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# Characterization of the Role of *LOB*-DOMAIN Genes and Brassinosteroids in Rice Architecture

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## Dedication

"Being a Latina in STEM means I no longer represent only myself; I represent my community, my culture and the values my family instilled in me"

- Latinas in STEM

I would like to thank my husband, family, friends, and community for their constant support and encouragement throughout my scientific career.

ABSTRACT OF THE DISSERTATION

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by

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Rice architecture has historically been manipulated to increase grain yields. Creating rice plants with an erect stature caused by upright leaves allows for higher-density planting. In rice, the plant hormone Brassinosteroid (BR) contributes to shoot architecture, mainly controlling plant height and leaf erectness. In *Arabidopsis*, *LATERAL ORGAN BOUNDARIES* (*LOB*) is expressed in organ boundaries and regulates organ separation by modulating BR accumulation. *LOB* is positively regulated by BRs and LOB activates expression of *BAS1*, which inactivates BR. This *LOB*-BR feedback loop limits growth in the organ boundaries. This dissertation focuses on characterizing *LOB* orthologs in rice and investigates the use of *LOB* and *BAS1* to manipulate local BR accumulation in the rice, with a goal of creating plants with an erect stature.

Chapter 1 investigates the site-specific expression of *AtLOB* in rice to manipulate BR accumulation. In rice, the *AtLOB* promoter drove expression in organ boundaries and lamina joints and was used to drive expression of *AtLOB* to modulate BR accumulation. *AtpLOB:LOB* plants had altered leaf shape and decreased leaf inclination, characteristics associated with BR-deficiency. These results suggest that *AtLOB* expression in a distinct

domain alters leaf morphology without other changes in shoot architecture caused by BR deficiency.

Chapter 2 examines the expression of *AtBAS1* under the *LOB* promoter in rice to repress BR signaling. *AtpLOB:BAS1* plants displayed characteristics associated with both reduced BR and enhanced BR signaling. *AtpLOB:BAS1* plants had upright leaves as well as elongated inflorescences with increase branching and long, slender grains. These results suggest that localized *AtBAS1* expression in rice alters leaf inclination and inflorescence architecture.

In Chapter 3, a genome-wide analysis of the rice *LBD* gene family was conducted to determine which *OsLBD*s are orthologous to *AtLOB*. Through phylogenetic analyses identified the *OsLBD* class Ia genes, *OsRa2*, *OsIG1*, *OsLOB4*, and *OsIAL1*, which had high sequence similarity to AtLOB protein sequence. All class Ia *OsLBDs* were expressed in lamina joints, inflorescences, and floral organs, suggesting that class Ia *OsLBD*s may have similar functions to *AtLOB*. Using BR hormone response data, class Ia *OsLBD*s were not BR regulated, suggesting they may not function in BR signaling.

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## Introduction

In 2011, the world population reached approximately 7 billion people and it is estimated that by the year 2050 it will exceed 9 billion (11). Agriculturally important cereal crops such as maize, wheat, and rice are an important source of calories for human and animal consumption, especially in the developing world (3). About half of the population relies on rice as its major source of nutrition, with Asia consuming more than 90% of rice (3). As the world population increases, food shortage and security have become serious global problems and will continue to be so, based on the growing population trends. To alleviate these issues, rice production must keep up with demand. In order for this to happen, scientists must find ways to increase rice grain production (38; 41; 62).

In the 1960s, there were significant biotechnological advances that addressed the problems of increasing population and food scarcity (40). This era was termed the "Green Revolution" (40). In 1966, the International Rice Research Institute (IRRI) bred a semi-dwarfed, high yielding rice variety termed "Miracle Rice", also known as IR8 (26; 40). The development of IR8 lead to an increase in rice grain production and this variety was eventually adopted worldwide (18; 40). After further studies, scientists identified *Semi-dwarf1* (*SD1*), which encodes a rate limiting enzyme of the gibberellin (GA) biosynthetic pathway, as the gene responsible for the IR8's short stature (3; 40). Many *sd1* alleles have been developed in numerous rice breeding programs in Asia to manipulate plant height in rice (2). Since the development of the IR8 rice variety, many rice lines have been bred to suit the needs of different countries (38; 60).

**The ideal rice plant**

Rice (*Oryza sativa*), a monocot, is an annual grass that has been used as a model crop plant for the cereals (35; 77). Rice is composed of hollow, jointed stems (culms), and side branches (tillers) (35; 77). The tillers bear panicles, which produce spikelets that develop into grains (35; 77). The rice leaf is divided into three regions along the proximal-distal axis: sheath, lamina joint, and blade (35; 77). The sheath is the proximal region that begins at the base of the node and wraps around the culm, protecting the developing leaves and SAM and also preventing water and dust from entering the culm (35; 77). The distal region of the leaf is the blade, which is flat and is the major site of photosynthesis (35; 77). The junction between the blade and sheath is the lamina joint (collar), which is the site of leaf bending (35; 77). The lamina joint is composed of two regions, the ligule and auricles. The ligule is an extension of the sheath into the lamina joint and forms a papery membrane in the inside of the lamina joint. The auricles are small appendages on either side of the base of the blade.

The growth of the rice plant is divided into three general stages: vegetative, reproductive, and grain filling/ripening stage (77). The vegetative stage occurs from germination to panicle initiation (35; 77). During the vegetative stage, numerous leaves and tillers are forming (35). During the reproductive stage, which ranges from panicle initiation to heading, culms are elongating, panicles are emerging, and flowering is occurring (35). In the last stage, heading to maturity, grain filling/ripening occurs (35; 78)

Since rice is a major food source for many countries, improvements to increase yield are essential. Manipulation of rice architecture to meet food demands has been a target of many rice breeding programs (60). While the IR8 variety increased yield production through its short stature, increased tillers, and erect leaves, scientists have been working on altering rice architecture to create a more productive rice plant (39; 42; 60; 76). IRRI proposed a new ideal plant with desirable traits to increase in grain yield The new ideal plant has the following traits: (1) high number of productive tillers; (2) each tiller produces high-yielding panicles, which bears 200-250 grains; (3) 90-100 cm in plant height, which prevents lodging by wind and rain(4) thick and sturdy stems; (5) dark green thick and erect leaves; for better use of solar energy and denser planting and (6) vigorous, deep roots systems (39).

Height and leaf angle are two traits that have been the focus of efforts to improve rice yields. Rice plant height is mainly determined by the length of the culms, the hollow jointed stems that bear panicles (77). The culms are composed of internodes, which quickly elongate during reproductive growth due to cell elongation and division (77). Semi-dwarf stature is an important agronomic trait, as short plants have reduced straw biomass and are resistant to lodging (31; 42).

Leaf erectness is a desirable trait that allows for high density planting (65). Rice plants with erect leaves have increased photosynthetic efficiency and require less growth space, which allows for higher density planting (39; 40; 54; 65; 70). Upright leaves increase the photosynthetic efficiency of the plant by optimizing canopy photosynthesis (39; 70). Upright leaves can allow sunlight to penetrate the lower leaves that are usually

shaded by drooping upper leaves and can also capture sunlight on both the adaxial and abaxial sides whereas "normal" drooping leaves capture light mainly on the adaxial surface (3; 39; 70).

**Plant hormones and rice shoot architecture**

Since the Green Revolution in the 1960s, hormone function in crops has been studied for its important role in agricultural improvement (3; 76). Altering hormone signaling has been an approach used to modify crops to suit the needs of different countries (3). Two hormones, which have been greatly studied because of their potential role in altering plant architecture, are gibberellins (GAs) and brassinosteroids (BRs) (3; 42). GAs and BRs are two groups of plant hormones that play an essential role in normal growth and development (55; 64). GAs are plant hormones that play a role in various developmental processes but in particular culm elongation in rice (3). Since the identification of the *SD1* gene in the IR8 variety, many GA-related dwarf rice mutants have been isolated (44; 64). Many rice varieties have been created that have mutations in the GA biosynthesis or metabolism pathway, resulting in plants with different severities of dwarfism (3).

Studies have shown that BR plays a role in various physiological and developmental processes such as stem elongation, cell expansion and division, tiller and leaf erectness, and plant height (73). BR-deficient mutant rice plants are dwarfed, have decreased fertility, smaller grains, reduced and compact tillering, and dark-green upright leaves (3; 19; 73). Some traits of BR-deficient rice are beneficial. For example, erect

leaves can result in increased yields because they allow dense planting and increase photosynthesis. However, other BR-deficient traits diminish these benefits. For example, as rice plants that are severely dwarfed lack productive tillers and fertile grains (19; 39).

The BR signaling and biosynthetic pathway has been studied in various plant species, uncovering the importance of BR in plant shoot architecture (19; 55). The BR plasma membrane receptor, *BRI1* (*Brassinosteroid-insensitive 1*), perceives BR at the cell surface and begins a series of biochemical events that lead to the activation of transcription factors that activate or repress BR-response genes (19; 27). Rice *bri1* mutants such as *d61* have upright leaves and are dwarfed due to reduced internode elongation (82). The altered internode elongation pattern in *bri1* mutants is due to a decrease in cell division and elongation caused by defects in BR perception (75; 82). *bri1* mutants have been studied in various plant species, *Arabidopsis*, rice, barley, tomato, and pea, and all resulted in dwarfed plants with BR insensitivity (12; 16; 53; 57; 82). BR-biosynthetic mutants have similar phenotypes to BR-signaling mutants, for example, *br-deficient dwarf2* (*brd2*) and *ebisu dwarf* (*d2*) are dwarfed with upright leaves and produce smaller grains (29; 30). Both mutants result in a reduction in BR biosynthesis intermediates but can be partially rescued with the addition of exogenous BR (29; 30).

**The shoot apical meristem and its role in plant architecture**

The shoot apical meristem (SAM) is a population of cells at the tip of the shoot axis that controls shoot architecture (79). The SAM continuously produces lateral organs, such as leaves and flowers, and is the ultimate source of all above ground organs

that develop after embryogenesis (8). The SAM contains a population of stem cells that divide to replenish themselves and supply a continuous source of daughter cells for the formation of new organ primordia (33). The SAM is organized into two zones: the central zone (CZ), which contains slowly dividing stem cells that remain undifferentiated, and the peripheral zone (PZ), which flanks the CZ and contains rapidly dividing cells that will be incorporated into lateral organs (10). Lateral organs and shoots initiate in a specific pattern from the PZ (68; 71). Proper stem cell maintenance in the SAM requires a negative feedback loop between WUSCHEL (WUS), a homeodomain transcription factor, and the CLAVATA (CLV) signaling pathway (4). *WUS* is expressed in a group of cells beneath the CZ and regulates stem cell identity (46) Loss-of-function *wus* mutants result in premature termination of the SAM, while ectopic expression of *WUS* results in plants with an enlarged meristem (46; 66). *CLV3*, which encodes a small peptide, is expressed in the CZ and is positively regulated by WUS (9; 21; 52). *clv3* mutants exhibit enlarged meristems, opposite to the phenotype of *wus* mutants (13). CLV3 binds to CLV1 receptor kinase to activate a signaling pathway that represses *WUS* transcription to limit meristem size (14; 21; 58).

Meristem maintenance and the formation of lateral organs involve the coordination of various processes in order for patterning and proliferation to happen properly (10). Class I *KNOX* (*Knotted-like homeobox*) genes are a family of transcription factors that play a large role in shoot meristem function (72). Class I *KNOX* genes are expressed in the meristem and down-regulated in lateral organ primordia (25). *KNOTTED1* (*Kn1)* in maize, is expressed in vegetative, inflorescence and floral

meristems (37). Dominant *Kn1* mutants, which have ectopic *Kn1* expression in the leaf, result in defects in proximal-distal patterning, with displacement of the sheath distally into the blade and corresponding formation of ectopic ligule and auricle in the blade domain (22). *SHOOTMERISTEMLESS (STM*), the *Kn1* homolog in *Arabidopsis*, is expressed in the SAM and functions in SAM formation and maintenance (4; 48). *stm* mutants produce cotyledons but fail to develop a SAM during embryogenesis (48). The expression patterns of class I *KNOX* genes and mutant phenotypes in different plant species have shown the importance of class I *KNOX* genes in meristem maintenance (4; 48).

**Functions of the boundary region**

Between the SAM and lateral organ primordia lies a distinct band of cells, which serves as a barrier to isolate populations of cells within their distinct domains (17; 34; 61). This boundary region between the SAM and organ primordia plays a significant role in the regulation of plant architecture (17; 61). The boundary is a region of limited growth, containing small, slowly dividing cells that are morphologically distinct from cells in adjacent regions (34; 61). Several boundary identity genes have been characterized to uncover the mechanisms that control boundary formation and organ separation.

In *Arabidopsis*, the *CUP-SHAPED COTYLEDON (CUC 1, 2, 3)* genes are expressed in the boundary between the SAM and lateral organ primordia (1; 28). *BRASSINAZOLE RESISTANT1* (*BZR1*), a key transcription factor in BR signaling,

represses the expression of *CUC* genes in the meristem and lateral organ primordia (23; 43). In the boundary, BZR1 levels are reduced, allowing for the expression of *CUC* and proper boundary formation (23). Mutant analysis revealed that *CUC* genes function redundantly and are involved in embryonic SAM formation and the separation of cotyledons (1). Single *cuc* mutants have subtle phenotypes, while *cuc1cuc2* double mutants lack a SAM and have defects in organ separation resulting in fused cotyledons, and floral organs (sepals, and stamens) (1). *LATERAL SUPPRESSOR (LAS)* is also expressed in the boundary during axillary meristem initiation and *las* mutants are unable to form axillary shoots during vegetative development (24). *JAGGED LATERAL ORGANS (JLO)* is another boundary gene that has been shown to play an important role in organ separation (6). Dominant-negative mutants resulting from expression of a fusion protein between JLO and the EAR domain, a transcriptional repression domain, displayed partially fused cotyledons and leaves that curled upwards and did not expand properly (6). The characterization of boundary identity genes demonstrates the importance of the boundary to maintain proper organ separation.

*LATERAL ORGAN BOUNDARIES* (*LOB*), the founding member of the *LOB*-domain (*LBD*) gene family, encodes a plant-specific transcription factor that is expressed in all organ boundaries during the vegetative and reproductive stages of development (69). While *lob* mutants resembled wild-type plants, closer examination of the paraclade junction, the junction between main stem, axillary stem, and cauline leaf, revealed organ separation defects resulting from subtle fusion between the cauline leaf and axillary meristem (5). Introduction of the wild-type *LOB* gene into *lob* mutants rescued the

fusion phenotype, showing *LOB* is responsible for the boundary separation defect (5). Transgenic plants that ectopically express *LOB* have shorter petioles and smaller rosette leaves compared to wild type, resembling BR-deficient mutants (5; 15; 69). These results suggests that *LOB* functions to limit growth in the boundary region (69).

**LOB negatively regulates BR signaling**

To investigate the relationship between *LOB* and BR, *Arabidopsis* seedlings were treated with exogenous BR and in response, *LOB* transcript levels were induced, revealing that *LOB* is BR regulated (5). A microarray experiment, conducted to identify downstream targets of LOB, identified *PHYB ACTIVATION TAGGED SUPPRESSOR1 (BAS1)* as a target of LOB transcriptional regulation (5). These interactions, in which *LOB* is positively regulated by BR and in turn, LOB activates expression of *BAS1*, which inactivates BR, result in a negative feedback loop to limit BR accumulation in organ boundaries (5). *LOB* regulates organ separation, in part, by modulating the accumulation of BRs at the organ boundaries. As such, LOB acts to limit growth in boundary cells (5; 34). These studies in *Arabidopsis* show BR to play an important role in the formation of organ boundaries by repressing organ boundary identity genes (23).

*BAS1* encodes a cytochrome p450 enzyme that inactivates BRs by C-26 hydroxylation (56). *BAS1* was found to be expressed in the basal region of young leaves and developing seeds, and in the paraclade junction, which overlaps with *LOB* expression in the boundaries (5). Expression of *BAS1* under the *LOB* promoter in the *lob* mutant background suppressed the organ fusion phenotype, restoring organ separation (5). This

demonstrates that the loss of BAS1 activity in *lob* mutants contributes to the fusion phenotype. In the wild-type background, *BAS1* expression under the *LOB* promoter resulted in plants that appeared to be morphologically normal (5). Under closer inspection however, the pedicels were longer, perhaps due to compensation for the reduction of BR in organ boundaries (5).

*CYP34A*s, the rice *BAS1* homologs, also inactivate BRs by C-26 hydroxylation (56). *CYP34A*s are expressed in shoot apices, leaf sheaths and blades, internodes, and panicles as well as roots (63; 74). Misexpression of *CYP734A*s in rice results in a BR-deficient phenotype that produced plants with erect leaves and reduced height (63). During the juvenile phase, RNA interference knockdown of *CYP734A* genes resulted in plants that exhibited defects in the lamina joint and produced rigid shoots that terminated after producing only a few leaves (74). During the reproductive stage, *CYP734A* RNAi plants had panicles with wider branch angles caused by the development of a pulvinus not found in wild type plants (74). Thus, modulation of BR levels via BR catabolism contributes to normal shoot development in rice.

**The *LATERAL ORGAN BOUNDARIES DOMAIN* gene family**

The LBD proteins comprise a family of plant specific transcription factors that share a conserved 100 amino-acid domain located near the N-terminus that is responsible for DNA binding (69). The LBD genes can be divided into two classes based on amino acid conservation within the LOB domain (69). Class I LBD proteins contain a zinc finger motif C-block, containing four conserved Cys residues, $CX_2CX_6CX_2C$, a GAS

block that starts with a $FX_6FG$ motif and ends with a $DPX_2G$ motif, and a Leucine zipper-like coiled-coil motif, $LX_6LX_3LX_6L$, that functions in protein-protein interactions (32; 45; 69). Class II LBD proteins have an incomplete leucine-zipper-like domain (50; 69).

