MERISTEM FORMATION (ROX). *rax1 rox* double mutants have fewer axillary stems than *rax1* single mutants and the expression patterns of *RAX1* and *ROX1* overlap. Lastly, *ROX* expression is controlled by RAX as the transcript level of *ROX* is lower in *rax* mutant plants (Yang et al. 2012).

3d. *LATERAL ORGAN FUSION 1/LATERAL ORGAN FUSION 2 (LOF1/2)*

LATERAL ORGAN FUSION 1 (LOF1) encodes a MYB transcription factor and is expressed on the adaxial side of the meristem-leaf boundary, paraclade junction (inflorescence node containing axillary stem and cauline leaf), and the base of floral organs (Lee et al. 2009). *Arabidopsis* plants harboring a mutation in the *LOF1* gene have

a fusion of the cauline leaf and axillary stem, suggesting that *LOF1* functions to separate these organs. Furthermore, non-epidermal cells in the fused region are enlarged and misaligned in *lof1* plants, suggesting *LOF1* is involved in regulating cell size in the boundary (Lee et al. 2009). *LATERAL ORGAN FUSION 2 (LOF2)* functions with *LOF1* as introduction of the *lof2* mutation enhances the *lof1* phenotype. *LOF1* also functions in concert with *CUC2* and *CUC3*, but not *CUC1*. *lof1 cuc2* and *lof1 cuc3* double mutants exhibit pedicel-stem fusions, as well as additional fusions between the axillary stem and primary stem, which are absent in *lof1, cuc2,* and *cuc3* single mutants (Lee et al. 2009). However, no pedicel-stem fusions were observed in *lob1 cuc1* double mutants (Lee et al. 2009). These data suggest that *LOF1* functions together with the *CUC* genes to separate lateral organs and axillary meristem formation.

Interestingly, the *LOF1* ortholog in tomato, *TRIFOLIATE (TF)*, has been implicated in leaflet and axillary meristem formation. *tf* tomato plants have less complex leaves and fewer axillary stems than wild-type tomato plants whereas plants over-expressing *TF* have more complex leaves and deeper leaf serrations (Naz et al. 2012). The *tf* phenotype provides further evidence that boundary genes play a role in complex leaf development.

3e. *JAGGED LATERAL ORGANS (JLO)*

JAGGED LATERAL ORGANS (JLO) encodes a LATERAL ORGAN BOUNDARIES-DOMAIN (LBD) protein and is expressed in the meristem-lateral organ boundary during embryogenesis. Transgenic plants expressing a fusion of JLO to the

EAR transcriptional repression domain, which is thought to result in a dominant negative function, also exhibit organ fusions. Plants mis-expressing *JLO* have higher levels of *STM* transcripts and lower levels of *PIN1* expression in leaves (Borghi et al. 2007). More recently, however, it was found that JLO interacts in a protein complex with AS1 (MYB-domain transcription factor) and AS2 (an LBD protein) (Rast and Simon 2012). Furthermore, the interaction between JLO and AS1 requires AS2, as activation of a yeast reporter gene only occurred when all three proteins were present. Taken together, JLO functions in a protein complex with AS1 and AS2 in the formation of lateral organs via coordination of auxin transport.

3f. *LATERAL ORGAN BOUNDARIES (LOB)*

LATERAL ORGAN BOUNDARIES was originally identified through an enhancer-trap screen and is expressed in all organ boundaries in *Arabidopsis* including the base of leaves, floral organs, pedicels and lateral roots (Shuai et al. 2002). Plants carrying a hypomorphic *lob* mutation exhibit a fusion between the cauline leaf and axillary stem in the paraclade junction (Bell et al. 2012). This phenotype suggests that a function of LOB is to separate lateral organs from the meristem. Over-expression of *LOB* results in plants that have shorter petioles and smaller rosette leaves than wild-type plants suggesting that *LOB* may function to limit growth in the boundary region, perhaps by regulating cell size and cell growth to control lateral organ separation (Shuai et al. 2002).

The *LOB* gene encodes an ~20-kD transcription factor that binds to DNA to differentially regulate genes involved in a wide variety of biological processes including

brassinosteroid responses (Husbands et al. 2007; Bell et al. 2012). Furthermore, it has been shown that LOB protein interacts with bHLH048 protein and this interaction reduces the *in vitro* DNA-binding ability of LOB (Husbands et al. 2007). LOB binds to the 5'-(G)CGGC(G)-3' sequence where the core 5'-CGGC-3' motif is required. This binding is enhanced when a guanine nucleotide is located on either side of the core motif (Husbands et al. 2007).

To regulate separation of the axillary stem and cauline leaf, LOB limits growth by directly regulating the expression of a brassinosteroid catabolic gene, *PHYB ACTIVATION TAGGED SUPPRESSOR (BAS1)* (Bell et al. 2012). Over-expression of *LOB* results in elevated levels of *BAS1* transcript. Furthermore, *BAS1* transcript levels are lower in *lob* mutants suggesting LOB positively regulates *BAS1* expression. *BAS1* contains the LOB-binding motif and the results of a chromatin-immunoprecipitation experiment and an electro-mobility shift assay suggested that LOB directly binds to the promoter of *BAS1*. Lastly, introduction of *BAS1* under the control of the *LOB* promoter is sufficient to restore the separation between the cauline leaf and axillary meristem in *lob* mutant plants (Bell et al. 2012). To date, the only developmental phenotype associated with *lob* plants is the aforementioned lack of separation of the axillary stem and cauline leaf.

LOB is the founding member of the plant specific, 43 member *LATERAL ORGAN BOUNDARIES DOMAIN (LBD)* gene family (Shuai et al. 2002). There are two classes of *LBD* genes based on their N-terminal protein sequence. Class I LBDs have a canonical $CX_2CX_6CX_3C$ zinc finger-like motif (also called the C-block), an $LX_6LX_3LX_6L$ leucine

zipper-like coiled-coil motif where X is any amino acid residue, and a GAS-block motif. Class II LBDs only have the zinc-finger-like motif (Shuai et al. 2002; Matsumura et al. 2009). The LBD family is conserved in all plant species and are involved in important developmental processes (Bortiri et al. 2006; Yang et al. 2006; Coudert et al. 2013; Wang et al. 2013; Zhang et al. 2014). LBD genes are involved in lateral root formation, pollen development, auxin response, defense against pathogens, callus induction, and anthocyanin synthesis in *Arabidopsis* (Okushima et al. 2007; Rubin et al. 2009; Fan et al. 2012; Feng et al. 2012; Thatcher et al. 2012; Kim et al. 2015). In maize, they have been implicated in inflorescence branching, female gametophyte development, and leaf formation (Bortiri et al. 2006; Evans 2007), and in rice they function to control patterning of the leaf, glume, female gametophyte, and hull (Li et al. 2008; Zhang et al. 2015). *LBD* genes have been implicated in hormone responses and leaf development in apple (Wang et al. 2013) and secondary phloem and xylem formation in *Populus* (Yordanov et al. 2010).

3g. *ORGAN BOUNDARY1 (OBO1)*

ORGAN BOUNDARY 1 (OBO1) was originally identified based on its expression pattern in meristem-lateral organ boundaries in *Arabidopsis* (Cho and Zambryski 2010). OBO1 is thought to act as a transcription factor due to its nuclear localization, and the demonstrated function of another family member, LIGHT-DEPENDENT SHORT HYPOCOTYLS 1 (LSH1) (Zhao et al. 2004). Plant over-expressing *OBO1* have altered petal numbers and stamenoid petals, including a fusion of the pollen-sac to the boundary

region of the petal base (Cho and Zambryski 2010). CUC1 directly regulates *OBO1* expression as well that of the related gene, *LIGHT-DEPENDENT SHORT HYPOCOTYLS 4* (Takeda et al. 2011). *LSH4* is expressed in the boundary cells of lateral organs and the SAM. Plants that mis-express *LSH4* have pleiotropic defects including wrinkled cotyledons, extra floral organs, and multiple flowers emanating from a single flower. *STM* and *WUS* expression is detected in these secondary flowers suggesting that *LSH4* induces ectopic meristems in floral organs (Takeda et al. 2011).

LSH1, a homolog of *LSH4* is involved in light regulation of developing seedlings. *LSH1* is expressed in the shoot apex (including the boundary region) and hypocotyls of seedlings irrespective of light source (Zhao et al. 2004). *Arabidopsis* plants with a dominant *lsh1-d* mutation had a more sensitive response to red, far-red, and blue-light. The sensitive light response of *lsh1-d* seedlings requires the red-light photoreceptor phytochrome A (phyA) (Zhao et al. 2004). Given that *LSH1* is expressed in the boundary and that its homologs, *LSH3* and *LSH4* have demonstrated boundary roles, the boundary could play a role in light perception.

4. Light signaling in plants

Plants utilize sunlight to produce energy for reproduction and growth through photosynthesis. In this process, chloroplasts convert sunlight to chemical energy in the form of glucose. The primary photosynthetic and chloroplast-rich organ in plants is the leaf. Plants therefore require mechanisms to regulate leaf orientation towards the sun for efficient light capture. For example, when plants are irradiated with blue-light (the

primary wavelength absorbed for photosynthesis), they bend their leaves towards the light to maximize light capture (Inoue et al. 2008). There are several mechanisms that plants utilize to alter leaf angle and structure in response to light. In *Arabidopsis*, asymmetric growth on the bottom (abaxial) side of the petiole pushes leaves upward toward the light (Polko et al. 2012). In contrast legumes utilize the pulvinus, a motor organ at the base of leaves that uses turgor pressure expand or contract to alter leaf position (Watanabe and Sibaoka 1973; Abe 1980; Vogelmann 1984). Interestingly, it has been shown that in *Medicago trucatula* and other legumes, the *elongated petiolule (elp1)* mutant fails to fold its leaflets because it lacks the pulvinus motor organ (Chen et al. 2012; Zhou et al. 2012). *ELP* is an ortholog of *LOB* and its transcript is detected in the basal region of young leaflets. Given that *AtLOB* is expressed at the base of lateral organs, the *LOB* orthologs *RA2* (*Maize*) and *ELP1* (*Medicago)* are expressed in the pulvinus, and *ra2* plants have a smaller pulvinus at the base of the inflorescence branches, it is clear that the pulvinus shares characteristics with boundary regions.

Although plants can detect and respond to a wide-range of light sources (Sharrock and Quail 1989; Somers et al. 1991; Somers et al. 1998; Gyula et al. 2003), the remainder of the Introduction will focus on responses to blue-light as LOB regulates blue-light response genes. The molecular mechanisms that control blue-light perception are well characterized. In *Arabidopsis,* the receptor protein PHOTOTROPIN (PHOT1) perceives blue-light, which causes it to undergo a conformational change that results in increased kinase activity and autophosphorylation (Tokutomi et al. 2008). Once phosphorylated, PHOT1 is internalized to the cytoplasm via clathrin-mediated endocytosis (Wang et al.

2008; Kaiserli et al. 2009; Roberts et al. 2011). PHOT1 phosphorylates NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3) (Pedmale and Liscum 2007), a component of a CULLIN3 complex. PHOT1 is polyubiquitinated upon blue light exposure, suggesting that NPH3 serves to mediate the degradation of PHOT1 (Roberts et al. 2011). Lastly, PHYTOCHROME KINASE SUBSTRATE 2 (PKS2) co-precipitates with PHOT1 and NPH3, suggesting it functions in a complex with these proteins to transduce a signal for proper blue-light response (Liscum and Briggs 1995; Motchoulski and Liscum 1999; Briggs 2002; Lariguet 2006; de Carbonnel et al. 2010). Following relay of the blue-light signal, physiological changes occur that include movement of the illuminated organs towards blue-light (Mullen et al. 2006; Inoue et al. 2008), a phenomenon termed phototropism (Inoue et al. 2010), which requires PHOT1, NPH3, and PKS2. Thus, when plants are irradiated with blue-light from above, their leaf angle is altered resulting in more erect leaves. However, this response has not been well characterized at the cellular level and it is not known which cells within the leaf perceive and respond to blue-light.

5. Contributions of this dissertation

This dissertation focuses around characterizing the role of LOB in plant development and responses to blue-light. In the first chapter, I show evidence that LOB negatively regulates blue-light response genes, *PHOT1, PKS2,* and *NPH3* and this regulation is important for a proper blue-light response. Plants with a hypomorphic mutation in *LOB* are more sensitive to short term blue-light response. Furthermore, *LOB* itself is regulated by blue-light through *PKS2* and *PHOT1,* suggesting a feedback loop is

present for proper LOB-mediated blue-light response. Importantly, the boundary region is a crucial player in blue-light response. Given the expression pattern of *LOB*, the adaxial leaf axil is involved in the hyponastic response to blue-light.

In the second chapter, I show that LOB directly regulates the expression of an *NPH3*-related gene *NAKED PINS IN YUC MUTANTS 1 (NPY1).* The transcript of *NPY1* is increased upon LOB induction. Furthermore, LOB directly binds to the promoter of *NPY1* and *NPY1* expression is altered in *lob-3* mutants suggesting that LOB directly regulates the expression of *NPY1*. However, *npy1-1* mutants do not have any developmental defects and do not have an altered phototropic response. Although the molecular mechanism of LOB regulating *NPY1* is clear, their biological relationship remains elusive.

Chapter III shows that the LOB protein interacts with HISTONE DEACETYLASE 3 (HDT3) for proper separation of the axillary stem and the cauline leaf and for a proper blue-light response. LOB interacts with HDT3 in yeast and in onion epidermal peels and their expression patterns partially overlap. *hdt3-1* mutants resemble *lob-3* mutants in that they are more sensitive to short term blue-light illumination. In a second allele, *hdt3-2, BAS1* transcript levels are lower in the paraclade junction and have an organ fusion of the axillary stem and cauline leaf. These data suggest that HDT3 is necessary for proper LOB function in separating the axillary stem from the cauline leaf and blue-light phototropism.

In Chapter IV, I identified a chemical that inhibits the *LOB* over-expression phenotype. The chemical, LAT24D02, inhibits the LOB over-expression phenotype,

however its mode of action is unclear. LAT24D02 application does not affect LOB's ability to regulate *BAS1* transcript. Furthermore, LAT24D02 does not alter brassinosteroid response suggesting that LAT24D02 acts to alter a brassinosteroid-independent LOB pathway. Lastly, analog analysis suggests that a substructure of LAT24D02 is responsible for its function.

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CHAPTER 1

LOB regulates *NPH3, PKS2,* and *PHOT1* for plant responses to blue-light

ABSTRACT

Leaves form at the periphery of the SAM and their positioning is critical for optimal light capture. Several factors regulate leaf angle, including hormone concentrations, light quality, and light intensity. The plant-specific transcription factor LATERAL ORGAN BOUNDARIES (LOB) functions at the leaf base and directly regulates the expression of genes involved in blue-light responses. Blue-light is one of the primary wavelengths utilized for photosynthesis and plants have evolved mechanisms that allow them to sense the direction of blue-light irradiation and respond accordingly. One blue-light response is leaf phototropism. When plants are illuminated with blue-light from above, the abaxial petiole cells elongate, resulting in upward movement of leaves toward the light source. LOB directly represses the expression of *PHOT1*, *PKS2*, and *NPH3*, which encode components of the photoreceptor complex. This suggests a role for LOB in modulating the blue-light response. Plants over-expressing *LOB* have reduced transcript levels of these genes and loss-of-function *lob* mutants exhibit an impaired blue-light phototropic response. Furthermore, *LOB* transcripts are increased upon blue-light treatment. These data are consistent with a negative feedback loop involving LOB regulation of the blue-light response. The localized pattern of *LOB* expression suggests that the leaf axil plays an important role in blue-light perception. These data indicate a novel role for LOB in regulating the blue-light response in plants.

Introduction

Leaves, the primary photosynthetic organ in plants, form at the periphery of the shoot apical meristem (SAM). Regulation of leaf position is critical to ensure optimal light capture. Plants have evolved mechanisms to detect the direction and intensity of a light source and respond accordingly (Neff et al. 2000; Briggs 2002). For example, when illuminated from above, the abaxial petiole cells expand causing leaves to grow more vertically. This vertical growth of leaves as a response to external stimuli is called the hyponastic response.

Blue-light is a major contributor to photosynthesis. Photoreceptors that perceive blue-light illumination include the cryptochromes and phototropins. There are two cryptochromes in *Arabidopsis* (*CRY1* and *CRY2),* which are involved in regulating gene expression, photomorphogenesis, and floral initiation (El-Assal et al. 2001; Cashmore 2003; Sancar 2003).

The two phototropins that perceive blue-light are PHOTOTROPIN1 (PHOT1) and its paralog PHOTOTROPIN2 (PHOT2) (Demarsy and Fankhauser 2009). *Arabidopsis* plants with a *phot1* loss-of-function mutation do not respond to blue-light illumination (Liscum and Briggs 1995). PHOT1 is necessary for hypocotyl bending towards blue-light, as well as several other phototropic responses, including stomata opening, chloroplast movement, and leaf positioning (Jarillo et al. 2001; Kagawa et al. 2001; Sakai et al. 2001; Inoue et al. 2008). PHOT1 is a Serine/Threonine kinase that belongs to the AGC family of proteins (cAMP-DEPENDENT PROTEIN KINASE, CGMP-DEPENDENT PROTEIN KINASE, and PHOSPHOLIPID-DEPENDENT

PROTEIN KINASE C) (Bogre et al. 2003). Although the signaling cascade downstream of PHOT1 is poorly understood, the mode of action of PHOT1 is well characterized. Following blue light exposure, PHOT1 is autophosphorylated and moves from the plasma membrane internally to the cytosol where it is targeted for degradation (Tokutomi et al. 2008; Demarsy and Fankhauser 2009). Recent biochemical evidence shows that PHOT1 interacts with other proteins including NON-PHOTOTROPIC HYPCOTOYL 3 (NPH3) and PHYTOCHROME KINASE SUBSTRATE 2 (PKS2) (Bardwell and Treisman 1994; Motchoulski and Liscum 1999; de Carbonnel et al. 2010). NPH3 is a membrane-associated BTB (BROAD COMPLEX, TAMTRACK, and BRIC A BRAC) protein that is involved in heterodimerization (Bardwell and Treisman 1994). NPH3 also contains the conserved NPH3 domain and is a member of the CULLIN3-RING-Ligase (CRL3) ubiquination complex. Based on its localization, NPH3 is proposed to function as a protein that aids in PHOT1 ubiquitination at the plasma membrane (Motchoulski and Liscum 1999; Pedmale and Liscum 2007; Roberts et al. 2011). Originally discovered in the same screen as *phot1-5*, *nph3-1* mutant hypocotyls fail to respond to unilateral blue-light (Motchoulski and Liscum 1999). Furthermore, it has been shown that *NPH3* is required for proper leaf positioning. *Arabidopsis* plants carrying an *nph3* loss-of-function mutation have less erect leaves than wild-type after blue-light illumination (Inoue et al. 2008; de Carbonnel et al. 2010). Lastly, *nph3-6 phot1-5* double mutants have a less erect leaf angle than either of the single mutants, suggesting they function in a similar pathway for proper blue-light response (de Carbonnel et al. 2010).

PKS2 encodes a membrane-localized protein that is phosphorylated by PHOT1. PKS2 interacts in a complex with PHOT1 and NPH3, and has a demonstrated role in the blue-light response pathway (de Carbonnel et al. 2010). After blue-light illumination, *Arabidopsis pks2-2* mutants have less erect leaves than wild-type and this phenotype is enhanced in *phot1-5 pks2-2* double mutants. The current model for blue-light perception is that after PHOT1 is autophosphorylated, it is internalized through clathrin-mediated endocytosis, where it phosphorylates PKS2, which transduces a signal for blue-light response. PHOT1 then interacts with the NPH3-CRL3 complex resulting in PHOT1 ubiquitination and degradation. Although the biochemical interaction of PHOT1, NPH3, and PKS2 is well characterized, the downstream events of this pathway are poorly understood.

Plant lateral organs are separated from the meristem by the boundary region (Rast and Simon 2008), which is composed of cells that are smaller and divide less frequently than the surrounding cells (Hussey 1971; Breuil-Broyer et al. 2004). Several studies in recent years have identified factors that are involved in boundary function in *Arabidopsis* including *CUP-SHAPED COTYLEDON (CUC), LATERAL ORGAN FUSION 1/2 (LOF1/2), LATERAL ORGAN BOUNDARIES (LOB)*, and *JAGGED LATERAL ORGAN (JLO). Arabidopsis cuc, lob*, and *lof1/2* mutants exhibit fusions of the lateral organs to the SAM and to each other, indicating they have defects in organ separation (Aida et al. 1997; Greb et al. 2003; Raman et al. 2008; Lee et al. 2009; Bell et al. 2012). Transgenic *Arabidopsis* plants expressing a fusion of JLO to the EAR transcriptional repression domain, which is thought to result in a dominant negative function, also exhibit organ

fusions (Borghi et al. 2007). The above data suggest that these boundary-expressed genes function in boundary formation and organ separation.

Originally identified through enhancer-trap screening, *LOB* is expressed in all boundary regions including the base of leaves, floral organs, pedicels, and lateral roots (Shuai et al. 2002). *Arabidopsis* plants homozygous for a hypomorphic *lob* mutation exhibit a fusion of the axillary stem to the cauline leaf, suggesting that the central function of *LOB* is to separate lateral organs from the SAM (Bell et al. 2012). Plants that mis-express *LOB* have short petioles and small rosette leaves, resembling brassinosteroid (BR)–deficient plants (Wang et al. 2001; Shuai et al. 2002). Recently, LOB has been shown to directly regulate *PHYB ACTIVATION TAGGED SUPPRESSOR (BAS1)*, a gene that codes for a BR-inactivating enzyme (Bell et al. 2012). Furthermore, BR application increased *LOB* expression suggesting a negative feedback loop functions to restrict BR levels in the boundary; BR increases *LOB* expression, which negatively regulates BR accumulation via increasing *BAS1* activity (Bell et al. 2012). *LOB* encodes an ~20 kDa transcription factor. The conserved LOB domain is a DNA-binding domain that binds to a core CGGC nucleotide sequence and binding efficiency is increased with a G on either side of the core sequence (Husbands et al. 2007).

To investigate the factors involved in the hyponastic response to blue-light, in this chapter we characterized the relationship between LOB and *NPH3, PKS2,* and *PHOT1*. Our results show that *NPH3, PKS2,* and *PHOT1* transcripts are altered in plants that have varying levels of LOB activity. LOB also binds directly to the promoter regions of *PKS2,* and *PHOT1* in yeast and *in planta. lob* mutants have altered hyponastic responses to both

long- and short- term blue-light illumination suggesting the axil is an important region for setting leaf angle after blue-light illumination. Lastly, *LOB* transcript is altered in plants following illumination by blue-light and in *pks2-2* and *phot1-5* plants, leading to a negative feedback loop that optimizes leaf growth for efficient light capture. Our study is the first, to our knowledge, that demonstrates the boundary region is important for proper blue-light response in leaves.

Results

Ectopic LOB activity alters *NPH3, PKS2,* and *PHOT1* transcript levels.

In a previous microarray experiment, components of the blue-light response pathway were identified as downstream targets of LOB (Bell et al. 2012). To further investigate the regulation of genes involved in blue-light response, we confirmed this regulation in an independent experiment in which LOB activity was induced using a dexamethasone-(DEX) inducible system. After 4 hours of DEX treatment, the transcript level of *NPH3, PKS2,* and *PHOT1* were >2.0-fold lower in 14-day-old *35S:LOB-GR* plants compared to MOCK-treated plants (Figure 1.1A-C). We next asked if this change in *NPH3, PKS2,* and *PHOT1* transcript following LOB-GR induction is dependent on protein synthesis. *35S:LOB-GR* plants were treated with DEX together with the translational inhibitor cycloheximide (CHX). *NPH3, PKS2,* and *PHOT1* transcript levels were reduced by >2.0-fold in these plants compared to CHX-treated *35S:LOB-GR* (Figure 1.1A-C) plants suggesting that LOB directly represses *NPH3, PKS2*, and *PHOT1* expression.

A time course of DEX treatment was conducted to determine how quickly the transcript levels of *NPH3, PKS2,* and *PHOT1* changed after LOB-GR induction. *35S:LOB-GR* plants were incubated with DEX for 0 hours, one hour, and two hours and *NPH3, PKS2,* and *PHOT1* transcript levels were assayed by RT-PCR. After 2 hours of induction, all three gene transcripts were reduced in DEX-treated seedlings compared to MOCK-treated seedlings (Figure 1.2).

NPH3, PKS2, and *PHOT1* transcripts are altered in loss-of-function *lob-3* mutants

NPH3, PKS2, and *PHOT1* transcript levels were reduced in response to ectopic *LOB* expression. To further investigate the regulation of *NPH3, PKS2*, and *PHOT1* by LOB, we assayed the transcript levels of these genes in a *lob-3* mutant using qRT-PCR. Transcript levels of *NPH3* and *PKS2* were 1.5- and 2.0-fold higher, respectively, in *lob-3* seedlings compared to wild-type, while no difference in *PHOT1* transcript levels was detected (Figure 1.1D). Interestingly, in inflorescences, *NPH3* and *PHOT1* transcripts were 1.7- and 4.2-fold lower, respectively, in *lob-3* mutants compared to wild-type (Figure 1.1E), while *PKS2* transcripts were not significantly different than wild-type. Finally, we examined the transcript level of these genes in paraclade junctions (cauline leaf node). There were no detectable differences in transcript levels of *NPH3, PKS2,* and *PHOT1* between *lob-3* and wild-type plants in paraclade junctions (Figure 1.3). These results suggest that LOB negatively regulates *NPH3* and *PKS2* in seedlings but may positively regulate *NPH3* and *PHOT1* in inflorescences.

Seedlings with higher levels of LOB activity resemble *phot1-5* loss-of-function plants

LOB over-expression plants have reduced *PHOT1* transcript compared to MOCK-treated plants, so we examined the response of these seedlings to unilateral blue-light. Etiolated *Col-0 Arabidopsis* plants respond to unilateral blue-light illumination by growing towards the blue-light source. The response to blue-light was not altered by DEX treatment, as *Col-0* seedlings grown on media supplemented with DEX grew towards the blue-light source as did control seedlings (Figure 1.4A-B). Etiolated *phot1-5* seedlings did not respond to unilateral blue-light (Figure 1.4C-D) as previously reported (Liscum and Briggs 1995). *35S:LOB-GR* seedlings grown on control media without DEX responded to unilateral blue-light by growing towards the blue-light source; however, *35S:LOB-GR* seedlings grown in the presence of DEX did not (Figure 1.4E-F). Furthermore, the transcript levels of *NPH3, PKS2,* and *PHOT1* in etiolated *35S:LOB-GR* seedlings were significantly lower in *35S:LOB-GR* seedlings grown on DEX-supplemented media compared to those grown on control media (Figure 1.4G), consistent with the failure of *35S:LOB-GR* seedlings to respond to blue-light.

LOB is associated with the promoter regions of *PKS2* and *PHOT1* in yeast and *in planta*

Repression of *NPH3*, *PKS2,* and *PHOT1* in DEX-induced *LOB-GR* did not require translation, suggesting that LOB directly regulates these genes. Furthermore, canonical LOB binding sites (Husbands et al. 2007) are present in exonic regions of *NPH3* and *PHOT1* and upstream of *PKS2* (Figure 1.5A). *NPH3* contains five putative

binding sites; one in each of the first, second, and fifth exons and, two in the fourth exon. Two putative binding sites separated by 23 bps are located ~600 bps upstream of the *PKS2* ATG. *PHOT1* contains three putative LOB binding sites; two in the first exon separated by five bps and a third in the fourth exon. We examined LOB binding to these sites using a Yeast-1-Hybrid assay. DNA fragments spanning the putative LOB-binding sites in *NPH3, PKS2,* and *PHOT1* were cloned upstream of a *HIS* reporter gene and integrated into the yeast genome. We cloned the LOB coding region as a translational fusion to the GAL4-activation domain (LOB-AD) and the resulting plasmid was transformed into yeast containing the integrated promoter:HIS constructs. Yeast containing the genomic region of *PKS2* and *PHOT1* fused to the *HIS* auxotrophic selectable marker and LOB-AD grew on selective media -HIS in the presence of 3-Amino 1, 2, 4-Triazole (3-AT), whereas the empty vector (AD) did not confer growth. These results indicate that LOB directly binds to the promoter region of *PKS2* and *PHOT1* in yeast (Figure 1.5B). We found no difference in growth of yeast containing LOB-AD and the genomic region of *NPH3* upstream of the *HIS* selectable marker gene with and without 3-AT.