The *LBD* gene family is comprised of 43 members in *Arabidopsis* and maize, and 35 members in rice (69; 83; 85). The putative *LOB* ortholog in maize is *Ramosa2* (*ZmRA2*), which controls inflorescence architecture (7). The *ra2* mutant has an increased number of long, indeterminate, upright branches (7). In rice, *OsRA2* is the presumed *LOB* ortholog based on its sequence similarity to *ZmRA2* (7). Phylogeny among the *Arabidopsis LBD*s position *LOB* to be in a clade with *ASYMMETRIC LEAVES2 (AS2)*, *LBD10*, *LBD25/DOWN IN DARK AND AUXIN1 (DDA1)*, and *LBD36/ASYMMETRIC LEAVES2-LIKE1 (ASL1)* (50; 51). Of the *LBD*s that are in the same clade as *LOB*, only *AS2* has been studied in maize and rice (20; 36; 49; 84). *AS2* functions in the specification of adaxial/abaxial organ polarity and floral development (36; 47; 80; 81). Ectopic expression of *AS2* caused adaxialization of the abaxial domain, while *as2* mutants had downward curled leaves with asymmetric lobing and altered floral organs (47; 59; 67; 81). The *AtAS2* ortholog in maize and rice is *INDETERMINATE GAMETOPHYTE1* (*ZmIG1/OsIG1*), which has been shown to have similar functions in shoot differentiation, lateral growth of leaf development, and floral development (49; 84). *Zmig1* mutants have abnormal leaves with defects in organ polarity as well as abnormal floral development (20). *OsIG1*-RNAi lines have alterations in height, blade development, leaf angles, and defects in floral organ (84). Misexpression of *OsIG1*

resulted in inhibited shoot differentiation and abnormal leaves that twist upward and lack auricles (49). Since there is functional conservation between *LBD* genes in *Arabidopsis*, maize, and rice, it is reasonable to hypothesize that function of uncharacterized *LBD* genes are also conserved in different plant species.

**Overview of the research in this dissertation**

In rice, BRs have been shown to contribute to shoot architecture, primarily controlling plant height and leaf erectness. BR-deficient rice plants have increased leaf erectness, which is an important agronomic trait, but they also are small in stature and have reduced fertility, which diminishes yield. The *Arabidopsis LOB* gene maintains proper organ separation, in part by modulating BR accumulation. Taking what has been learned about *LOB* function in *Arabidopsis*, I have extended the studies of *LOB* to an agronomically important crop plant, rice. Altering hormone signaling in precise tissues may be a useful strategy for directing phenotypic changes that are beneficial for agriculture.

Chapter One investigates whether the LOB-BR interaction found in *Arabidopsis* is present in rice. The *AtLOB* promoter, which was found to drive expression in organ boundaries and lamina joint, drove *AtLOB* expression to modulate BR signaling. Localized misexpression of *LOB* produced rice plants with wide, erect leaves and no other alterations in plant architecture.

Chapter Two examines the outcome of expressing *AtBAS1* in rice to repress BR signaling in the organ boundaries. Using the *AtLOB* promoter to drive *AtBAS1* in rice

organ boundaries, produced plants with altered shoot architecture associated with BR deficiency as well as enhanced BR signaling. Transgenic plants had decreased leaf inclination as well as longer inflorescences with longer, slender grains.

In chapter three, I characterized putative *LOB* orthologs in rice to investigate the possibility that these genes regulate BR accumulation in rice as they do in *Arabidopsis.* Phylogenetic analysis confirmed four *OsLBD* genes, *OsRA2*, *OsIG1*, *OsLOB4*, and *OsIAL1*, to be the most closely related to *AtLOB.* Since only the LOB domain at the N-terminal region is conserved among the LBD proteins, the variable C-terminal region can give these genes diverse functions making it difficult to identify *AtLOB* orthologs solely on the basis of phylogenetic analyses. Therefore, these genes were examined for both pattern of expression and possible regulation by BR. While *OsRA2*, *OsIG1*, *OsLOB4*, and *OsIAL1* were expressed in tissues similar to *AtLOB*, suggesting that similar functions to *AtLOB*, they were not regulated by BR suggesting BR regulation may not be conserved among *AtLOB* and rice orthologs.

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# Chapter 1

## Expression of *Arabidopsis LATERAL ORGAN BOUNDARIES* in Organ Boundaries Results in Altered Leaf Architecture in *Oryza sativa*


### Abstract

In maize, rice and other cereals, improved yields have been achieved by increasing planting density. High-density planting has been made possible in part, due to changes in plant architecture, principally an increase in leaf erectness. In rice, the plant hormone brassinosteroid (BR) has been shown to regulate shoot architecture, primarily controlling plant height and leaf erectness. BR-deficient rice plants are dwarfed and have reduced fertility, but also have increased leaf erectness compared to wild-type plants. In *Arabidopsis*, LATERAL ORGAN BOUNDARIES (LOB), a plant-specific transcription factor, regulates organ separation by modulating BR accumulation at the organ boundaries. LOB positively regulates expression of *BAS1*, which encodes an enzyme that inactivates BRs. This study investigated whether the LOB-BR interaction found in *Arabidopsis* is present in rice and explored the use of *AtLOB* gene to modulate local BR signaling to manipulate shoot architecture. Repressing BR signaling only in the organ boundaries is predicted to result in increased leaf erectness without the other detrimental effects on shoot architecture that BR deficiency causes. The *AtLOB* promoter drove *GUS* expression in rice organ boundaries, shoot/root and blade/sheath junctions, suggesting similarity between the rice blade-sheath junction and the leaf-meristem boundary. To modulate BR signaling in rice, the *AtLOB* promoter was used to drive expression of the

*AtLOB* gene. Expressing *AtLOB* in a restricted pattern may result in a reduction in local BR levels in boundary regions, resulting in erect leaves. During the heading stage, *AtpLOB:LOB* rice plants showed *LOB* transcript expression in dissected lamina joints and displayed characteristics associated with a reduction of BR such as altered leaf shape and decreased leaf inclination. These results indicate that expression of *AtLOB* in a distinct domain alters leaf angle and shape without other detrimental effects on fertility and plant height, which is caused by overall BR deficiency.

**Introduction**

About half of the world's population relies on rice as the major source of nutrition; therefore, increases in rice grain production are essential in order to meet the needs of the growing population (16; 17; 19; 29). Developing an understanding of the processes that regulate plant architecture may allow eventual manipulation of agronomic traits for crop improvement (26). Hormone function in crop plants has been studied for its important role in agricultural improvement (2; 38). Altering hormone signaling in precise tissues of the plant may be a useful strategy for directing phenotypic changes that are beneficial for agriculture (38). Plant hormones in particular affect leaf erectness, which is an important agronomic trait in cereals (2; 38). Plants with erect leaves require less growth space, allowing for higher density planting, which contributes to increased grain yields per acre (17; 23; 30; 33). Along with denser planting, upright leaves can also increase the photosynthetic efficiency of the plant (2; 16; 33). Erect leaves will allow sunlight to penetrate the lower leaves that are usually shaded by drooping upper leaves

(2; 16; 33). They also capture sunlight on the adaxial and abaxial surface compared to normal drooping leaves that capture light on the adaxial surface (2; 16; 33).

Brassinosteroid (BR), a plant steroid hormone, has been shown to play an important role in various physiological and developmental processes in rice (6; 36). BRs regulate cell expansion and division, which affects shoot architecture (36; 41). BR-deficient rice plants have reduced stature, decreased fertility, smaller grains, compact tillering, and erect leaves (2; 6; 36). Leaves in particular are affected by reduced BR levels, which results in leaves that are upright, dark-green, and having wider and shorter blades (37). While half of these traits are beneficial to increasing yield due to their compact stature, severe dwarfness, and infertility diminish the benefits (6; 16).

In *Arabidopsis*, *LATERAL ORGAN BOUNDARIES* (*LOB*), the founding member of the *LOB*-domain gene family, encodes a plant-specific transcription factor, that is expressed in the boundary between the shoot apical meristem (SAM) and initiating lateral organs (32). Loss-of-function *lob* mutants have defects in organ separation causing a subtle fusion of the paraclade junction, the boundary between the cauline leaf and axillary stem (4). *LOB* regulates organ separation, in part, by modulating the accumulation of BRs in the boundary (4). *Arabidopsis* plants that ectopically express *LOB* have short petioles and small rosette leaves, resembling BR-deficient mutants (32). Furthermore, *LOB* transcript levels were induced when treated with exogenous BR (4). A microarray experiment was performed to identify downstream targets of LOB using an inducible LOB-overexpression line (4). This microarray identified *PHYB ACTIVATION TAGGED SUPPRESSOR1 (BAS1)*, a cytochrome P450 that inactivates BR, as a target of LOB

transcriptional regulation (4; 25). These interactions result in a feedback loop where *LOB* is positively regulated by BR and LOB activates expression of *BAS1,* which inactivates BR (4). This feedback loop limits BR accumulation in organ boundaries resulting in the boundary cells being smaller and having limited cell division (4; 12).

The relationship between LOB and BR in the boundary region has only been studied in *Arabidopsis* (4). I am extending these studies to rice, an agronomically important crop. Using a transgenic approach, a construct containing the reporter gene *beta-glucuronidase* (*GUS),* driven by the *Arabidopsis* promoter *AtpLOB,* was expressed in rice to determine whether the *AtLOB* promoter can drive boundary expression in rice, similar to *Arabidopsis* (32). The *AtpLOB* drove expression in a restricted pattern in rice, confined to the shoot/root base and the blade-sheath junction, suggesting that LOB may play a role in this boundary and that there is similarity between the meristem/leaf and blade/sheath boundaries. The *AtpLOB* was also used to express the *AtLOB* gene in rice to investigate functional conservation between *Arabidopsis* and rice. If LOB function is conserved in rice, AtLOB should activate the rice *BAS1* ortholog and repress BR signaling in the organ boundaries. This is expected to result in more erect leaves without other detrimental effects on fertility and plant height associated with BR deficiency (10). Ten independent $T_2$ *pLOB:LOB* transformants were analyzed during the seedling and heading stage (when the panicle emerges from the leaf sheath) for characteristics associated with BR deficiency. During the heading stage, *AtpLOB:LOB* transgenic lines displayed characteristics associated with a reduction of BR in the leaves, resulting in

erect leaves. Expressing *AtLOB* in a restricted pattern resulted in plants with altered leaves and no other changes in plant architecture caused by BR deficiency.

## Material and Methods

### Plant material, growth conditions, and transformations

*Oryza sativa* ssp. *japonica* cultivar Kitaake seeds were transformed by the UCR transformation facility using calli induced from mature seeds as starting material for *Agrobacterium*-mediated transformation (http://ptrc.ucr.edu/services.html) (34). Transformed plants were selected on Murashige and Skoog (MS) medium containing hygromycin (HYG) (24). Positive transformants were confirmed by PCR using *hptII* (HYG) primers (Table 1.1). Wild-type (*Oryza sativa* ssp. *japonica* cultivar Kitaake), *AtpLOB:GUS*, and *AtpLOB:LOB* seeds were surface sterilized in 95% EtOH for 5 min, followed by 1% NaClO for 5 min, and rinsed three times with sterile water. Seeds were fully submerged in $H_2O$ and imbibed overnight at room temperature. Seeds were then transferred to a dark incubator at 30°C for four days. For greenhouse-grown plants, seeds were sterilized, fully submerged in $H_2O$ at room temperature for three days, then transferred to 1-gallon pots containing UC Soil Mix II (http://agops.ucr.edu/soil/). The day after sowing, iron sulfate premade mix (30% iron, 18% sulfate, Brandt) was added to the soil. Plants were grown in a greenhouse at 30°C under 16-hr-light and 8-hr-dark conditions.

To identify positive transformants in subsequent generations of independent *AtpLOB:LOB* lines ($T_2$, L1-22) seeds were grown as previously described above. To confirm the presence of the transgene, PCR genotyping using T-DNA-specific primers was used (Table 1.1).

**Constructs**

The *pLOB:GUS:3'Intergenic region* (*IGR)* and *pLOB:LOB:3'IGR* fusions are described in (4). The *pLOB:LOB:3'IGR* contains 5-kb of the *LOB* promoter region, the *LOB* coding region and 2.5-kp IGR 3′ to the *LOB* stop codon amplified from genomic DNA (4). In *pLOB:GUS:3'IGR,* the *LOB* coding region is replaced with *GUS*. The 7.5-kb fragment containing *pLOB:GUS:3'IGR* was excised from the pCB302 backbone using *Kpn*I and *Pst*I restriction enzymes and cloned into pCAMBIA 1300 binary vector (www.pCambia.org). An 8.1-kb fragment containing *pLOB:LOB:3'IGR* was excised from pCB302 plasmid using *Kpn*I and *Pst*I restriction enzymes and cloned into pCAMBIA 1300 binary vector (www.pCambia.org).

***GUS* expression and histological analysis**

For GUS histochemical staining, plant tissues of interest were harvested and completely submerged in GUS stain solution (100 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mg/ml X-Gluc, 100 µg/ml chloramphenicol, 2 mM potassium ferricyanide, 2 mM ferrocyanide). Submerged tissues were placed in a vacuum chamber for 10 min, then incubated in the dark at 37°C for 24-72 hours. After

incubation, the GUS stain solution was replaced with 70% EtOH. Tissues were then mounted on a glass slide in 50% glycerol and viewed using a compound microscope.

For sectioning of GUS-stained tissues, the protocol by Rojas-Pierce and Springer was modified as described (28). Stained tissues were placed in ice-cold fixative (4% w/v paraformaldehyde, 4% v/v DMSO, in 1x Phosphate buffered saline, PBS) and vacuum infiltrated for 15 min. Fixative was replaced and left overnight at 4°C. Tissues were then dehydrated through an ethanol series at 4°C (30%, 40%, 50%, 60%, 70%, and 85%), 60 min each step, and left in 95% EtOH at 4°C for an overnight incubation. Two 30 min incubations of 100% EtOH at room temperature were performed, followed by a 60 min incubation of 100% EtOH + 0.1% Eosin. The EtOH was replaced with Citrisolv using a series of Citisolv/EtOH mixtures (25% Citrisolv/75% EtOH, 50% Citrisolv/50% EtOH, 75% Citrisolv/25% EtOH) at room temperature, 30 min for each step, followed by two 100% Citrisolv incubations for 60 min each. Paraplast wax chips replaced 25% of the Citrisolv volume and left overnight. The 75% Citrisolv/25% wax was incubated at 42°C until the wax chips melted completely. An additional 25% volume of Paraplast chips was added and samples were incubated at 60°C for several hours. The Citrisolv/wax mixture was replaced with freshly melted wax twice a day for four days and incubated at 60°C. After the fourth day, the tissues were poured into molds and left to harden. Once hardened, wax blocks containing individual tissues were cut and mounted on wood blocks to prepare for sectioning. Blocks were stored at 4°C until needed. Tissues embedded in the wax blocks were cut into 8-µm sections and the resulting ribbons were placed on slides in a drop of water and placed on a slide warmer at 42°C overnight. To

deparafinize the sections, slides were soaked in Citrisolv for 10 min twice. Tissues were rehydrated through an ethanol series (100%, 95%, 80%, 70%, 50%, and 30%) for 10 min each and then transferred to water. Tissues were mounted under a cover glass in an aqueous mounting media and dried overnight. Slides were viewed on a compound microscope (LEICA) with dark field or DIC optics and a SPOT camera (Diagnostic Instruments).

**Expression analysis**

Total RNA was isolated from samples during the seedling and heading stage using the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's guidelines. For semi-quantitative RT-PCR analysis, cDNA was synthesized from 2-4 µg of total RNA using an oligo-dT primer, and either SuperScript III or SuperScript IV reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was diluted and used as a template in PCR reactions to detect *LOB* transcripts. *OsACTIN1* (*Os03g50885*) (Table 1.1) was used as a control and the PCR cycling conditions were as follows: denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. To detect *LOB* transcripts, two *LOB* primer sets were used (Table 1.1). PCR conditions for $LOB^1$ amplification were as follows: denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s. PCR conditions for $LOB^2$ amplification were as follows: denaturation at 95°C for 2 min, followed by 33 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s.

**Phenotypic analysis**

Greenhouse-grown seedlings were analyzed for phenotypic traits associated with BR in the months of November and December. Plant height was measured from the base of the soil to the highest point of the plant. Photographs were taken of lamina joints and the angle of the lamina joints were measured using imageJ. The tiller-to-tiller angle was measured from photographs taken of the whole plant using imageJ. Tiller angle was measured between the outermost tiller on the left side and the outermost tiller on the right. Using a standard ruler, the sheath length, and blade length/width were measured.

**Root analysis**

Wild-type, *AtpLOB:GUS*, and *AtpLOB:LOB* seeds were fully submerged in $H_2O$, imbibed overnight at room temperature, and then transferred to a dark incubator at 30°C for 4 days. Uniform seedlings were transferred to media plates containing half strength MS medium (pH 7.5) (24). Plates were positioned vertically and placed in a growth chamber at 30°C under 16-hr-light and 8-hr-dark conditions. As soon as the second leaf began to unroll, root lengths were measured using a caliper.

**Results**

***AtpLOB* was found to drive expression in a restricted pattern in rice**

In *Arabidopsis LOB* regulates organ separation by modulating BR accumulation at the organ boundaries (4). To determine whether the *Arabidopsis LOB* promoter,

*AtpLOB*, drives expression in organ boundaries in rice similar to its expression in *Arabidopsis*, a T-DNA construct containing the *AtpLOB* fused to the *GUS* reporter gene (*AtpLOB:GUS*) was transformed into rice (Fig. 1.1A). Of twelve $T_2$ heterozygous positive transformants that were obtained, four lines were identified to have a single T-DNA insertion based on the 3:1 segregation ratio of Mendelian inheritance of single transgenic locus. All lines showed similar pattern of *GUS* expression and one *AtpLOB:GUS* line was used for detailed expression analysis and as a control in phenotypic analysis.