To assay LOB binding to *NPH3, PKS2* and *PHOT1 in planta,* we performed a Chromatin Immunoprecipitation (ChIP) experiment using anti-LOB antibodies in *35S:LOB-GR* seedlings. We detected enrichment of *PHOT1* BSI in samples immunoprecipitated from DEX-treated LOB-GR, compared to MOCK-treated seedlings. There was no significant enrichment of BSII in DEX-treated samples compared to MOCK-treated samples. As a control, we performed ChIP with an anti-GST antibody and

found no difference in relative enrichment of *PHOT1*-binding site I (BSI) in MOCK-treated and DEX-treated samples (Figure 1.5C). We also detected enrichment of BS1 of *PKS2* in DEX-treated samples compared to MOCK-treated samples. Again, there was no difference in enrichment between DEX-treated and MOCK-treated samples using an anti-GST antibody (Figure 1.5C). However, no enrichment was detected of any of the binding sites of *NPH3* in DEX-treated compared to MOCK-treated samples (Figure 1.5E). These results suggest that LOB directly binds to *PKS2* and *PHOT1 in planta*.

lob-3 mutants have altered blue-light hyponastic response in short term blue light exposure

Arabidopsis plants that are illuminated with blue-light from above respond by increasing abaxial petiole growth so that leaves grow at a more upright angle – so called the hyponastic response (Inoue et al. 2008). The alteration in *NPH3, PKS2,* and *PHOT1* transcripts in *lob-3* plants prompted us to examine *lob-3* loss-of-function mutants for altered blue-light induced leaf inclination. Thirteen-day-old *lob-3* mutants grown in white light exhibited a larger petiole angle (60.6°) than wild-type plants (46.5°) (Figure 1.6A). However, after 24 hours of illumination with blue-light from above, *lob-3* mutants exhibited a more erect leaf (24.1°) than wild-type (33.2°) (Figure 1.6B) suggesting that *lob-3* plants were more sensitive to blue-light. The change in angle following 24 hours of blue-light illumination was significantly different in *lob-3* plants compared to wild-type (Figure 1.6C), which is consistent with increased *NPH3* and *PKS2* transcripts in *lob-3* compared to wild-type seedlings (Figure 1.1). To test the genetic relationship between

LOB and *PKS2* and *PHOT1,* we characterized the response to 24 hours of blue-light illumination in *lob-3 phot1-5* and *lob-3 pks2-2* double mutants. Under white light conditions, *lob-3 phot1-5* and *lob-3 pks2-2* seedlings displayed petiole angles not significantly different than *lob-3* plants (Figure 1.6A). After 24 hours of blue light exposure, *lob-3 pks2-2* and *lob-3 phot1-5* seedlings exhibited a petiole angle of 34.9° and 39.2° (Figure 1.6B). These double mutants showed a reduced response to blue-light illumination compared to *lob-3* plants (Figure 1.6C). These data are consistent with the requirement of *PKS2* and *PHOT1* for the blue-light hyper-response of *lob-3* plants.

lob mutants have defects in the long-term hyponastic blue-light response

We next asked if *lob-3* plants have an altered response to long-term blue light exposure. Plants were grown in white light and then transferred to a blue-light chamber where they were illuminated with blue-light from above for 5 days, 8 hours. After long-term blue-light illumination, wild-type plants displayed an average petiole angle of 15.1° (Figure 1.7A and I), whereas *phot1-5* plants exhibited a reduced response, resulting in a less erect leaf with an average petiole angle of 36.9° (Figure 1.7E and I). Relative to wild-type and *phot1-5*, *lob-3* mutants showed an intermediate leaf inclination phenotype with an average petiole angle of 22.5° (Figure 1.7D and I). A second *lob* allele, *lob::DsE*, which is in the *Ler* ecotype, also displayed a less erect leaf phenotype compared to the wild-type *Ler* (Figure 1.8). *lob-3* plants that were transformed with a functional copy of *LOB* exhibited a leaf angle similar to wild-type plants (Figure 1.7F). *nph3-1* mutants also showed a less erect leaf phenotype compared to wild-type plants with an average petiole

angle of 18.4° (Figure 1.7C and I). *pks2-2* mutants did not have a significantly different petiole angle than wild-type (Figure 1.7B and I). This difference in petiole angle between wild-type and *lob-3* mutants could be seen after 3 days of blue-light illumination (Figure 1.9). *lob-3 pks2-2* plants were not significantly different in petiole angle than *lob-3* mutants (Figure 1.7F), suggesting that *PKS2* and *LOB* are not the only factors involved in the blue-light response. *lob-3 phot1-5* double mutants resembled *phot1-5* single mutants (Figure 1.7F) suggesting that *LOB* and *PHOT1* function in a similar pathway in the blue-light response.

LOB is positively regulated by blue-light

As previously reported, *LOB* is positively regulated by brassinosteroid (BR), acting in a negative feedback loop (Bell et al. 2012). We asked if *LOB* is also regulated by blue-light. We measured *LOB* transcript levels in 14-day-old wild-type seedlings after blue-light illumination for 1 hour, 4 hours and 24 hours. After 1 hour of blue-light illumination, *LOB* transcripts were ~1.6-fold increased. Levels stabilized to an approximately 1.4-fold increase after 24 hours of blue light exposure (Figure 1.10A). These data suggest that blue-light acts to rapidly increase *LOB* transcripts. We next asked if *PHOT1* and *PKS2* impact *LOB* transcript levels. Indeed, *LOB* transcripts were reduced ~4.0-fold and ~2.0-fold in 14-day-old *pks2-2* and *phot1-5* plants, respectively (Figure 1.10B). From these data, *LOB* transcript is positively regulated by blue-light likely downstream of PKS2 and PHOT1.

Other boundary genes do not play a role in leaf responses to blue-light

LOB is expressed in all organ boundaries including the leaf axil. That *lob* mutants have a less erect leaf phenotype after blue-light illumination suggests the axil is important for leaf responses to blue-light. To determine if other boundary-expressed genes also play a role in blue-light phototropism, we measured the leaf angle of several boundary mutants after unilateral blue-light illumination from above. We examined response to blue-light inclination in *cuc1-13, cuc2-3, las-4, lof1-1,* and *lof1-1 lof2-1* plants. None of these mutants displayed a significantly different leaf angle compared to wild-type plants, suggesting this boundary mediated hyponastic response is specific to *LOB* (Figure 1.11).

As previously reported, LOB negatively regulates BR levels via direct regulation of *BAS1*. Furthermore, BRs play a role in blue-light phototropism (Nakamoto et al. 2006). We asked if BRs play a role in the *lob-3* mediated blue-light hyponastic response. To test this, we measured the petiole angle in transgenic plants that express *BAS1* under the *LOB* promoter, in the wild-type and *lob-3* mutant background. *pLOB:BAS1* has been previously shown to suppress the fusion phenotype in paraclade junctions in *lob-3* mutants. *pLOB:BAS1* plants in the wild-type background displayed a petiole angle not significantly different than wild-type (Figure 1.12). In the *lob-3* background, *pLOB:BAS1* plants did not exhibit a significant difference in petiole angle than *lob-3* plants (Figure 1.12). These data suggests that *LOB* mediated blue-light hyponastic response is independent of BRs.

Discussion

Plants require light in order to conduct photosynthesis to create sugars for energy storage. Therefore, it is necessary for the plant leaves to orient themselves to capture an optimum amount of sunlight. Some plants, such as sunflower, alfalfa, and mallow have mechanisms to alter their leaf positioning for optimal light capture (Greer and Thorpe 2009; Denison et al. 2010; Vandenbrink et al. 2014). Furthermore, plants have the ability to sense shade and respond accordingly (Franklin and Whitelam 2005). After shading, stem-bearing plants increase stem growth to avoid the shade whereas rosette plants, like *Arabidopsis*, increase cell elongation of the abaxial petiole cells causing a more erect leaf (Reed et al. 1993; Morelli and Ruberti 2000).

Blue-light photoreceptors detect blue-light (the primary wavelengths used for photosynthesis) and allow for phototropic responses. In leaves, this response is to alter the petiole angle to orient the leaf toward the light source. Previous data suggests that the molecular mechanisms involved in the blue-light response in leaves are dependent on the fluence rate of blue-light (de Carbonnel et al. 2010). *phot1-5* plants have a less erect leaf angle than wild-type after low-intensity blue-light illumination from above but the defect is less severe after high-intensity blue-light illumination. This suggests there is functional redundancy with *PHOT1* in mediating the hyponastic response to high-intensity blue-light. Our results clearly indicate a role for *LOB* and the boundary region in blue-light mediated hyponastic response as *lob* plants have altered responses to blue-light illumination. Furthermore, *NPH3, PKS2,* and *PHOT1* transcripts are altered in plants with varying levels of LOB activity and LOB binds to *PKS2* and *PHOT1* suggesting LOB

directly regulates the expression of these genes. Puzzlingly, *NPH3* and *PHOT1* transcripts were lower in inflorescences of *lob-3* plants compared to wild-type plants suggesting that LOB contributes positively to their regulation in inflorescences, or the presence of a compensatory mechanism. Another possibility is that LOB may negatively regulate a negative regulator of *NPH3* and *PHOT1* in inflorescences.

Given that *PHOT1* transcript is decreased in *LOB* over-expression plants (Figure 1.1) and that LOB binds to the first exon of *PHOT1* (Figure 1.5), it is possible that LOB negatively regulates *PHOT1* by physically inhibiting its transcription. For *PKS2,* the data suggests that LOB binds upstream of the *PKS2* ATG site (Figure 1.5) to negatively regulate its expression. Although *NPH3* transcript is decreased in *35S:LOB-GR* plants treated with CHX and DEX, compared to CHX treated *35S:LOB-GR* plants, we could not detect direct binding of LOB to the promoter region of *NPH3.* A possible explanation for this discrepancy may be that LOB binding to *NPH3* requires a cofactor that is only present in the boundary region. A second explanation is that the LOB-DNA binding interaction is transient or weak.

lob-3 plants exhibit a more sensitive response to short-term (24 hours) blue-light illumination from above suggesting that LOB plays a role in determining the leaf angle in response to blue-light. *lob-3 pks2-2* and *lob-3 phot1-5* double mutants were less sensitive than *lob-3* plants demonstrating that *PHOT1* and *PKS2* contribute to the hypersensitive phenotype. We were unable to generate the *lob-3 nph3-1* double mutant due to close linkage of these genes (within 0.70 cM). Furthermore, *PKS2* and *NPH3* transcript is increased in *lob-3* mutants compared to wild-type plants. We could not detect a

significant difference in *PHOT1* transcript levels, but its broad expression may preclude detection of changes in transcript levels in the boundary. We propose a model in which LOB negatively regulates *PHOT1, PKS2* and *NPH3*, therefore, the increase in blue-light response in *lob-3* mutants contributes to the hypersensitive response in these plants. Other factors are likely to be involved since a complete rescue of the wild-type response was not observed. Interestingly, *phot1-5 phot2-1* double mutant plants exhibit a less erect leaf angle than *phot1-5* plants after high intensity blue-light illumination suggesting that *PHOT2* plays a role in leaf responses to high intensity blue-light (de Carbonnel et al. 2010). However, according to our microarray study, *PHOT2* transcript levels are not significantly different after LOB induction suggesting that LOB does not regulate *PHOT2* transcript levels, although further experiments are required to support these data (Bell et al. 2012).

Intriguingly, *lob* plants display a less erect leaf angle than wild-type after long-term exposure to blue light from above. Furthermore, *LOB* transcript is increased rapidly after blue-light illumination and reduced in *pks2-2* and *phot1-5* plants, indicating that blue-light positively regulates LOB transcript levels. These data are consistent with a negative feedback loop involving blue-light and LOB, to regulate leaf angle allowing optimum capture of blue-light. These data have two implications. Firstly, *LOB* is involved in the blue-light mediated hyponastic response in plants and that the boundary region at the leaf axil is important for proper blue-light response. It is puzzling that *phot1-5* and *pks2-2* mutants do not have an altered response to short-term blue-light illumination. One explanation for this difference is that our experiments were carried out

with a higher fluence rate (30 μM/m^2s) of blue-light (de Carbonnel et al. 2010). A second possibility is that we illuminated plants with blue-light for a different length of time than previous studies (24 hours versus 5 days 8 hours in previous experiments) (de Carbonnel et al. 2010).

How is the leaf axil involved in the blue-light response of leaves? The fact that *LOB* is expressed in the boundary (Shuai et al. 2002), yet *lob* mutants have altered blue-light hyponastic responses (Figures 1.6-8) is perhaps explained by a mobile signal that moves from the boundary to the abaxial petiole cells to regulate cell growth. Plants use mobile signals, like hormones that are produced in one area of the plant to affect growth and response in another part of the plant (Eckhardt 2015). One such signal is ethylene (Bleeker 2000). Ethylene is an integral plant hormone that is also involved in the hyponastic response to flooding and blue-light response (Millenaar et al. 2005; Bailey-Serres and Voesenek 2008). Furthermore, plants grown on media supplemented with an ethylene biosynthesis inhibitor show petiole angles similar to *lob-3* mutants (Figure 1.13). Additionally, *lob-3* mutants display an attenuated hyponastic response following ethylene treatment. This suggests that *LOB* plays a role in ethylene-mediated hyponastic response (Figure 1.13). Further experiments are needed to tease apart the *LOB,* ethylene, and hyponastic response connection.

A second hormone that is involved in plant responses to blue-light is auxin (Halliday et al. 2009). Plants that have been treated with an auxin transport inhibitor show a reduced response to blue-light suggesting that auxin transport is necessary for proper blue-light responses (Jensen et al. 1998). Could auxin play a role with LOB for

leaf responses to blue-light? *PKS2* is a direct target of LOB and is expressed in the leaf blade and at the base of the leaf, which correlates with auxin distribution (Figure 1.1 and 1.5) (de Carbonnel et al. 2010). One hypothesis is that LOB regulates *PKS2*, which affects auxin distribution and thus the leaf angle in response to blue-light. Another, non-mutually exclusive hypothesis is that LOB regulates genes that are involved in auxin biosynthesis or transport, which can affect the hyponastic response to blue-light. LOB directly regulates the expression of *NAKED PINS IN YUC MUTANTS (NPY1)*, a gene involved in auxin transport (Cheng et al. 2007) lending support that LOB may regulate auxin transport in the hyponastic response to blue-light (Chapter 2). More studies are needed to test these hypotheses.

Our data show that *lob* mutants have a reduced hyponastic response to long-term exposure to blue light – although it is not as severe as *phot1-5* mutants. If LOB negatively regulates *PHOT1,* then lowering *PHOT1* transcript levels in a *lob-3* mutant could rescue the wild-type phenotype. However, after 24 hours of blue-light illumination, *lob-3 phot1-5* double mutants displayed a leaf angle significantly different than wild-type plants suggesting that there are other factors involved in the blue-light hyponastic response pathways. The *CRYPTOCHROMES (CRY)* family is composed of two members, *CRY1* and *CRY2,* and are involved in a variety of blue-light responses including photomorphogenesis, flowering time, and photoperiod determination (El-Assal et al. 2001; Cashmore 2003; Sancar 2003). However, none of these genes are differentially regulated by LOB according to our microarray study (Bell et al. 2012). Further experiments are needed to fully tease apart this pathway.

Materials and methods

Plant materials and growth conditions:

lob-3, *pLOB:BAS1 Col-0, pLOB:BAS1 lob-3, 35S:LOB-GR* (Bell et al. 2012), *pks2-2* (de Carbonnel et al. 2010), *phot1-5* (Liscum and Briggs 1999), *nph3-1* (Alonso et al. 2003), *lof1-1, lof1-1 lof2-1* (Lee et al. 2009), *las-4* (Greb et al. 2003), *cuc2-3,* and *cuc1-13* (Aida and Tasaka 2006) are in the Col-0 background. *ET22* is in the L*er* background. For plants grown on media, seeds were sterilized for 5 minutes with 95% EtOH, 5 minutes with a 20% Bleach/0.01% Tween20 solution, and rinsed 5 times with sterile water. They were then sown on Murashige and Skoog (MS) Media (pH 5.7) (Murashige and Skoog 1962) and stratified at 4°C in the dark for 2 days, then transferred to a growth chamber with 120 μM/m^2s white light in a 16 hour light/8 hour dark cycle at 22°C. For soil-grown plants, seeds were sterilized with 95% EtOH for 5 minutes before sowing on Sunshine Mix soil with 10 μM Marathon. They were illuminated with 115 μM/m^2s white light at 22°C. All light conditions were measured using a Li-COR LI-250A light meter.

Generation of double mutants and transformation:

Following crosses between *lob-3* plants and *phot1-5* or *pks2-2,* in the F2 generation, PCR genotyping was carried out as previously described (Shuai et al. 2002; Han et al. 2008; de Carbonnel et al. 2010). For transformations, *Arabidopsis* plants were grown under standard conditions as previously described (Shuai et al. 2002). Binary vectors were transformed into GV3101 *Agrobacterium* using standard procedures, and

Arabidopsis was transformed by floral dip (Clough et al. 1998). Transformants were selected either by BASTA spray (Finale, AgrEvo) or growth on MS media supplemented with 50 μM phosphinothricin (Sigma).

For induction of *35S:LOB-GR* seedlings, dexamethasone and cycloheximide were used at 5 μM and 10 μm concentrations, respectively.

Blue-light experiments:

Short-term blue-light experiments were carried out by transferring 13-day-old *Arabidopsis* plants to a growth chamber with Blue LED lights (Sunslighting) at an intensity of 30 $\mu M/m^2s$ for 24 hours. For long-term petiole angle experiments, plants were grown under white light until Stage 1.01 (Boyes et al. 2001) and transferred to a growth chamber with Blue LED lights at an intensity of 30 $\mu M/m^2s$, temperature of 22°C for 5 days 8 hours. Leaf angles were measured using ImageJ software. Angles are calculated by using the vertical growth vector and the petiole vector. To test the effect of blue-light on *LOB* transcript, *Col-0* plants were grown on soil under white light for 13 days and transferred to a chamber with Blue LED lights at an intensity of 30 $\mu M/m^2s$ for 1 hour, 4 hours, or 24 hours.

For hypocotyl phototropic measurements, seedlings were grown on vertical plates in the dark for 4 days before unilateral blue light exposure of 3 $\mu M/m^2s$ for 24 hours. Media were supplemented with MOCK treatment (control plates) or DEX.

Transcript analysis:

Total RNA was isolated with TRIzol reagent (LifeTechnologies). RT-PCR was conducted as described previously. Primers for *ACT2, UBC9,* and any gene specific

primers are shown in Table 1.1. qRT-PCR assays using SYBR GREEN were conducted on a BioRad Icycler using BioRad iQ5 Software. Relative transcript was calculated by the Pfaffl method (Pfaffl 2004). Significance was measured by student t-test.

Chromatin immunoprecipitation:

13-day-old *35S:LOB-GR* seedlings were induced by flooding with either 15 μM DEX or MOCK treatment. Induction was confirmed by transcript analysis of downstream target *BAS1*. ChIP was carried out as previously described using an anti-LOB antibody or anti-GST (Saleh et al. 2008). Relative binding was calculated by comparing DEX to MOCK samples. All data were normalized to control gene *ACT2.* Primers for binding sites are listed in Table 1.1.

Yeast-1-Hybrid:

The 700-bp region including the putative LOB binding site I of *PHOT1,* the entire 3-kb *NPH3* gene and promoter including putative LOB binding sites I-V, and the 1.1-kb region including putative LOB binding site I upstream of *PKS2* (Figure 1.5A) were PCR amplified with B1R and B4 Gateway adapters. Using the BP reaction, these fragments were cloned into the pDONR-P4-P1R entry vector (Invitrogen). Then, using the LR Clonase reaction, these fragments were inserted upstream of the HIS auxotrophic selectable marker using the pMW #2 vector (Deplancke et al. 2004). PHOT1-pMW#2, PKS2-pMW#2, and NPH3-pMW#2 were transformed into yeast as described (Deplancke et al. 2006). The LOB-AD and Empty-AD plasmid were transformed with a lithium acetate protocol (Clontech).

Ethylene treatments:

Col-0 and *lob-3* plants were grown on MS media for 13 days under white light in the presence of MOCK (water) or 5 mM Aminoethoxyvinylglycine (AVG). Plants were then subjected to either air or 5 ppm ethylene gas. Leaf petiole angles were measured with imageJ. Ethylene treatments were performed with assistance from Dr. Paul Larsen (UCR).

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Figure 1.1. Transcript levels of blue-light response genes are regulated by LOB. A-C) Relative transcript level of *NPH3* (A), *PKS2* (B), and *PHOT1* (C) in *35S:LOB-GR* plants treated with either mock, cycloheximide (CHX), dexamethasone (DEX), or DEX+CHX. n=2 biological replicates. D-E) Transcript level of *NPH3, PKS2,* and *PHOT1* in seedlings (D) and inflorescences (E) in WT and *lob-3 Arabidopsis* plants. n = 3 biological replicates. Error bars indicate SE. * $p < 0.05$. ** $p < 0.01$. Significance was determined by student t-test.

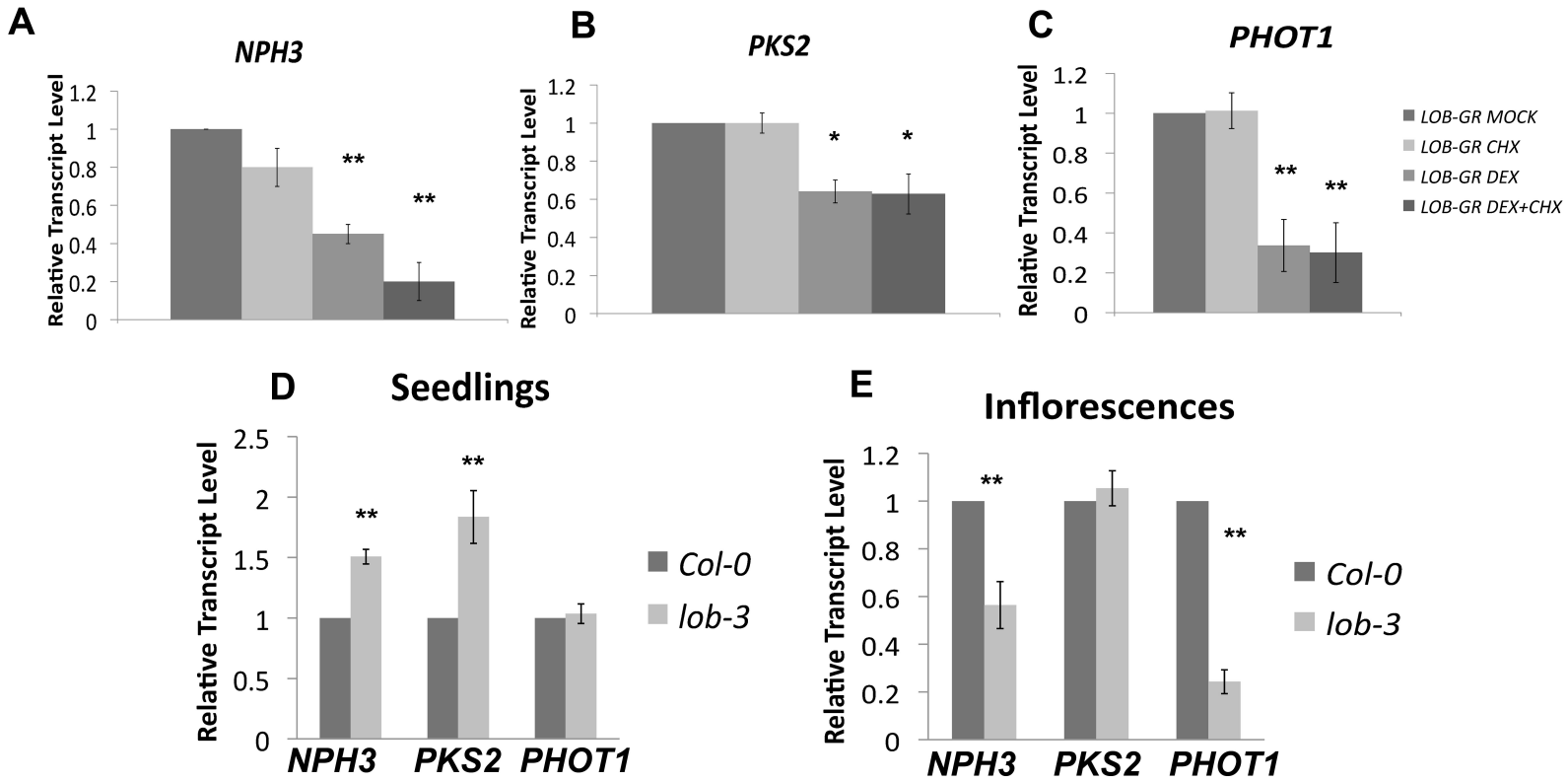

A
NPH3
Relative Transcript Level
1.2
1
0.8
0.6
0.4
0.2
0
**
**
B
PKS2
Relative Transcript Level
1.2
1
0.8
0.6
0.4
0.2
0
*
*
C
PHOT1
Relative Transcript Level
1.2
1
0.8
0.6
0.4
0.2
0
**
**
LOB-GR MOCK
LOB-GR CHX
LOB-GR DEX
LOB-GR DEX+CHX
D
Seedlings
Relative Transcript Level
2.5
2
1.5
1
0.5
0
**
**
NPH3
PKS2
PHOT1
Col-0
lob-3
E
Inflorescences
Relative Transcript Level
1.2
1
0.8
0.6
0.4
0.2
0
**
**
NPH3
PKS2
PHOT1
Col-0
lob-3

Figure 1.2. Transcript level of *NPH3, PKS2,* and *PHOT1* in a timecourse of DEX or MOCK treatment. Semi-quantitative RT-PCR of *PKS2, NPH3,* and *PHOT1* transcript from *35S:LOB-GR* seedlings supplemented with MOCK or DEX treatment for 1 hour and 2 hours. 25 cycles for UBC9 were used. *PKS2, NPH3,* and *PHOT1* were amplified with 28 cycles.

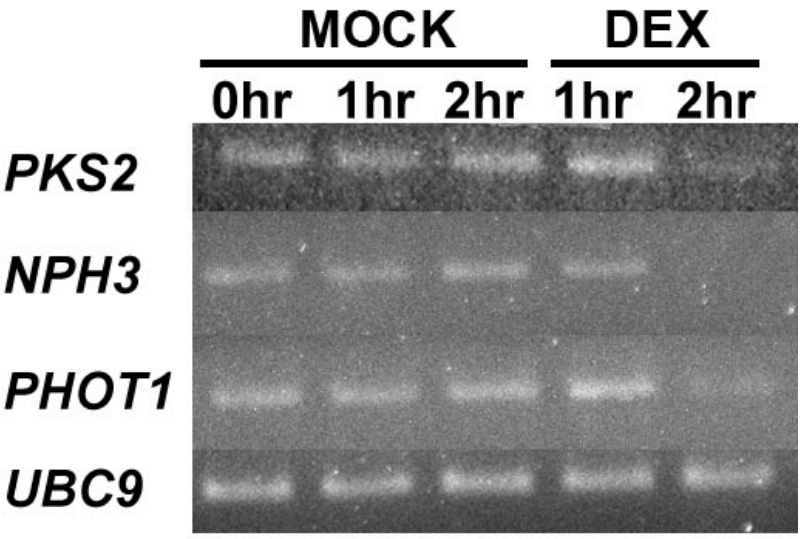

MOCK
DEX
0hr
1hr
2hr
1hr
2hr
PKS2
NPH3
PHOT1
UBC9

Figure 1.3. *NPH3, PKS2,* and *PHOT1* transcript levels in paraclade junctions. qRT-PCR of *NPH3, PHOT1,* and *PKS2* transcripts collected from the paraclade junction of *WT* and *lob-3 Arabidopsis* plants. Error bars indicated SE. Data represents the average of 3 biological replicates. Bars represent standard error. Significance was measured by student t-test.

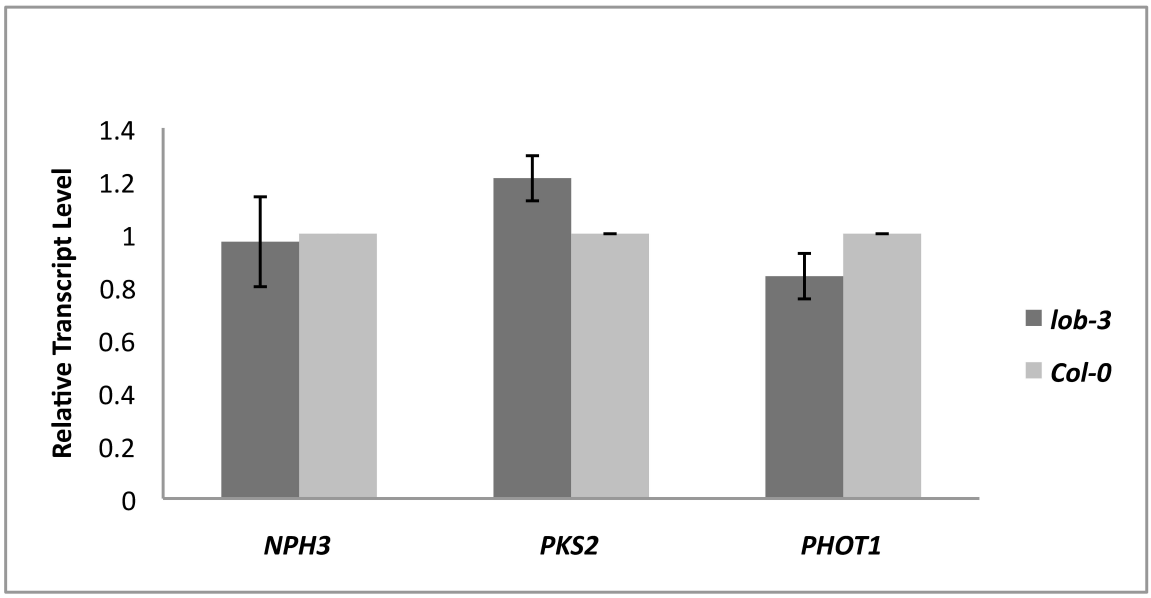

Relative Transcript Level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NPH3
PKS2
PHOT1
lob-3
Col-0

Figure 1.4. *LOB* mis-expression alters responses to blue-light. A,C,E) Plants grown on control media or B,D,F) media supplemented with DEX for 4 days in the dark then illuminated with blue-light on the right for 24 hours. A-B) *Col-0* plants C-D) *phot1-5* plants E-F) *35S:LOB-GR* plants. Images are representatives of n = 30. I) Relative transcript level measured by q-RT-PCR of *NPH3, PKS2,* and *PHOT1* in *35S:LOB-GR* plants supplemented with mock or DEX treatment. Data represents the average of 3 biological replicates. Error bars indicated SE. ** $p < 0.01$. Significance was measured by student t-test.