*GUS* activity was observed in a restricted pattern, confined to the shoot-root boundary and the blade-sheath junction (lamina joint) during different developmental stages (Fig. 1.2). During early stages of development, 2-4 days after germination, *GUS* was expressed at the tip of the coleoptile and the base of the shoot/root, a boundary between the shoot and root (Fig. 1.2, B-D). At the second leaf stage, *GUS* expression was faintly detected in the lamina joint of the developing second leaf (Fig. 1.2E). As the sheath and blade elongated, *GUS* activity was increasingly visible in the lamina joint of the second leaf (Fig. 1.2F). In mature leaves, *GUS* activity was more prominent in the lamina joint (Fig. 1.2, G and H). The *GUS* expression pattern in the rice blade/sheath junction confirms that this region can be considered a boundary within the leaf.

In *Arabidopsis*, *LOB* is expressed at the base of axillary branches, flowers, floral organs, as well as the anthers of flowers (32). In rice *AtpLOB:GUS* plants, *GUS* activity was detected in the anthers of the florets during early developmental stages and activity

decreased in later stage panicles with mature florets (Fig. 1.2, I and J). *GUS* expression was not detected at the base of floral organs (data not shown).

To look at the developmental timing of *LOB* expression, cross-sections of *AtpLOB:GUS* plants at the two-week stage were performed. In longitudinal sections of the shoot apex, there was no *GUS* activity detected in the SAM or the boundary between the SAM and leaf primordia, P1-P3 (Fig. 1.3A). While there was no *GUS* expression in the SAM and Plastochron 1 (P1; describes the primordium that is the first leaf from the SAM) (21) through P3 boundary, *GUS* was expressed in the boundary between the shoot and root, which was seen in 4-day-old seedlings (Fig. 1.2D; 3, C and D). In cross sections of the shoot apex and leaf primordia, *GUS* activity was not detected in P3 and P4, but it was detected in P5 leaves (Fig. 1.3B). *GUS* expression in the shoot/root and sheath/blade boundary suggests *AtpLOB* regulation may be conserved.

**Overall phenotypic characterization of mature *AtpLOB:LOB* transgenic rice plants**

In *Arabidopsis*, *LOB* limits BR accumulation in organ boundaries through a feedback loop where *LOB* is positively regulated by BR and LOB activates expression of *BAS1*, which inactivates BR (4). *AtpLOB* was found to drive expression in rice blade/sheath junction, the site of leaf bending, suggesting a similarity between the *Arabidopsis* leaf/meristem boundary and the presence of a conserved regulatory mechanism. Since *AtpLOB* drove expression in the lamina joint, *AtpLOB*:*AtLOB* should reduce BR in localized regions. We hypothesize that this will result in altered leaf angles.

To test this hypothesis, a T-DNA construct containing the *Arabidopsis LOB* promoter fused to the *LOB* coding region and 3'integenic region, *AtpLOB:LOB:3'IGR* , was transformed into rice (Fig. 1.1B). Twenty-two independent $T_2$ heterozygous transformants were obtained and analyzed for characteristics associated with BR deficiency (Table 1.2). For controls, one homozygous *AtpLOB:GUS* line was used along with wild-type. For each independent *AtpLOB:LOB* line, the lamina joints of immature leaves were dissected and pooled and analyzed for *LOB* transcript accumulation by RT-PCR. Of the twenty-two transformants, one line (L18) had very high levels of *LOB* transcript accumulation, four lines (L3, L16, L19, and L22) had moderate levels, five lines (L4, L6, L7, L14, and L15) had low levels, and twelve remaining lines had very little or undetectable transcript (Fig. 1.4). Ten independent lines showing the highest levels of *LOB* transcript were selected for further analysis.

Mature BR-deficient rice plants are dwarfed in stature with compact tillering and dark-green upright leaves that are short and wide (2; 6; 36). If LOB's function is conserved in rice, BR signaling in the organ boundaries and lamina joint should be reduced. This might result in alteration at the site of leaf bending, producing plants with erect leaves and no other detrimental effects associated with BR deficiency (10). The ten independent $T_2$ heterozygous *AtpLOB:LOB* lines were phenotypically characterized for traits correlated with BR deficiency such as height and tiller angle (Table 1.2). Of these ten lines, three lines (L4, L14, and L18) showed an increase in height, whereas L15 was shorter compared to wild-type (Fig. 1.5, A and C-G, Table 1.2). Studies have shown that dwarfing in BR-deficient plants is caused by altered elongation patterns in the culms (9;

10; 41). *AtpLOB:GUS* lines did not show any *GUS* activity in the node or internodes of culms at any stage (data not shown). Therefore, *AtLOB* is not expected to affect the development of the culms. The changes in plant height in the four lines may be caused by a compensatory effect due to site-specific reduction of BR or from the random insertion of the T-DNA in the genome that may disrupt the function of other genes. Nine of the ten lines did not show a significant difference in tiller-to-tiller angle compared to wild-type, suggesting *AtLOB* does not affect tiller angle (Fig. 1.5B, Table 1.2).

**Expression of *AtLOB* in a discrete domain alters leaf angle and blade width in mature plants**

In rice, the *AtLOB* promoter drives expression in organ boundaries, specifically in the lamina joints, suggesting *AtLOB* may affect leaf inclination. *LOB* transcript levels were examined in lamina joints of mature leaves of *AtpLOB:LOB* plants during the heading stage of development. Results showed only four lines (L14, L15, L16, and L17) having moderate *LOB* transcript accumulation in the lamina joints, while the other six lines had very little or undetectable transcript (Fig. 1.6A). Leaf angles of the lowest leaf and flag leaf (leaf below the panicle) were measured to determine if the *AtpLOB:LOB* transgene affects leaf angles. Only two (L14 and L15) of the four lines, which had moderate *LOB* transcript accumulation in the lamina joint, had leaf angles that were more erect than wild-type at both the lowest and flag leaf (Fig. 1.6, B-H, Table 1.2). The pattern of *GUS* activity and the expression of *AtLOB* transcripts in the lamina joint, strongly suggests that *AtLOB* may alter the leaf angles in rice.

BR mutants have wider, shorter blades along with shorter sheaths (10). Reduced BR affects the organization of cell files as well as cell division and elongation in the epidermal layer resulting in altered leaves (10; 11; 37). If the *AtpLOB:LOB* transgene is modulating BR in rice, it is possible that *AtLOB* will also cause abnormal leaf morphology. To determine if the sheaths and blades are affected, sheaths and blades were measured (Fig. 1.7, Table 1.2). In five of the ten lines, sheath lengths were longer in the lowest and flag leaf position (Fig. 1.7A). Only one line (L15) had shorter flag leaf sheaths compared to wild-type (Fig. 1.7A). Comparing the blade lengths to wild-type, four lines had longer blades at the lowest and flag leaf position (Fig. 1.7B). The sheath and blade lengths were not consistent among the majority of the lines, suggesting that the variations in lengths within the transgenic lines may be caused the position effects of the transgene. When observing the blade widths in comparison to wild-type, eight of the ten lines had wider blades at the lowest and flag leaf position (Fig. 1.7C). These results suggest that the transgene *AtpLOB:LOB* causes abnormal leaf morphology.

Only one line (L4) showed significant difference throughout the entire leaf (sheath length, blade length and width) at both the lowest and flag leaf position (Fig. 1.7, Table 1.2). While L18 had differences in the sheath length and blade width at both the lowest and flag leaf position (Fig. 1.7, Table 1.2). L4 and L18, two of the three lines that were significantly taller than wild-type, had longer leaves suggesting that the increase in height was a result of the increase in lengths. While L4 and L18 had moderate to low levels of *LOB* transcript, the changes in leaf length can be caused by a compensatory

effect due to the site-reduction of BR or by position effects of the transgene (Fig 1.5A and 1.7).

### *pLOB:LOB* does not alter plant architecture during the seedling stage

In *Arabidopsis*, *LOB* is expressed at the base of lateral roots and when ectopically expressed, results in shortened roots (4; 32). In rice, BR affects the development of the roots, as BR-deficient rice plants have shortened roots (10; 11). Rice *pLOB:GUS* lines did not show any expression in the roots (data not shown); therefore the transgene *AtpLOB:LOB* transgene is not expected to affect root elongation. While the majority of the lines did not show any difference in root elongation compared to wild-type, there were three lines whose roots were elongated (Fig. 1.9). The inconsistency of root length among the transgenic lines can be due to a compensatory effect due to the reduction of BR in other parts of the plant or by position effects of the transgene.

Altered shoot architecture during the heading stage of the transgenic plants prompted us to conduct phenotypic analysis at the seedling stage in order to determine how early changes were apparent since the *AtpLOB:LOB* transgene was expressed very early in development. Two *AtpLOB:LOB* lines (L14 and L15) were selected for further analysis because they displayed an erect leaf phenotype, altered height and leaf characteristics during the heading stage (Table 1.2). The spatial expression pattern of *AtLOB* in the fourth leaf during the seedling stage in $T_2$ heterozygous L14, L15, and wild-type were also observed (Fig. 1.9). *LOB* transcript accumulation was measured in the sheath (1 cm below the lamina joint), lamina joint, and blade (1 cm above the lamina

joint) using semi-quantitative RT-PCR (Fig. 1.9C). In L14, *LOB* transcripts were not detected in the sheath and lamina joint, but a weak level of expression was detected in the blade. In L15, *LOB* transcripts were not detected in any of the samples. The lack of transcript accumulation in both lines can be due to the low abundance of *LOB* transcript present at this stage. Plant height and the angle of the third leaf of heterozygous L14 and L15 and their nontransgenic siblings (SIBs) were measured. Due to these lines segregating, their nontransgenic siblings were analyzed as controls. L14 and L15 showed no significant difference to their SIBs or wild-type (Fig. 1.9A and B). This data suggests that at the seedling stage, there are no alterations in shoot architecture but as the plants enter the heading stage, LOB activity in the lamina joint alters leaf inclination.

**Discussion**

Cereals such as maize, wheat, and rice are the most important staple foods, with 50% of human population relying on rice as their major source of nutrition (15; 39). Due to the increasing growth of population, there has been a need to improve crop production to meet food demands (18). Over the years, plant hormones have shown to play a major part in manipulating crops to improve agricultural crop production (38). Modifying hormone accumulation in precise plant tissues may be a useful strategy for directing phenotypic changes that are beneficial for agriculture (38). In rice and other cereals, leaf erectness is a desirable trait that allows for high-density planting and grain yield (23; 30; 33).

Organ boundaries in plants play a significant role in the regulation of plant architecture (5; 27). Organ boundaries serve as a barrier to isolate population of cells within their distinct domains and defects in boundary formation resulted in the fusion of organs (5; 27). BRs have also been shown to play an important role in organ boundaries (4; 7) In *Arabidopsis*, *CUP-SHAPED COTYLEDON (CUC 1, 2, 3)* genes, boundary identity genes, are expressed in the boundary between the SAM and cotyledons (1; 8). It has been shown that *BRASSINAZOLE RESISTANT1* (*BZR1*), a key transcription factor in BR-signaling pathway, represses the expression of *CUC* genes (7; 20). *cuc* mutants have defects in organ separation resulting in fused cotyledons and floral organs (sepals and stamens) (1). BR-hypersensitive mutants have increased BR signaling, which represses *CUC* genes, causing organ fusion due to the overgrowth of the boundary (7). In the organ boundaries of wild-type plants, *BZR1* is expressed at low levels, allowing *CUC* genes to be expressed and result in proper organ formation (7).

*LOB* regulates organ separation, in part, by modulating the accumulation of BRs in the boundary (4). *lob* mutants have defects in organ separation causing a subtle fusion at the paraclade junction (4). Taking what has been learned about the relationship between *LOB* and BR, this study investigated whether the *LOB*-BR interaction found in *Arabidopsis* is present in rice and used the *AtLOB* gene to modulate BR in rice organ boundaries and blade/sheath junction.

During leaf development in grasses, the junction between the blade and sheath is composed of a linear band of cells, perpendicular to the proximal-distal axis of the leaf, known as the preligule band (31; 35). Studies of the ligule region in maize have shown

that the preligule band does not develop until leaf P7 (14). One study used laser capture microdissection and RNA-sequencing (LM-RNAseq) to identify a suite of genes that are specifically expressed in the preligular region (14). *In situ* hybridization of genes that are expressed in the preligular band, were also expressed in the boundary between the SAM and initiating lateral primordia and axillary meristems (14). *GRMZM2G393433*, a maize gene similar to *Arabidopsis CUC2*, is highly upregulated in the preligular region relative to surrounding blade and sheath regions, and is also expressed in the boundaries between SAM and initiating primordia, and tassel branches (14). Two genes for BR biosynthesis, *ZmBrd1* and *ZmD11*, were also found to be upregulated in the preligular region (14). Using *in situ* hybridization, *ZmBrd1* and *ZmD11* transcripts were not detected in the emerging ligule region, possibly due to the low abundance of transcript, but were detected in the boundary between the SAM and lateral primordia (14). This data suggests that genes expressed in the ligule region also expressed in other developing boundaries at the SAM and branch meristems (14).

When expressed in rice, the *Arabidopsis LOB* promoter drove expression in a restricted pattern, confined to the shoot/root, blade/sheath junction, and anthers , which suggests similarity between the expression of *LOB* in rice and *Arabidopsis*. While there was no *GUS* expression detected at the SAM/lateral organ primordia boundary in *pLOB:GUS* plants, it is possible that there is low abundance of transcript that could not be detected using the *GUS* reporter system. Using a technique that is are more sensitive to transcript accumulation, such as *in situ* hybridization, can determine if the *Arabidopsis LOB* promoter is expressed in the meristem/leaf boundary in rice. The data from the LM-

RNA seq indicates that a number of genes are specifically expressed in the meristem/leaf boundary and the preligule region are also expressed in expressed in the meristem/leaf boundary.

Studies haves shown BRs to contribute to the formation and maintenance of organ boundaries in *Arabidopsis* and rice (7; 10). *BZR1*, which regulates BR-responsive genes, has been shown to repress organ-boundary identity genes in *Arabidopsis* (7; 20). *bzr1-1D*, a dominant BR-hypersensitive *Arabidopsis* mutant, has organ-fusion defects caused by the overgrowth of the organ boundary due to the increase in cell elongation and size (7). The organ boundary defect in *bzr1-1D* exhibited a decrease in the angle between the stem and lateral branch (7). In rice, leaf erectness is caused by a reduction of BR in the lamina joint, the boundary between the blade and sheath (10; 13). *BZR1* has similar functions in rice, as it also affects rice organ boundaries (3). *OsBZR1P206L*, a dominant *OsBZR1* mutant hypersensitive to BR, had wider leaf angles compared to wild-type (3). In contrast, *OsBZR1* RNAi plants had reduced BR response causing an erect leaf phenotype (3).

*AtLOB* promoter activity in the lamina joint during the seedling and mature stage suggests *AtLOB* driven by *AtpLOB* will express *AtLOB* in this distinct domain and might affect leaf inclination. While *LOB* transcript accumulation in the lamina joint was found in four of the ten independent hemizygous transformants, two lines (L14 and L15) displayed an erect leaf phenotype compared to wild-type at the heading stage. At the seedling stage, there were no alterations in leaf angle in L14 and L15 in comparison to wild-type, suggesting the effects of *AtLOB* in the lamina joint occurs later in

development. The erect-leaf phenotype in *AtpLOB:LOB* plants can be due to the disruption of cell elongation/division due to the inactivation of endogenous BRs on the adaxial surface of the lamina joint (33). Many rice BR mutants exhibit the erect leaf phenotype, such as *Osbri1* (*d61*), which encodes a mutant BR receptor (22; 41). Overexpressing *IBH1*, a transcription factor that mediates BR regulation, exhibited erect leaves caused by the reduced cell elongation in the adaxial side of the lamina joint (42). These results suggest that the decrease in leaf inclination in *AtpLOB:LOB* plants is perhaps caused by the disruption of cell elongation/division due to the inactivation of endogenous BRs in the lamina joint.

Along with a decrease in leaf inclination, *AtpLOB:LOB* lines also displayed alterations in leaf morphology. Seven of the ten lines exhibited wider leaves at both the lowest and flag leaf position. BR-deficient mutants have wider leaf blades, which are a result of the disruption of the organization of cell files as well as cell division and elongation in the epidermal layer (10; 11; 37). Mutants in *Osdwarf4*, a BR-biosynthetic gene, have wider leaves resulting from defects in the arrangement and longitudinal elongation of the epidermal cells (11). The wider-leaf phenotype in the transgenic lines suggests that the organization of cell in the epidermis is disrupted by the decrease in BR.

Along with the wide-leaf phenotype, three *AtpLOB:LOB* lines exhibited longer sheaths. Of these three lines, two lines had an increase in height compared to wild-type. Only one line with shorter sheaths was reduced in overall height compared to wild-type. These results suggest that the alterations in sheath length play a role in overall plant height in the transgenic lines. Sheath length has been shown to be affected by BR (11;

40). BR-deficient mutants have been shown to affect the development of the sheaths by causing a disruption of the organization of the epidermal cells in the sheaths (11). *GSK2* encodes a rice ortholog of the kinase *BIN2,* which acts in the BR-signaling pathway (20). *GSK2* overexpression plants resemble BR-deficient plants, shortened in height with reduced sheath length caused by defects in cell organization (20; 37). The changes in sheath length in *AtpLOB:LOB* could be due to defects in cell elongation/division, similar to BR-deficient mutants (20; 37). The alterations in sheath length in *AtpLOB:LOB* is an unexpected trait that may be a result of compensation for reduced BR in the lamina joint by increasing growth and/or BR levels in other parts of the leaf such as the sheath and blade.

As a control, a low copy homozygous *AtpLOB:GUS* line was used. This transgene should not cause any alterations in phenotype due the *AtLOB* promoter driving expression of a reporter gene and not a functional gene. While all the plants were grown and measured at the same time, the *AtpLOB:GUS* line showed variations in traits that were significantly different from wild-type. A possible reason for this is the random insertion of T-DNA into the genome. The transgene can be inserted into functional genes and disrupt their function causing alterations in developmental processes which can lead to changes in phenotype.

Modulating BR in precise tissues may be a useful strategy for directing phenotypic changes that are beneficial for agriculture. This research will allow us to develop an understanding of the processes regulating shoot architecture in rice. Producing rice plants with erect shoot architecture could allow for higher density planting

that will result in an increase in biomass and grain yield that can benefit farmers. Understanding the role of BR signaling in rice and being able to modulate BR in specific tissues can lead to an important mechanism for crop improvement.

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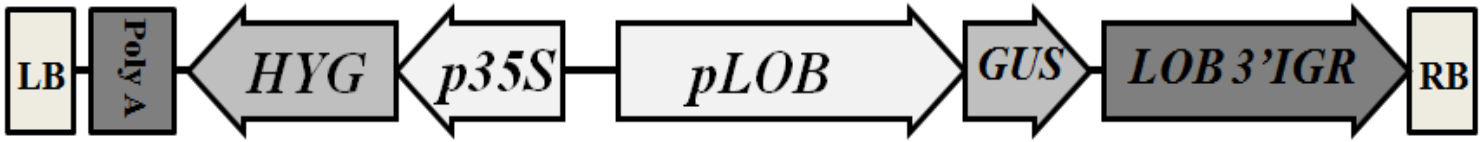
**Figure 1.1. Structure of *pLOB:GUS:LOB 3'IGR* and *pLOB:LOB:LOB 3'IGR* T-DNA constructs inserted into pCAMBIA1300 binary vector.**

A. *pLOB:GUS:LOB 3'IGR* T-DNA

B. *pLOB:LOB:LOB 3'IGR* T-DNA

LB=Left T-DNA border; poly-A=*35S* polyA; HYG=*Hygromycin phosphotransferase* II (*hpt*II), Hygromycin resistant gene (plant resistance selectable marker); p35S =Cauliflower mosaic virus *35S* promoter; *pLOB= AtLOB* promoter; *GUS*= β-glucuronidase; *LOB=AtLOB* coding sequence, cDNA; *LOB 3'IGR =LOB* 3' intergenic region; RB =Right T-DNA border.

**A.**


LB
Poly A
HYG
p35S
pLOB
GUS
LOB 3'IGR
RB

**B.**

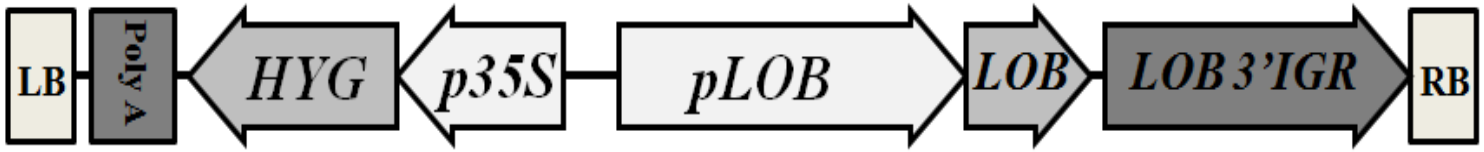

LB
Poly A
HYG
p35S
pLOB
LOB
LOB 3'IGR
RB

**Figure 1.2. *AtpLOB* drives expression in rice organ boundaries.**

Spatial and temporal *GUS* expression in various organs of *AtpLOB:GUS* transgenic rice plants.

A. 1-day-old seedling.

B. 2-day-old seedling.

C. 3-day-old seedling.

D. 4-day-old seedling.

E and F. Vegetative juvenile stage.

G. Lamina joint of a mature plant.

H. Adaxial side of lamina joint of a mature plant.

I. Spikelet during four developmental stages

J. Floral organs.

Sh=Sheath; LJ=Lamina joint; Bl=Blade; Cp=Carpel; An=Anther; Fl=Filament

Scale bars = 0.5 cm (A-D, G & H), 1 cm (E & F), 0.2 cm (I), and 0.1 cm (J).

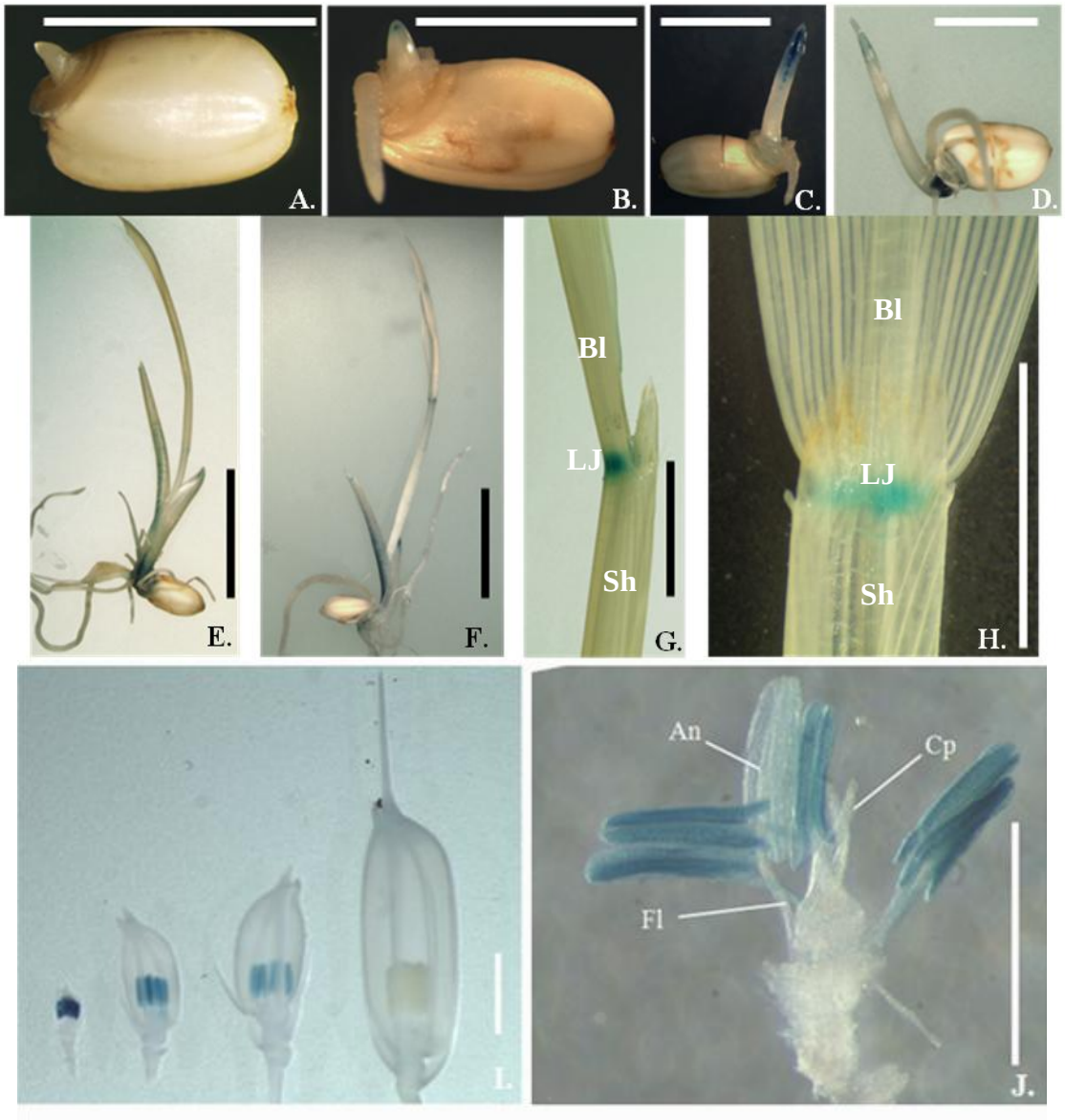

A.
B.
C.
D.
E.
F.
Bl
LJ
Sh
G.
Bl
LJ
Sh
H.
I.
An
Cp
Fl
J.

**Figure 1.3. *GUS* activity in cross sections of *AtpLOB:GUS* seedlings during the seedling stage.**

A. Longitudinal section of the shoot apex.

B. Cross section of shoot apex and leaf primordium.

C - D. Longitudinal section of the shoot and root junction. Panel D represents magnified view of boxed region in C.

Sa=Shoot apical meristem; VB=Vascular bundle; Sho=Shoot; and Ro=Root.

Scale bars = 0.1 mm (A), 0.2 mm (B & D), and 0.5 mm (C).

.

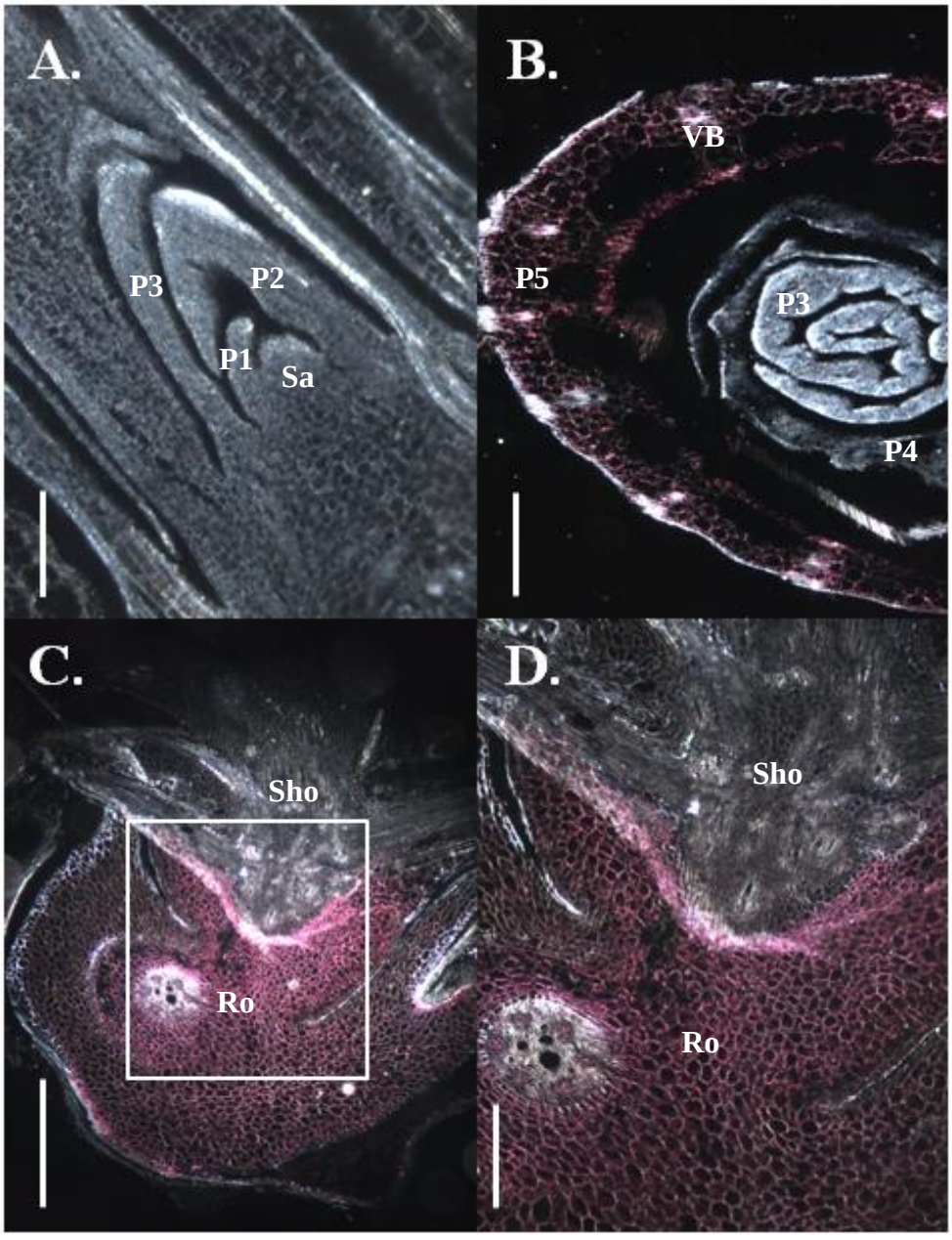

A.
P3
P2
P1
Sa
B.
VB
P5
P3
P4
C.
Sho
Ro
D.
Sho
Ro

**Figure 1.4. *LOB* transcript accumulation in lamina joints of $T_2$ heterozygous *AtpLOB:LOB* transgenic plants.**

RT-PCR analysis of *LOB* transcript levels in dissected lamina joints of pooled T2 *AtpLOB:LOB* (LOB=L;L1-L22), wild-type (WT), and *AtpLOB:GUS*, during the tillering stage. *Act1* (*Os03g50885*) was used as a control. Black bars represent intervening lanes that were excised.

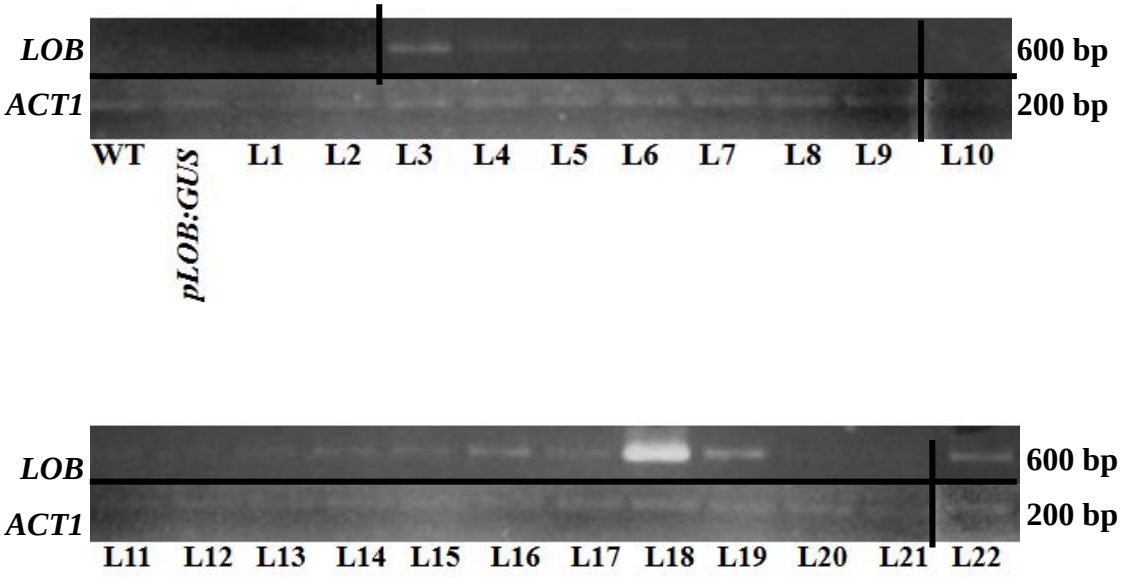

LOB
ACT1
600 bp
200 bp
WT
pLOB:GUS
L1
L2
L3
L4
L5
L6
L7
L8
L9
L10
LOB
ACT1
600 bp
200 bp
L11
L12
L13
L14
L15
L16
L17
L18
L19
L20
L21
L22

**Figure 1.5. Phenotypic characterization of $T_2$ heterozygous *AtpLOB:LOB* transgenic plants during the heading stage.**

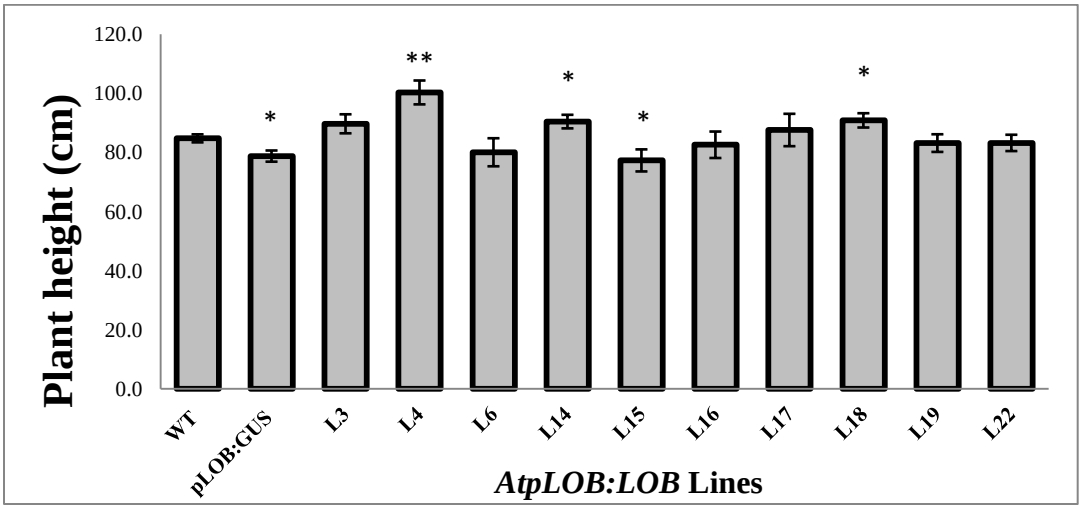
A. Quantitative measurement of plant height.

B. Tiller to tiller angle. Tiller angle was measured between the outermost tiller on the left side and the outermost tiller on the right.