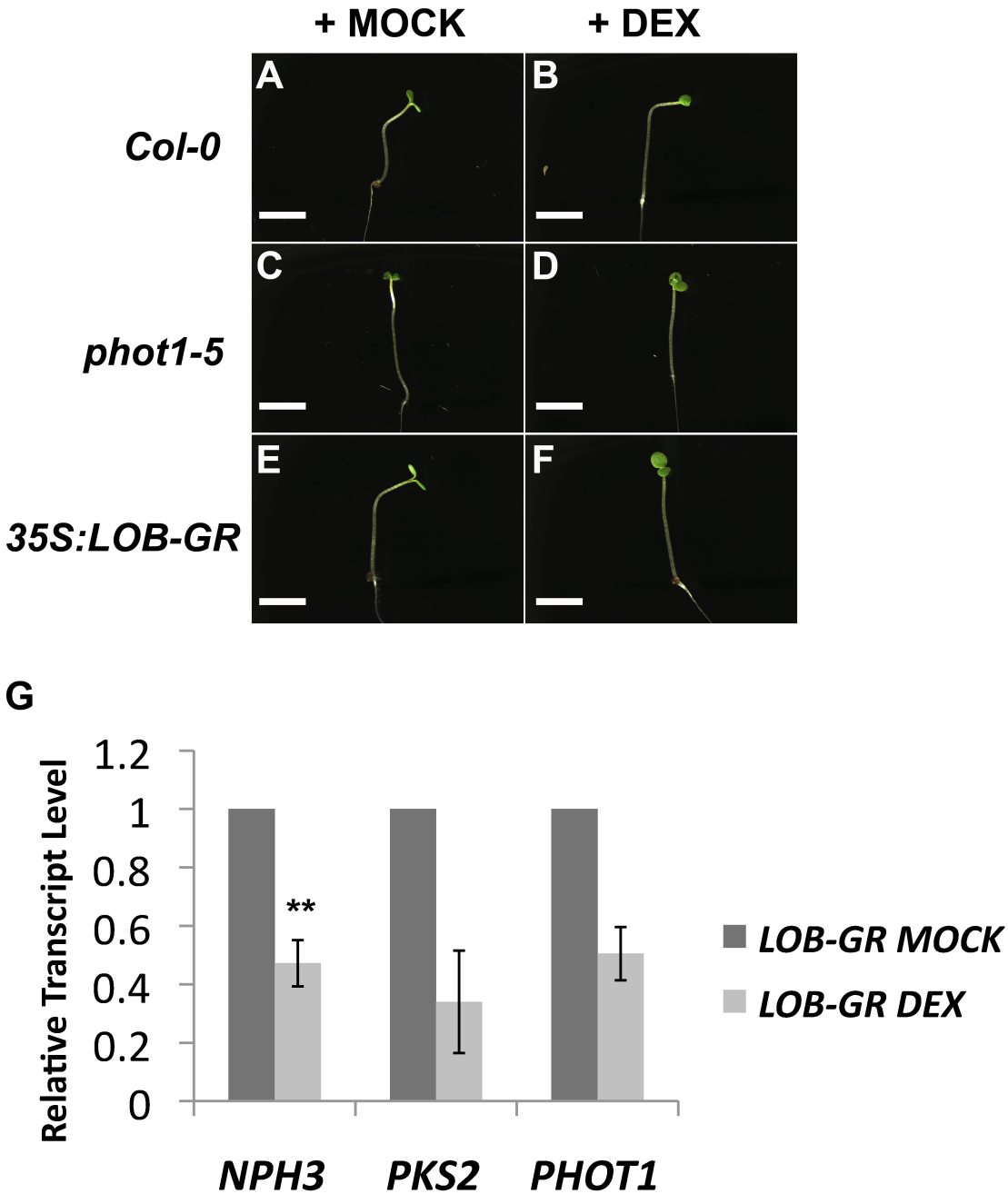

+ MOCK
+ DEX
A
B
Col-0
C
D
phot1-5
E
F
35S:LOB-GR
G
Relative Transcript Level
1.2
1
0.8
0.6
0.4
0.2
0
**
NPH3
PKS2
PHOT1
LOB-GR MOCK
LOB-GR DEX

Figure 1.5. LOB association with the genomic regions of blue-light response genes. A) Schematic representations of the *NPH3, PKS2,* and *PHOT1* gene. White boxes represent exons. For *PKS2, b*lack boxes represent exons of the upstream gene. Carrots represent the translation start codon. Bars indicate putative LOB-binding sites. B) Yeast-1-Hybrid results. Yeast were grown on selection media without 3-AT or 30 mM 3-AT for 3 days. C) Relative enrichment by ChIP analysis of *NPH3, PKS2,* and *PHOT1*. BS – binding site denoted in Figure 3A. Data represents the average of 3 biological replicates.. ** = $p < 0.01$. Bars represent standard error. Significance was measured by student t-test.

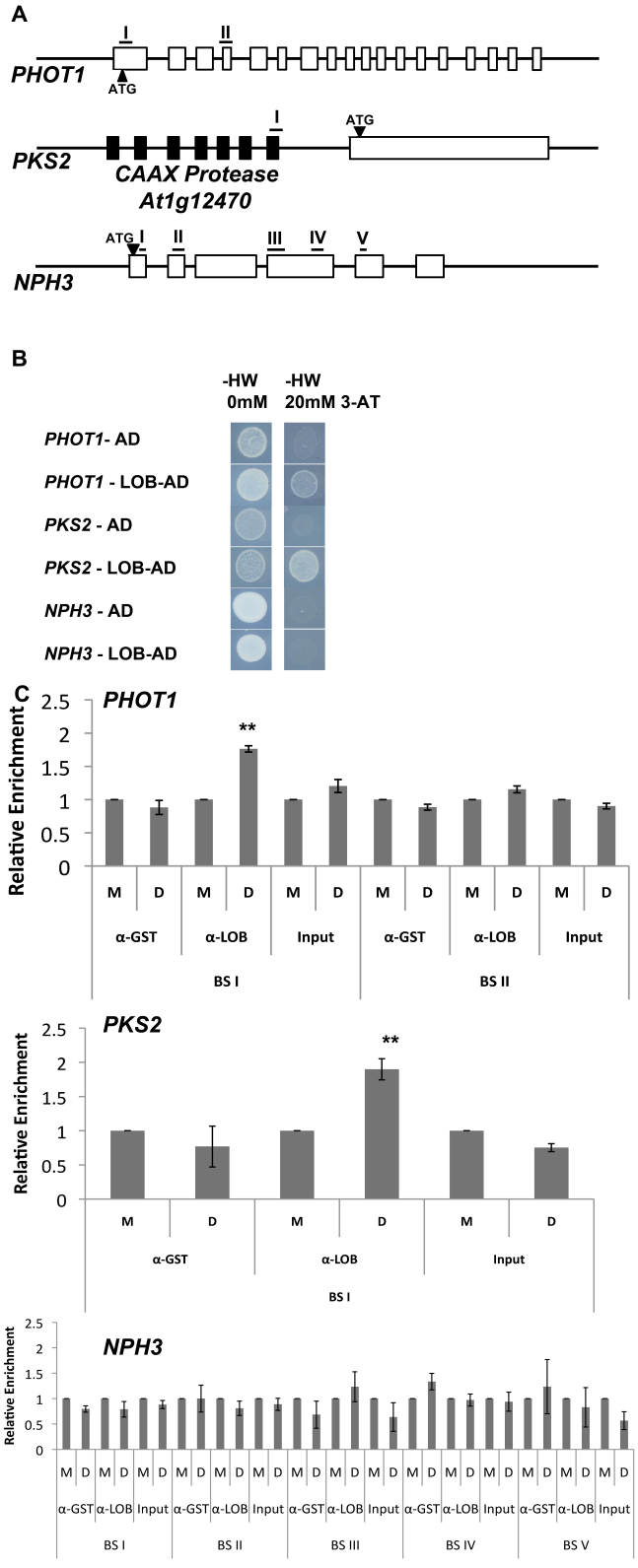

A
PHOT1
ATG
I
II
PKS2
CAAX Protease
At1g12470
ATG
I
ATG
NPH3
I
II
III
IV
V
B
-HW
0mM
-HW
20mM 3-AT
PHOT1- AD
PHOT1 - LOB-AD
PKS2 - AD
PKS2 - LOB-AD
NPH3 - AD
NPH3 - LOB-AD
C
PHOT1
Relative Enrichment
2.5
2
1.5
1
0.5
0
**
M
D
α-GST
α-LOB
Input
BS I
BS II
PKS2
Relative Enrichment
**
BS I
NPH3
Relative Enrichment
α-GST
α-LOB
Input
BS I
BS II
BS III
BS IV
BS V

Figure 1.6. *LOB* plays a role in short term blue-light response. A) Adaxial petiole angle of *Col-0, lob-3, lob-3 phot1-5, lob-3 pks2-2* seedlings grown for 13 days under white light. B) Adaxial petiole angle after 24 hours of 30 $\mu M/m^2s$ of blue-light illumination. C) Difference of petiole angle shown in panels A and B. n = 25. ** = $p<0.01$. Bars represent standard error. Significance was measured by student t-test.

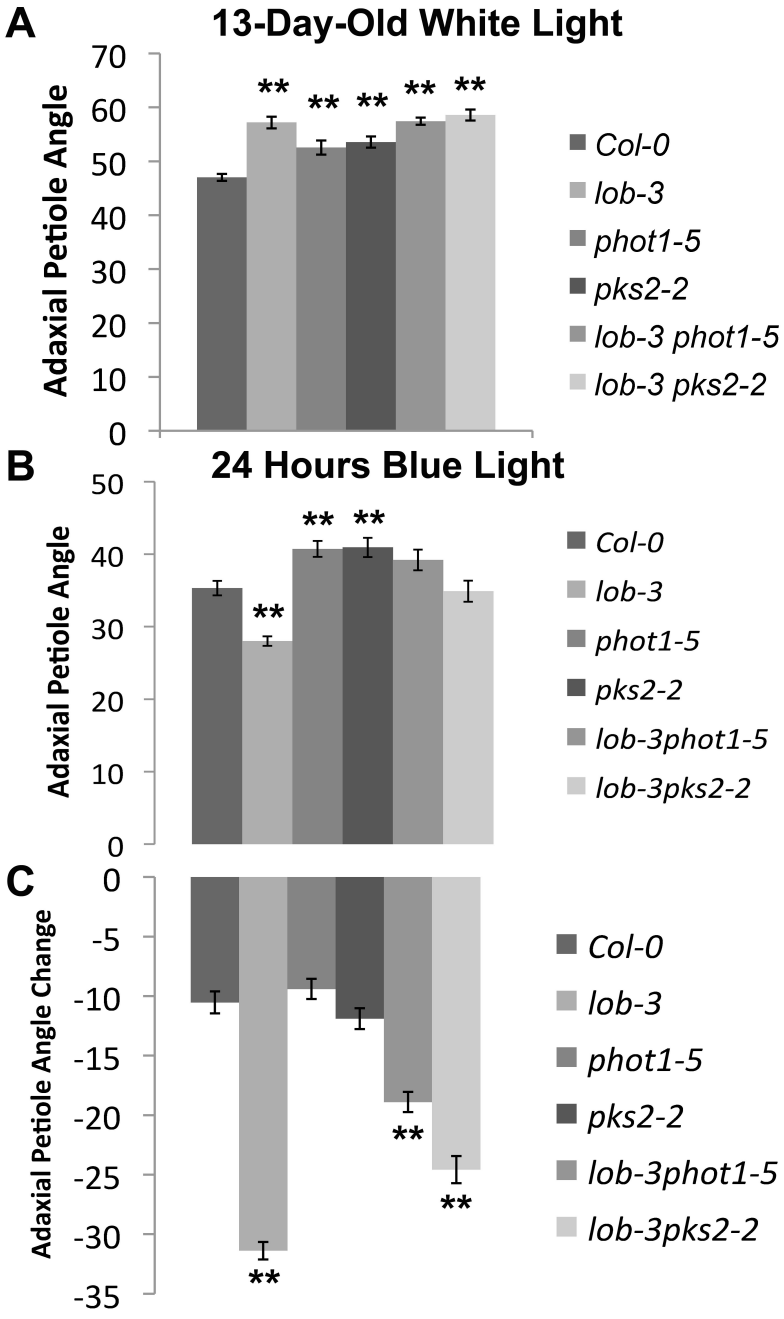

A
13-Day-Old White Light
Adaxial Petiole Angle
70
60
50
40
30
20
10
0
**
**
**
**
**
Col-0
lob-3
phot1-5
pks2-2
lob-3 phot1-5
lob-3 pks2-2
B
24 Hours Blue Light
Adaxial Petiole Angle
50
40
30
20
10
0
**
**
**
Col-0
lob-3
phot1-5
pks2-2
lob-3phot1-5
lob-3pks2-2
C
Adaxial Petiole Angle Change
0
-5
-10
-15
-20
-25
-30
-35
**
**
**
Col-0
lob-3
phot1-5
pks2-2
lob-3phot1-5
lob-3pks2-2

Figure 1.7. *lob-3* plants have altered leaf angles after long-term blue light exposure. (A-F) 14-day old seedlings illuminated with blue-light from above for 5 days 8 hours. A) *Col-0;* (B) *pks2-2;* (C) *nph3-1*; (D) *phot1-5;* (E) *lob-3*; and (F) *pLOB:LOB lob-3.* I) Quantitative leaf angles of A-F. ** = $p < 0.01$. * = $p < 0.05$. $24<n<32$. Bars represent standard error. Significance was measured by student t-test.

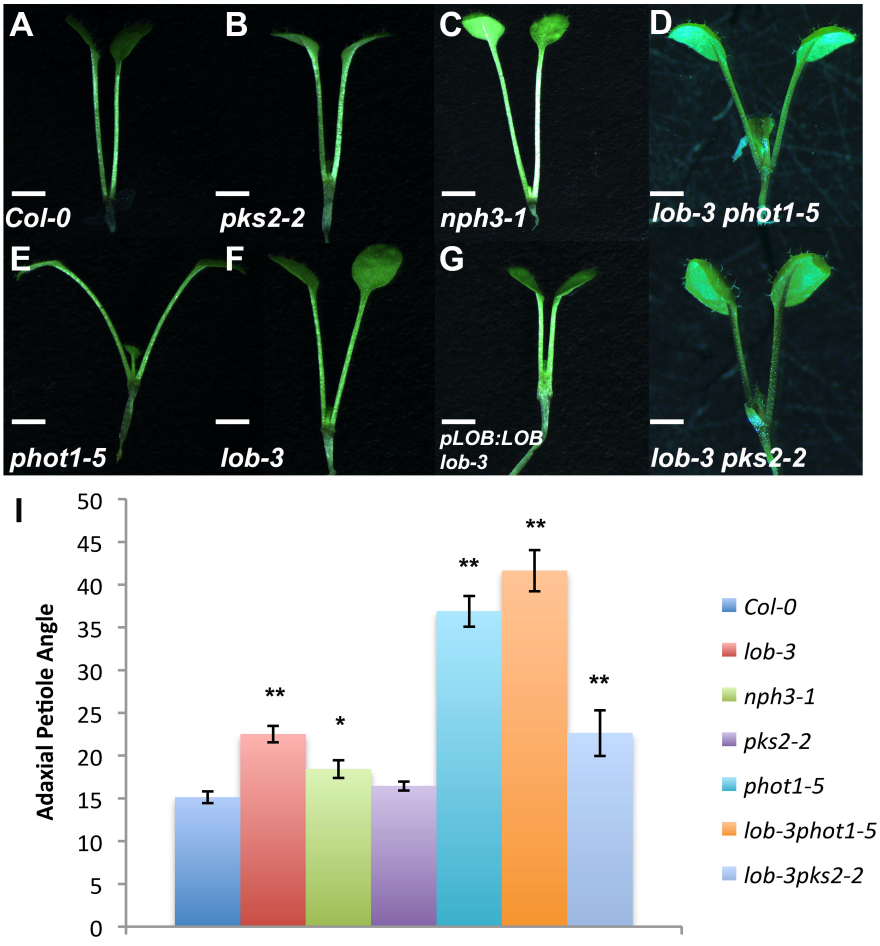
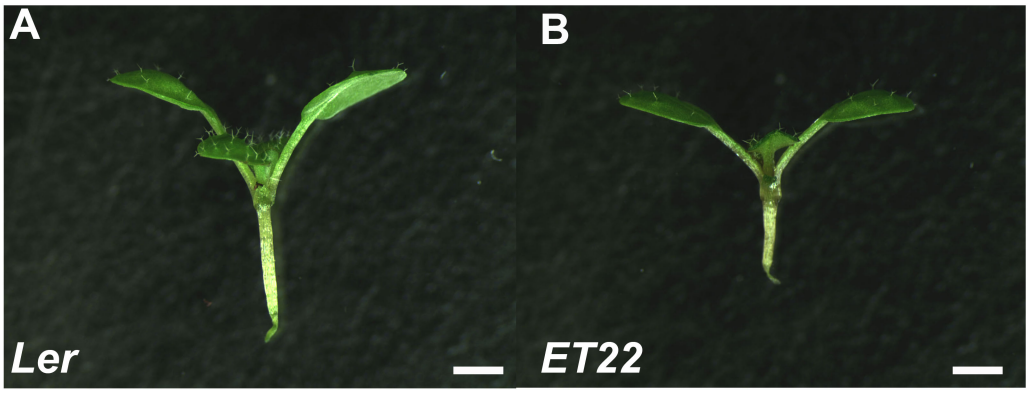

A
Col-0
B
pks2-2
C
nph3-1
D
lob-3 phot1-5
E
phot1-5
F
lob-3
G
pLOB:LOB
lob-3
lob-3 pks2-2
I
Adaxial Petiole Angle
50
45
40
35
30
25
20
15
10
5
0
**
*
**
**
**
Col-0
lob-3
nph3-1
pks2-2
phot1-5
lob-3phot1-5
lob-3pks2-2

Figure 1.8. *lob::DsE* plants have a less erect phenotype after blue-light response. A) *Ler* and B) *lob::DsE* after 5 days 8 hours of 30 μM/m^2s of blue-light illumination from above. C) Quantitative measurements of *Ler* and *ET22* plants. N = 32. ** = $p < 0.05$. Bars represent standard error. Significance was measured by student t-test.


A
Ler
B
ET22

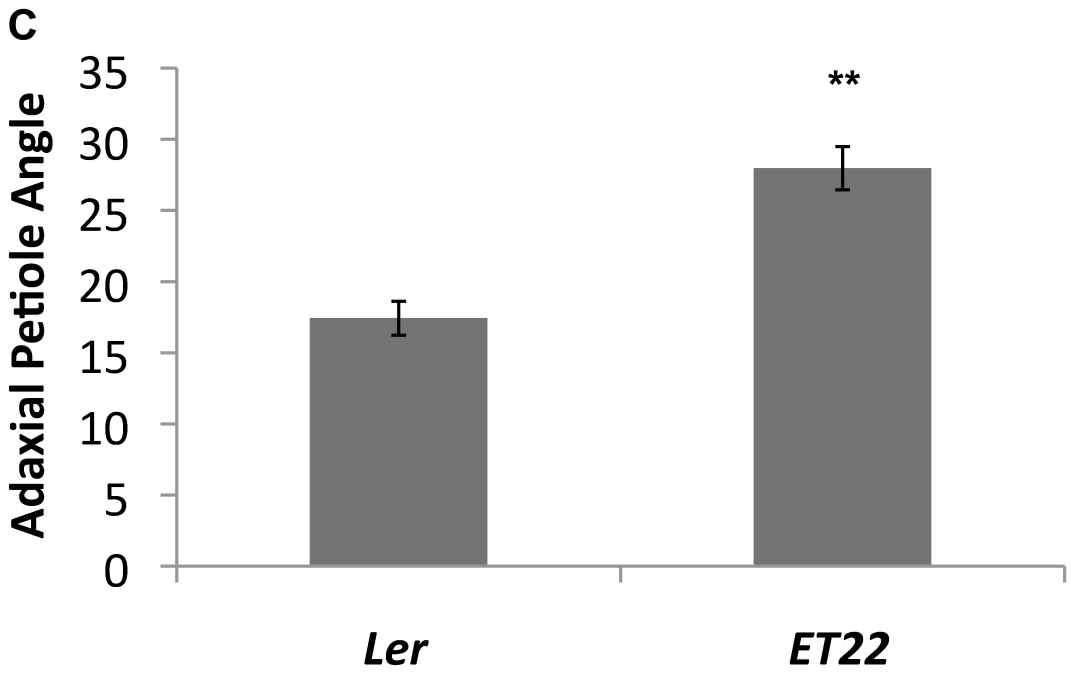

C
Adaxial Petiole Angle
35
30
25
20
15
10
5
0
**
Ler
ET22

Figure 1.9. Changes in leaf angle in response to blue-light time-course. A-I) Seedlings grown under 30 μM/m^2s for 3 days (A,D,G), 4 days (B,E,H) and 5 days (C,F,I). *Col-0* (A-C), *lob-3* (D-F), and *phot1-5* (G-I).

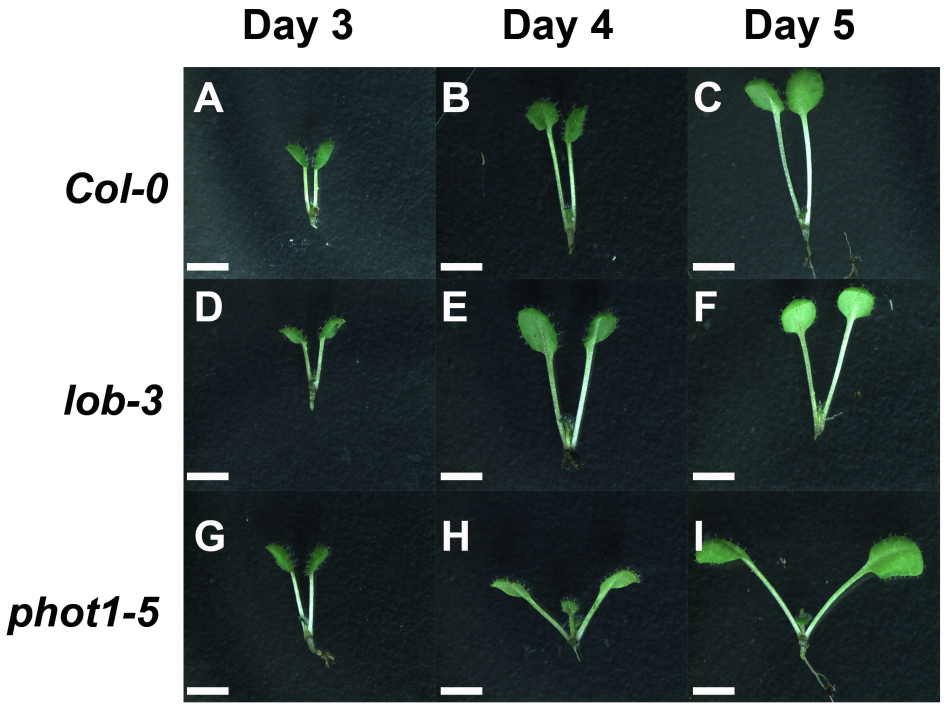

Day 3
Day 4
Day 5
Col-0
lob-3
phot1-5
A
B
C
D
E
F
G
H
I

Figure 1.10. *LOB* transcript levels are altered after blue-light illumination and in blue-light response gene mutants. A) *LOB* transcript levels in 14-day-old wild-type above ground organs after 0 hour, 1 hour, 4 hour, and 24 hours of blue-light illumination. B) qRT-PCR of *LOB* transcript in wild-type, *pks2-2* and *phot1-5* seedlings. Data represents the average of 3 biological replicates. Error bars indicate SE. * $p < 0.05$. ** $p < 0.01$. Significance was measured by student t-test.

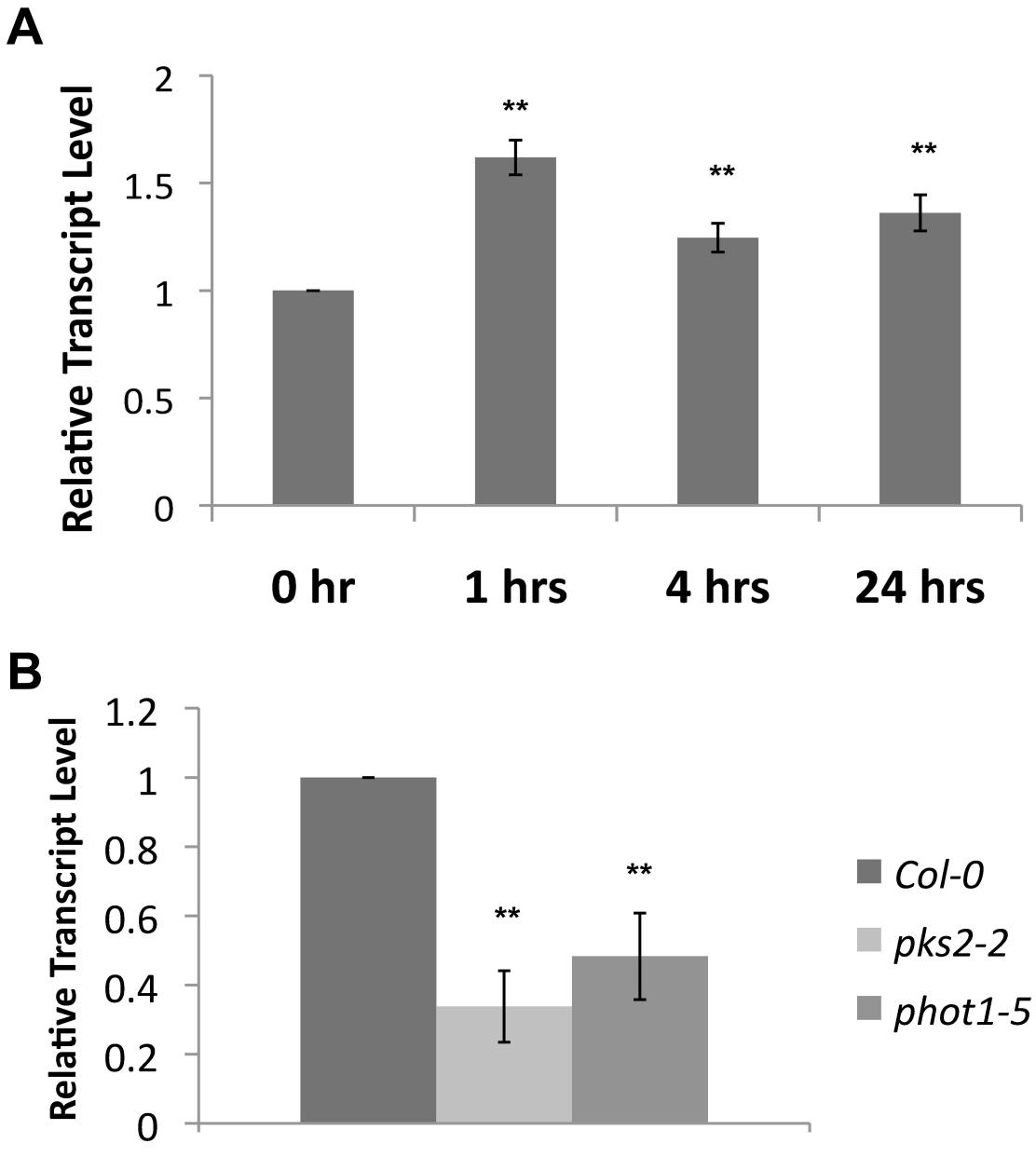

A
Relative Transcript Level
2
1.5
1
0.5
0
**
**
**
0 hr
1 hrs
4 hrs
24 hrs
B
Relative Transcript Level
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Col-0
pks2-2
phot1-5

Figure 1.11. Blue-light response in boundary mutants. Adaxial petiole angle of boundary mutants after 5 days 8 hours of unilateral blue-light from above. Error bars indicated SE. 24<n<32 plants. ** $p < 0.01$. Significance was measured by student t-test.

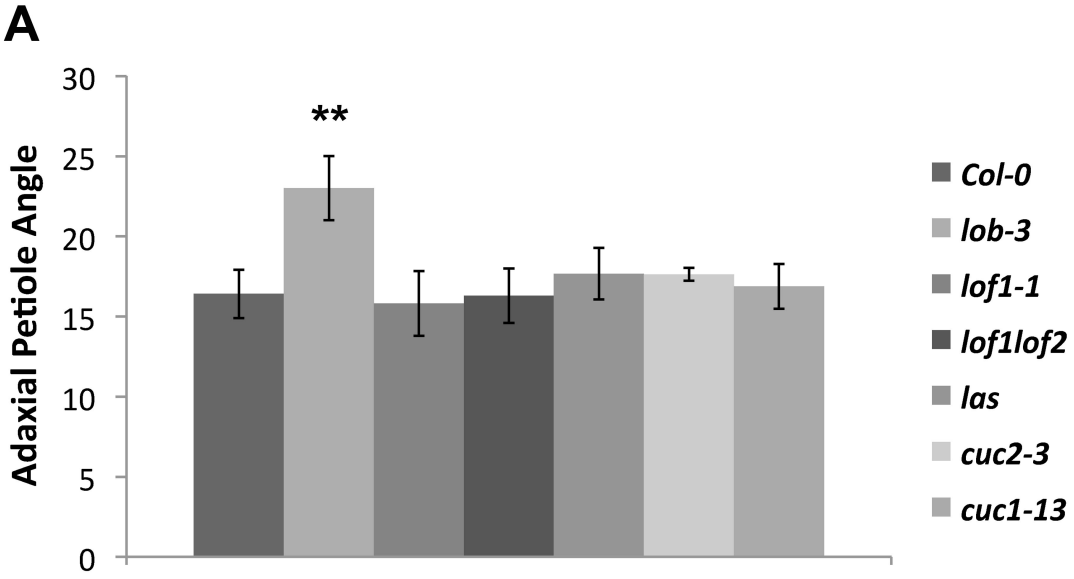

A
Adaxial Petiole Angle
30
25
20
15
10
5
0
**
Col-0
lob-3
lof1-1
lof1lof2
las
cuc2-3
cuc1-13

Figure 1.12. *LOB*-mediated blue-light response is independent of brassinosteroid levels. A-B) Petiole angle of *Col-0, lob-3, pLOB:BAS1 Col-0,* and *pLOB:BAS1 lob-3* after A) 24 hours and B) 5 days 8 hours of blue-light illumination. ** $p < 0.01$. $16<n<32$. Bars represent standard error. Significance was measured by student t-test.