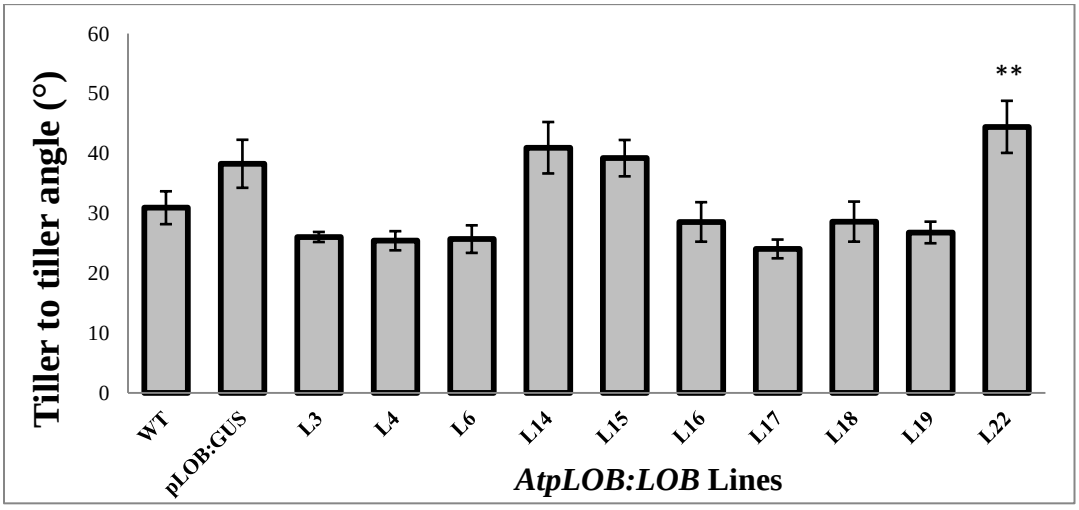
C-G. Gross morphology of wild-type and three *AtpLOB:LOB* transgenic lines.

Values are means ± SE (n=2-9 plants). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

**A.**


Plant height (cm)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
*
**
*
*
*
WT
pLOB:GUS
L3
L4
L6
L14
L15
L16
L17
L18
L19
L22
AtpLOB:LOB Lines

**B.**


Tiller to tiller angle (°)
60
50
40
30
20
10
0
**
WT
pLOB:GUS
L3
L4
L6
L14
L15
L16
L17
L18
L19
L22
AtpLOB:LOB Lines

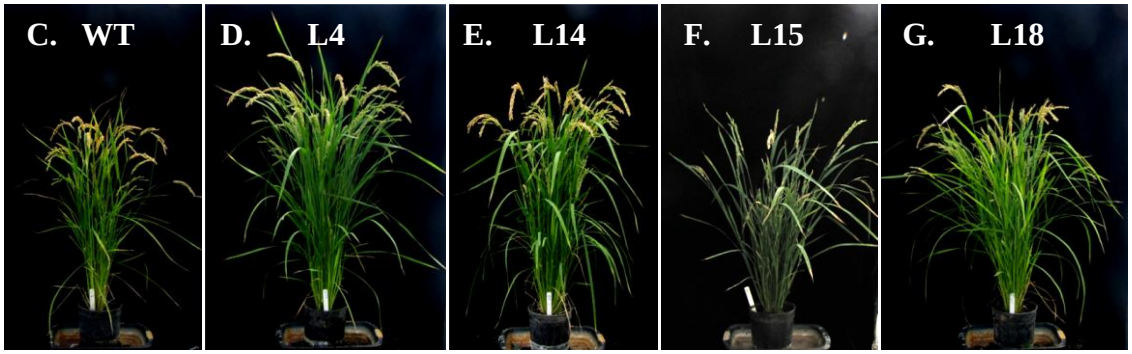

C. WT
D. L4
E. L14
F. L15
G. L18

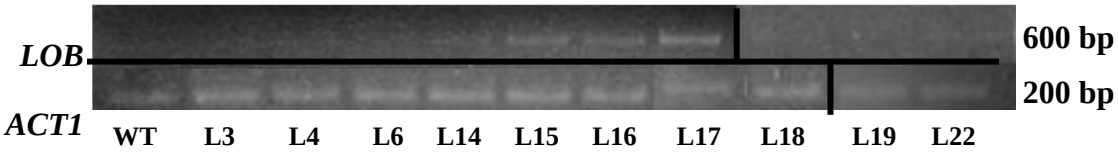
**Figure 1.6. Characterization of the lamina joint of mature leaves of $T_2$ heterozygous *AtpLOB:LOB* transgenic plants during the heading stage.**

A. RT-PCR analysis of *LOB* transcripts in isolated lamina joints of wild-type and *AtpLOB:LOB* lines. *Act1* was used as a control. Black bars represent intervening lanes that were excised.

B. Quantification of the lamina joint angle of the lowest and flag leaf on three random tillers per plant. Angles were measured from vertical using ImageJ. Values are means ± SE (n=2-9 plants, 3 tillers/plant). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

C-H. Close up view of the lowest lamina joint (C-E) and the flag leaf (F-H) of wild-type and the two *AtpLOB:LOB* transgenic lines with altered angles, L14, and L15.

**A.** ***AtpLOB:LOB* Lines**




**B.**

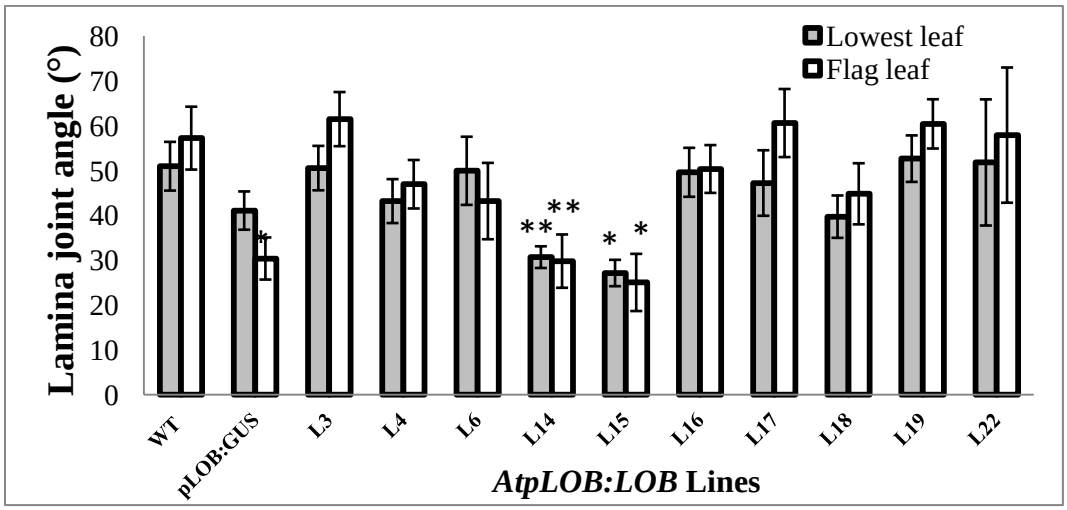




**Figure 1.7. Quantitative characterization of the lowest and flag leaves of wild-type, *pLOB:GUS*, and T2 heterozygous *AtpLOB:LOB* transgenic plants during the heading stage.**

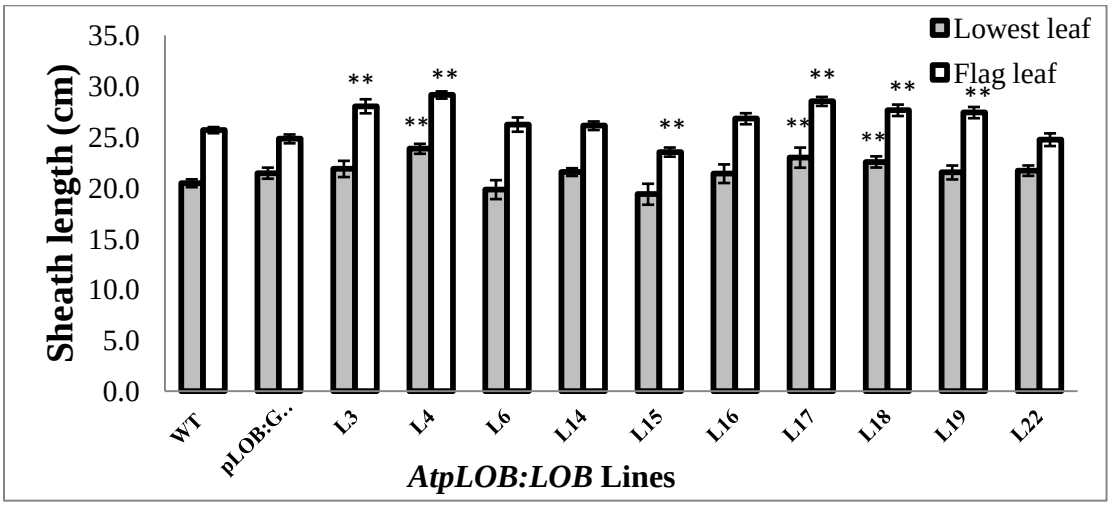
A. Leaf sheath lengths measured from the base of the sheath to the lamina joint.

B. Leaf blade lengths measured from the lamina joint to the tip of the blade.

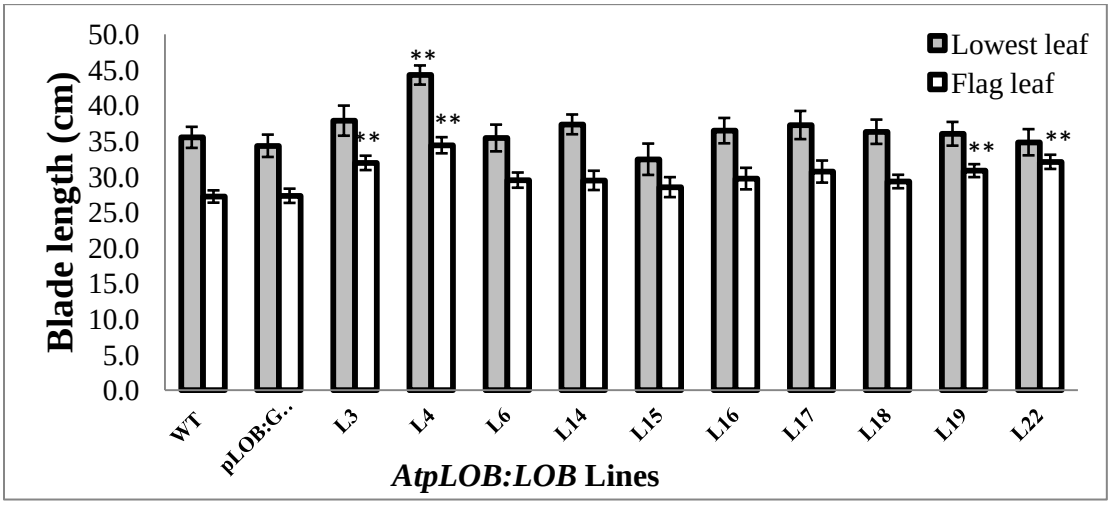
C. Leaf blade width measured at the widest part of the blade.

Values are means ± SE (n=2-9 plants, 3 tillers/plant). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

**A.**


Lowest leaf
Flag leaf
Sheath length (cm)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
WT
pLOB:G..
L3
L4
L6
L14
L15
L16
L17
L18
L19
L22
AtpLOB:LOB Lines

**B.**


Lowest leaf
Flag leaf
Blade length (cm)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
WT
pLOB:G..
L3
L4
L6
L14
L15
L16
L17
L18
L19
L22
AtpLOB:LOB Lines

**C.**

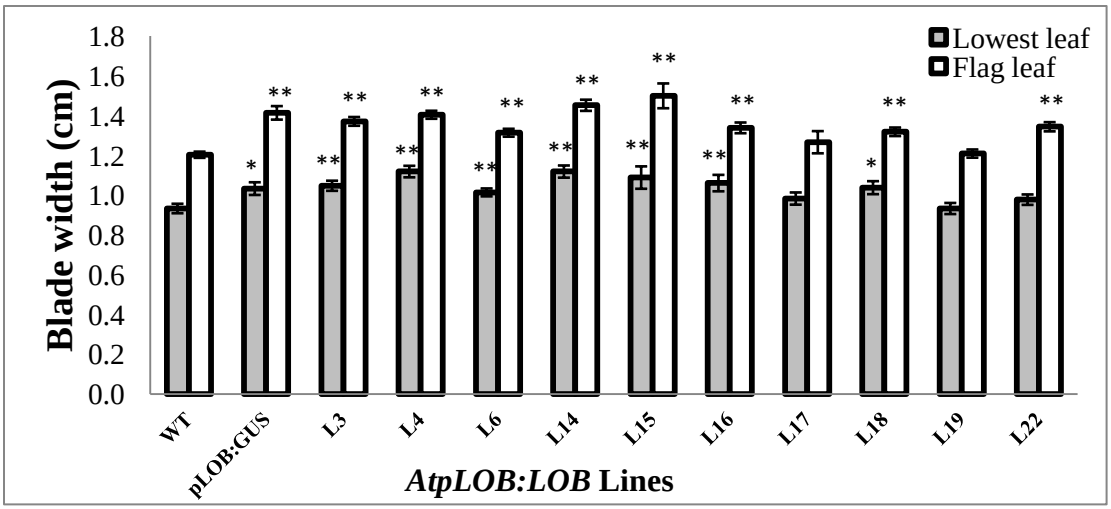

Lowest leaf
Flag leaf
Blade width (cm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
pLOB:GUS
L3
L4
L6
L14
L15
L16
L17
L18
L19
L22
AtpLOB:LOB Lines

**Figure 1.8. Root length of $T_2$ heterozygous *AtpLOB:LOB* transgenic plants.**

Seedlings were grown vertically grown on half strength MS medium and measured when the second leaf began to unroll.

Values are means ± SE (n=14-30 plants, L15 n=4). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

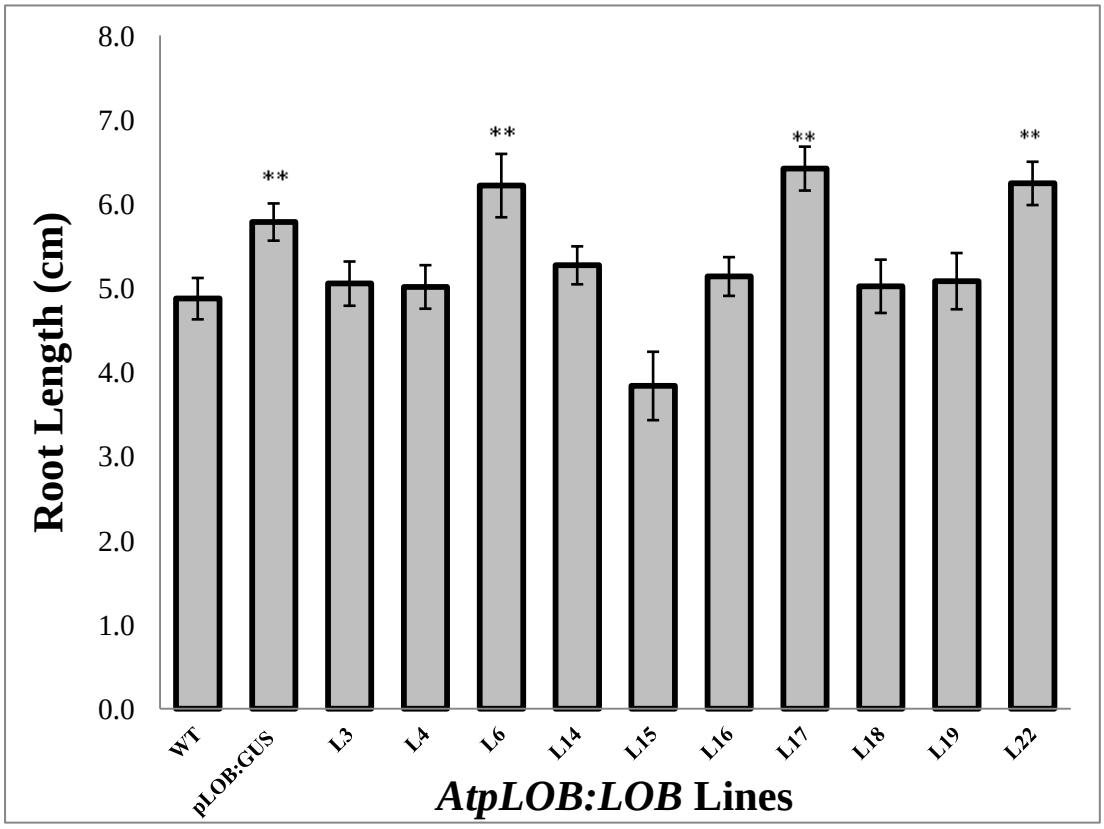

Root Length (cm)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
**
**
**
WT
pLOB:GUS
L3
L4
L6
L14
L15
L16
L17
L18
L19
L22
AtpLOB:LOB Lines

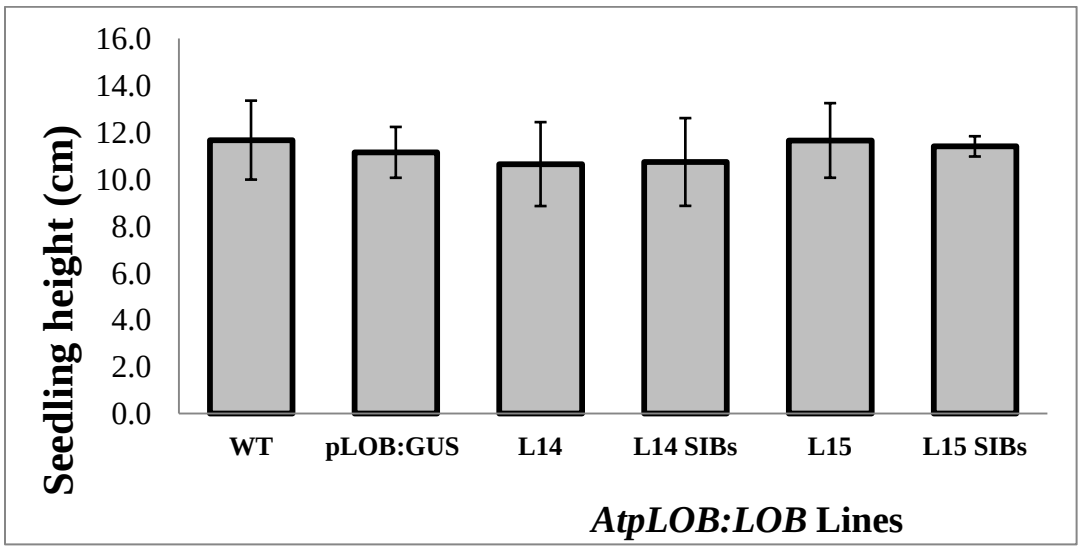
**Figure 1.9. Phenotypic characterization of $T_2$ heterozygous *AtpLOB:LOB* transgenic plants, L14 and L15, and their nontransgenic siblings (SIBs) during the fourth leaf stage.**

A. Quantitative measurement of plant height.

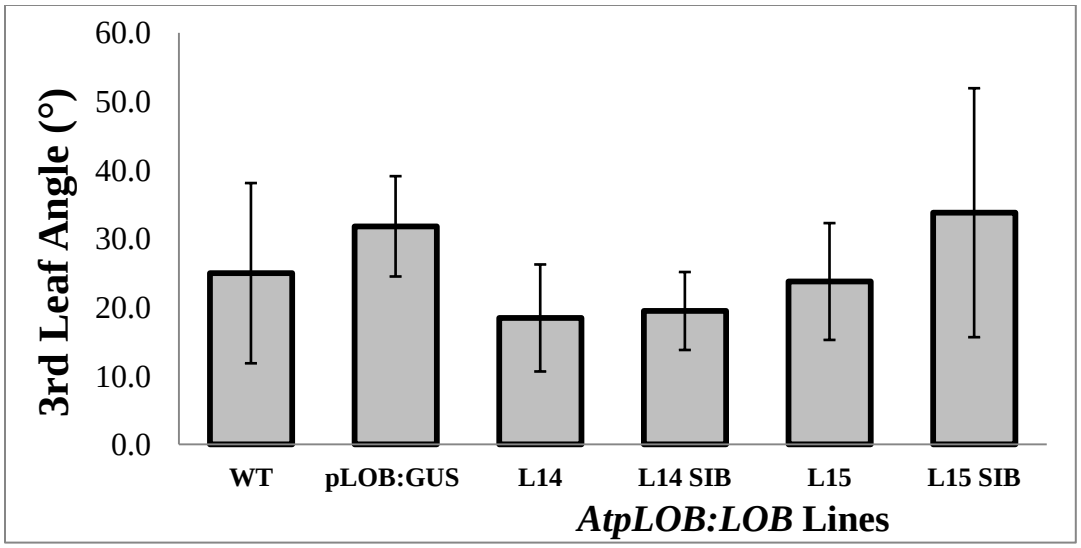
B. Quantitative measurement of the lamina joint angle of the third leaf. Close up images of the lamina joint were taken and the lamina joint angle was measured using imageJ. Values are means ± SE (n=15-21 plants for L14 and L15 lines, n=4 for SIBs). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

C. RT-PCR analysis of *LOB* transcripts in dissected fourth leaf tissues. S=Sheath (1cm below the lamina joint); LJ, Lamina joint; BL= Blade (1cm above the lamina joint). *Act1* was used as a control. Black bars represent intervening lanes that were excised.

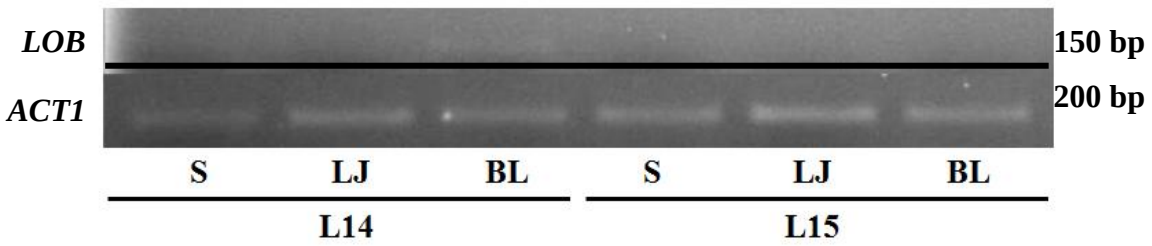
**A.**




**B.**




**C.**




**$T_2$ Heterozygous *AtpLOB:LOB* Lines**

**Table 1.1. Oligonucleotide sequences**

| Purpose | Gene | Primer Name | Sequence (5'-3') |
|---|---|---|---|
| GENOTYPE | *HYG* | HYG FWD | GCCATGTAGTGTATTGACCGATT |
| GENOTYPE | *HYG* | HYG REV | AGTTTAGCGAGAGCCTGACCTAT |
| RT-PCR | *ACTIN* | ACT1 FWD | GCCGTCCTCTCTCTGTATGC |
| RT-PCR | *ACTIN* | ACT1 REV | GCCGTTGTGGTGAATGAGTA |
| RT-PCR | *LOB*[1] | EMB028 | GAATTCATGGCGTCGTCATCAAACTC |
| RT-PCR | *LOB*[1] | EMB029 | AAGCTTCATGTTACCTCCTTGCTGATCATG |
| RT-PCR | *LOB*[2] | qLOB-F | GGCGTCGTCATCAAACTCAT |
| RT-PCR | *LOB*[2] | qLOB-R | CGTTGCTTGCTCCAAAGATT |

Table 1.2 Quantitaive measurements of WT, *pLOB:GUS*, and 10 independent T2 heterozygous *pLOB:LOB* transgenic lines

| | | | | | ***AtpLOB:LOB* independent lines** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **WT** | ***pLOB:GUS*** | **L3** | **L4** | **L6** | **L14** | **L15** | **L16** | **L17** | **L18** | **L19** | **L22** |
| **Seedling Stage** | **4th Leaf Stage** | **Height (cm)** | 11.7 ± 0.4 | 11.1 ± 0.3 | - | - | - | 10.6 ± 0.4 | 11.7 ± 0.3 | - | - | - | - | - |
| | | **3rd Leaf Angle (°)** | 25.0 ± 3.4 | 31.8 ± 1.7 | - | - | - | 18.4 ± 1.8 | 23.7 ± 1.9 | - | - | - | - | - |
| | **2nd Leaf Stage** | **Root Length (cm)** | 4.9 ± 0.2 | 5.8 ± 0.2** | 5.1 ± 0.3 | 5.0 ± 0.3 | 6.2 ± 0.4** | 5.3 ± 0.2 | 3.8 ± 0.5 | 5.1 ± 0.2 | 6.4 ± 0.3** | 5.0 ± 0.3 | 5.1 ± 0.3 | 6.2 ± 0.3** |
| **Heading Stage** | **Overall** | **Height (cm)** | 84.7 ± 1.4 | 78.7 ± 1.9* | 89.6 ± 3.2 | 100.3 ± 4.0** | 80.0 ± 4.7 | 90.4 ± 2.3* | 77.3 ± 3.7* | 82.6 ± 4.5 | 87.6 ± 5.5 | 90.8 ± 2.4* | 83.1 ± 3.0 | 83.2 ± 2.8 |
| | | **Tiller to Tiller (°)** | 30.9 ± 2.7 | 38.3 ± 1.5 | 26.0 ± 0.8 | 25.4 ± 1.6 | 25.7 ± 2.3 | 40.9 ± 4.3 | 39.2 ± 3.0 | 28.6 ± 3.3 | 24.0 ± 1.6 | 28.6 ± 3.3 | 26.8 ± 1.8 | 44.4 ± 4.3* |
| | **Lowest Leaf** | **Leaf Angle (°)** | 51.0 ± 2.4 | 41.1 ± 4.3 | 50.6 ± 5.0 | 43.2 ± 4.9 | 49.9 ± 7.6 | 30.7 ± 2.4** | 27.2 ± 2.9* | 49.6 ± 5.5 | 47.2 ± 7.3 | 39.7 ± 4.7 | 52.7± 5.2 | 51.8 ± 14.1 |
| | | **Sheath Length (cm)** | 20.4 ± 0.4 | 21.4 ± 0.5 | 21.9 ± 0.8 | 23.8 ± 0.5** | 19.8 ± 0.9 | 21.5 ± 0.4 | 19.4 ± 1.0 | 21.4 ± 0.9 | 23.0 ± 1.0* | 22.5 ± 0.6** | 21.5 ± 0.7 | 21.7 ± 0.5 |
| | | **Blade Length (cm)** | 35.5 ± 1.5 | 34.3 ± 1.6 | 37.8 ± 2.1 | 44.3 ± 1.3** | 35.4 ± 1.9 | 37.3 ± 1.4 | 32.4 ± 2.2 | 36.4 ± 1.8 | 37.2 ± 2.0 | 36.3 ± 1.7 | 36.0 ± 1.7 | 34.8 ± 1.8 |
| | | **Blade Width (cm)** | 0.9 ± 0.02 | 1.0 ± 0.03* | 1.0 ± 0.03** | 1.1 ± 0.03** | 1.0 ± 0.02* | 1.1 ± 0.03** | 1.1 ± 0.06** | 1.1 ± 0.04** | 1.0 ± 0.03 | 1.0 ± 0.03* | 0.9 ± 0.03 | 1.0 ± 0.03 |
| | | **Sh/Bl Ratio** | 1.7 ± 0.06 | 1.5 ± 0.05* | 1.7 ± 0.05 | 1.8 ± 0.04 | 1.8 ± 0.05 | 1.7 ± 0.06 | 1.7 ± 0.06 | 1.9 ± 0.12 | 1.6 ± 0.06 | 1.6 ± 0.06 | 1.7 ± 0.05 | 1.6 ± 0.09 |
| | | **Bl Length/Width** | 37.9 ± 1.2 | 33.1 ± 0.8** | 35.8 ± 1.3 | 39.7 ± 0.9 | 34.7 ± 1.5 | 33.3 ± 0.8** | 29.6 ± 0.7** | 34.5 ± 1.3 | 38.1 ± 2.5 | 35.2 ± 1.7 | 38.4 ± 1.1 | 35.7 ± 1.8 |
| | **Flag Leaf** | **Leaf Angle (°)** | 57.2 ± 7.0 | 30.4 ± 4.7** | 61.5 ± 6.0 | 47.0 ± 5.4 | 43.2 ± 8.5 | 29.8 ± 5.9** | 25.0 ± 6.4* | 50.4 ± 5.3 | 60.6 ± 7.6 | 44.8 ± 6.8 | 60.4± 5.5 | 57.9 ± 15.1 |
| | | **Sheath Length (cm)** | 25.7 ± 0.3 | 24.8 ± 0.4 | 28.0 ± 0.7** | 29.1 ± 0.4** | 26.2 ± 0.7 | 26.1 ± 0.4 | 23.5 ± 0.4** | 26.8 ± 0.5 | 28.5 ± 0.4** | 27.6 ± 0.6** | 27.4 ± 0.6** | 24.7 ± 0.6 |
| | | **Blade Length (cm)** | 27.2 ± 0.9 | 27.3 ± 1.0 | 31.9 ± 1.0** | 34.4 ± 1.1** | 29.5 ± 1.1 | 29.5 ± 1.4 | 28.5 ± 1.4 | 29.7 ± 1.5 | 30.7 ± 1.6 | 29.3 ± 0.9 | 30.8 ± 0.9** | 32.1 ± 1.0** |
| | | **Blade Width (cm)** | 1.2 ± 0.02 | 1.4 ± 0.03** | 1.4 ± 0.02** | 1.4 ± 0.02** | 1.3 ± 0.02** | 1.5 ± 0.03** | 1.5 ± 0.06** | 1.3 ± 0.03** | 1.3 ± 0.06 | 1.3 ± 0.02** | 1.2 ± 0.02 | 1.3 ± 0.02** |
| | | **Sh/Bl Length** | 1.1 ± 0.06 | 1.1 ± 0.03 | 0.9 ± 0.08 | 0.9 ± 0.08 | 0.9 ± 0.07* | 0.9 ± 0.10 | 1.0 ± 0.13 | 0.9 ± 0.08* | 0.9 ± 0.15 | 0.8 ± 0.07** | 0.9 ± 0.08 | 1.0 ± 0.10 |
| | | **Bl Length/Width** | 22.6 ± 0.7 | 19.5 ± 0.9 | 22.3 ± 0.7 | 24.5 ± 0.8 | 22.5 ± 0.9 | 20.7 ± 1.2 | 19.3 ± 1.4* | 22.3 ± 1.1 | 24.5 ± 1.3 | 22.3 ± 0.7 | 25.6 ± 0.8** | 24.1 ± 1.0 |

## Chapter 2

## *Arabidopsis BAS1* Expression in Rice Organ Boundaries Alters Shoot Architecture


### Abstract

Understanding hormone function in crop plants is important for altering shoot architecture for agricultural improvement. In particular, upright leaves caused by altered leaf angles leads to plants with an erect stature. Plants with erect leaves require less growth space, allowing for higher-density planting, which contributes to increased grain yields per acre. In rice, the plant hormone brassinosteroid (BR) plays an important role in the control of plant architectural traits, especially plant height and leaf erectness. BR-deficient plants are dwarfed with reduced fertility, but also have increased leaf erectness. The *Arabidopsis BAS1* gene, *AtBAS1*, and its rice orthologs *CYP734A*s, encode a cytochrome P450 enzyme that inactivates BR. Previous studies have shown overexpressing *CYP734A*s in rice resulted in an overall BR-deficient phenotype. This study investigated the site-specific expression of *AtBAS1* in rice to repress BR signaling in the organ boundaries. Expressing *AtBAS1* in a restricted pattern may result in a reduction in local BR levels in boundary regions, leading to increased leaf erectness without other changes in plant architecture. Expression of *AtBAS1* under the *AtLOB* promoter, which drives expression in rice organ boundaries, produced plants with altered shoot and root architecture during the seedling stage. As the plants matured, alterations in the shoot architecture became more apparent. *AtpLOB:BAS1* plants displayed characteristics associated with a reduction of BR as well as enhanced BR signaling such

as the production of upright leaves and elongated inflorescences with increased branching and long, slender grains. These results indicate that mis-expressing *AtBAS1* in a restricted pattern in rice alters leaf inclination as well as inflorescence architecture without detrimental effects on shoot architecture that result from BR deficiency.

**Introduction**

Rice, an important staple food around the world, especially in developing countries, has had an increase in crop yield production over the past decades as a result of altering shoot architecture (18-20; 30). Rice traits that have lead to the growth in rice production are the formation of a large number of productive tillers, dark, wide, and erect leaves, and vigorous, deep root systems (2; 18; 28). Plants with upright leaves have increased photosynthetic efficiency and can be planted at a high-density resulting in an increase in biomass and grain yield (18; 19; 24; 36). Upright leaves allow sunlight to penetrate the lower leaves that are usually shaded by the upper leaves, optimizing canopy photosynthesis (2; 36). In addition to preventing shading of the lower leaves, erect leaves can capture sunlight on both the adaxial and abaxial sides compared to drooping leaves that capture light only on the adaxial surface (2; 36).

Understanding hormone function in crop plants has played an important role for agricultural crop improvement (2; 44). Altering hormone signaling in precise tissues of the plant, has been a useful strategy for directing phenotypic changes that are beneficial for agriculture (44). Gibberellin (GA), a plant hormone, plays a role in the regulation of various developmental processes, in particular stem elongation (2; 32). In 1966, the

International Rice Research Institute (IRRI), bred a semi-dwarfed, high-yielding rice variety known as IR8, which lead to an increase in rice grain production (2; 19). Studies identified *Semi-dwarf1* (*SD1)*, which encodes a rate limiting enzyme of the GA biosynthetic pathway, to be responsible for the alterations in the IR8 shoot architecture (2). Semi-dwarfed rice plants have shown to be beneficial to increasing yield due to its resistance to lodging caused by wind and rain (2; 20; 46). Since these findings, many semi-dwarf rice varieties have been created with mutations in the GA biosynthetic and metabolism pathway due to their agronomic importance (2; 32).

Another plant steroid hormone that plays a major role in rice is brassinosteroid (BR) (11; 41). BR affects various physiological and developmental processes such as stem elongation, cell expansion and division (41). BR-deficient rice plants have altered shoot architecture, including reduced height, decreased fertility, smaller grains, reduced and compact tillering, and dark-green upright leaves (2; 11; 41). While half these traits are beneficial to increasing yield, the other traits diminish the benefits.

The Springer lab has identified several *Arabidopsis* genes that are expressed in the meristem-organ boundary and function in organ separation and meristem maintenance. *LATERAL ORGAN BOUNDARIES* (*LOB*), a plant-specific transcription factor, is expressed in a distinct band of cells that defines the boundary between the shoot apical meristem (SAM) and initiating lateral organ primordia (35). Loss-of-function *lob* mutants have organ separation defects resulting from subtle fusion between the cauline leaf and axillary meristem (6). *Arabidopsis* plants that ectopically express *LOB* have stunted growth resembling BR-deficient mutants (6; 9; 35). To study the relationship

between *LOB* and BR, seedlings were treated with exogenous BR and in response, *LOB* transcript levels were induced, revealing that it is regulated by BR (6). To identify downstream targets of LOB, a microarray experiment was performed using an inducible LOB-overexpression line. This experiment identified *PHYB ACTIVATION TAGGED SUPPRESSOR1 (BAS1)* as a target of LOB transcriptional regulation (6). *BAS1* encodes a cytochrome P450 enzyme that inactivates BR by C-26 hydroxylation (26). *LOB* regulates organ separation, in part, by modulating the accumulation of BR at the organ boundaries (6). These interactions result in a feedback loop where LOB regulates BR signaling in organ boundaries by activating *BAS1*, which inactivates BR (6).