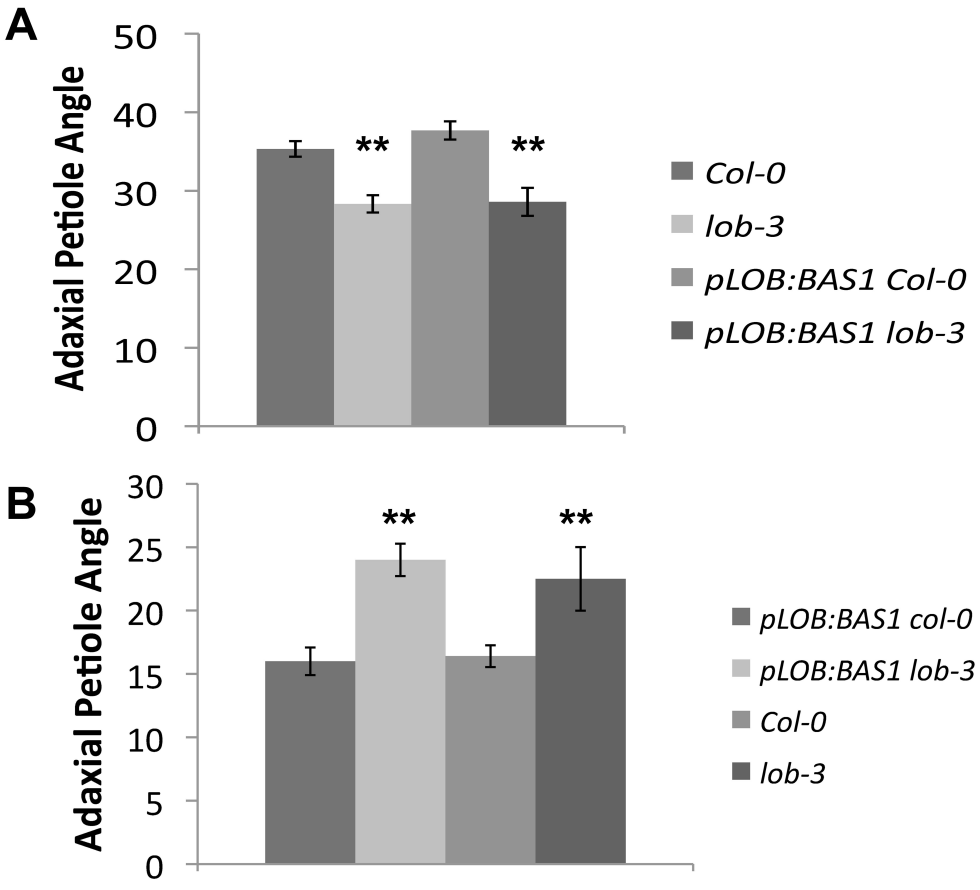

A
Adaxial Petiole Angle
50
40
30
20
10
0
**
**
Col-0
lob-3
pLOB:BAS1 Col-0
pLOB:BAS1 lob-3
B
Adaxial Petiole Angle
30
25
20
15
10
5
0
**
**
pLOB:BAS1 col-0
pLOB:BAS1 lob-3
Col-0
lob-3

Figure 1.13 *lob-3* plants fail to respond to ethylene treatment. *Col-0* and *lob-3* plants grown on media for 13 days followed by 24 hours of 5ppm ethylene gas. n = 24. ** = $p < 0.05$. Error bars represent standard error. Significance was measured by student t-test.

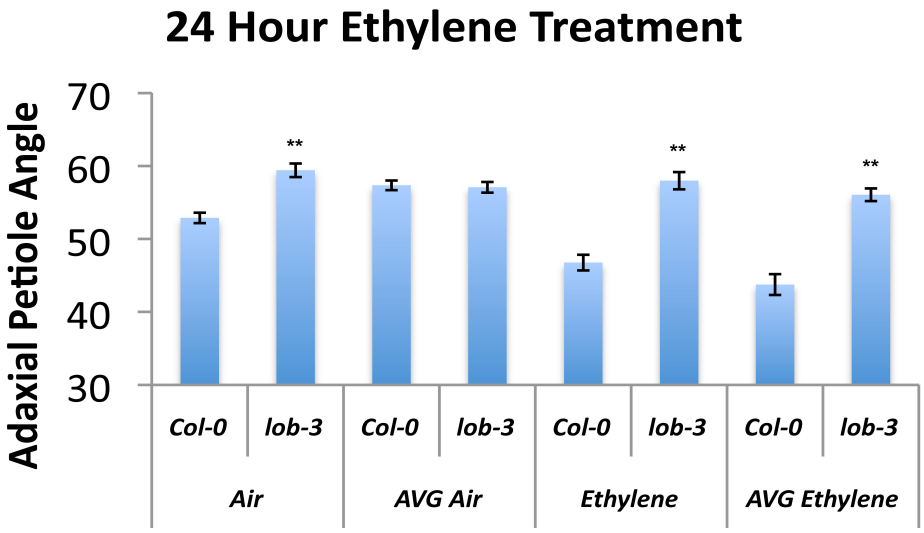

24 Hour Ethylene Treatment
Adaxial Petiole Angle
70
60
50
40
30
**
**
**
Col-0
lob-3
Col-0
lob-3
Col-0
lob-3
Col-0
lob-3
Air
AVG Air
Ethylene
AVG Ethylene

Table 1.1 List of oligonucleotide sequences.

Primer Name	Sequence 5' → 3'	Tm (°C)
qRT-NPH3 FP	TCCCTGTGTAAGCCCATCTAA	55.4
qRT-NPH3 RP	AGACTCCATCTTGGTCCTGAAG	56.1
qRT-PKS2 FP	AGCCAGAGTTTGTTGCTTCAG	55.7
qRT-PKS2 RP	GCAGCCAAGAGTAGCGAGAA	57.2
qRT-PHOT1 FP	CACTGATCCTAGGCTTCCCG	57.2
qRT-PHOT1 RP	GTGGTTAGATCAGTCTCTGGACC	56.4
qACT2 - FP	GCACCCAGTTCTACTCACAG	55.2
qACT2 - RP	CAACATACATGGCAGGGAC	53.5
qUBC9 - FP	GATAGCCCTTATTCTGGAGGAG	54.1
qUBC9 - RP	TTGGATGGAACACCTTCGT	54
PHOT1 BSI FP	ACCTCATGGATGGCTCTGAA	55.9
PHOT1 BSI RP	GTGGCTTTCCCGTCTTTGT	55.7
PHOT1 BSII FP	ATCGCAGAGAAACTCGCAAA	54.8
PHOT1 BSII RP	CCAGCACTTGCATACATAATCG	54.1
PKS2 BSI FP	TGATGTGGGAATCTGAGAGC	54.3
PKS2 BSI RP	ACCAAGATTGGCCTCTAAGC	54.9
NPH3 BSII FP	TGCCTATGATTAGGTTTGTTGC	53.4
NPH3 BSII RP	CCATAAACTTTCTCTAGTTCCAGCTT	54.8
NPH3 BSIII FP	CAGCATAATCCCTCCACAGAA	54.4
NPH3 BSIII RP	GTTCGAATTGCATCCCTACG	53.8
NPH3 BSIV FP	TGGCTAAAGCATTGCTGATCT	54.7
NPH3 BSIV RP	AGCATGAGAGGAAACGGCTA	56
NPH3 BSV FP	TGTACGATGTTGATCTTGTTCAGAG	54.8
NPH3 BSV RP	AAGCCTCGCCACTCTCATTT	56.8
pNPH3 Y1H1 attB1R	GGGGACTGCTTTTTTGTACAAACTTG TGCAGTGATTACACGAACGA	66.7
pNPH3 Y1H1 attB4	GGGGACAACTTTGTATAGAAAAG TTGGTTCCTACGAGCGAGAAGA	65.7
pPKS2 Y1H1 attB1R	GGGGACTGCTTTTTTGTACAAAC TTGAGCTATGTCGTGTGGGTTCC	67.5
pPKS2 Y1H1 attB4	GGGGACAACTTTGTATAGAAAA GTTCAGTTTCTCAACGTCGATTCC	64.4
pPHOT1 Y1H1 attB1R	GGGGACTGCTTTTTTGTACAAA CTTGACCAGAGTTCCTCACGCCTA	67.9
pPHOT1 Y1H1 attB4	GGGGACAACTTTGTATAGAAAAGT TGTCAAACCATCCATCTACCACA	65
qRT-LOB FP	GGCGTCGTCATCAAACTCAT	55.4
qRT-LOB RP	CGTTGCTTGCTCCAAAGATT	54
RT-NPH3 FP	CGTTACCGGAATCTGCTAGG	55
RT-NPH3 RP	TCGTTCTGAAGCTCGACGTA	55.7
RT-PKS2 FP	GCCAGATCCAGAAGTTCCAA	54.8
RT-PKS2 RP	TTCGAATCTTCTTCACTGTGG	52.5
RT-PHOT1 FP	GTCAGGCCGAAGAAACTCTG	55.7
RT-PHOT1 RP	TTCGAATCTTCTTCACTGTGG	52.5

Chapter 2

LATERAL ORGAN BOUNDARIES (LOB) directly regulates the expression of *NAKED PINS IN YUC MUTANTS 1 (NPY1)*

Abstract

Lateral organs are formed at the periphery of the meristem and separation of these organs from the meristem is necessary for correct plant development. The boundary region is a small group of cells that separates lateral organs from the shoot apical meristem. *LATERAL ORGAN BOUNDARIES (LOB)* is expressed in the boundary and encodes a transcription factor that differentially regulates gene expression. LOB has a demonstrated role in the separation of lateral organs and blue-light mediated hyponastic response. In a previous microarray experiment, *NAKED PINS IN YUC MUTANTS 1 (NPY1)* was identified as a putative target of LOB. *NPY1* transcript is increased in plants that have higher LOB activity, indicating that LOB regulates *NPY1* in a positive manner. In addition, *NPY1* transcripts are lower in inflorescences of *lob* mutants than in wild-type inflorescences and LOB directly binds to the promoter region of *NPY1*, suggesting that *NPY1* is a direct target of LOB. However, *npy1-1* single mutants did not exhibit any developmental abnormalities and *npy1-1* did not enhance the organ fusion observed in *lob-3* single mutants. Furthermore, *npy1-1* single mutants did not exhibit a blue-light phototropic defect suggesting *NPY1* does not play a demonstrated role in the phototropic response to blue-light. These data suggest that LOB directly regulates *NPY,* however the biological relevance of this relationship is unclear.

Introduction

Leaves are integral and necessary plant organs and their primary purpose is to create energy for the plant by utilizing light from the sun. Leaves form at the periphery of the shoot apical meristem (SAM), which is located at the tip of the plant and has two important functions. The first function is to create lateral organs and the second is to maintain a reservoir of pluripotent stem cells. These stem cells are located in a small region of the SAM called the central zone where they divide and their daughter cells in the peripheral zone differentiate to form lateral organs (Szymkowiak and Sussex 1996). Plant lateral organs are separated from the meristem by the boundary region (Braybrook and Kuhlemeier 2010).

The boundary is composed of cells that are smaller and divide more slowly than the surrounding cells (Callos and Medford 1994). Due to its morphological location and function, the boundary region has a distinct and important role during lateral organ development. In recent studies, several genes have been shown to be specifically expressed in the boundary region, including *JAGGED LATERAL ORGANS (JLO)*, *CUP SHAPED COTYLEDON (CUC),* and *LATERAL ORGAN FUSION 1/2* (*LOF1/2)*. *Arabidopsis cuc* and *lof1/2* mutants have fusions of the lateral organs to the SAM and to each other, demonstrating their role in lateral organ separation (Aida et al. 1997; Greb et al. 2003; Lee et al. 2009). Transgenic plants expressing a fusion of JLO to the EAR transcriptional repression domain, which is thought to result in a dominant negative function, also exhibit organ fusions (Borghi et al. 2007). These data suggest that boundary-expressed genes function in boundary formation and organ separation.

Another gene that has been shown to be involved in the separation of lateral organs is the *Arabidopsis thaliana* gene *LATERAL ORGAN BOUNDARIES (LOB)*. Originally identified by its expression pattern in an enhancer-trap screen, *LOB* is expressed in all boundary regions including the base of leaves, floral organs, pedicels, and lateral roots (Shuai et al. 2002). *Arabidopsis* plants containing a hypomorphic *lob* mutation exhibit a slight fusion of the axillary stem to the cauline leaf, suggesting that LOB functions to separate lateral organs from the SAM (Bell et al. 2012). Over-expression of *LOB* results in plants that have shorter petioles and smaller rosette leaves than wild-type plants, suggesting that *LOB* may function to limit growth in the boundary region, perhaps by regulating cell size and cell growth to control lateral organ separation (Shuai et al. 2002).

The *LOB* gene encodes an ~20 kDa protein that binds to DNA and acts as a transcription factor to differentially regulate genes involved in a wide variety of biological processes including brassinosteroid responses (Husbands et al. 2007; Bell et al. 2012). The DNA binding recognition site for LOB has been identified as a core 5'-CGGC-3' nucleotide sequence; binding efficiency is increased with a G on either side of the core sequence (Husbands et al. 2007).

The initiation of lateral organs requires the proper transport of the phytohormone auxin. Plants fail to form floral organs if auxin transport is blocked by treatment with the inhibitor 1-N-naphthylphthalamic acid (NPA) (Reinhardt et al. 2000; Vanneste and Friml 2009). Furthermore, plants harboring mutations in an auxin-transport protein fail to form inflorescences (Okada et al. 1991; Serrano-Cartagena et al. 1999; Scanlon 2003).

Interestingly, when exogenous auxin is applied to the flank of the meristem of these mutant plants, initiation of lateral organs occurs (Reinhardt et al. 2000).

Auxin must be transported to the correct location in the SAM periphery in order for lateral organs to form. Transport is carried out by family members of the PIN-FORMED (PIN) family of proteins (Krecek et al. 2009). PIN1 is a trans-membrane protein and is polarly localized to the plasma membrane such that it exports auxin to the site of leaf primordia initiation. Compared to the surrounding cells, auxin response is higher at the site of initiating primordia (Reinhardt et al. 2003; de Reuille et al. 2006; Jonsson et al. 2006; Smith and Bayer 2009). Auxin has also been implicated in boundary formation. A small percentage of *Arabidopsis* plants that have been treated with synthetic auxin have fusions of the cotyledons (Hadfl et al. 1998; Furutani et al. 2004). Furthermore, after auxin application, the percentage of fused cotyledons was increased in *cuc1,* but not *cuc2* seedlings compared to wild-type suggesting that auxin negatively regulates *CUC2* activity (Furutani et al. 2004).

Previously, a microarray was performed to identify genes that were differentially expressed in response to over-expression of *LOB* (Bell et al. 2012). This experiment identified a number of putative LOB targets including *NON PHOTOTROPIC HYPOCOTYL 3 (NPH3)* and *NAKED PINS IN YUC MUTANTS 1 (NPY1).* The NPH3 protein is localized to the plasma membrane and contains a BTB (BROAD COMPLEX, TAMTRACK, and BRIC A BRAC) domain that is involved in the heterodimerization of proteins (Bardwell and Treisman 1994). Based on protein localization and the presence of a BTB domain, NPH3 is proposed to function as a scaffold protein that can link other

proteins at the plasma membrane (Motchoulski and Liscum 1999). *NPH3* was initially identified based on its role in light responses (Motchoulski and Liscum 1999) and more recently has been shown to be involved in regulating leaf positioning. Plants carrying an *nph3-6* knock-out mutation form leaves that are less erect than wild-type leaves (de Carbonnel et al. 2010) and are unable to respond to blue-light (Sakai et al. 2000). A related gene, *NPY1*, functions with the protein kinase PINOID (PID) to regulate auxin-mediated flower development. PID functions to localize the PIN auxin-efflux proteins, thus determining the direction of auxin movement (Friml et al. 2003; Kleine-Vehn et al. 2009). *npy1* and *pid* single mutants have altered floral organ numbers, whereas *npy1 pid* double mutants completely lack floral organs and cotyledons (Cheng et al. 2007). *npy1* also enhances the phenotype of the auxin-deficient *yucca3 yucca4* double mutant, which has a defect in auxin biosynthesis (Zhao et al. 2001; Zhao et al. 2002) The *npy1 yuc3 yuc4* triple mutant has a more severe phenotype than the *yuc3 yuc4* double mutant and resembles the severe auxin-transport deficient *pin1* mutant. These data suggest that *NPY1* has a role in auxin response (Cheng et al. 2007).

Several lines of evidence suggest that LOB regulates *NPY1* expression. *NPY1* RNAs were increased in seedlings that over-express *LOB* and the *NPY1* promoter region contains the LOB-binding site 5'-(G)CGGC(G)-3' (Husbands et al. 2007; Bell et al. 2012). Furthermore, *NPY1* expression profiles partially overlap with *LOB* (Furutani et al. 2004; Winter et al. 2007). Data from this Chapter suggests that *NPY1* is directly activated by LOB and that *NPY1* expression in flowers is altered in *lob-3* mutants. However *npy1-1* mutants do not have fusions of lateral organs nor do they have altered responses to blue-

light, suggesting that *NPY1* does not have a demonstrated role in blue-light-mediated hyponastic responses nor the separation of lateral organs.

Results

Ectopic LOB activity alters *NPY1* transcript levels.

In a previous microarray experiment, *NPY1* was identified as a downstream target of LOB. To confirm the microarray data, we performed an independent experiment in which LOB activity was induced using a dexamethasone (DEX) inducible system (Bell et al. 2012). We measured the transcript level of *NPY1* by quantitative RT-PCR (qRT-PCR) After 4 hours of DEX treatment, the transcript level of *NPY1* was >2-fold higher in 14-day-old *35S:LOB-GR* plants compared to MOCK-treated plants (Figure 2.1A). We next asked if this change in *NPY1* transcript following LOB-GR induction was dependent on protein synthesis. *35S:LOB-GR* plants were treated with DEX together with the translational inhibitor cycloheximide (CHX). *NPY1* transcript levels were increased by >2 fold in these plants compared to CHX treated *35S:LOB-GR* (Figure 2.1A) plants.

NPY1 transcripts are altered in loss-of-function *lob-3* mutants

NPY1 transcript levels were higher in response to ectopic *LOB* expression (Figure 2.1). To further investigate the regulation of *NPY1* by LOB, we assayed the transcript levels of *NPY1* in the *lob-3* mutant using qRT-PCR. The transcript levels of *NPY1* were not significantly different in *lob-3* seedlings and paraclade junctions compared to wild-

type. In inflorescences however, *NPY1* transcripts were 3.2-fold lower, in *lob-3* mutants compared to wild-type (Figure 2.2), These results suggest that LOB positively regulates *NPY1* in inflorescences.

LOB is associated with the promoter regions of *NPY1 in planta*

NPY1 transcript levels are altered in response to *LOB* over-expression in a translation-independent manner suggesting that LOB directly regulates *NPY1*. Furthermore, canonical LOB-binding sites are present in the promoter region of *NPY1* (Figure 2.3A). Two putative binding sites, separated by 38 bps, are located ~1.5 kb upstream of the *NPY1* translation start site. Additional binding sites are located in the 5'UTR (600bp upstream of the *NPY1* translation start site), in the first exon, in the second exon, and in the fourth exon. We examined LOB binding to these sites using a chromatin immunoprecipitation assay using anti-LOB antibodies in *35S:LOB-GR* seedlings incubated with DEX or MOCK solution, followed by qPCR. We detected enrichment of *NPY1*-binding site I (BSI) in samples immunoprecipitated from DEX-treated compared to MOCK-treated seedlings. There was no significant enrichment of any other binding site in DEX-treated samples compared to MOCK-treated samples. As a control, we performed ChIP with an anti-GST antibody and found no difference in relative enrichment of BS1 in MOCK-treated and DEX-treated samples (Figure 2.3C). These results suggest that LOB directly binds to the promoter of *NPY1 in vivo*.

NPY1 expression in flowers is altered in *lob-3* mutants

To understand the control of *NPY1* expression by LOB, we examined the expression of *NPY1* in flowers of wild-type and *lob* plants using promoter:*GUS* reporter constructs. In wild-type plants, *pNPY1:GUS* expression was observed at the base of the floral organs and throughout the carpel. Strong GUS staining was observed at the base of the carpel and the boundary of the stigma and style of the carpel (Figure 2.4A). In seedlings, GUS staining was observed throughout the cotyledon, leaf petiole, and throughout the SAM and hypocotyl (Figure 2.4C). In paraclade junctions, GUS expression was observed in the cauline leaf but not the axillary or primary stem (data not shown) This pattern of expression partially overlaps with that of *LOB* (Shuai et al. 2002), consistent with the positive regulation of *NPY1* by LOB. Compared to wild-type, weaker GUS activity was observed at the base of the floral organs and at the boundary of the stigma and style in *NPY1:GUS lob-3* plants (Figure 2.4B). These data suggest that LOB positively regulates *NPY1* expression at the base of the floral organs. No difference in the expression pattern of *pNPY1:GUS* was observed between *lob-3* and wild-type plants in other tissues (data not shown).

npy1-1 plants do not have an altered hyponastic response to blue-light

Given that *NPY1* is positively regulated by LOB and *lob* plants have an altered hyponastic response to blue-light, we asked if *npy1-1* plants have an altered response to blue-light illumination. Plants were grown in white light and then transferred to a blue-light chamber where they were illuminated with blue-light from above for 5 days, 8

hours. Wild-type plants had an average petiole angle of 15.1° (Figure 2.5A), whereas *lob-3* plants had a less erect leaf angle with an average petiole angle of 22.5°. *npy1-1* mutants did not have a significantly different petiole angle (15.8°) than wild-type (Figure 2.5A). We then asked if *npy1-1* mutants had an altered response to short-term exposure to blue light. *npy1-1* plants were grown in white light for 13 days followed by illumination with blue-light for 24 hours. After blue-light illumination, wild-type leaves had an average petiole angle of 35.3°, while *lob-3* plants had a more erect leaf angle of 28.3° (Figure 2.5B). *npy1-1* plants had an adaxial leaf angle of 38.1°, which is not statistically different from the petiole angle of wild-type plants (Figure 2.5B). These results indicate that *NPY1* does not play a demonstrative role in the hyponastic response to blue-light.

npy1 plants have a normal paraclade junction phenotype

Since *lob* plants have a fusion between the axillary stem and cauline leaf and LOB positively regulates *NPY1*, we asked if *npy1-1* mutants had defects in the separation of the axillary stems and cauline leaves. *lob-3* had an average length of contact between the axillary stem and cauline leaf of 0.83 mm, 0.8 mm, and 0.91 mm for the first three paraclade junctions compared to 0.22 mm, 0.3 mm, and 0.3 mm for wild-type plants (Figure 2.6). *npy1-1* plants had an average contact length not significantly different (0.24 mm, 0.24 mm, and 0.33 mm) than wild-type plants indicating that *NPY1* does not play a demonstrative role in separation of the axillary stem and cauline leaf. To understand the genetic relationship between *LOB* and *NPY1,* we examined *lob-3 npy1-1* double mutants for axillary stem-cauline leaf fusion. Compared to *lob-3* plants, there was no significant

difference in contact length between the axillary stem and cauline leaf of *lob-3 npy1-1* double mutants (Figure 2.6).

Discussion

Microarray data identified *NPY1* was a putative target of LOB. To characterize this relationship, we examined the transcript level of *NPY1* in *LOB* over-expression plants and *lob-3* mutants. *NPY1* transcript was significantly increased in *35S:LOB-GR* plants compared to control plants even in the presence of the translational inhibitor, CHX. This indicates that LOB regulates *NPY1* independently of translation, consistent with LOB directly regulating *NPY1* expression. To test this, we examined LOB occupancy of the *NPY1* promoter using a ChIP assay. Binding sites in the *NPY1* promoter were enriched in samples derived from *LOB* over-expression plants precipitated with a LOB-antibody compared to control samples. Taken together, these data suggest that LOB directly regulates *NPY1* expression.

What is the biological relevance for the regulation of *NPY1* by LOB? *npy1-1* mutants do not exhibit a fusion between the axillary stem and cauline leaf compared to wild-type plants, nor do they have an altered hyponastic response to blue-light, two phenotypes that *lob* mutants exhibit. A possible explanation for the lack of observable phenotype is that NPY1 may be functionally redundant. *NPY1* is a member of the *NPH3* family, which functions as scaffolding proteins in a variety of processes (Motchoulski and Liscum 1999; Pedmale and Liscum 2007; Li et al. 2011). Possibly, another NPH3 family member functions with NPY1 in the separation of lateral organs. Recently, it has

been shown that *NPY3* and *NPY5* play a role in PIN1 distribution and flower development. *npy1 npy3 npy5* triple mutants resemble *pin1* mutants and PIN1 distribution is altered in the triple mutant compared to wild-type plants (Furutani et al. 2014). However, *NPY3* and *NPY5* were not differentially regulated in the *35S:LOB-GR* microarray experiments. Additional experiments are required to test this possibility.

That LOB regulates *NPY1* transcript provides a link between auxin and *LOB* function. Lateral organs are formed at the periphery of the SAM and this process requires proper auxin transport. One hypothesis is that LOB is involved in the separation of lateral organs by regulating *NPY1,* which regulates auxin transport. We could find no evidence that LOB regulation of *NPY1* is involved in the separation of the axillary stem and cauline leaf. *NPY1* transcripts are not significantly different in *lob* mutant paraclade junctions compared to wild-type plants and *npy1* mutants do not have a fusion of the axillary stem and cauline leaf. Could LOB regulate *NPY1* for proper development of other organs? One possibility is that LOB regulates *NPY1* for proper floral organ development. *npy1-1* plants have altered floral organ development and *NPY1* transcript is significantly decreased in inflorescences. However, we did not observe a difference in floral organ number or development in *lob-3 npy1-1* double mutants compared to single *npy1* mutants (data not shown). A biological role for *NPY1* regulation by LOB is not clear and further experiments are required.

Materials and Methods

Plant materials and growth conditions:

lob-3, *35S:LOB-GR* (Bell et al. 2012), and *npy1-1* (Cheng et al. 2007) are in the Col-0 background. For plants grown on media, seeds were sterilized for 5 minutes with 95% EtOH, 5 minutes with a 20% bleach/0.01% Tween20 solution, and rinsed 5 times with sterile water. They were then sown on Murashige and Skoog (MS) media (pH 5.7) (Murashige and Skoog 1962) and stratified at 4°C in the dark for 2 days, then transferred to a growth chamber with 120 μM/m^2s white light in a 16 hour light/8 hour dark cycle at 22°C. For soil-grown plants, seeds were sterilized with 95% EtOH for 5 minutes before sowing on Sunshine Mix soil with 10 μM Marathon. They were illuminated with 115 μM/m^2s white light at 22°C. All light conditions were measured using a Li-COR LI-250A Light Meter.

Generation of double mutants and transformations:

Following crosses between *lob-3* plants and *npy1-1,* F_2 plants were genotyped using PCR as previously described (Shuai et al. 2002; Cheng et al. 2007). For transformations, *Arabidopsis* plants were grown under standard conditions as previously described (Shuai et al. 2002). Binary vectors were transformed into GV3101 *Agrobacterium* using standard procedures, and *Arabidopsis* was transformed by floral dip (Clough 1998). Transformants were selected either by BASTA spray (Finale, AgrEvo) or growth on MS media supplemented with 50μM phosphinothricin (Sigma). A total of six *pNPY1:GUS* single-locus lines in *Col-0* wild type background and seven single-locus lines in *lob-3* background were examined for expression patterns. Pictures are representatives of these lines. Line 2 was used for *pNPY1:GUS Col-0* and line 7 was used for *pNPY1:GUS lob-3*.

For induction of *35S:LOB-GR* seedlings, dexamethasone and cycloheximide were used at 5 μM and 10 μm concentrations, respectively.

To generate the *pNPY1:GUS* construct, an ~4.1 kb DNA fragment upstream of the *NPY1* translational start site and the first codon was amplified using *pNPY1F* and *pNPY1R* primers with introduced restriction enzyme sites and subcloned into a *TOPO-TA* vector (Invitrogen). Plasmids were confirmed by sequencing (UCR Core Facility). *pNPY1* was then ligated to pCB308 (Xiang et al. 1999) to create a translational fusion and positive clones were confirmed by restriction digest. Primers used are shown in Table 2.1

Histological studies:

For GUS staining, plants were stained and cleared as previously described (Geisler et al. 2002). Images were taken on a Leica MZ12.

Blue-light experiments:

For long-term blue-light phototropism experiments, plants were grown under white light until Stage 1.01 (Boyes et al. 2001) and transferred to a growth chamber with blue LED lights (Sunslighting) at an intensity of 30 $\mu M/m^2s$, temperature of 22°C for 5 days 8 hours. Short-term blue-light experiments were carried out by transferring 13-day-old *Arabidopsis* plants to a growth chamber with blue LED lights at an intensity of 30 $\mu M/m^2s$ for 24 hours. Leaf angles were measured using ImageJ software. Angles are calculated by using the vertical growth vector and the petiole vector.

Transcript analysis:

Total RNA was isolated with TRIzol reagent (LifeTechnologies). RT-PCR was conducted as described previously. Primers for *ACT2* and any-gene specific primers are

shown in Table 2.1. qRT-PCR assays using SYBR GREEN were conducted on a BioRad Icycler using BioRad iQ5 Software. Relative transcript was calculated by the Pfaffl method (Pfaffl 2004). Significance was determined by student t-test analysis.

Chromatin immunoprecipitation:

13-day-old *35S:LOB-GR* seedlings were induced by flooding with either 15 μM DEX or MOCK- (control) treatment. Induction was confirmed by transcript analysis of downstream target *BAS1*. ChIP was carried out as previously described (Saleh et al. 2008) using an anti-LOB antibody (Bell et al. 2012) or anti-GST (Santa Cruz Biotechnology). Relative binding was calculated by comparing DEX-treated to MOCK-treated samples. All data were normalized to control gene *ACT2.* Primers for binding sites are listed in Table 2.1.

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Figure 2.1. Transcript level of *NPY1* is regulated by LOB. A) Relative transcript levels of *NPY1* in *35S:LOB-GR* plants treated with either MOCK (control), cycloheximide (CHX), dexamethasone (DEX), or DEX+CHX. *ACT2* was used as a control. n=2 biological replicates. Error bars indicate SE. * $p < 0.05$. ** $p < 0.01$. Significance was determined by student t-test analysis.

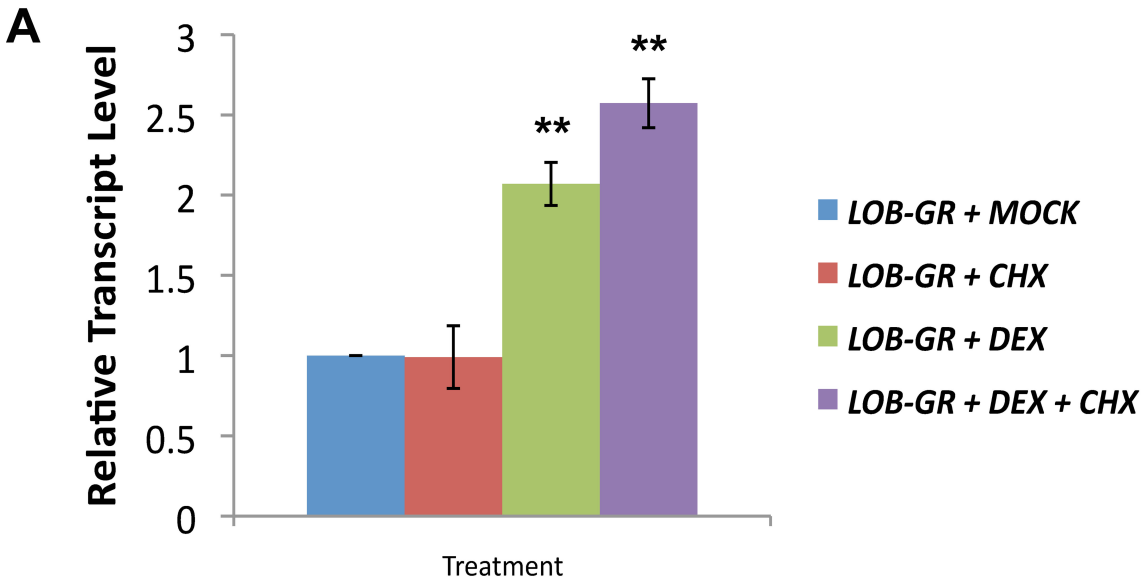
A
Relative Transcript Level
3
2.5
2
1.5
1
0.5
0
**
**
Treatment
LOB-GR + MOCK
LOB-GR + CHX
LOB-GR + DEX
LOB-GR + DEX + CHX

Figure 2.2. *NPY1* transcript levels are altered in *lob-3* mutants. A) Relative transcript levels of *NPY1* in seedlings, paraclade junctions, and inflorescences of wild-type and *lob-3 Arabidopsis* plants. *ACT2* was used as a control. Error bars indicate SE. Data represents the average of 3 biological replicates. ** = $p < 0.01$. Significance was determined by student t-test analysis.

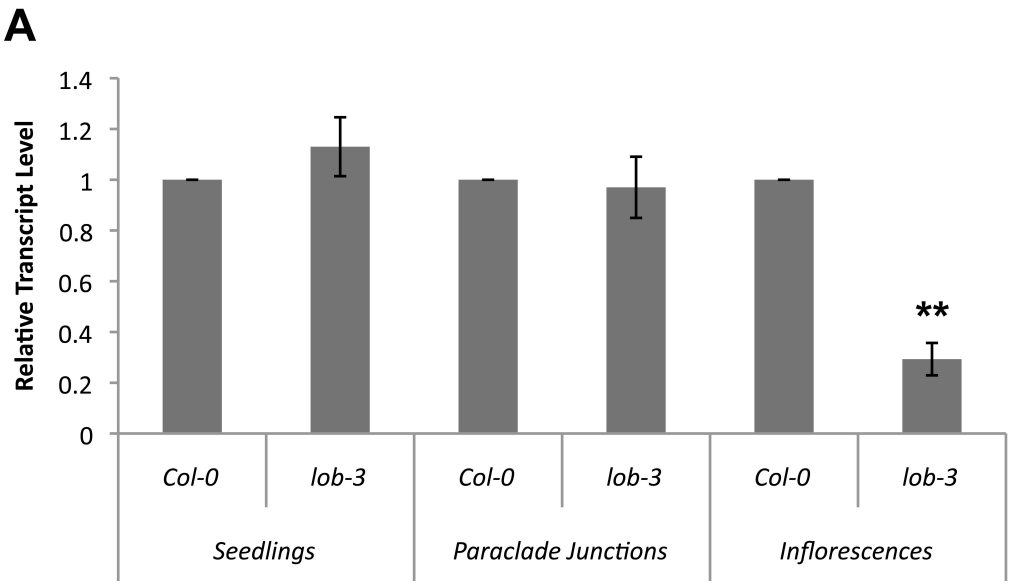
A
Relative Transcript Level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
Col-0
lob-3
Col-0
lob-3
Col-0
lob-3
Seedlings
Paraclade Junctions
Inflorescences

Figure 2.3. LOB association with the genomic regions of *NPY1*. A) Schematic representations of the *NPY1* gene. White boxes represent exons. Arrowhead represents the translation start codon. Bars indicate putative LOB binding sites. B) Relative enrichment by ChIP analysis of LOB. BS – binding site denoted in Figure 3A. Data represents the average of 3 biological replicates. ** = $p < 0.01$. Bars represent standard error.

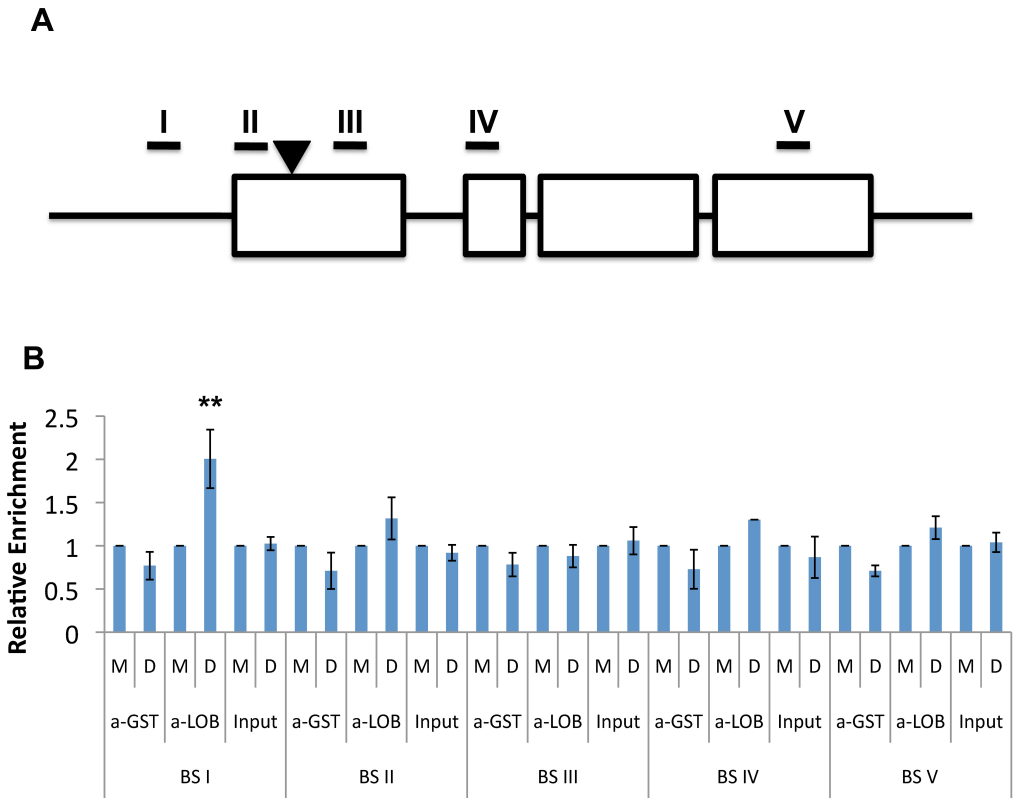
A
I
II
III
IV
V
B
Relative Enrichment
2.5
2
1.5
1
0.5
0
**
M
D
a-GST
a-LOB
Input
BS I
BS II
BS III
BS IV
BS V

Figure 2.4. *NPY1* expression is altered in *lob-3* inflorescences. GUS activity of *NPY1:GUS* in floral buds of (A) wild-type and (B) *lob-3* plants. C) *GUS* activity of *NPY1:GUS* in wild-type seedlings. All tissues were stained overnight. Scale bar = 0.5 mm in A and B; 200 μm in C.

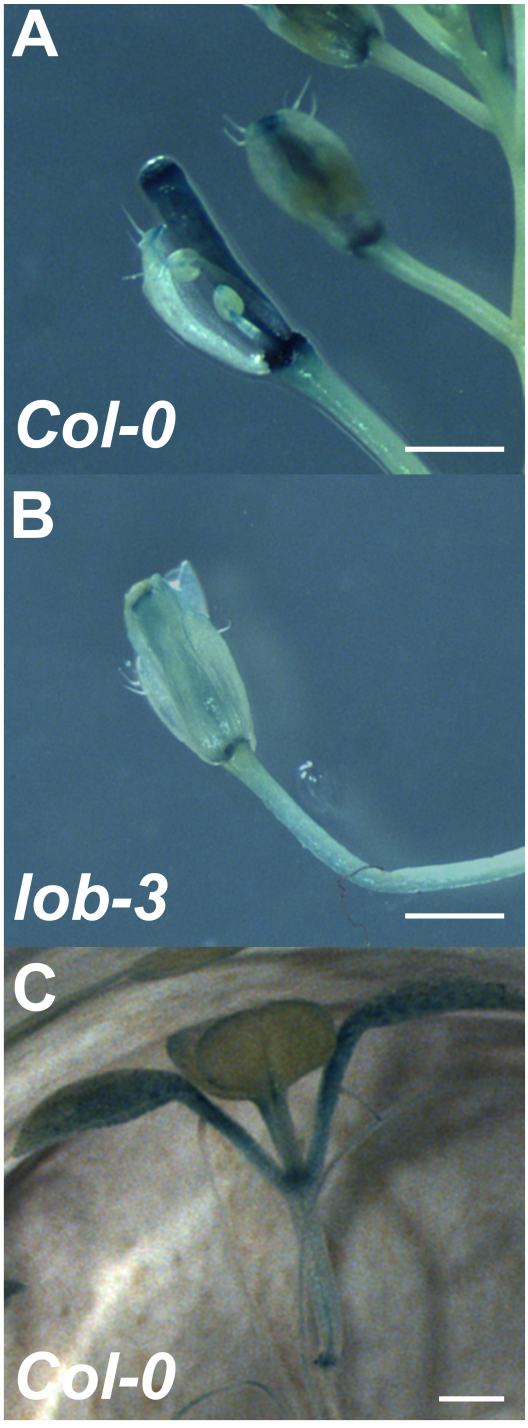
A
Col-0
B
lob-3
C
Col-0

Figure 2.5. *npy1-1* mutants do not have altered blue-light responses. A-B) Adaxial petiole angle of *Col-0, lob-3,* and *npy1-1* seedlings after 5 days 8 hours (A) or 24 hours (B) of 30 $\mu M/m^2s$ of blue-light illumination. n = 25. ** = $p<0.01$. Bars represent standard error. Significance was determined by student t-test analysis.

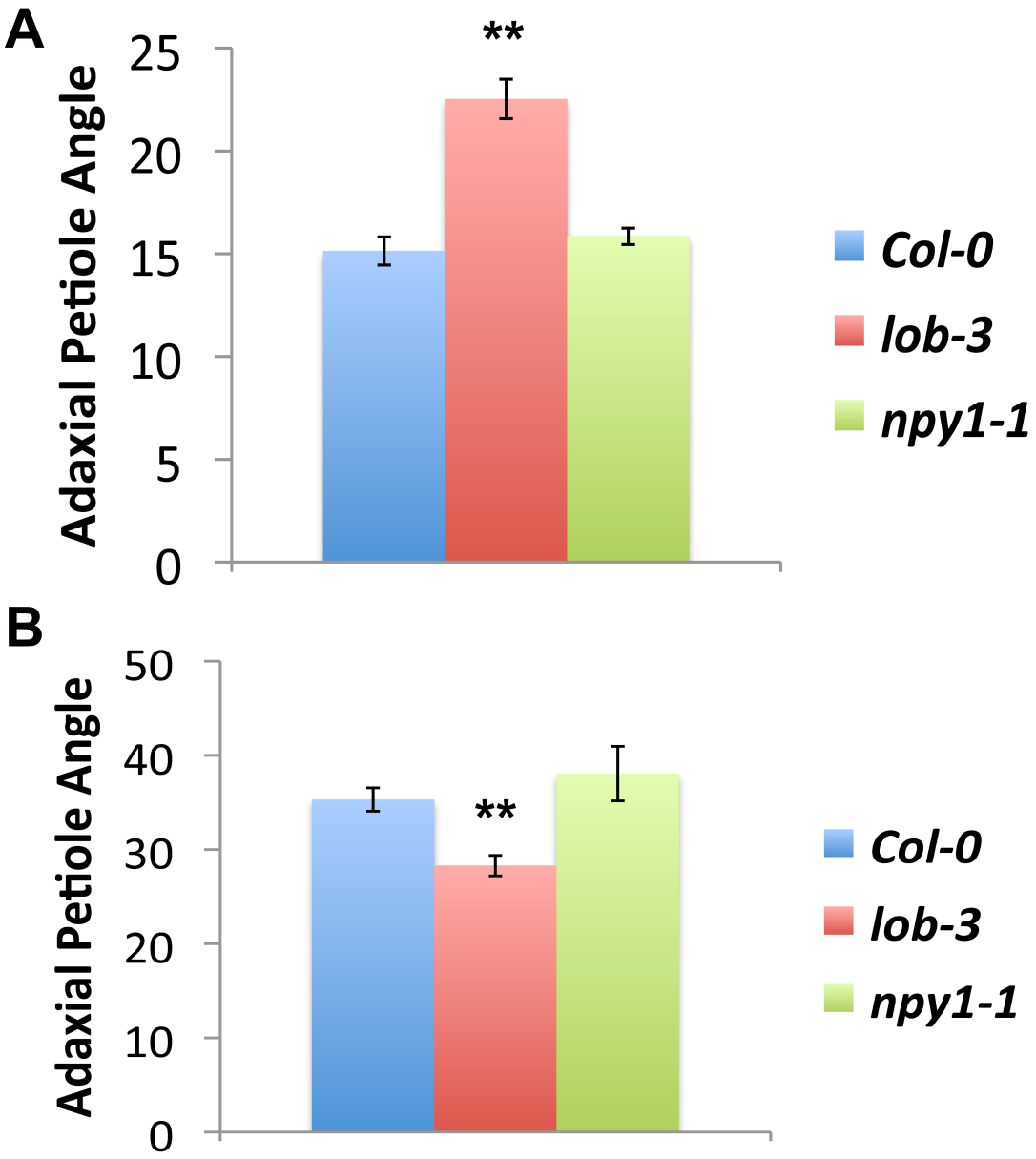
A
Adaxial Petiole Angle
25
20
15
10
5
0
**
Col-0
lob-3
npy1-1
B
Adaxial Petiole Angle
50
40
30
20
10
0
**
Col-0
lob-3
npy1-1

Figure 2.6. *npy1-1* plants resemble wild-type plants. Quantitative data of the length of contact between the axillary stem and cauline leaf of the first through third paraclade junctions *Col-0, lob-3, npy1-1,* and *lob-3 npy1-1*. n = 25. ** = $p < 0.01$, bars indicate SE. Significance was determined by a student t-test.

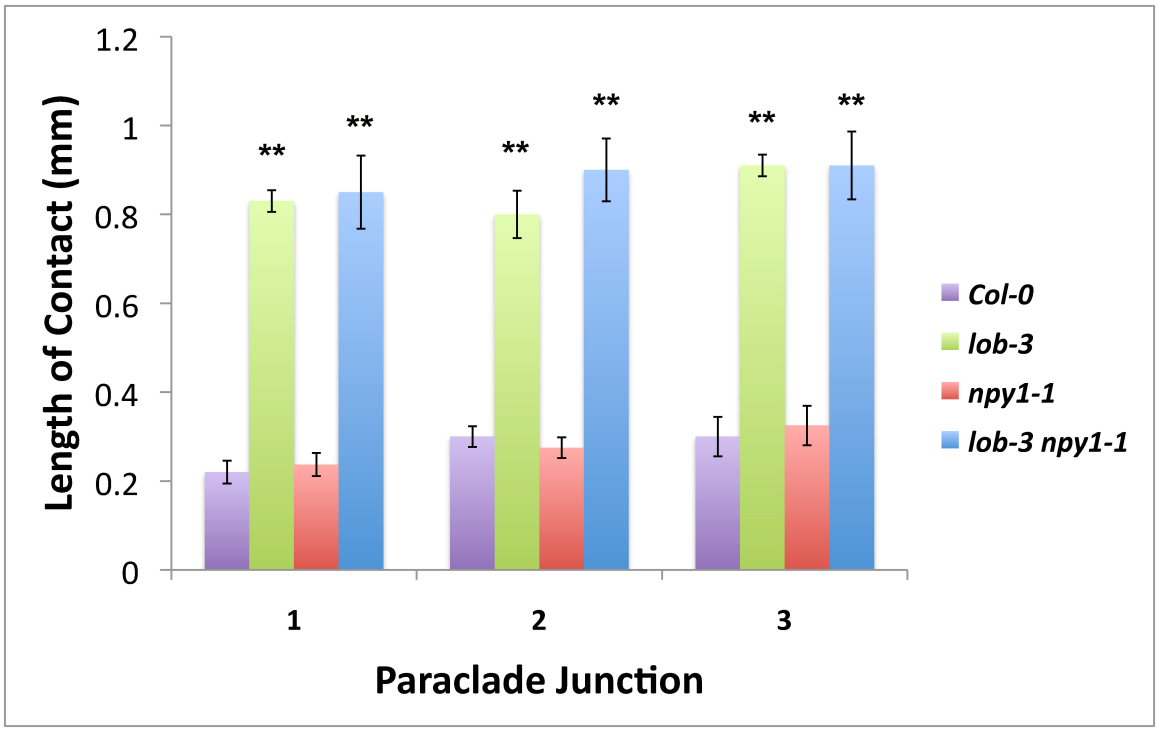
1.2
1
0.8
0.6
0.4
0.2
0
Length of Contact (mm)
**
**
**
**
**
**
1
2
3
Paraclade Junction
Col-0
lob-3
npy1-1
lob-3 npy1-1

Table 1. List of oligonucleotide sequences. Restriction enzyme sites are indicated in bold.

Primer Name	Sequence (5' – 3')	Tm (°C)
qNPY1-BF	TGGAGCTGATGAGACGGTGA	58
qNPY1-BR	ACACGTTTCTCATCCGCCATG	57.9
qACT2 - FP	GCACCCAGTTCTACTCACAG	55.2
qACT2 - RP	CAACATACATGGCAGGGAC	53.5
qUBC9 - FP	GATAGCCCTTATTCTGGAGGAG	54.1
qUBC9 - RP	TTGGATGGAACACCTTCGT	54
NPY1-CHIPIF	AAATGTGACCTCGCCAACAT	55
NPY1-CHIPIR	AGAGTTCTCGTTGAGCTGATTATG	54.6
NPY1-CHIPIIF	TTTCTTGCTCAGCTCCACCT	56.5
NPY1-CHIPIIR	CACTACGAGTGGTGGTGCAA	57.4
NPY1-CHIPIIIF	GTTTCTCCCCACCGATTCAC	55.8
NPY1-CHIPIIIR	GGGAAAGGAGAGATTAGCAACA	54.5
NPY1-CHIPIVF	CGTTGGGACGGACTACAAAA	55.1
NPY1-CHIPVR	CGTTTGCTTTAGTTCCGCTAC	54.2
NPY1-CHIPVF	TGAGATTTGAGCCAAACAGAGA	54.1
NPY1-CHIPVR	AGGCTTTGTCTTTGAGGGAAT	54.4
pNPY1-F	**GGATCC**TACATGTGAGATTGTTACTG	55
pNPY1-R	**GGATCC**CATATTTCTTCGTCTTGT	54.7

Chapter 3

Identification and characterization of HISTONE DEACETYLASE 3 (HDT3) as a protein interactor of LATERAL ORGAN BOUNDARIES (LOB)

Abstract

Lateral organs, such as leaves, form at the periphery of meristems and are separated from the meristem by a boundary region. Plants that have defects in boundary formation fail to fully separate forming organs from the meristem and have lateral organ fusions. *LATERAL ORGAN BOUNDARIES (LOB)* encodes a transcription factor that functions to separate lateral organs and mediate leaf responses to blue-light. Previous reports show that LOB interacts with the basic-helix-loop-helix protein bHLH048 and this interaction alters the *in vitro* binding ability of LOB. To identify additional LOB interacting proteins, we carried out a Yeast-2-Hybrid screen and found that LOB interacts with HISTONE DEACETYLASE 3 (HDT3) in yeast and *in vivo.* Consistent with LOB-HDT3 interaction, the expression patterns of *LOB* and *HDT3* overlap. Furthermore, lesions in *HDT3* result in phenotypes similar to those displayed in *lob* mutants. Taken together, HDT3 interacts with LOB to separate the cauline leaf from the axillary stem and is involved in the blue-light response in leaves.

Introduction

Lateral organs, such as leaves and flowers, form at the periphery of apical meristems. Plants require separation of lateral organs from these meristems for proper growth and development (Rast and Simon 2008). Plants that fail to separate these two distinct domains have fused organs or fail to form reproductive structures (Endrizzi et al. 1996; Aida et al. 1997; Aida et al. 1999; Lee et al. 2009). Plants separate lateral organs and the shoot apical meristem by the establishment of a boundary region (Nakata and Okada 2013).

Several genes have been implicated in boundary specification including *LATERAL ORGAN BOUNDARIES (LOB)* (Aida et al. 1997; Aida et al. 1999; Shuai et al. 2002; Greb et al. 2003; Borghi et al. 2007; Lee et al. 2009; Bell et al. 2012). *LOB* is the founding member of the 43 member plant-specific *LATERAL ORGAN BOUNDARIES DOMAIN (LBD)* gene family and encodes a DNA-binding transcription factor (Shuai et al. 2002; Husbands et al. 2007). *LOB* is expressed in all boundary regions including the base of lateral roots, leaves, flowers, floral organs, and at the junction of the cauline leaf and axillary stem (Shuai et al. 2002). Plants that harbor a hypomorphic allele of *lob* fail to fully separate the axillary stem and cauline leaf, suggesting that LOB functions in plant development to separate lateral organs (Bell et al. 2012). As transcription factors, LBD proteins bind to DNA in a preferential manner. LBDs bind to the 5'-(G)CGGC(G)-3' DNA motif where the core 5'-CGGC-3' is required. Inclusion of a guanine nucleotide on either side of the core 5'-CGGC-3' sequence increases the binding affinity of LBD proteins (Husbands et al. 2007). Furthermore, it has been shown that LOB interacts with

bHLH048 and this interaction reduces the *in vitro* DNA-binding affinity of LOB (Husbands et al. 2007).

Transcription factors modulate the expression of target genes by activation or repression of transcription. DNA is packed around histone proteins in nucleosomes to make chromatin; the degree of packing impacts gene expression. Condensed chromatin is typically associated with gene repression while loosely packed chromatin is associated with gene activation (Bird and Wolffe 1999; Verdone et al. 2005). In a condensed chromatin state, the transcriptional machinery cannot physically access DNA. These physical constraints are removed when the chromatin is in a loosely packed state. In the past several decades, researchers have worked to understand how post-translational modifications of histones affect gene expression. Histone modifications alter the degree of chromatin condensation thus affecting gene expression (Jenuwein and Allis 2001; Turner 2002; Josselyn and Frankland 2015). Two post-translational modifications that impact chromatin accessibility are histone acetylation and methylation (Zhang and Reinberg 2001).

Histones are acetylated at specific residues and the degree of acetylation determines the state of chromatin packing. Highly acetylated histones are associated with loosely packed chromatin. The degree of histone acetylation is regulated by HISTONE ACETYL TRANSFERASEs, proteins that add acetyl groups to specific residues on histones. Conversely, histone-deacetylases remove acetyl groups from histones (Wu et al. 2000; Wu et al. 2003; Hollender and Liu 2008). There are 18 known histone deacetylases in *Arabidopsis* (Alinsung et al. 2009). Four of these genes belong to the HD2 family of

proteins, which include HD2A/HDT1, HD2B/HDT2, HD2C/HDT3, and HD2D/HDT4 (Wu et al. 2000; Dangl et al. 2001).

Histone deacetylases play a role in a variety of developmental processes including leaf formation, flowering time, and seed development (Zhou et al. 2004; Pontes et al. 2007). Interestingly, HDT1 and HDT2 have been implicated in establishment of adaxial-abaxial leaf polarity in conjunction with the *LBD* gene *ASYMMETRIC LEAVES 2 (AS2)*. Plants treated with a chemical inhibitor of the HD2 family of histone deacetylases (Trichostatin A) have radialized leaves resembling *as2* mutants (Yoshida et al. 1990; Byrne et al. 2000; Semiarti et al. 2001; Xu et al. 2003; Ueno et al. 2007). Furthermore, altering HDT1/2 levels through RNA-interference affects AS2 function (Ueno et al. 2007).

HD2C/HDT3 has been implicated in abscisic acid (ABA) response (Sridha and Wu 2006). *HDT3* is expressed constitutively in plants and the HDT3 protein is localized to the nucleus, consistent with its role as a transcriptional regulator. Plants over-expressing *HDT3* have decreased responses to ABA and transcript levels of several ABA-responsive genes are decreased in *HDT3* over-expressing plants. Furthermore, ABA represses *HDT3* expression (Sridha and Wu 2006).

Given that transcription factors typically work in complexes and LOB differentially regulates target gene expression, it is likely that LOB interacts with other proteins to regulate gene expression (Edwards et al. 1998; Fan and Dong 2002; de Folter et al. 2005). It was previously shown that an interaction with the basic helix-loop-helix protein bHLH048 reduces the DNA-binding ability of LOB (Husbands et al. 2007). To

gain insight into how LOB regulates transcription of its target genes, I conducted a Yeast-II-hybrid screen (Bartel and Fields 1995) resulting in the identification of several putative LOB-interacting partners. One protein, HDT3, was chosen for further study. The expression pattern of *LOB* and *HDT3* partially overlap and plants harboring a mutation in the *HDT3* gene resemble *lob* mutant plants. Two different alleles of *hdt3* showed defects in organ separation and leaf responses to blue-light. *hdt3-2* plants have a fusion of the axillary stem and cauline leaf. *hdt3-1* plants have a more sensitive phototropic response to blue-light. Lastly, LOB and HDT3 proteins interact *in vivo.*

Results

Yeast II hybrid assay to identify LOB-interacting proteins

To identify undiscovered proteins that interact with LOB, we screened ~$1x10^6$ protein interactions using a *GAL4*-based yeast-two-hybrid system. The full-length *LOB* coding sequence was cloned into vector pAS2 allowing *LOB* to be expressed as a translational fusion to the GAL4 binding domain (BD) (Figure 3.1A). The *LOB-BD* construct was transformed into yeast and tested to confirm that it did not activate reporter gene expression alone. This yeast strain was transformed with a GAL4-activation domain tagged library, made from cDNA derived from RNA isolated from wild-type and *ap1 cal1* inflorescence tips (Kempin et al. 1995; Clontech). This library was chosen for the screen because it was made from a source enriched for cells that express LOB and therefore should contain LOB-interacting proteins. After transformation, yeast were

plated on minimal media (SD) lacking tryptophan (to select for the LOB-BD plasmid), leucine (to select for library plasmid), histidine + 3 Amino Triazole (3-AT), and adenine (to select for protein interaction). Of the ~$1x10^6$ screened interactions, 206 individual colonies grew on selection media. After a secondary screen, 29 colonies grew on selective media and represented 9 unique clones. These were selected for further analysis (Table 3.1). LOB-interacting proteins fell into several classes, including translation initiation factors, reticulon proteins, a 14-3-3 protein, and a histone deacetylase. Given that LOB functions as a transcription factor, we examined the relationship with HDT3, a histone deacetylase, because of its predicted role in transcriptional regulation. To confirm the interaction, yeast were re-transformed with plasmids that contained the *LOB-BD* and *HDT3-AD* and plated on selective media –Trp/-Leu. In addition, these yeast were dotted on minimal media –Trp/-Leu/-His/-Ade + 3-AT. Yeast containing the LOB-BD and HDT3-AD plasmids grew on selective media –Trp/-Leu/-His/-Ade + 3-AT whereas yeast containing LOB and the empty activation domain plasmid, pGADT7*,* did not (Figure 3.1B). To understand which domain of LOB is required for the interaction with HDT3, plasmids encoding the LOB domain-BD (LBD-BD) and C-terminal domain of LOB fused to the BD (C-term-BD) (Figure 3.1A) were co-transformed into yeast along with plasmids encoding HDT3-AD. Neither yeast harboring LBD-BD and HDT3-AD nor C-term-BD and HDT3-AD constructs grew on selective media (Figure 3.1B).