One group of cytochrome P450s, *CYP34A*s, is found in multiple plant species and functions to inactivate BR (27; 39). *In silico* analysis revealed the rice genome contains four *BAS1* homologs, (*CYP734A2*, *CYP734A4*, *CYP734A5*, and *CYP734A6*), which also function to inactivate BR (31; 43). *CYP734A2*, *CYP734A4*, and *CYP734A6* are expressed in various parts of the shoot (shoot apices, leaf sheaths and blades, internodes, and panicles) as well as roots, whereas *CYP734A5* expression was at very low levels in the shoot and was highly expressed in the roots (31; 43). RNA interference knockdown of *CYP734A* genes resulted in plants that had defects in the blade/sheath boundary (lamina joint), with a discontinuous ligule, moving into the blade (43). *CYP734A* RNAi plants also produced stiff shoots that terminated after producing only a few leaves (43). Tissue sections of the SAM revealed *CYP734A* RNAi plants to have boundary defects, as the boundary between the SAM and P1 was shallower compared to wild-type (43). In the reproductive stage, *CYP734A* RNAi plants had panicles with wider branch angles caused

by the development of a pulvinus that was not found in wild-type plants (43). Overexpression of *CYP734A*s in rice resulted in plants with erect leaves and reduced height, resembling traits similar to BR-deficient plants (31). Overexpressing rice *CYP34A* genes in *Arabidopsis* resulted in a BR-deficient phenotype (26). This data suggests that the functions of *CYP34A*s are conserved within different plant species (27; 39).

While broadly overexpressing *CYP734A*s in rice resulted in an overall BR-deficient phenotype (39), expression of *CYP734A*s in a restricted pattern should result in site-specific reduction in BR levels. Repressing BR signaling only in the blade/sheath boundary is expected to result in plants with erect leaves. The *AtLOB* promoter, which was found to be expressed in a restricted pattern in rice similar to *Arabidopsis* (Chapter 1), was used to drive expression of *AtBAS1* in rice (35). Expressing *AtBAS1* in the organ boundaries should inactivate BR specifically at the site of leaf bending, resulting in plants with erect leaves. Sixteen independent heterozygous $T_2$ *AtpLOB:BAS1* lines were analyzed for characteristics associated with BR deficiency such as erect leaves and altered plant height and four $T_3$ homozygous lines were analyzed in greater detail. Overall, *AtpLOB:BAS1* transgenic lines had erect leaves, which is a trait associated with BR deficiency, but they also had alterations in other parts of the shoot that are typically associated with an increase in BR. These results suggest that localized misexpression of *AtBAS1* does alter rice shoot architecture.

## Material and Methods

### Plant material, growth conditions, and transformations

*Oryza sativa* ssp. *japonica* cultivar Kitaake seeds were transformed by the UCR transformation facility using calli induced from mature seeds as starting material for *Agrobacterium*-mediated transformation (http://ptrc.ucr.edu/services.html) (37). Transformed plants were selected on Murashige and Skoog (MS) medium containing hygromycin (HYG) (25). Positive transformants were confirmed by PCR using *hptII* (HYG) primers (Table 2.1). Wild-type (*Oryza sativa* ssp. *japonica* cultivar Kitaake), *AtpLOB:GUS*, and *AtpLOB:BAS1* seeds were surface sterilized in 95% EtOH for 5 min, followed by 1% NaClO for 5 min, and rinsed three times with sterile water. Seeds were fully submerged in $H_2O$ and imbibed overnight at room temperature. Seeds were then transferred to a dark incubator at 30°C for four days. For greenhouse-grown plants, seeds were sterilized, and then fully submerged in $H_2O$ at room temperature for three days. After the three days, seedlings were transferred to 1-gallon pots containing UC Soil Mix II (http://agops.ucr.edu/soil/) supplemented with iron sulfate premade mix (30% iron, 18% sulfate, Brandt) the day after sowing. Plants were grown in a greenhouse at 30°C under 16-hr-light and 8-hr-dark conditions.

To select for positive transformants in subsequent generations of *AtpLOB:BAS1* lines ($T_2$ B1-16; $T_3$ B3, B6, B8, and 10), seeds were grown as previously described above. PCR genotyping using T-DNA-specific primers was used to confirm the presence of the transgene (Table 2.1). To identify $T_3$ homozygotes for B3, B6, B8, and B10, ten

progeny lines from each of the four $T_2$ heterozygous lines were used. Twenty seedlings per progeny line were tested for the presence of the transgene using PCR genotyping and T-DNA-specific primers (Table 2.1). Lines with all seedlings positive for the presence of the transgene were identified as a homozygous line.

**Constructs**

The *AtpLOB:BAS1: 3'Intergenic region* (*IGR)* contains 5-kb of the *LOB* promoter region, the *BAS1* coding region and 2.5-kb intergenic region 3′ to the *LOB* stop codon amplified from genomic DNA (6). A 9.1-kb fragment containing *pLOB:BAS1:3'IGR* was excised from pCB302 plasmid using *Kpn*I and *Pst*I restriction enzymes and cloned into pCAMBIA 1300 binary vector (www.pCambia.org).

**Expression Analysis**

Total RNA was isolated from samples at various developmental stages using the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's guidelines. For semi-quantitative RT-PCR analysis, cDNA was synthesized from 2-4 µg of total RNA using an oligo-dT primer, and either SuperScript III or SuperScript IV reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was diluted and used as a template in PCR reactions to detect *BAS1* transcripts. *OsACTIN1* (*Os03g50885*) (Table 2.1) was used as a control and the PCR cycling conditions were as follows: denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. To detect *BAS1* transcripts, two *BAS1* primer sets were used (Table 2.1). PCR conditions for

*BAS1*[1] (Table 2.1) amplification were as follows: denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s. PCR conditions for *BAS1*[2] (Table 2.1): denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 30 s.

**Phenotypic analysis**

Greenhouse-grown seedlings were analyzed for phenotypic traits associated with BR during the months of January and February (main shoot measurements) and May and June (overall plant measurements). Plant height was measured from the base of the soil to the highest point of the plant. Photographs were taken of lamina joints and the angle of the lamina joints were measured using imageJ. The number of tillers per plant was measure by cutting the plant 3 inches from the soil. Images were taken from above and tillers were counted. The tiller-to-tiller angle was measured from photographs taken of the whole plant using imageJ. Tiller angle was measured between the outermost tiller on the left side and the outermost tiller on the right. Using a standard ruler, the sheath length, blade length/width, main shoot height, panicle height, internode lengths, and length of primary branches were measured. Using a caliper, the grain length/width, panicle internodes, and pedicels were measured.

**Root analysis**

Wild-type, *AtpLOB:GUS,* and *AtpLOB:BAS1* seeds were fully submerged in $H_2O$, imbibed overnight at room temperature, and then transferred to a dark incubator at 30°C

for 4 days. Uniform seedlings were transferred to media plates containing 0.5X MS medium (pH 7.5) (25). Plates were positioned vertically and placed in a growth chamber at 30°C in 16-hr-light and 8-hr-dark conditions. As soon as the second leaf began to unroll, root lengths were measured using a caliper.

**Lamina Inclination assay**

The lamina joint inclination assay was performed as described (45). Sterilized seeds were fully submerged in water and placed in a dark incubator at 30°C for 3 days. Uniform seedlings were transferred to magenta boxes with 0.5X MS medium (pH 7.5) and placed in a 30°C incubator with 16-hr-light and 8-hr-dark conditions for 5 days. The second leaf was excised and floated on distilled water overnight. 24-epi-brassinolide (BL), the most bioactive BR (21), in 95% EtOH was added to a final concentration of 0.001 µM, 0.01 µM, 0.1 µM, and 1 µM followed by 2 days of incubation. Images of each leaf were captured and the lamina angles were measured using ImageJ (http://imagej.nih.gov/ij/).

**Results**

**Shoot architecture of $T_2$ heterozygous *AtpLOB:BAS1* lines**

Three rice *BAS1* homologs (*CYP734A2*, *CYP734A4*, and *CYP734A6*) are expressed in the shoot apices, leaf sheaths and blades, internodes, panicles, and roots (31; 43). Overexpressing *CYP734A*s in rice produced dwarfed plants with upright leaves,

characteristics of BR-deficiency (31). To investigate the result of mis-expressing *CYP734A*s in a restricted domain, a transgene containing the *AtBAS1* CDS under the control of the *AtLOB* promoter, which was found to drive expression in rice lamina joints (Chapter 1), was transformed into rice (Fig. 2.1). Expressing *AtBAS1* in a restricted domain, specifically the lamina joint, should inactivate endogenous BR. Sixteen independent $T_2$ heterozygous *AtpLOB:BAS1* lines were analyzed along with two controls, a homozygous *AtpLOB:GUS* line and wild-type (Fig. 2.2, Table 2.2). *AtBAS1* transcript accumulation in dissected lamina joints of thirteen of the sixteen lines was analyzed by RT-PCR (Fig. 2.2A). Insufficient RNA was gathered for three lines B2, B6, and B7. Of the thirteen lines analyzed, five lines (B1, B3, B8, B9, and B14) had moderate levels of transcript accumulation, five lines (B5, B10, B11, B12, and B16) had low levels, and three lines (B4, B13, and B15) had little to no detectable level of *AtBAS1* transcript accumulation.

Rice mutants deficient in BR are dwarfed in stature with upright leaves (2; 11; 41). To determine if *AtBAS1* causes phenotypic alterations similar to those seen in BR mutants, plant height and leaf angle were measured in mature plants (Fig. 2.2B and C, Table 2.2). Plants in eleven of the sixteen independent $T_2$ heterozygous *AtpLOB:BAS1* lines were taller compared to wild-type, resembling plants with enhanced BR signaling, which is contrasting the BR-deficiency phenotype. The reduced stature in BR-deficient plants is primarily due to the altered elongation patterns in the culms (13; 14; 48). In *AtpLOB:GUS* lines, there was no *GUS* activity in the node or internodes of culms at any stage (Chapter 1) suggesting the expression of *AtBAS1* in $T_2$ heterozygous *AtpLOB:BAS1*

lines will not affect the development of the culms. The changes in plant height in the $T_2$ heterozygous *AtpLOB:BAS1* lines may be caused by a compensatory effect due to site-specific reduction of BR similar to what was found in *Arabidopsis pLOB:BAS1* transgenic plants (6). In *Arabidopsis*, the expression of *BAS1* in the *LOB* domain in *lob* mutants rescued the organ-fusion phenotype (6). In the wild-type background, *pLOB:BAS1* transgenic plants appeared to be morphologically normal but did have longer pedicels perhaps due to the plant compensating for the reduction of BR in organ boundaries (6).

In rice, the *AtLOB* promoter was found to drive expression in the blade/sheath boundary (Chapter 1), suggesting *AtBAS1* expression in the lamina joint will inactivate BR, which should alter leaf angles. To investigate the possible effects of *AtBAS1* on leaf inclination, angles of the lowest leaf and flag leaf (leaf below the panicle) were measured (Fig. 2.2C). Of the sixteen independent $T_2$ heterozygous *AtpLOB:BAS1* transgenic lines, nine lines exhibited a decrease in leaf angle at the lowest and/or flag leaf position compared to wild-type. The altered leaf angles in the transgenic *AtpLOB:BAS1* lines strongly suggested that the site-specific expression of *AtBAS1* caused alterations in leaf inclination in rice.

**$T_3$ Homozygous *AtpLOB:BAS1* alters shoot height during the seedling stage.**

For a more detailed analysis of traits associated with localized BR-deficiency, four independent $T_3$ homozygous *AtpLOB:BAS1* lines (B3, B6, B8, and B10) were used. $T_2$ heterozygous *AtpLOB:BAS1* lines B3 and B8 were selected because they displayed an

erect-leaf phenotype as well as an increase in height while B6 and B10 only had an erect-leaf phenotype compared to wild-type during the heading stage (when the panicle emerges from the leaf sheath). In order to determine when changes in shoot architecture are apparent due to *AtBAS1*, characterization of these traits was conducted at an earlier developmental stage using $T_3$ homozygous *AtpLOB:BAS1* lines (B3, B6, B8, and B10).

As early as 2 days after germination, *GUS* driven by the *AtLOB* promoter displayed expression at the shoot/root boundary (Chapter 1). At the second leaf stage, *GUS* activity was observed in a restricted pattern, confined to the blade/sheath junction of the second leaf (Chapter 1). Due to early *GUS* activity in the shoot/root and blade/sheath boundaries in *AtpLOB:GUS* plants, plant height and the angle of the third leaf were measured in $T_3$ homozygous *AtpLOB:BAS1* lines during the seedling stage (Table 2.2). Surprisingly, three of the four lines had an increase in height, which is the opposite of what is found in rice mutants with decreased BR (Fig. 2.3A). A possible explanation is that the transgenic plants are compensating for the reduction of BR in the boundaries (33).

The angle of the third leaf was measured during the seedling stage but none of the four *AtpLOB:BAS1* lines were significantly different from wild-type (Fig. 2.3B). Examining the spatial expression pattern of *AtBAS1* in the fourth leaf of *AtpLOB:BAS1* lines can determine if the lack of altered angles correlates with *BAS1* expression levels. *AtBAS1* transcript accumulation was measured in dissected leaf tissues: sheath (1cm below the lamina joint), lamina joint, and blade (1 cm above the lamina joint) using semi-quantitative RT-PCR (Fig. 2.3D). In all four transgenic lines, *AtBAS1* transcript

accumulation was detected in all leaf tissues. While the expression of *AtBAS1* was present in all parts of the leaf, none of the four independent $T_3$ *AtpLOB:BAS1* lines showed any alterations in leaf angle at the seedling stage suggesting the effects of *AtBAS1* in the lamina joint becomes apparent as the plant matures.

In rice, BR has been shown to affect the development of the roots (14). BR-deficient mutants such as *BR-deficient dwarf1* (*brd1*) have shortened roots and a decrease in the formation of crown roots compared to wild-type (14; 15). *Arabidopsis LOB* is expressed at the base of lateral roots and when ectopically expressed, results in shortened roots (6; 35). Rice *pLOB:GUS* lines did not show any expression in the roots but there was expression at the shoot/root boundary (Chapter 1), therefore *AtBAS1* is not expected to alter root architecture. Root lengths were measured at the seedling stage, and two (B6 and B10) of the four lines had decreased root elongation compared to wild-type (Fig. 2.3C). The variation of root length among the *AtpLOB:BAS1* lines can be due to the compensatory effect of reduced BR at the shoot/root boundary.

**Overall phenotypic characterization of mature $T_3$ *AtpLOB:BAS1* transgenic rice plants.**

To determine the spatial expression pattern of *AtBAS1* during the heading stage, transcript accumulation of *AtBAS1* was analyzed using RNA samples from different tissues from the four *AtpLOB:BAS1* (17). *AtBAS1* transcript was detected in the sheath (1 cm below the lamina joint), lamina joint, and blade (1 cm above the lamina joint) of mature leaves at the lowest and flag leaf position (Fig. 2.4). The uppermost node and

internode, and immature panicles had little to no detectable *AtBAS1* transcript accumulation (Fig. 2.4).

As the *AtpLOB: BAS1* plants entered the heading stage, phenotypic characteristics associated with BR-deficiency were more visible. Characteristics associated with BR-deficiency are reduced height and compact tillering with fewer number of tillers (41), so we examined these traits in *AtpLOB:BAS1* plants (Table 2.2). Of the four $T_3$ *AtpLOB:BAS1* homozygous lines examined, none showed a significant difference in height compared to wild-type (Fig. 2.5). In BR-deficient plants, plant height is correlated with altered internode patterning of the culm (41). In the case of the transgenic lines, *AtBAS1* was not expected to alter internode patterning due to the lack of detectable *AtBAS1*expression in the internodes and nodes (Chapter 1). When looking more closely at the tillers, two (B3 and B10) of the four lines had an increase in tiller-to-tiller angle and only one line (B3) had a significant increase in the number of tillers compared to wild-type (Fig. 2.6). While these traits are opposite of what is typically found in BR-deficient mutants, the low frequency of the number of *AtpLOB:BAS1* lines (two of four lines) having these traits suggests *AtBAS1* is not affecting the tillers and may be caused by transgene position effects (40).

**_AtBAS1_ alters leaf inclination and sheath length in $T_3$ _AtpLOB:LOB_ mature plants.**

Due to *AtpLOB:GUS* patterning and expression of *AtBAS1* transcripts in the lamina joint, leaf inclination of mature leaves was measured. Leaf angles of the lowest and flag leaf were measured to determine if the *AtpLOB:BAS1* transgene causes upright

leaves (Fig. 2.7, Table 2.2). Three of the four lines (B6, B8, and B10) had decreased leaf angles at the lowest leaf position, while at the flag leaf position, all lines had decreased leaf angles compared to wild-type.

BR-deficient plants have short, wide leaf blades along with shorter sheaths, while plants with enhanced BR-signaling have long, thin blades with longer sheaths (14; 42). Reduced BR affects the organization of cell files as well as cell division and elongation in the epidermal layer resulting in altered leaves (14; 15; 42). During the heading stage, the expression of *AtBAS1* in the sheath and blade of transgenic lines, just below and above the lamina joint respectively, suggests that these tissues may be affected. To determine if leaf morphology is affected by *AtBAS1*, sheath and blade length/width were measured (Fig. 2.8 and Table 2.2). Two (B8 and B10) of the four lines exhibited shorter sheath lengths in the lowest leaves, while the flag leaves had longer sheaths in three (B3, B6, and B10) of the four lines (Fig. 2.8A). The variation in sheath length among the lowest and flag leaves suggests that the internode pattering of the culm maybe be affected. One line (B3) had longer leaf blades at the lowest leaf position, while B10 had shorter flag leaf blades compared to wild-type (Fig. 2.8B). At the lowest leaf position, none of the four lines displayed wider leaves but at the flag leaf position, one line (B3) had wider leaves compared to wild-type (Fig. 2.8C). The blade lengths and widths were not consistent among the majority of the lines at both the lower and flag leaf position, suggesting that the variations in blade lengths and widths within the transgenic lines are caused the position effects of the transgene.