LOB expression partially overlaps with *HDT3* expression

Consistent with LOB and HDT3 interaction in *vivo,* the expression pattern of the genes that encode these proteins should overlap. We examined expression patterns using promoter:*GUS* reporter gene fusions. As previously reported, *LOB* is expressed in boundaries throughout plant development. To generate p*HDT3:GUS* plants, the 2.4-kB promoter region of *HDT3* was cloned upstream of the *GUS* gene in pCB308 and transformed into *Col-0* wild-type plants. We compared expression resulting from this construct to that of *pLOB:GUS* (Shuai et al. 2002). In roots, p*LOB:GUS* expression was observed at the base of the lateral root (Figure 3.2A). *HDT3:GUS* expression was observed throughout the root including the boundary between the primary root and lateral root (Figure 3.2D). *LOB:GUS* expression was observed in the paraclade junction as previously reported (Figure 3.2B). Strong *HDT3:GUS* expression was observed in paraclade junctions and throughout the cauline leaf, primary, and axillary stems (Figure 3.2E). In flowers, *LOB:GUS* expression was observed at the base of the floral organs (Figure 3.2C). *HDT3:GUS* expression was observed throughout all floral organs (Figure 3.2F). These data indicate that HDT3 and LOB expression patterns overlap; consistent with our hypothesis that LOB and HDT3 interact *in vivo.*

LOB interacts with HDT3 in onion epidermal peels

To understand the interaction of LOB and HDT3, we used a Bi-molecular Fluorescence Complementation (BiFC) assay in onion epidermal peels, using the LOB

protein fused to the N-terminal eYFP protein and HDT3 fused to the C-terminal eYFP protein. 16 hours after bombardment, eYFP signal was observed in nuclei of onion epidermal cells transiently transformed with LOB-eYFP^{N} and HDT3-eYFP^{C} fusion proteins (Figure 3.3A). However, no signal was detected when LOB-eYFP^{N}/eYFP^{C}, eYFP^{N}/HDT3-eYFP^{C}, and eYFP^{N}/eYFP^{C} were co-transformed by bombardment into onion epidermal cells (Figure 3.3A). These data suggest that LOB and HDT3 interact in onion epidermal cells.

Characterization of *HDT3* mutations

To determine if loss of *HDT3* impacted plant development, we examined phenotypes of *hdt3* mutants. We obtained seed from two T-DNA insertions in *HDT3* from the *Arabidopsis* Biological Resource Center (Alonso et al. 2003). *hdt3-1* SALK_12799C) contained an insertion in the *HDT3* gene located 500 bp downstream from the translational start codon in the fourth exon. *hdt3-2* (SALK_002860) contained a T-DNA insertion located in the 5'-Untranslated region (UTR) (Figure 3.4A). As previously reported, the full-length *HDT3* transcript was not detected in cDNA derived from *hdt3-1* plants (Sridha and Wu 2006; Luo et al. 2012). However, we detected partial *HDT3* transcript by RT-PCR using primers that anneal downstream of the T-DNA insertion site. No transcript was detected using primers that anneal upstream of the T-DNA insertion site (Figure 3.4B). This suggests that the T-DNA insertion in *hdt3-1* plants prevents the full *HDT3* T-DNA transcript from being synthesized, although a transcript downstream of the T-DNA insertion is still present. A possible explanation for

the presence of a partial transcript is that a promoter element in the T-DNA might allow for the transcription of the downstream region. In *hdt3-2* plants, the transcript level of *HDT3* was reduced in paraclade junctions compared to wild-type, suggesting that the T-DNA negatively affects the transcript level of *HDT3* (Figure 3.4C).

hdt3 mutants have defects in organ separation

lob mutants have a fusion of the axillary stem and cauline leaf (Bell et al 2012). We reasoned that if LOB interacts with HDT3, then HDT3 could function in the separation of lateral organs. *hdt3-2* plants did not exhibit abnormalities of vegetative structures, floral organs, or roots compared to wild-type. However, *hdt3-2* plants exhibited a fusion between the axillary stem and cauline leaf (Figure 3.5A-C). This fusion was quantified by measuring the length of contact between the cauline leaf and axillary stem in the paraclade junction in *hdt3-2* plants. In wild-type plants, the axillary stem contacts the cauline leaf base for 0.2 mm in the first, second, and third paraclade junctions, respectively, while the degree of contact between these two organs in *lob-3* mutants is 0.8 mm, 0.6 mm, and 0.97 mm in the first, second and third paraclade junctions, respectively. *hdt3-2* mutants, had an average degree of contact of 0.6 mm between the axillary stem and cauline leaf, which is a significant difference compared to wild type (Figure 3.5D). These data demonstrate that *HDT3* plays a role in the separation of the axillary stem and cauline leaf. *hdt3-1* plants did not exhibit a significant difference in the degree of contact of the axillary stem and cauline leaf compared to wild-type plants (Figure 3.5E).

LOB directly regulates the transcription of *BAS1* and this regulation is necessary for proper lateral organ formation (Bell et al. 2012). Considering *hdt3-2* mutant plants have fusion of the axillary stem and cauline leaf, we asked if HDT3 regulates *BAS1* transcript levels. To test this, we determined *BAS1* transcript levels in excised paraclade junctions from *Col-0, lob-3,* and *hdt3-2* plants. Similar to *lob-3* plants, *BAS1* transcript levels were significantly lower in the paraclade junction of *hdt3-2* plants compared to wild-type (Figure 3.5F).

hdt3-1 mutants are more sensitive to blue-light illumination

More recently, it has been shown that LOB directly regulates blue-light response genes for proper phototropic response (Chapter 1). We asked whether HDT3 is involved in the LOB-mediated blue-light response pathway. *hdt3-1* plants were grown in white light for 13 days followed by illumination with blue-light for 24 hours. When grown in white light, 13-day-old wild-type plants had an adaxial petiole angle of 46.5° while *lob-3* plants had an adaxial petiole angle of 60.3°. *hdt3-1* leaves were less erect than wild-type but more erect than *lob-3* plants (Figure 3.6A). *lob-3 hdt3-1* double mutants resembled *lob-3* single mutants (Figure 3.6A) suggesting that they function in similar pathways in setting the petiole angle to white light. After exposure to blue light for 24 hours, wild-type leaves inclined toward the light source, undergoing a change in petiole angle of 13°, whereas *lob-3* plants underwent an ~32° change in angle. *hdt3-1* plants resembled lob mutants, with a change in angle of 30°. This demonstrates that HDT3 is involved in the blue-light hyponastic response. *lob-3 hdt3-1* double mutants did not have an enhanced

petiole angle after blue-light illumination from above (Figure 3.6C) suggesting that they function in similar pathways for plant responses to blue-light. To determine if the increased sensitivity to blue-light observed in *hdt3-1* plants was due to altered levels of blue-light factors, we examined levels of *NPH3, PKS2,* and *PHOT1* transcripts in these mutants using qRT-PCR. No significant differences in *NPH3, PKS2,* and *PHOT1* transcript levels were detected in *hdt3-1* plants compared to wild-type (Figure 3.6D).

Etiolated *35S:LOB-GR* plants grown in the presence of DEX do not respond to unilateral blue-light (Chapter 1), consistent with the reduced levels of *NPH3, PKS2,* and *PHOT1* transcript in these plants. We asked if HDT3 is required for this aspect of the *LOB* over-expression phenotype. When grown on DEX, *35S:LOB-GR hdt3-1* plants resembled *35S:LOB-GR* plants, in that they did not to exhibit blue-light phototropism suggesting that *HDT3* is not required for the *LOB* over-expression phenotype (data not shown). Taken together, these data show that *HDT3* is involved in lateral organ separation and blue-light responses.

Discussion

Previous studies reported that bHLH048 interacts with LOB and this interaction alters the binding affinity of LOB to DNA (Husbands et al. 2007). To identify additional proteins that interact with LOB, we conducted a yeast-2-hybrid screen and identified HDT3 as a protein interactor of LOB. We further showed that the entire LOB protein is required for its interaction with HDT3.

Furthermore, we have demonstrated that HDT3 and LOB interact in onion epidermal peels and shown that HDT3 is involved in lateral organ separation and blue-light response. Plants that have decreased *HDT3* transcripts fail to fully separate the axillary stem from the cauline leaf and this phenotype resembles that of plants carrying a hypomorphic *lob-3* allele. Furthermore, the transcript level of *BAS1* is significantly lower in the paraclade junction of *hdt3-2* mutant plants suggesting that the lack of separation phenotype in *hdt3-2* is due to altered *BAS1* levels.

It is seems unlikely that HDT3 interacts with LOB to directly regulate *BAS1* expression. *HDT3* encodes a histone deacetylase, which is involved in the negative regulation of gene expression. However, LOB has been shown to directly activate *BAS1* transcription (Bell et al 2012). Therefore, we propose a model in which LOB interacts with HDT3 to negatively regulate a negative regulator of *BAS1* (Figure 3.7). This model suggests that LOB not only directly regulates *BAS1* transcripts but also indirectly regulates *BAS1* expression. These two modes of regulating *BAS1* transcript levels could allow for several layers of regulation of *BAS1* activity.

How does HDT3 regulate response to blue-light? *hdt3-1* plants were more sensitive to blue-light-induced leaf inclination, yet we did not detect changes in the transcript levels of blue-light response genes in these plants compared to wild-type (Figure 3.6). We cannot discount the possibility that LOB interacts with HDT3 to directly regulate other genes involved in the blue-light response. One hypothesis is that LOB functions in an HDT3-independent repressive complex with other proteins to negatively regulate the transcript of blue-light response genes. A second hypothesis is that LOB and

HDT3 interact in a single complex along with other proteins and that functional HDT3 is not necessary for this complex to function, perhaps because of the presence of redundant HDAC proteins (Wu et al. 2000). Further characterization of LOB-binding partners is required to distinguish these two properties. Regardless, we show that HDT3 is involved in the blue-light hyponastic-response pathway (Figure 3.7).

Materials and Methods

Yeast II hybrid assay:

The *LOB-BD, LBD-BD,* and *LOB C-term-BD* constructs were described previously (Husbands et al. 2007). A yeast-2-hybrid cDNA library made from RNA isolated from wild-type and *ap1cal1* (Kempin et al. 1995) inflorescences was used as the prey. *LOB-BD, LBD-BD, LOB C-term-BD*, and *pGAD-T7* constructs were transformed into yeast strain AH109 (Clontech, Mountain View, CA) using the lithium acetate protocol (Clontech) with some modifications, described here. Transformed yeast cells were resuspended in YPD, rather than the recommended in TE buffer, then plated on both minimal media –Trp/-Leu to select for both plasmids and to evaluate transformation efficiency, and minimal media –Trp/-Leu/-His/-Ade + 3AT for stringent selection of interaction (Clontech). Plasmids were extracted by a CTAB protocol (Murray and Thompson 1980) with some modifications. Following growth overnight, yeast cells from 1.5 ml of liquid culture grown in selective media were pelleted by centrifugation for 1 minute at 13,000 rpm. The yeast pellet was then resuspended in 600 μL of CTAB

buffer/1 μL β-mercaptoethanol and flash-frozen by liquid nitrogen. The pellet was then defrosted and sonicated for 20 seconds at high power to break open cells. After sonication, the solution was extracted with phenol/chloroform and the DNA then precipitated with isopropyl alcohol. DNA was then pelleted followed by resuspension with 100 μL of TE buffer. cDNA clones were amplified using primers flanking the insertion and PCR products were analyzed by restriction digest to identify unique interactors. Primers used for PCR are shown in Table 3.2.

GUS staining expression analysis:

The *pLOB:GUS* line has been previously described (Shuai et al. 2002). To generate the *pHDT3:GUS* construct, an ~2.3-kb DNA fragment upstream of the *HDT3* translational start site and the first two codons was cloned into the binary vector *pCB308* (Xiang et al. 1999) to create a translational fusion with the *GUS* gene using introduced restriction sites. Primers used for cloning are shown in Table 3.2. A total of five independent, single-locus transgenic lines were characterized for *pHDT3:GUS* expression. Images in Figure 3.2 are from lines four and six. Plant tissues were stained for GUS activity in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid and were cleared in 70% (v/v) ethanol as previously described (Geisler et al. 2002). Images of GUS stained plants were captured by a Leica MZ12 microscope or Leica DMR stereoscope (UCR).

Bi-molecular fluorescence complementation:

For N-terminal YFP-tagged LOB, PCR-amplified *LOB* cDNA was introduced into plasmid pENTR/D-TOPO (Invitrogen) to produce an entry clone, and the cloned ORF was transferred into BiFC destination vector pSAT4(A)-DEST-nEYFP-N1 to

produce LOB-YFP^{N} (Citovsky et al. 2006). The same procedure was used for C-terminal YFP-tagged HDT3 constructs using pSAT5(A)-DEST-cEYFP-N1 to produce HDT3-eYFP^{C} (Citovsky et al. 2006). Primers used for cloning are shown in Table 3.2. The above plasmids and respective empty vectors were loaded onto 1.1-μm tungsten particles and bombarded using the Bio-Rad PDS-1000/He Particle Delivery System (Bio-Rad) according to manufacturer's instructions. Fluorescence was observed 16 hours after bombardment using a Yokogawa CSU-W1 confocal microscope (UCR, Rasmussen Lab). Merged images were prepared using ImageJ.

Plants and growth conditions:

All plants used in this study are in the *Colombia* (*Col-0*) ecotype. *lob-3* is described previously (Bell et al. 2012). *hdt3-1* (SALK_12799C) and *hdt3-2* (SALK_002860) were obtained from the ABRC (Alonso et al. 2003). Primers to confirm T-DNA insertions are shown in Table 3.2. Plants were grown on sunshine mix soil with osmocote and Marathon. Images of paraclade junction phenotypes were captured on a Leica MZ12 microscope (UCR). Measurements of contact length between stems and cauline leaf were made using a digimatic caliper (model 700-113; Mitutoyo). Petiole angle measurements were calculated by ImageJ.

Transcript analysis:

To characterize the affect of the T-DNA insertion in *hdt3-1* and *hdt3-2* mutants as well as *BAS1* transcript levels in *hdt3-2* mutants, total RNA was extracted by TRIzol reagent and precipitated by 100% EtOH. 4 μg of RNA was used for cDNA synthesis as previously described (Lin et al. 2003). *APT1* was amplified for 23 cycles and amplicon a

and b regions of *HDT3* were amplified for 35 cycles. Primers shown in Table 3.2. To detect *PHOT1, PKS2*, and *NPH3* transcripts, total RNA was extracted from 13-day-old *hdt3-1* or wild-type seedlings. *qPCR* was carried out using a Bio-Rad iCycler and analysis was performed by Bio-Rad iQ5 software. Relative transcript levels were calculated by the Pfaffl method (Pfaffl 2004). Significance was determined by a student t-test.

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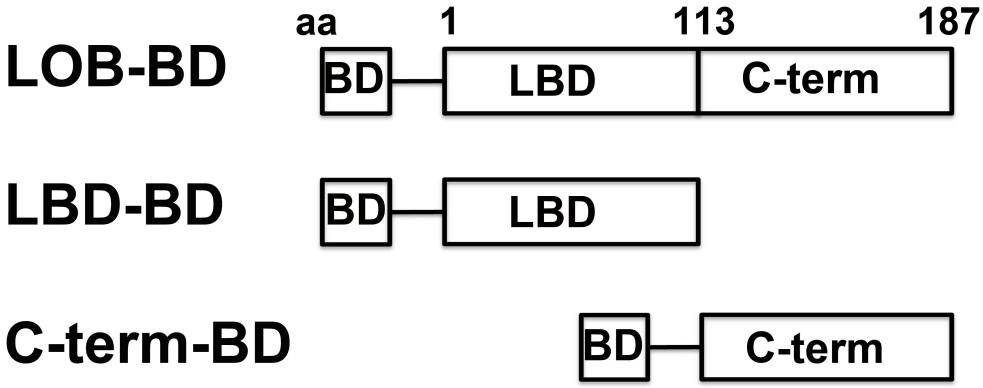
Figure 3.1. HDT3 and LOB interact in yeast. A) Cartoon representation of the LOB protein highlighting the LBD and C-terminal domains fused to the GAL4-BD. B) Yeast-2-Hybrid showing HDT3 and LOB interact. Yeast containing respective AD and BD fusion proteins were dotted on selection media –WL or –WLAH.

A



B

Bait	Prey	-WL	-WLAH +3AT
BD	AD		
LOB-BD	AD		
BD	HDT3-AD		
LOB-BD	HDT3-AD		
LBD-BD	HDT3-AD		
C-term-BD	HDT3-AD		

Figure 3.2. *HDT3* expression partially overlaps with *LOB*. A-C) *pLOB:GUS* expression in roots (A), paraclade junctions (B), and floral organs (C). D-F) *pHDT3:GUS* expression in roots (D), paraclade junctions (E), and floral organs (F). Roots were stained for 6 hours and paraclade junctions and inflorescences were stained overnight. Pictures are representations of n = 25. Line 4 and 6 were used for pictures. Scale bar = 100 μm in A and D; 1 mm in B and E ; 0.5 mm in C and F.

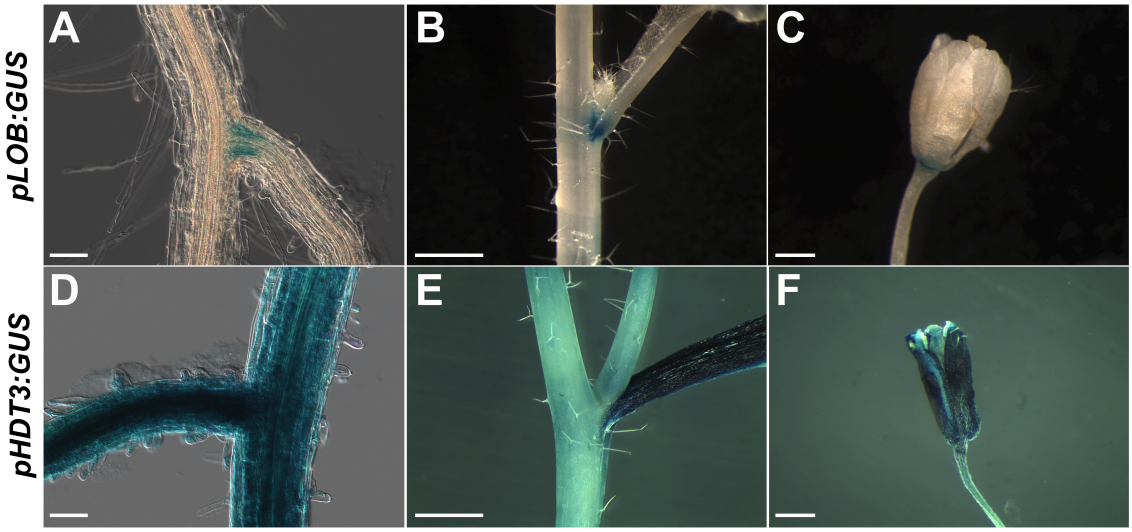

pLOB:GUS
pHDT3:GUS
A
B
C
D
E
F

Figure 3.3. LOB and HDT3 interact in onion epidermal peels. Left panels are bright-field images of onion epidermal cells 16 hours post-bombardment. Middle panels are YFP signal and right panels are merged images. nYFP = N-terminal of YFP. cYFP = C-terminal of YFP. LOB-nYFP = LOB protein fused to the N-terminal of YFP. HDT3-cYFP = HDT3 fused to the C-terminal of YFP.

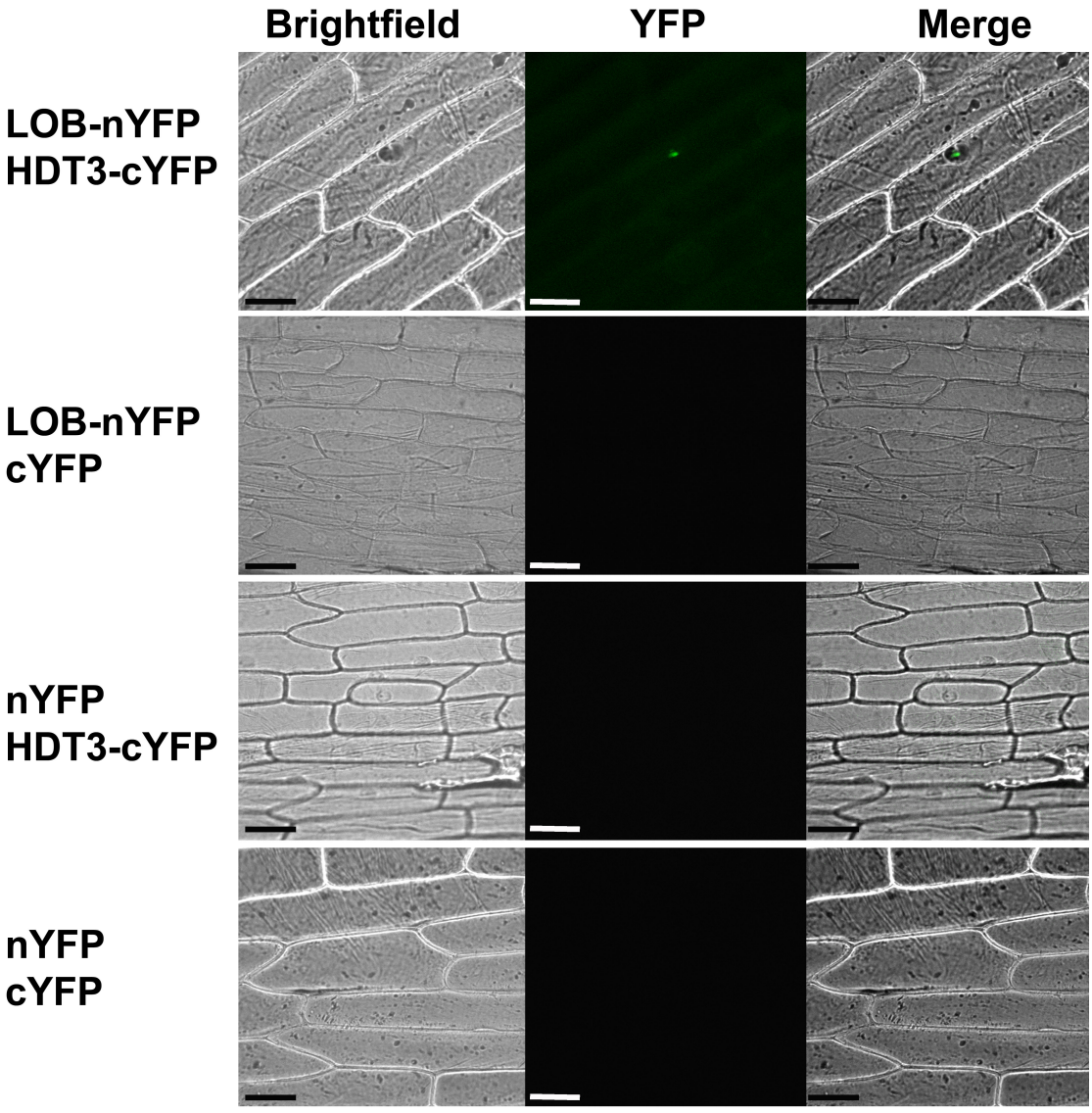
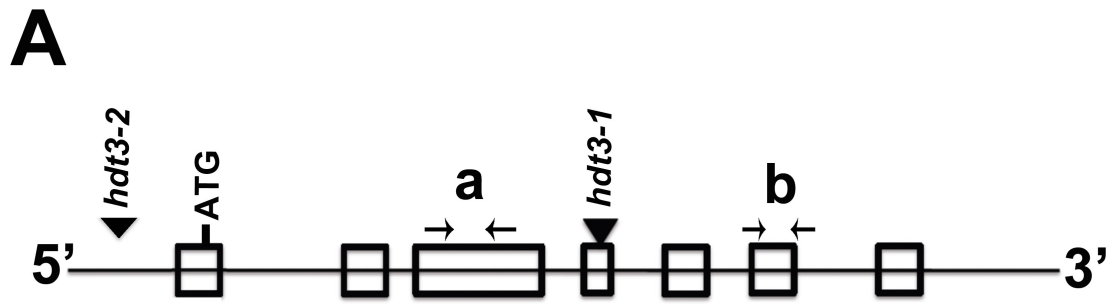
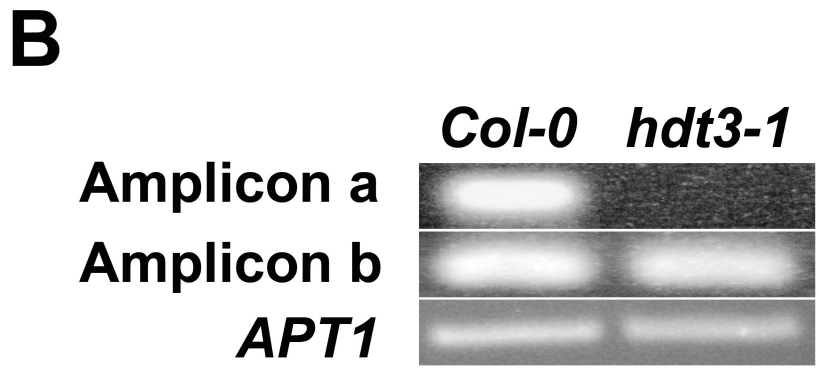

Brightfield
YFP
Merge
LOB-nYFP
HDT3-cYFP
LOB-nYFP
cYFP
nYFP
HDT3-cYFP
nYFP
cYFP

Figure 3.4. Characterization of *hdt3* alleles. A) Cartoon representation of the *HDT3* transcript and the location of the T-DNA insertions (arrowhead). Amplicon a is upstream of the *hdt3-1* T-DNA insertion and amplicon b is downstream. B) RT-PCR of amplicon a and b in wild-type (*Col-0)* and *hdt3-1* plants. *APT1* was amplified for 35 cycles and amplicon a and b regions of *HDT3* were amplified for 35 cycles. C) qPCR of *HDT3* transcript in dissected paraclade junctions of wild-type and *hdt3-2* plants. * = $p < 0.1$. n = 3 biological replicates.


A
hdt3-2
ATG
a
hdt3-1
b
5'
3'


B
Col-0
hdt3-1
Amplicon a
Amplicon b
APT1

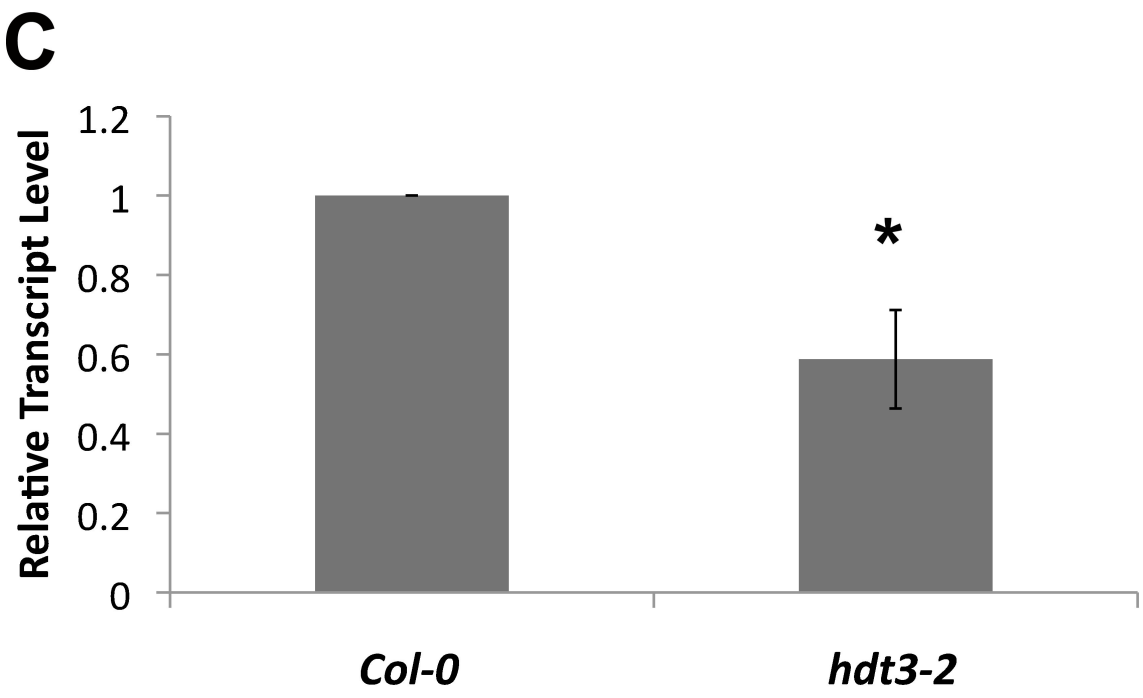
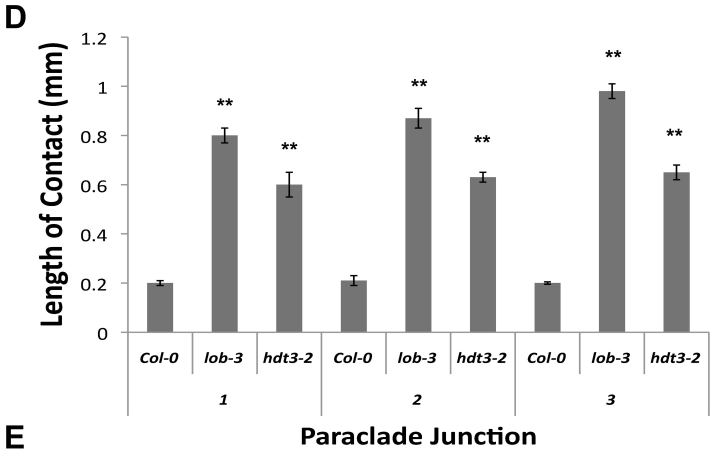
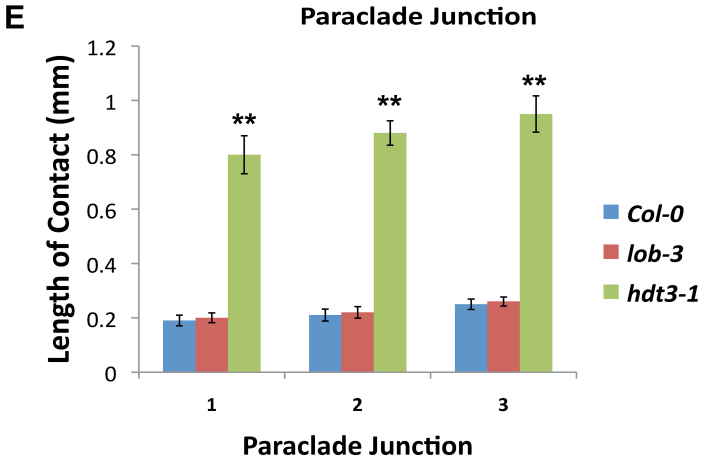

C
Relative Transcript Level
1.2
1
0.8
0.6
0.4
0.2
0
*
Col-0
hdt3-2

Figure 3.5. *hdt3-2* plants resemble *lob-3* mutants. A-C) Paraclade junctions of *Col-0* (A), *lob-3* (B), *hdt3-2* (C). D) Quantitative data of the length of contact of the axillary stem and cauline leaf of the first, second, and third paraclade junctions. n = 30. E) *BAS1* transcripts in dissected paraclade junctions of *Col-0, lob-3,* and *hdt3-2.* F) Quantitative data of the length of contact of the axillary stem and cauline leaf of the first, second, and third paraclade junctions in *Arabidopsis*. n = 24. ** = $p < 0.01$, bars indicate SE. Data represents the average of 3 biological replicates.


A
B
C
Col-0
lob-3
hdt3-2


D
Length of Contact (mm)
1.2
1
0.8
0.6
0.4
0.2
0
**
Col-0
lob-3
hdt3-2
1
2
3
Paraclade Junction


E
Length of Contact (mm)
1.2
1
0.8
0.6
0.4
0.2
0
**
1
2
3
Paraclade Junction
Col-0
lob-3
hdt3-1

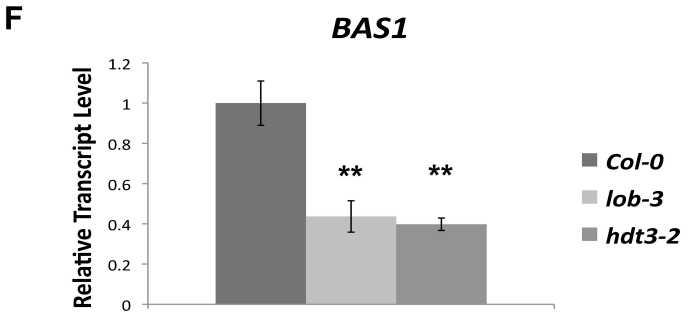

F
BAS1
Relative Transcript Level
1.2
1
0.8
0.6
0.4
0.2
0
**
Col-0
lob-3
hdt3-2

Figure 3.6. *hdt3-1* mutants have a phototropic response similar to *lob-3* mutants. A) Adaxial petiole angle of 13-day-old plants grown in white light conditions. B) Adaxial petiole angle after 24 hours of blue-light illumination from above. C) Change in petiole angle after 24 hours of blue-light illumination from above. D) Transcript levels of blue response genes *PHOT1, PKS2,* and *NPH3* in *hdt3-1* plants. ** = $p < 0.01$. A-C, n = 24 plants. D, data represents the average of 3 biological replicates. Significance was determined by a student t-test.

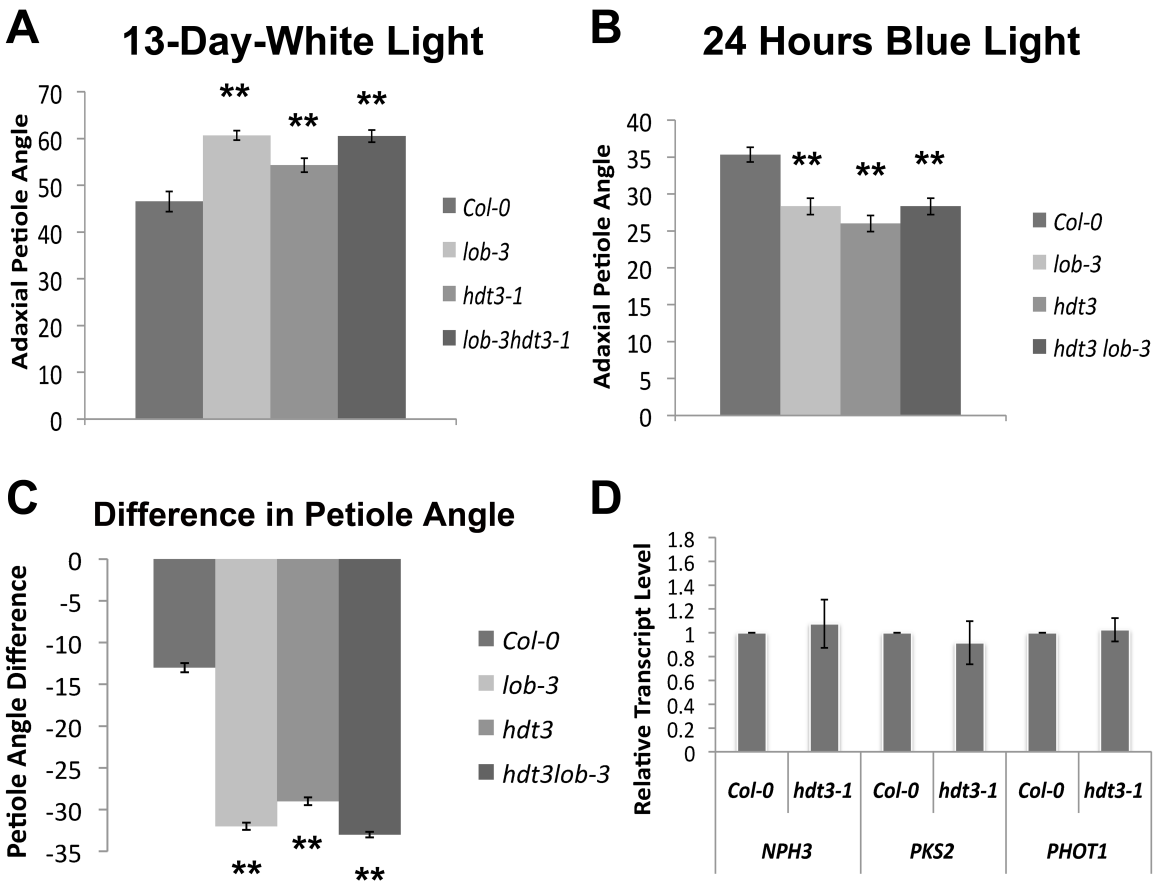

A
13-Day-White Light
Adaxial Petiole Angle
70
60
50
40
30
20
10
0
**
**
**
Col-0
lob-3
hdt3-1
lob-3hdt3-1
B
24 Hours Blue Light
Adaxial Petiole Angle
40
35
30
25
20
15
10
5
0
**
**
**
Col-0
lob-3
hdt3
hdt3 lob-3
C
Difference in Petiole Angle
Petiole Angle Difference
0
-5
-10
-15
-20
-25
-30
-35
**
**
**
Col-0
lob-3
hdt3
hdt3lob-3
D
Relative Transcript Level
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Col-0
hdt3-1
Col-0
hdt3-1
Col-0
hdt3-1
NPH3
PKS2
PHOT1

Figure 3.7. Model for LOB/HDT3 interaction.

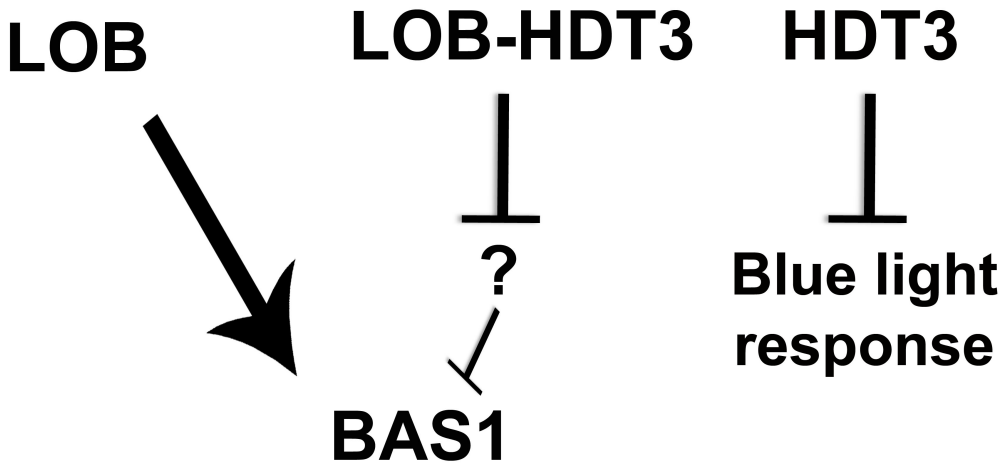

LOB
LOB-HDT3
HDT3
?
Blue light
response
BAS1

Table 3.1. LOB-interacting proteins identified in the Yeast-2-Hybrid screen.

Locus	Gene Name	Putative Function
At5g10450	*GRF6*	14-3-3 Protein Involved in BR Response
At5g54770	THI1	Thiamine Biosynthetic Gene
At1g53850	PAE1	alpha-5 E1 Subunit of 20s Proteosome
At3g14290	PAE2	20S Proteosome alpha E2 Subunit
At5g02740	HDT3	Histone Deacetylase
At5g59880	ADF4	Actin Depolymerizing Factor
At2g27710	N/A	60S Acidic Ribosomal Protein Family
At3g10260	N/A	Reticulon Family Protein
At5g67510	N/A	Translation Protein SH3-like Family Protein

Table 3.2. List of oligonucleotide sequences. Restriction sites are indicated in bold.

Primer Name	Sequence (5' → 3')	Tm (°C)
5'AD LD-insert screening oligo	CTATTCGATGATGAAGATACCCCACCAAACCC	61.4
3'AD LD-insert screening oligo	GTGAACTTGCGGGGTTTTTCAGTATCTACGAT	61.5
xma1-pHDT3F	**CCCGGG**CCTAGGAAACCGCCATTGT	67.1
xma1-pHDT3R in frame	**CCCGGG**TCCATTGTTGTGCGAGGTAGT	65.8
HDT3 Gateway FP	CACCATGGAGTTCTGGGGTGTTGAAG	61.4
HDT3 Gateway RP	AGCAGCTGCACTGTGTTTGG	58.8
LOBgateway F2	CACCATGGCGTCGTCATCAAACTC	73.4
LOBgateway R	CATGTTACCTCCTTGCTGATCAT	63.9
LBa1	TGGTTCACGTAGTGGGCCATCG	73
HDT3-RTR	CACTGTGTTTGGCCTTTGTG	54.9
SALK_002860-FP	TCATGGCCCAACTAAAGGAG	60.07
SALK_002860-RP	GATGTGCACGAGCCTGTCTA	60.02
qHDT3 FP	GAATGGGAGCGTTTTCTTCTCT	55.1
qHDT3 RP	ACTACCGTCAGCGTCATCATCT	57.8
HDT3-RTF	GAACCCAAGAAGAGGTCTGC	55.5
HDT-RUP	CTCCTCGTCTCCAGAGTTTTC	54.7
HDT3-R	AGGGTTCTTGGAGGAGTTGG	56.6
qRT-NPH3 FP	TCCCTGTGTAAGCCCATCTAA	55.4
qRT-NPH3 RP	AGACTCCATCTTGGTCCTGAAG	56.1
qRT-PKS2 FP	AGCCAGAGTTTGTTGCTTCAG	55.7
qRT-PKS2 RP	GCAGCCAAGAGTAGCGAGAA	57.2
qRT-PHOT1 FP	CACTGATCCTAGGCTTCCCG	57.2
qRT-PHOT1 RP	GTGGTTAGATCAGTCTCTGGACC	56.4
qACT2 - FP	GCACCCAGTTCTACTCACAG	55.2
qACT2 - RP	CAACATACATGGCAGGGAC	53.5
qUBC9 - FP	GATAGCCCTTATTCTGGAGGAG	54.1
qUBC9 - RP	TTGGATGGAACACCTTCGT	54
qBAS1-F	CAATCATAGCGGTCCATCAT	52.4
qBAS1-R	GGAGCCAAGTGAAAGGTGAA	55.2

Chapter 4

Chemical genetics as a tool to understand LATERAL ORGAN BOUNDARIES function in *Arabidopsis thaliana*

Abstract

Aerial lateral organs form at the periphery of the Shoot Apical Meristem (SAM) and are separated from the meristem by a boundary region. One gene involved in boundary formation is *LATERAL ORGAN BOUNDARIES (LOB)*. *LOB* is expressed in all organ boundaries, yet *lob* plants have only a subtle organ separation defect, in which the base of the cauline leaf is fused to the axillary stem. LOB is the founding member of the 43-member LATERAL ORGAN BOUNDARIES DOMAIN (LBD) family and functions as a transcription factor that differentially regulates gene expression. Given that LOB is expressed in all organ boundaries in *Arabidopsis* yet mutants have a limited phenotype, it is likely that other proteins act redundantly with LOB, masking other LOB functions. We sought to overcome possible redundancy using chemical genetics. We conducted a screen to identify molecules that inhibit *LOB* function and identified one chemical, LAT24D02 that inhibited the *LOB* over-expression phenotype of plants grown in the dark. By analyzing analogs of LAT24D02, we identified a substructure that is correlated with the inhibition of *LOB* over-expression activity. LAT24D02 did not affect the *LOB* over-expression phenotype in light-grown seedlings and furthermore, does not affect the ability of LOB to regulate a known downstream target, *BAS1*. The data from this Chapter suggests that LAT24D02 acts downstream of LOB in an uncharacterized LOB pathway.

Introduction

Genetics is a major tool to study biological systems. Two methods to study gene function are forward and reverse genetics. In a forward genetic screen, a scientist induces a mutation in an organism and looks for a phenotype of interest. Common mutagens are chemicals (i.e. EMS), X-ray or fast neutron radiation, and transposons (Greene et al. 1994; Greene et al. 2003; Belfield et al. 2012). Conversely, in a reverse-genetics study, the phenotype resulting from loss-of-function of a specific gene is studied. However, a null mutation does not always result in an observable phenotype due to redundancy, the phenomenon where more than one gene codes for proteins involved in a similar process (Nowak et al. 1997). What are the mechanisms that lead to redundancy? Genome duplication events result in the presence of multiple copies of the same gene, resulting in the creation of gene families. Typically, genes that have similar sequences have a similar function. Thus, if there are genes with redundant functions in a single pathway, mutations in multiple genes are required to obtain an observable phenotype.

Several methods have been developed to overcome redundancy, such as chemical genetics (Stockwell 2000; Hicks and Raikhel 2014). The principle of chemical genetics is to use small molecules that perturb the function of a protein leading to an observable phenotype. In the case of two proteins that have similar sequence and function, a chemical that binds to a conserved region may perturb both proteins resulting in a loss-of-function phenotype. An example that demonstrates the use of chemical genetics to overcome genetic redundancy is the identification of the Abscisic Acid (ABA) receptor in plants (Park et al. 2009). Prior genetic experiments were unsuccessful in identifying the

ABA receptor and its function. However, by conducting a chemical genetic screen, the ABA receptor was identified using an ABA analog, pyrabactin 3 (Park et al. 2009). In a second example, researchers were able to use the chemical bikinin to characterize the brassinosteroid perception pathway. Brassinosteroids are perceived by a membrane-bound protein, BRI1, which activates members of the GSK-3-like kinase family of proteins (Wang et al. 2006). Bikinin targets a subset of these kinases including four proteins that previously had an uncharacterized role in the brassinosteroid response pathway (De Rybel et al. 2009). Furthermore, chemical genetics has been used to study cancer biology (Mayer et al. 1999), vertebrate development (Wheeler and Brandii 2009), neurobiology (Koh and Crews 2002) and bacterial resistance (Poole 2004). One chemical identified through a chemical genetics project to study cancer biology, monastrol, is an antagonist of mitosis in mammals that inhibits the motility of kinesin Eg5. Previous to these experiments, most molecules used to study cancer-targeted tubulin (Mayer et al. 1999).

There are several advantages to using chemical genetics to study protein function in plants. This approach can be much faster than generation of higher-order mutants, which can take months or even years. Secondly, it is possible to observe pleiotropic phenotypes depending on the concentration of the chemical that is applied. A chemical could lead to multiple phenotypes at high concentration, but at low concentrations may result in plants with fewer, or milder phenotypes. This allows for dosage-dependent responses to be characterized, which can serve as a basis for enhancer/suppressor screens (Aghajan et al. 2010). For example, it is possible to assay for mutated plants that are

resistant or susceptible to a chemical. Additionally, a chemical can be applied to the whole plant by growth on media or in a temporal fashion by direct application to the organs of interest (Wahl 1985). Chemical genetics is also reversible as removal of a chemical can lead to reversion of a potential phenotype (Wulff and Arenkiel 2012). Another advantage is that differing chemical concentrations can lead to a spectrum of phenotypes. For example, application of a low concentration of a chemical to a plant might result in a mild phenotype, while application of a high concentration a more severe phenotype. A major advantage of chemical genomics is the potential to disrupt genes for which there are no available mutant strains or that are required early in development. For example, if a gene is required for both embryogenesis and floral organ development, a mutation may cause early lethality, which will mask its role in floral organ development. For example, T-DNA mutants are widely used to study a variety of biological responses in *Arabidopsis*, however, T-DNA inactivation lines may cause embryonic lethality (Kim et al. 2005).

The pipeline for utilizing chemical genetics is as follows: 1) A forward phenotypic screen is conducted to identify chemicals that cause a phenotype of interest. 2) A secondary screen is conducted to discard chemicals that do not repeat the initial screen results. 3) Finally, a variety of methods are used to discover the target of the chemical of interest, which is typically the bottleneck in the process. Once a chemical is identified, the goal is to identify the protein that the chemical affects (Cong et al. 2012). One way to do this is to perform column chromatography where a chemical is affixed to beads in a column and a protein extract from the organism under study is run through the

column. Protein binding to the chemical will result in retention in the column. This protein can then be identified by mass-spectrometry (Nuhse et al. 2003). A second technique used in target discovery is mutant analysis. The concept behind this approach is that mutations that disrupt the protein target will have altered sensitivity to the chemical (Cong et al. 2012). One way to carry this out is to apply the chemical to a mutagenized population and to screen for plants that do not respond to the chemical. The mutation can then be mapped and the corresponding gene characterized. This step is often time consuming and expensive, thus it is important to discard non-selective compounds in step two mentioned above (Cong et al. 2012).

The *Arabidopsis LATERAL ORGAN BOUNDARIES (LOB)* gene is one of 43 members of the *LATERAL ORGAN BOUNDARIES DOMAIN (LBD)* gene family (Shuai et al. 2002). Members of the LBD family bind to DNA in a sequence-specific manner to regulate gene expression. Furthermore, interaction with a bHLH protein reduces the ability of LOB to bind DNA (Husbands et al. 2007). LOB is expressed in all organ boundaries including the leaf - shoot apical meristem boundary, the base of floral organs, roots, and at the base of cauline leaves in the paraclade junction (Shuai et al. 2002). To date, the only reported developmental defect in hypomorphic *lob-3* mutants is a failure of separation of the cauline leaf and axillary stem in the paraclade junction (Bell et al. 2012). This suggests that a major role of LOB is to separate lateral organs from each other. The fact that *LOB* is expressed in all organ boundaries, yet *lob* mutants have only a limited phenotype suggests that there are other proteins that function redundantly with *LOB* (Husbands et al. 2007; Bell et al. 2012).

LOB negatively regulates brassinosteroid levels in the boundary region to separate lateral organs (Bell et al. 2012). Plants that over-express *LOB* are dwarfed, resembling brassinosteroid synthesis mutants and have altered brassinosteroid responses (Shuai et al. 2002; Bell et al. 2012). Furthermore, LOB directly activates the expression of a brassinosteroid-catabolic enzyme.

In this Chapter, I report a chemical genetics screen to identify chemicals that inhibited the *LOB* over-expression phenotype. Of the 9,600 chemicals that were screened, one, LAT24D02, was selected for further analysis. Although this chemical affected the *LOB* over-expression phenotype, it did not directly inhibit the ability of LOB to regulate a downstream target gene of LOB. These results suggest that this chemical affects another unidentified LOB dependent pathway.

Results

Chemical genetic screen for inhibitors of the *LOB* over-expression phenotype

Wild-type Arabidopsis seedlings that are grown in the dark (etiolated) form an apical hook (Darwin and Darwin 1880), which protects the shoot apical meristem as the seedling grows towards the soil's surface. However, etiolated seedlings that over-express LOB lack an apical hook. To induce LOB activity, we use a steroid-based LOB inducible construct, in which the LOB protein is fused to the glucocorticoid receptor (GR) and driven by the strong *35S* promoter (Bell et al. 2012). The LOB-GR protein fusion is sequestered in the cytosol by HEAT SHOCK PROTEIN 90 (HSP90). In the presence of a

steroid such as dexamethasone (DEX), the LOB-GR protein is released from HSP90 and translocates to the nucleus where LOB is free to act as a transcription factor (Picard et al. 1988). *35S:LOB-GR* plants grown in the presence of DEX fail to form an apical hook (Bell et al. 2012) (Figure 4.1) and this phenotype is used as a marker for LOB activity. The goal for this chemical screen was to identify chemicals that inhibit LOB activity by screening for *LOB* over-expression plants that form an apical hook.

LAT24D02 inhibits the *LOB* over-expression phenotype

To identify chemicals that inhibit the *LOB* over-expression phenotype, ~ 5-10 seeds were sown in each well of 96-well plates containing Murashige and Skoog media (MS) supplemented with 25μM of a chemical from the LATCA library (Zhao et al. 2007), LifeChemicals (LifeChemicals Inc.) or the solvent DMSO as a control, then stratified in the dark at 4°C for 2 days. These plates were transferred to the dark for 4 days at 21°C. 3600 chemicals from the LATCA library (Zhao et al. 2007) and 6000 chemicals from the LifeChemicals library (LifeChemicals Inc.) were screened for their ability to inhibit the *LOB* over-expression phenotype and two chemicals were identified (Figure 4.2). One chemical was termed LAT24D02 after the library (LATCA), plate number (24) and well position (D02), and selected for further analysis. *35S:LOB-GR* plants grown on control plates (no DEX) had an apical hook but when grown on 5 μM DEX did not (Figure 4.2A-B). However, *35S:LOB-GR* plants grown on 5 μM DEX and 25 μM LAT24D02 had an apical hook (Figure 4.2D) indicating that this chemical ameliorated this aspect of the *LOB* over-expression phenotype. We then asked if

LAT24D02 had an effect on non-induced *35S:LOB-GR* plants. *35S:LOB-GR* plants when grown on a control plates and 25 μM LAT24D02 had an apical hook, similar to wild-type but also had wider hypocotyls (Figure 4.2C). The effect on hypocotyl width observed in *35S:LOB-GR* seedlings grown in the presence of LAT24D02 suggests that LAT24D02 has other effects in addition to inducing apical hook formation in *LOB* over-expressed seedlings.

Chemical analogs suggest 3-fluoro-benzene substructure in LAT24D02 is responsible for inhibiting the *LOB* over-expression phenotype.

We next tried to determine whether a particular substructure of LAT24D02 was responsible for the reversion of apical hook formation in *35S:LOB-GR* plants grown on DEX and LAT24D02. To address this question, we screened analogs of LAT24D02 for their ability to revert the *35S:LOB-GR* phenotype (Figure 4.3). We tested four different chemical analogs of LAT24D02 for their effect on etiolated *35S:LOB-GR* seedlings grown in the presence of DEX . None of these chemicals caused apical hook formation, indicating that they did not inhibit the *LOB* over-expression phenotype (Figure 4.3). In comparing the structures of LAT24D02 and the analogs, the two substructure unique to LAT24D02 is a fluorine on the third carbon of the benzene ring. Interestingly, LAT24F02 did not induce apical hook formation in DEX treated *35S:LOB-GR* seedlings. Two differences between LAT24D02 and LAT24F02 is the position of the fluorine in the benzene ring and an ethyl-ester group instead of a methyl ester group. LAT24D02 has a fluorine attached to the third carbon of the benzene ring and a methyl-ester group

whereas LAT24F02 has a fluorine attached to the second carbon of the benzene ring and an ethyl-ester group. Therefore, the attachment of a fluorine atom to the third carbon of the benzene ring and the presence of a methyl-ester group in LAT24D02 is important for its ability to induce apical hook formation in *LOB* over-expressing plants.

LAT24D02 does not affect the *LOB* over-expression phenotype in light-grown seedlings

We examined LAT24D02 effects on the *LOB* over-expression phenotype at various stages of development. *35S:LOB-GR* plants were grown in the light on media supplemented with control solution or DEX. As previously described (Bell et al. 2012), *35S:LOB-GR* plants grown in the presence of DEX are smaller than those grown on control plates (Figure 4.4A-B). *35S:LOB-GR* plants grown on plates supplemented with DEX and LAT24D02 were indistinguishable from plants over-expressing *LOB* (Figure 4.4C). These data indicate that LAT24D02 does not inhibit the *LOB* over-expression phenotype in the light. One possible explanation is that LAT24D02 may affect the *LOB* over-expression phenotype in a dark growth-dependent manner.

LAT24D02 does not affect the GR-inducible system

LAT24D02 could suppress the phenotype of *35S:LOB-GR* plants grown on media supplemented with DEX if it inhibited the GR-DEX inducible system. To test this possibility, 25 μM LAT24D02 was applied to plants over-expressing a shoot apical meristem gene, *SHOOT MERISTEMLESS (STM)* (Endrizzi et al. 1996) using the DEX-

inducible system. If LAT24D02 inhibits some aspect of the DEX inducible system, such as blocking interaction between GR and DEX, then the *STM* over-expression phenotype would be inhibited when *35S:STM-GR* plants are grown on media supplemented with DEX and LAT24D02. As previously reported (Gallois et al. 2002), *35S:STM-GR* plants grown on 5 μM DEX do not develop leaves whereas *35S:STM-GR* plants grown on control plates resemble WT (Figure 4.5A-C). *35S:STM-GR* plants grown in the presence of DEX failed to form leaves regardless of the presence or absence of LAT24D02 (Figure 4.5C-D). Given that LAT24D02 had no effect on the *STM* over-expression phenotype, it is unlikely that LAT24D02 affects the GR-DEX inducible system.

LOB function is not altered by LAT24D02

How does LAT24D02 function at a molecular level? One possibility is that LAT24D02 interacts with LOB, or a protein complex involving LOB, and this interaction inhibits LOB function. To test this possibility, we grew *35S:LOB-GR* plants in the presence of LAT24D02 and DEX and observed the transcript accumulation of a direct target of LOB, *PHYB ACTIVATION TAGGED SUPPRESSOR (BAS1)* (Bell et al. 2012). As previously shown (Bell et al. 2012), *BAS1* transcript levels were highly increased in *35S:LOB-GR* plants grown in the presence of DEX (Figure 4.6). *35S:LOB-GR* plants grown on media supplemented with 5 μM DEX and 25 μM LAT24D02 had increased *BAS1* transcript compared to MOCK treated *35S:LOB-GR* seedlings. Therefore, LAT24D02 does not affect the induction of *BAS1* by LOB and likely acts to alter the function of a protein downstream of LOB.

LAT24D02 likely affects a LOB-brassinosteroid independent pathway

Since LAT24D02 did not inhibit LOB up-regulation of downstream target *BAS1*, LAT24D02 may affect the function of a protein downstream of LOB. To test this possibility, we detected transcript levels of a brassinosteroid-response gene, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 22* (*TCH4)*, after brassinosteroid application (Xu et al. 1995). *TCH4* transcript levels were measured by RT-PCR in wild-type plants that were grown on media and then flooded with 100 nM epi-brassinolide, 25 μM LAT24D02, or both. *TCH4* transcript was increased in wild-type seedlings incubated with 100 nM epi-brassinolide compared to MOCK-treated plants (Figure 4.7). *TCH4* transcript level was increased in seedlings incubated with 100 nM epi-brassinolide and 25 μM LAT24D02 compared to MOCK-treated plants indicating that LAT24D02 did not affect the TCH4 induction by epi-brassinolide. These results suggest that LAT24D02 does not affect brassinosteroid response. Therefore, LAT24D02 must affect some other non-brassnoisteroid response - *LOB* specific pathway.

Discussion

The primary goal of this chapter was to identify chemicals that can alter LOB activity. Considering *LOB* is expressed in all organ boundaries in *Arabidopsis,* yet the only developmental phenotype observed in *lob* mutants is a lack of separation of the axillary stem and cauline leaf, it is likely that other proteins function redundantly with LOB (Shuai et al. 2002; Bell et al. 2012). Since chemical genetics can be a powerful

approach to study functionally redundant genes (Stockwell 2000), we used this approach to identify chemicals that can inhibit the *LOB* over-expression phenotype in the hopes of identifying additional tools to study LOB function.

The experiments carried out in this Chapter show that LAT24D02 inhibits the *LOB* over-expression phenotype in the dark and that the 3-fluoro-benzene substructure of LAT24D02 is likely responsible for its action. However, LAT24D02 does not directly affect LOB function and does not affect BR response downstream of LOB. Interestingly, LAT24D02 does not affect the *LOB* over-expression phenotype in the light (Figure 4.5). One possible explanation for this observation is that LAT24D02 exclusively inhibits the dark-dependent *LOB* over-expression phenotype. Potentially, LOB could be involved in different pathways depending on light source. LOB has been found to interact with different proteins (bHLH048 and HDT3) and perhaps LOB interactions could be dictated by light source (Husbands et al. 2007). Furthermore, *LOB* transcript is regulated by blue-light (Chapter 1). One can imagine that LOB could function in a different pathway under blue-light illumination (a component of white light) than in the dark. Therefore, LAT24D02 might affect the *LOB* over-expression phenotype only in the dark if LOB has dark-specific functions. Further experiments are required to tease apart the potential different light source-dependent LOB functions.

Lastly, LAT24D02 does not affect the LOB-dependent brassinosteroid response. Given that LOB regulates brassinosteroid levels and that LAT24D02 inhibits the *LOB* over-expression phenotype, we tested the possibility that LAT24D02 affects brassinosteroid levels (Bell et al. 2012). However, we show that LAT24D02 does not

affect brassinosteroid levels (Figure 4.7). Therefore, LAT24D02 must affect some other LOB-dependent pathway. LOB is involved in the blue-light response pathway. LOB directly regulates blue-light response gene and *LOB* is in-turn regulated by blue-light (Chapter 1). LAT24D02 could be involved in the LOB-blue-light pathway, although this is unclear. The loss of apical hook in *35S:LOB-GR* seedlings grown on DEX is attributed to a loss of brassinosteroid levels and the relationship of brassinosteroids and blue-light response is not well characterized. Further experiments are necessary to untangle the function mode of action of LAT24D02.

Materials and methods

Plant materials and growth conditions:

All plants are in the *Col-0* ecotype. *35S:LOB-GR* plants were previously described (Bell et al. 2012). *35S:STM-GR* plants were previously described (Gallois et al. 2002). For all light-grown plants, seeds were sterilized with 95% EtOH for 5 minutes, followed by a solution of 20% bleach + 0.01% Tween-20 for 5 minutess, then washed five times with water before plating on Murashige and Skoog plates (Murashige and Skoog 1962). Seedlings were grown for 14 days under 120 $\mu M/m^2s$ white light.

Chemical genetics screen:

One μL of a 2.5 mM chemical solution was pipetted into two 96-well plates using the Biomex FX Robot in the UCR Core facility. One plate contained 99 μL of Murashige and Skoog (MS) media supplemented with 5 μM DEX and the second plate contained 99 μL of Murashige and Skoog (MS) control (DMSO) media. Five - ten seeds were sown in

each well. As a control, one row of each 96-well plates contained MS media with no chemical, DMSO, nor DEX added. Plates with *35S:LOB-GR* seeds were then placed in the dark for two days at 4°C to stratify. Seeds were transferred to 21°C and incubated for 4 days in the dark. Presence or absence of apical hook was observed under a Leica MZ12 microscope.

Analog analysis:

LAT24D02 analogs present in our libraries were identified using ChemMine (Backman et al. 2011).

RNA isolation and RT-PCR:

RNA was isolated using TRIAZOL reagent. For reverse-transcriptase mediated PCR, cDNA was synthesized from 4ug of RNA using Oligo(dT) and Superscript Reverse Transcriptase (Invitrogen). Gene specific primers are listed in Table 4.1. Northern-blot hybridization was carried out as previously described with gene specific probes for *BAS1* (Martienssen et al. 1989).

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Figure 4.1. Schematic representation of chemical genetic screen for inhibitors of the *LOB* over-expression phenotype. Left panel: *35S:LOB-GR* plant grown in the absence of DEX, exhibiting an apical hook. Right panel: *35S:LOB-GR* plant grown in the presence of DEX, showing no apical hook. The screen was designed to identify chemicals that cause *35S:LOB-GR* plants grown in the presence of DEX (right panel) to have an apical hook (left panel).

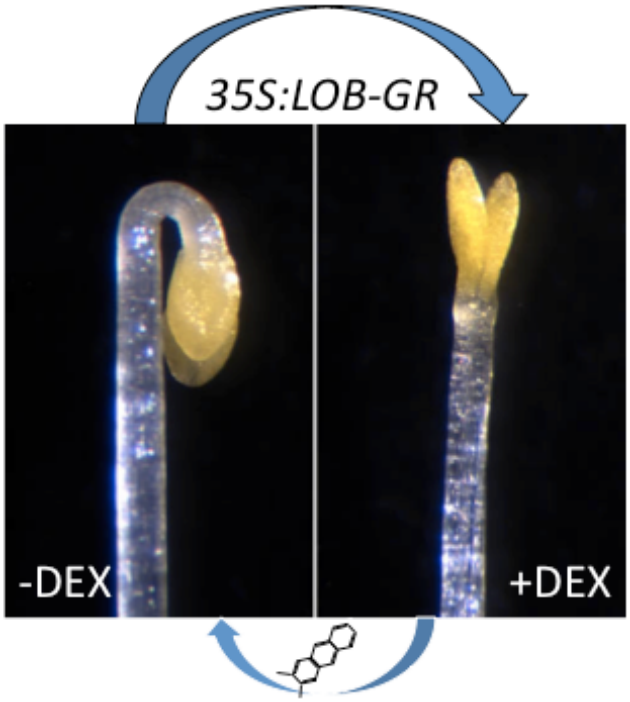

35S:LOB-GR
-DEX
+DEX

Figure 4.2. Phenotype of plants grown in the presence of LAT24D02. A) *35S:LOB-GR* plant grown on control plate showing an apical hook. B) *35S:LOB-GR* plant in the presence of 5 μM DEX showing a loss of apical hook. C) *35S:LOB-GR* plant supplemented with 2.5 μM LAT24D02 showing an apical hook. D) *35S:LOB-GR* plant supplemented with 5 μM DEX and 2.5 mM LAT24D02 showing an apical hook. Bar = 1mm.

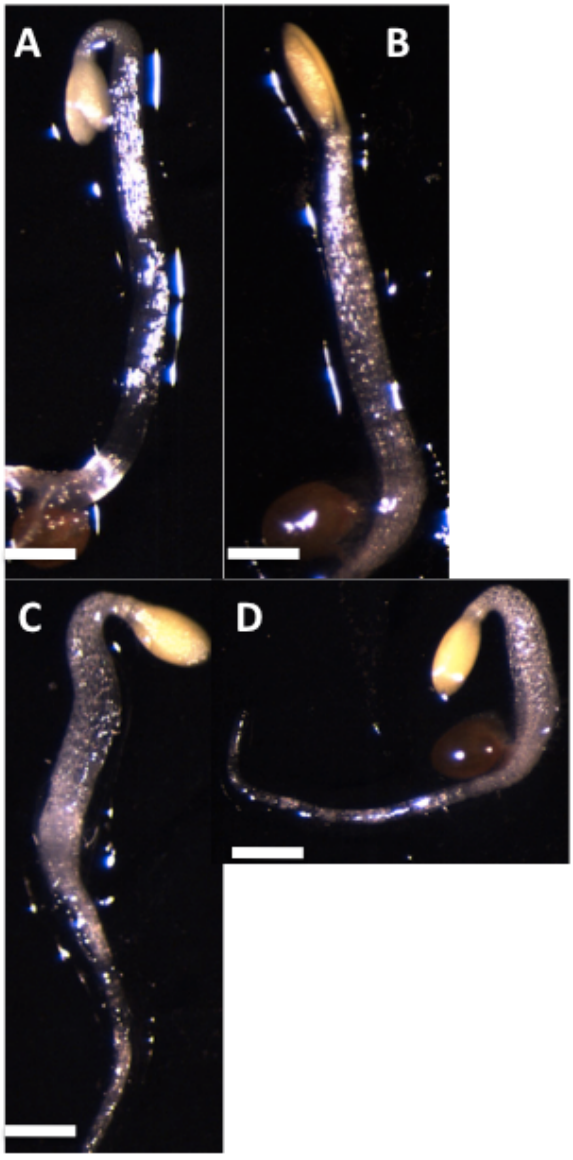

A
B
C
D

Figure 4.3. Chemical analogs suggest a substructure is responsible for inhibiting the *LOB* over-expression phenotype. Different analogs of LAT24D02 and representative phenotypes of *35S:LOB-GR* plants when grown on associated chemical and DEX. Only LAT24D02 causes a loss of apical hook upon *LOB* over-expression.

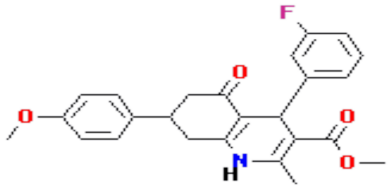
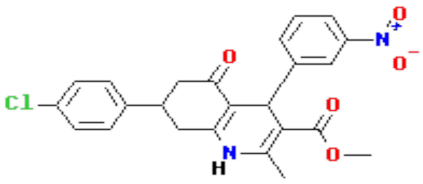
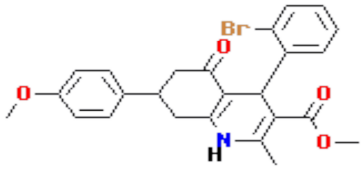
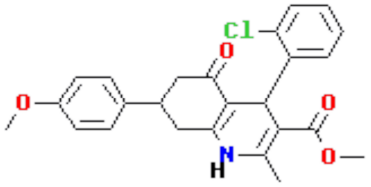
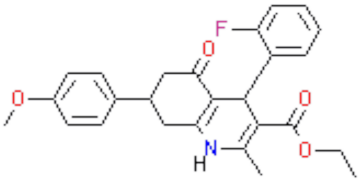
Chemical Name	Structure	Apical Hook
LAT24D02		YES
LAT26G11		NO
LAT012H02		NO
LAT13A04		NO
LAT24F02		NO

Figure 4.4. LAT24D02 does not affect the *LOB* over-expression phenotype of plants grown in the light. A) *35S:LOB-GR* plant grown on control media. B) *35S:LOB-GR* plant grown in the presence of 5 μM DEX. C) *35S:LOB-GR* plant grown on MS media supplemented with 5 μM DEX and 2.5 mM LAT24D02.


A
B
C

Figure 4.5. Dexamethasone does not affect the GR system. All seedlings are grown in the presence of 5 μM DEX. A-B) *Col-0* wild-type plants grown on DMSO and 2.5 mM LAT24D02, respectively. There is no difference in the size of seedlings. C-D) *35S:LOB-GR* seedlings in the presence of DMSO or 2.5 mM LAT24D02. E-F) *35S:STM-GR* plants in the presence of DMSO or 2.5 mM LAT24D002.

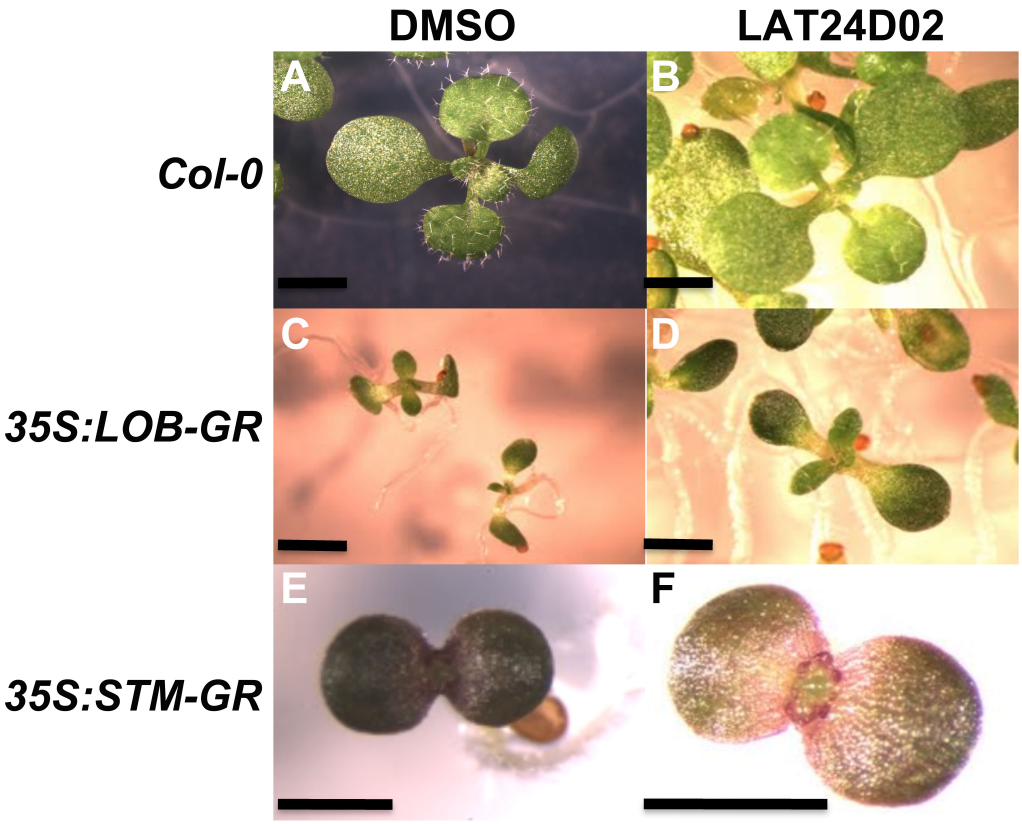

DMSO
LAT24D02
Col-0
35S:LOB-GR
35S:STM-GR
A
B
C
D
E
F

Figure 4.6. LAT24D02 does not directly affect LOB activity. Northern blot of *BAS1* RNA extracted from 14-day old plants grown media supplemented with control, 2.5 mM LAT24D02, 5 μM DEX, or both 2.5 mM LAT24D02 and 5 μM DEX. *BAS1* RNA is upregulated in plants grown on DEX and LAT24D02 + DEX.

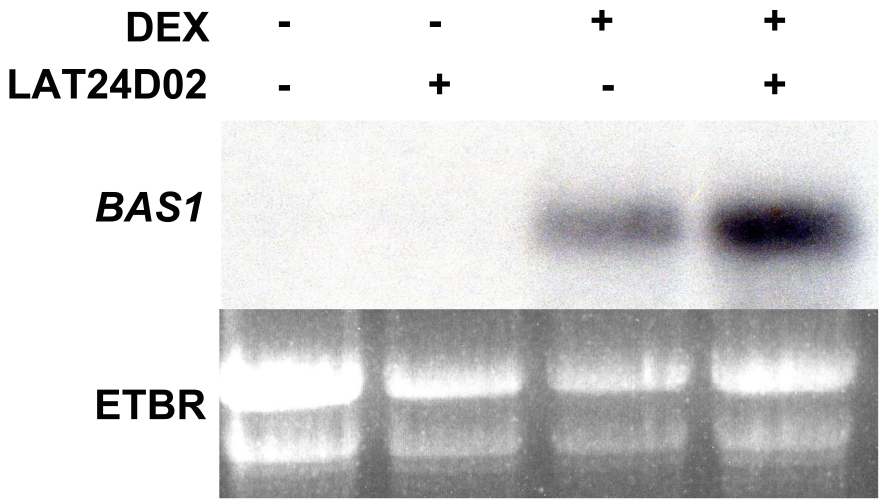

DEX - - + +
LAT24D02 - + - +
BAS1
ETBR

Figure 4.7. LAT24D02 does not affect brassinosteroid response. Semi-quantitative PCR of *TCH4* transcript in wild-type *Col-0* plants after treatment with 100 nM epi-brassinolide and 2.5 mM LAT24D02 for 4 hours. PCR products were run on a gel after 28 cycles for *TCH4* and 22 cycles for *APT1*.

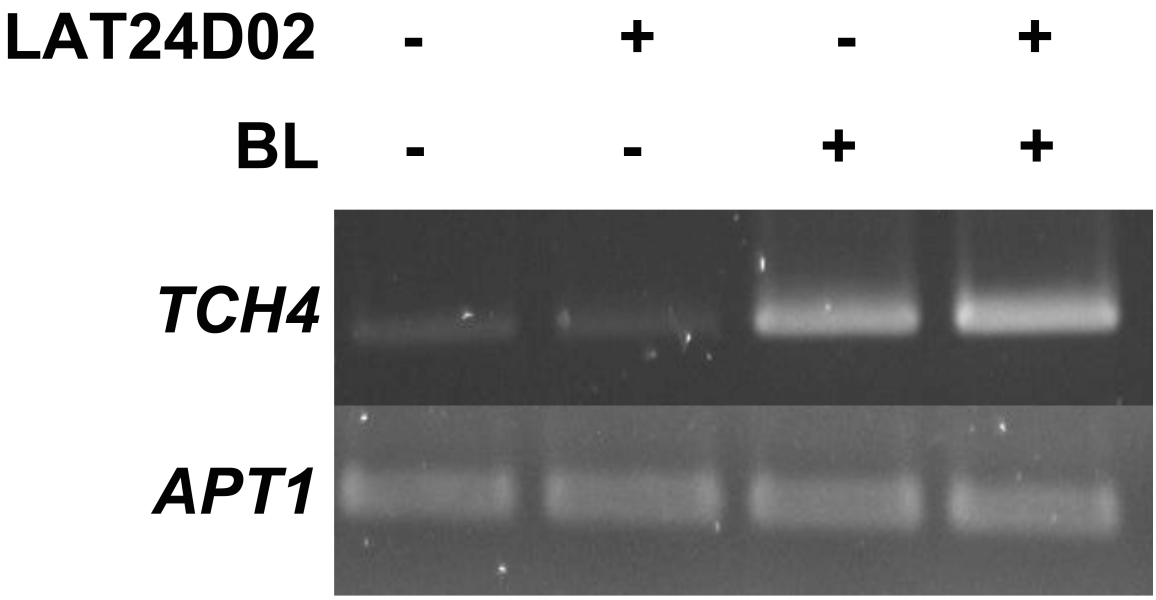

LAT24D02 - + - +
BL - - + +
TCH4
APT1

Table 4.1. List of oligonucleotide sequences.

Primer Name	Sequence (5'→3')	(Tm)°C
RT-PCR BAS1 F	5'-GGCGGAGACAAAACGCTAT-3'	55.5
RT-PCR BAS1 R	5'-CGGTAGGTGCATGCTGATAA-3'	59.72
TCH4-3	5'-GTTGAGTCAAGCTCTTGTGACAACC-3'	62.2
TCH4-4	5'ATGGCGATCACTTACTTGCTTCCTC-3'	64.56
BAS1 Probe F	5'CCCGGGAGTCGTTGTTGAAGCTGATAGAGC-3'	65
BAS1 Probe R	5'-GTTGAGTCAAGCTCTTGTGACAACC-3'	65

Conclusions

The shoot apical meristem (SAM) in plants is responsible for two actions: 1) the formation of lateral organs at its periphery and 2) the renewal of meristem cells (Braybrook and Kuhlemeier 2010). Lateral organs emerge from founder cells in the peripheral zone of the SAM. To replenish cells lost to organ formation, daughter cells derived from stem cells in the central zone divide and move into the peripheral zone. (Szymkowiak and Sussex 1996). Aerial lateral organs, such as leaves, branches, and flowers, are produced at the flanks of the SAM (Braybrook and Kuhlemeier 2010) and their initiation requires the phytohormone Auxin (Reinhardt et al. 2000; Vanneste and Friml 2009). Lateral organs are separated from the SAM by a small band of cells called the boundary region.

Several genes are expressed in the boundary and have demonstrated roles in the separation of lateral organs including *LATERAL ORGAN BOUNDARIES (LOB)* (Rast and Simon 2008; Bell et al. 2012). *LOB* is the founding member of the 43-member, plant specific LBD gene family and is expressed in all organ boundaries including the base of lateral roots, floral organs, and at the junction of the cauline leaf and axillary stem junction (Shuai et al. 2002). Plants carrying a hypomorphic *lob* mutation exhibit a fusion between the cauline leaf and axillary stem in the paraclade junction suggesting that one function of LOB is to separate lateral organs. LOB interacts with the basic helix-loop-helix protein bHLH048 and this interaction alters the binding affinity of LOB *in vitro*. LOB binds to the 5'-(G)CGGC(G)-3' sequence where the core 5'-CGGC-3' motif is required. This binding is enhanced when a Guanine nucleotide is located on either side of

the core motif (Husbands et al. 2007). LOB functions to differentially regulate several target genes including genes that are involved in modulating brassinosteroid levels (Bell et al. 2012).

In this dissertation, I have demonstrated that LOB regulates the expression of *NPH3, PKS2,* and *PHOT1* and that LOB has an important role in regulating leaf inclination responses to blue-light. After blue-light illumination from above, asymmetric growth of abaxial petiole cells results in a more erect leaf. This change in leaf inclination in response to blue-light is called the hyponastic response and requires the activities of *NPH3, PKS2,* and *PHOT1* (Inoue et al. 2008; de Carbonnel et al. 2010). *lob* plants exhibit altered hyponastic responses to both short and long term blue-light illumination. Given that *LOB* is expressed in the leaf axil, these data suggest that the axil is an important component of the blue-light hyponastic response. We hypothesize that following blue-light perception in the leaf axil, a signal moves from the axil to the abaxial petiole cells to regulate cell growth. Although the identity of the proposed signal is unknown, a strong candidate is the hormone ethylene, which has been implicated in regulation of hyponasty in response to various external stimuli (Millenaar et al. 2005; Bailey-Serres and Voesenek 2008). Furthermore, we have shown that LOB plays a role in the ethylene mediated hyponastic response as *lob-3* plants exhibit an attenuated response following ethylene treatment (Chapter 1). What role does LOB play in the hyponastic response to blue-light? One possibility is that the LOB regulation of *NPH3, PKS2*, and *PHOT1* is solely responsible for the blue-light response defects in *lob* plants. A second possibility is that the altered blue-light response in *lob* plants is due to defects in regulation of genes

involved in ethylene response. LOB could also play a role in regulating some combination of blue-light and ethylene response genes to regulate hyponastic growth. Interestingly, an ethylene response factor, *CYTOKININ RESPONSE FACTOR 1 (CRF1)*, is positively regulated by LOB (Bell et al. 2012). This regulation may have an important role in the blue-light hyponastic response providing a link between blue-light, ethylene, and LOB.

There are several common themes for LOB function. Previous reports show that LOB is involved in a negative feedback loop to regulate brassinosteroid levels in the boundary. *LOB* expression is positively regulated by brassinosteroids and LOB directly activates the expression of a BR catabolic gene, *BAS1*, which negatively regulates brassinosteroid levels (Bell et al. 2012). In this dissertation, data show that LOB negatively regulates *NPH3, PKS2,* and *PHOT1*, which are involved in the blue-light hyponastic response. *LOB* is also positively regulated by blue-light. These feedback loops allow for relatively rapid response to stimuli, followed by the eventual stabilization of a response. For example, plants will grow towards blue-light, however if they are too close to the light source, they may overheat leading to death. Therefore, it is important for plants to mediate their proximity to a light source. Several external stimuli affect leaf angle including heat, flooding, and shade (van Zanten et al. 2009; Keller et al. 2011; Rauf et al. 2013). It would be interesting to see if LOB plays a role in regulating the leaf angle in response to these stimuli.

Thus far, LOB has been implicated in regulating two hormone-related processes, brassinosteroid signaling and the ethylene-mediated hyponastic response (Bell et al.

2012). This dissertation suggests that LOB is associated with another hormone, auxin. LOB directly regulates *NPY1*, a gene that interacts with proteins involved in auxin transport (Cheng et al. 2007). Possibly, LOB regulates *NPY1* to control auxin transport to contribute to the separation of lateral organs. Given that auxin plays a role in increasing cell size and cell division (Perrot-Rechenmann 2010) and the boundary is composed of cells that grow slowly and, divide infrequently compared to the surrounding cells (Callos and Medford 1994; Gaudin et al. 2000), we hypothesize that LOB regulates *NPY1* to keep auxin outside of the boundary region. Expression of an auxin reporter gene is reduced in boundary cells compared to the surrounding cells (Vernoux et al. 2010) suggesting that the boundary region has lower levels of auxin. Future studies should focus on the connection of auxin regulation and LOB in the boundary.

How does LOB function at a molecular level? Previous studies show that LOB is a transcription factor that differentially regulates gene expression (Husbands et al. 2007; Bell et al. 2012). In this dissertation, I report the identification of LOB interacting proteins. We identified HISTONE DEACETYLASE 3 (HDT3) as a binding partner of LOB in yeast and *in vivo* and show that HDT3 has a function in organ separation and leaf responses to blue-light. Interestingly, *BAS1* transcript is reduced in paraclade junctions of *hdt3* plants compared to wild-type. Histone deacetylases negatively regulate gene expression, therefore it seems unlikely that HDT3 directly regulates *BAS1* expression (Wu et al. 2000; Wu et al. 2003; Hollender and Liu 2008). One hypothesis is that HDT3 negatively regulates a negative regulator of *BAS1*, which would be consistent with reduced *BAS1* levels in *hdt3* paraclade junctions compared to wild-type. Despite the

defects in blue-light response observed in one *hdt3* allele, transcript levels of LOB targets *NPH3, PKS2,* and *PHOT1* were not significantly different in *hdt3* plants compared to wild-type. There are several possible explanations of these data. Firstly, HDT3 may play a role in a blue-light response pathway that is independent of *NPH3, PKS2,* and *PHOT1*. Secondly, LOB is in a complex with HDT3 and other proteins, but HDT3 may not be necessary for its function, perhaps because of the presence of redundant HDAC proteins. Future projects should focus on the mechanism of HDT3 function, especially in characterizing its target genes involved in organ separation and blue-light response.

The fact that *LOB* is expressed in all organ boundaries, yet *lob* mutants have only a limited phenotype suggests that there are other proteins that function redundantly with *LOB* (Husbands et al. 2007; Bell et al. 2012). To further characterize the function of LOB, we carried out a chemical genetic screen to identify chemicals that inhibit LOB activity. We identified one chemical, LAT24D02 that inhibits the *LOB* over-expression phenotype, however the mode of action of LAT24D02 is unclear. It does not affect the ability of LOB to regulate a direct target gene suggesting that it acts downstream of LOB in an uncharacterized LOB dependent pathway. The obvious future goal is to identify the protein target of LAT24D02. This could be done using column chromatography with protein extracts from *Arabidopsis* or by mutant analysis. Once the protein target is identified, its relationship to LOB could then be characterized.

To further characterize the molecular function of LOB, future studies should be carried out as follows:

1) Further exploration of LOB's target genes. The microarray data suggests that LOB regulates several genes including *TREHALOSE-6-PHOSPHATE PHOSPHATASE J (TPPJ)* (Bell et al. 2012). The *TPPJ* ortholog in maize, *RAMOSA3 (RA3),* is involved in inflorescence branching. Furthermore, the maize *ra3* mutant resembles *ra2*, which is thought to be orthologous to *LOB* (Bortiri et al. 2006; Satoh-Nagasawa et al. 2006). It would be interesting to study the biological relevance of LOB regulation of *TPPJ* in *Arabidopsis.*

2) Understand the role of HDT3 in LOB function. This dissertation shows that LOB interacts with HDT3, however, it is unknown which genes are regulated by this HDT3-LOB interaction. One future direction is to characterize genes that are regulated by both HDT3 and LOB through microarrays or ChIP. If LOB and HDT3 physically interact to regulate a subset of LOB targets, genes that are regulated by both LOB and HDT3 would be good candidates.

3) Further characterize the role of the boundary in blue-light responses. Since LOB is expressed in the boundary and *lob* mutants show altered responses to blue-light, the boundary is an important player in the blue-light response. Ethylene is a candidate hormone that could link the boundary to the hyponastic response, especially because preliminary evidence suggests that LOB regulates *CRF1* transcript levels. One future experiment is to observe the response of *crf1* mutants to blue-light.

4) Understand the molecular mechanisms of the pulvinus in other plant species. Legumes utilize the pulvinus, a motor organ at the base of leaves, which uses turgor pressure to expand or contract to alter leaf position (Watanabe and Sibaoka 1973; Abe

1980; Vogelmann 1984). It has been shown that in *Medicago,* the *elongated petiolule (elp1)* mutant fails to fold its leaflets because it lacks the pulvinus motor organ (Chen et al. 2012; Zhou et al. 2012). *ELP* is an ortholog of *LOB* and its transcript is detected in the basal region of young leaflets. Given that *AtLOB* is expressed at the base of lateral organs, the *LOB* orthologs *RA2* (*Maize*) and *ELP1* (*Medicago)* are expressed in the pulvinus, and *ra2* plants have a smaller pulvinus at the base of the inflorescence branches, it is clear that the pulvinus shares characteristics with boundary regions. It would be important to know what genes are expressed in the pulvinus and their role in regulating blue-light responses.

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