**Mature $T_3$ *AtpLOB:BAS1* transgenic lines have altered inflorescence architecture**

An important factor in increasing grain yield pertains to the panicle such as the increase in the number of spikelets per panicle and grain weight, due to grain filling (20; 28). BRs have also been shown to contribute to the development of the panicle and grains (14; 38). BR-deficient rice mutants have reduced fertility with shorter panicles and smaller grains, while plants with increased BR have longer panicles and much larger grains (2; 11). All four *AtpLOB:BAS1* lines displayed an increase in panicle length and the number of grains per panicle compared to wild-type in randomly selected panicles. This is surprising, as there was little to no *AtBAS1* transcript detected in immature panicles and longer panicles are traits that correlate with enhanced BR signaling (Fig. 2.4, Fig. 2.9, and Table 2.2). It may be possible that *AtBAS1* is expressed at an earlier time than what was analyzed in the panicles, which would cause reduced BR very early in panicle development. The reduction of BR in early panicle development may cause the plant to compensate and increase BR throughout the panicle.

For a closer examination of the effects of *BAS1* on grain morphology, the lengths and widths of the grains from $T_3$ *AtpLOB:BAS1* plants were measured (Figure 2.10, Table 2.2). In two (B6 and B10) of the four lines grains were significantly longer, while all lines had grains with reduced width compared to wild-type (Fig. 2.10C and D). All lines displayed a grain length to width ratio larger than wild-type, meaning the *AtpLOB:BAS1* lines have longer, slender grains (Fig. 2.10B and E). A significant increase in 1,000-grain weight was observed in two (B6 and B10) of the four lines, compared to wild-type

(Fig. 2.10A). The increase in grain weight of line B6 and B10 was correlated with increased grain length. These results suggest that the transgene *AtpLOB:BAS1* causes an increase in overall panicle length and the production of slender grains with more mass than wild-type.

BR, which plays a role in cell division and elongation, functions in internode elongation (16). In BR-deficient mutants, shortened tillers that result from altered internode elongation patterning cause a decrease in height (14; 41). The *brassinosteroid-dependent 1* (*brd1*) rice mutant, which has defects in BR biosynthesis, is severely dwarfed, with shortened internodes and abnormal leaf morphology (23). While none of the *AtpLOB:BAS1* lines exhibited changes in overall height, the internode lengths were examined to determine if the internode elongation pattern was disrupted. Measurement of the main shoot of *AtpLOB:BAS1* lines revealed that these lines were not significantly different from wild-type (Fig. 2.11A). The relative ratio of each internode (I to V) to the overall height was similar in the main shoot of the transgenic plants and wild-type (Fig. 2.11B and C). Overall, the internode patterning of the main shoot was not disrupted in any of the *AtpLOB:BAS1* lines, which is consistent with their height not being altered.

*AtpLOB:BAS1* lines have longer panicles that produce long, slender grains, resembling rice plants with increased BR. Initial studies were done on panicles from random tillers was examined. The main shoot panicle, which is the first inflorescence to emerge, in more detail (Table 2.2). All four *AtpLOB:BAS1* transgenic lines produced longer main shoot panicles compared to wild-type (Fig. 2.12B). Three lines (B3, B6, and

B8) had longer primary branches on the main shoot with an increase in the number of grains per main panicle compared to wild-type (Fig. 2.12C and G).

Next, the internode lengths between the lowest eight primary branches (counting acroptetally) on the main shoot panicle were measured (Fig. 2.12D). The internode lengths in wild-type were relatively evenly distributed, ranging from 9-16% in each category. In line B3 and B6, about 75% and 71%, respectively, of internodes ranged in lengths from <1.0 mm to 5.0 mm and >25.1 mm. In line B8, more than 30% of the internodes were <1.0 mm in length and about 50% of the internodes ranged in lengths from 10.1-15 mm and >25.1 mm. In line B10, a little more than 30% of the internodes were <1.0 mm in length and about 45% of the internodes ranged in lengths from 15.1-30 mm. Compared to wild-type, all *AtpLOB:BAS1* lines showed more variation in the internode patterning of the primary branches on the main shoot panicle.

The number of primary branches per panicle significantly increased in all *AtpLOB:BAS1* lines compared to wild-type (Fig. 2.12E). While the numbers of secondary branches in all transgenic lines were comparable to wild-type, the number of lateral spikelets per primary branch increased in lines B3 and B6, but the length of the pedicels was only different in B3 (Fig. 2.12E and F).

### *AtpLOB:BAS1* transgenic lines are sensitive to exogenous BR

In *Arabidopsis*, *LOB* expression is positively regulated by BR and LOB activates expression of *BAS1*, which inactivates BR (6). This feedback loop limits BR accumulation in organ boundaries (6). *AtBAS1* activity in the lamina joint and the erect-

leaf phenotype in *AtpLOB:BAS1* plants suggest that *AtBAS1* is inactivating endogenous BRs. To determine if these plants were sensitive to exogenous BR, the lamina inclination assay, used to determine sensitivity to exogenous BR, was conducted (45). This bioassay measures the changes in leaf angle due to the application of exogenous BR (45). If *AtBAS1* inactivates BR, the leaf angles in *AtpLOB:BAS1* lines should not change with the addition of exogenous BR. In the absence of exogenous BL, wild-type plants have a leaf angle of 22°. As BL concentrations increased from 0.001, 0.01, 0.1 and 1 μM, the wild-type leaf angle increased from 30°, 70°, 124°, and 130°, respectively (Fig. 2.13). All four *AtpLOB:BAS1* transgenic lines had increases in leaf angle as the concentration of BL increased, similar to wild-type's response to BL (Fig. 2.13). Comparing wild-type and the transgenic lines, there were no significant differences of leaf angle at the varying concentrations of BL. The results of the lamina inclination assay confirm that the sensitivity of the transgenic lines to exogenous BR is similar to wild-type. A possible explanation is that the sit-specific activity of *AtBAS1* during the seedling stage is inadequate to inactivate exogenous BR when saturated with BR.

**Discussion**

Over the years there have been numerous studies demonstrating the importance of BR in plant biological processes (9; 11). BRs are essential for various plant physiological and developmental processes such as cell expansion and division, skotomorphogenesis, and senescence (1; 41). Research characterizing BR responses in rice has allowed an increase in crop yield production over the past decades due to BR

altering shoot architecture (18-20; 30). Two main rice traits that are affected by BRs are plant height and leaf angle (14). Plants that are reduced in stature are more resistant to lodging by wind and rain (11). Upright leaves can increase photosynthetic efficiency and allow planting at a high-density resulting in an increase in biomass and grain yield (24; 33; 36).

Organ boundaries in plants play a significant role in the regulation of plant architecture. Boundary cells serve as a barrier to isolate population of cells within their distinct domains and allow proper meristem maintenance (10; 29). Studies haves shown BRs to contribute to the formation of organ boundaries by repressing organ boundary genes to maintain the limited cell size and division in the boundary (12). *Arabidopsis* mutants hypersensitive to BR displayed organ-fusion phenotypes. BRASSINAZOLE RESISTANT1 (BZR1), a key transcription factor in the BR-signaling pathway, has been shown to repress expression of organ-boundary identity genes in *Arabidopsis* (12; 21). *Arabidopsis bzr1-1D*, a dominant BR-hypersensitive mutant, has fused organs due to the overgrowth of the boundary region (12). The organ-fusion defects in *bzr-1D* are caused by the activation of BR signaling, which results in an increase in cell elongation and size (12). The defects in organ boundaries in *bzr1-1D* cause the stem to bend towards the lateral branch resulting in an smaller angle between the two (12). In rice, *OsBZR1P206L*, a dominant *OsBZR1* mutant with hypersensitivity to BR, resulted in increased leaf bending (4). In contrast, *OsBZR1* RNAi plants had reduced BR response causing erect leaves (4).

In *Arabidopsis*, *LOB* regulates organ separation by modulating BR accumulation at the organ boundaries (6). *LOB* is positively regulated by BR and LOB activates expression of *BAS1*, which inactivates BR (6). This feedback loop limits BR accumulation in organ boundaries resulting in the boundary cells being smaller and having limited cell division (6). Because *LOB* is expressed in a discrete domain in *Arabidopsis* and controls BR accumulation, the *AtLOB* promoter was expressed in rice and drove expression in a restricted pattern confined to the shoot/root and the blade/sheath boundaries (Chapter 1). This suggested that there is a similarity between the meristem/leaf and blade/sheath boundaries and that *AtBAS1* under the *AtLOB* promoter can be used to repress BR signaling in the blade/sheath boundary, resulting in plants with erect leaves.

Groups of cytochrome P450 enzymes have been shown to play a major role in the biosynthesis of BR (5). BR biosynthesis mutants have phenotypic alterations such as compact stature, dwarfed, small, dark green leaves, delayed flowering and reduced fertility (34). *DWARF4* (*DWF4*), a cytochrome P450 enzyme that catalyzes a rate limiting step in BR biosynthesis, has been shown to play a role in shoot architecture, in particular regulating height, in various plant species (7; 21). In *Arabidopsis*, *dwf4* mutants have defects in cell elongation resulting in dwarfed plants with short, round leaves (3). Overexpressing *DWF4* resulted in the production of taller plants with increased number of branches, siliques, and seeds compared to wild-type (8). To study the maize *DWF4* gene, *ZmDWF4* was constitutively expressed in *Atdwf4* mutants and was able to rescue the mutant phenotype (22). While these plants resembled wild-type,

they were also taller with an increase in the number of branches and seeds (22). In rice, *dwf4* mutants were semi-dwarfed with erect leaves and no alterations in leaf or grain morphology (33). These studies on *DWARF4* enzyme, suggest conserved function of among these plant species. This indicates a possibility that the function of *BAS1* is conserved and expressing *AtBAS1*, a cytochrome P450 enzyme, specifically in the lamina joint in rice, should result decrease BR levels and erect leaves.

One study expressed sterol C-22 hydroxylases active in BR biosynthesis from different plant species (Zm-CYP724B4, At-CYP90B1, and Os-CYP90B2) in rice (47). These genes were under the control of an *Arabidopsis* promoter that is expressed in only the stems, leaves, and roots in rice (47). These transgenic rice plants exhibited an increase in BR levels only in the stems and leaves, resulting in an increase in height, the number of tillers and grains, seed weight, leaf length, and leaf angles (47). These phenotypic changes in the transgenic plants caused an increase in grain yield per plant compared to wild-type plants (47). These data suggest that altering BR in specific regions can lead to changes in shoot architecture that can be beneficial to agricultural crops.

*CYP34A*s, the rice *BAS1* homologs, encode a group of cytochrome P450 enzymes that inactivate BRs by C-26 hydroxylation (26). Misexpression of *CYP734A*s (*CYP734A2*, *CYP734A4*, and *CYP734A6*) in rice results in an overall BR-deficient phenotype; erect leaves and reduced height (31). Expressing *CYP734A*s in a restricted pattern in rice should repress BR signaling in local regions. This study investigated the expression of *AtBAS1* in rice organ boundaries to repress BR. Expressing *AtBAS1* in a

restricted pattern altered leaf inclination as well as inflorescence architecture without other detrimental effects on shoot architecture that BR deficiency causes.

As a control, a low copy homozygous *AtpLOB:GUS* rice line was analyzed along with wild-type. The *AtpLOB:GUS* transgene was not expected to cause any alterations in phenotype due to the *LOB* promoter driving expression of a reporter gene and not a functional gene. While all the plants were grown and analyzed at the same time, *AtpLOB:GUS* plants displayed variations in phenotypic traits that were significantly different from WT (Table 2.2). *AtpLOB:GUS* plants had longer roots at the seedling stage, while at the heading stage *AtpLOB:GUS* plants were shorter with compact tillering and had an increased number of primary branches per panicle. *AtpLOB:GUS* plants also had an increased number of round grains per panicle. These traits are characteristics of both enhanced BR signaling and reduced BR. A possible reason for this is the random insertion of T-DNA into the rice genome. The transgene can be inserted into genes and disrupt their function causing alterations in developmental processes, which can lead to changes in phenotype.

*AtLOB* promoter activity in the lamina joint of the second leaf during the seedling stage (Chapter 1) suggests *AtBAS1* driven by *AtpLOB* will express *AtBAS1* in this distinct domain and might affect leaf inclination early in development. In *AtpLOB:BAS1* plants, *AtBAS1* transcript was expressed in the sheath (1cm below the lamina joint), lamina joint, and blade (1cm above the lamina joint) early and late in development. At the seedling stage, there were no alterations in leaf angle in comparison to wild-type, suggesting the effects of *AtBAS1* in the lamina joint may occur later in development. During the

heading stage all *AtpLOB:BAS1* lines had plants with erect leaves at either the lowest or flag leaf position or both compared to wild-type. Overexpressing *IBH1*, a transcription factor that mediates BR regulation of cell elongation, resulted in plants with erect leaves caused by the reduced cell elongation in the adaxial side of the lamina joint (49). The decrease in leaf inclination in *AtpLOB:BAS1* plants is perhaps caused by the disruption of cell elongation/division due to the inactivation of endogenous BRs on the adaxial surface of the lamina joint.

While all four *AtpLOB:BAS1* lines exhibited erect leaf angles, which is a trait associated with BR-deficiency, these plants also displayed phenotypes linked to enhanced BR signaling such as increase sheath length and alterations in inflorescence architecture (15; 47). Rice mutants with BR deficiency affect the development of the sheaths by causing a disruption of the organization of the epidermal cells in the sheaths (15). *CYP734A* overexpression lines have shortened leaf sheaths resembling BR-deficient phenotypes (31). This unexpected phenotypic trait of longer sheaths in *AtpLOB:BAS1* plants may result from compensation for the reduction of BR in lamina joints by increasing BR in other parts of the leaf. Cross-sections of the sheaths can determine if there is a defect in the cell division/elongation as a result of BR reduction in the lamina joint.

BR-deficient rice mutants have reduced fertility with shorter panicle and grains, while plants with increased BR have longer panicles and much larger grains (2; 11). All *AtpLOB:BAS1* lines had panicles that were longer as a result of the increase in the number and length of primary branches. In rice *AtpLOB:GUS* plants, there was no *GUS*

activity detected at the base of the inflorescences and floral organs (Chapter 1). While there was very little to no detection of *AtBAS1* transcript in immature panicles, taken from a development stage later than the *GUS* expression analysis, it is possible that *AtBAS1* will alter inflorescence architecture. *AtBAS1* may be expressed at an earlier time than what was analyzed and at a level that was not detectable using the GUS reporter system. Arabidopsis *pLOB:BAS1* transgenic plants have longer pedicels but overall are morphologically normal, this is comparable to the increase in length of the primary branches found in the rice *AtpLOB:BAS1* transgenic plants (6).

All *AtpLOB:BAS1* lines exhibited an increase in the number of grains per panicle, which seems to correlate with the increase in the length and number of primary branches. Transgenic plants overexpressing *DWARF AND LOW-TILLERING* (*DLT*), a positive regulator in BR response in rice, resulted in altered grains that were longer but decreased in width (42). The grains had a ratio of length to width that revealed they were longer and slender compared to those of WT. *AtpLOB:BAS1* grains did not resemble grains from BR-deficient mutants; instead, they were comparable to plants with enhanced BR-signaling. In rice *AtpLOB:GUS* plants, *GUS* activity was detected in the anthers of the florets during early developmental stages and decreased as the panicles developed and florets matured (Chapter 1). This suggests *AtBAS1* may reduce BR in developing florets and affect early floral development producing longer, slender grains in *AtpLOB:BAS1* plants.

Understanding hormone function in crop plants is important for altering shoot architecture for agricultural improvement. Producing rice plants with erect shoot

architecture can allow for high-density planting that will result in increased plant biomass and grain yields. Studies on BR have shown that it plays a major role in shoot architecture, in particular lamina joint bending. Using a transgenic approach, the *AtpLOB:BAS1* transgene was expressed in rice and produced plants with erect leaves and elongated inflorescences with increased branching and long, slender grains in the absence of detrimental effects on fertility and plant height caused by BR deficiency. These plants can lead to the development of useful rice lines for growers, as erect plants with an increase number of grains will allow higher density planting, contributing to increased grain yields per acre.

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**Figure 2.1. Structure of the *AtpLOB:BAS1:LOB 3'IGR* T-DNA construct inserted into pCAMBIA1300 binary vector.**

LB=Left T-DNA border; poly-A=*35S* polyA; HYG=*Hygromycin phosphotransferase* II (*hpt*II), Hygromycin resistant gene (plant resistance selectable marker); p35S =Cauliflower mosaic virus *35S* promoter; *pLOB= AtLOB* promoter; *BAS1=AtBAS1* coding sequence cDNA; *LOB 3'IGR =LOB* 3' intergenic region; RB =Right T-DNA border.


LB
Poly A
HYG
p35S
pLOB
BASI
LOB 3'IGR
RB

**Figure 2.2. Characterization of sixteen independent $T_2$ heterozygous *AtpLOB:BAS1* transgenic plants (B1-B16), wild-type (WT), and *AtpLOB:GUS* during the heading stage.**

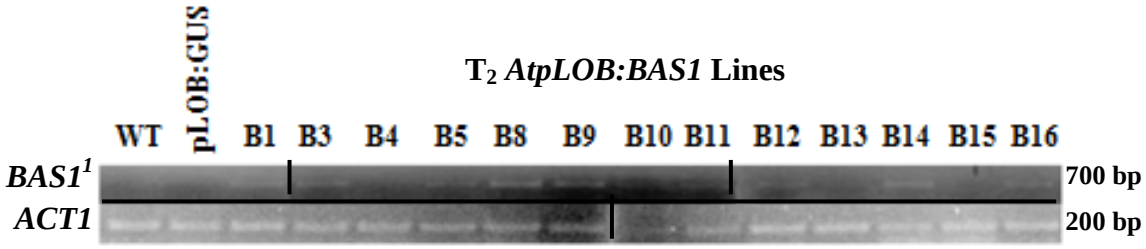
A. *AtBAS1* transcript accumulation in dissected lamina joints. *Act1* (*Os03g50885*) was used as a control. Black bars represent intervening lanes that were excised.

B. Quantitative measurements of plant height.

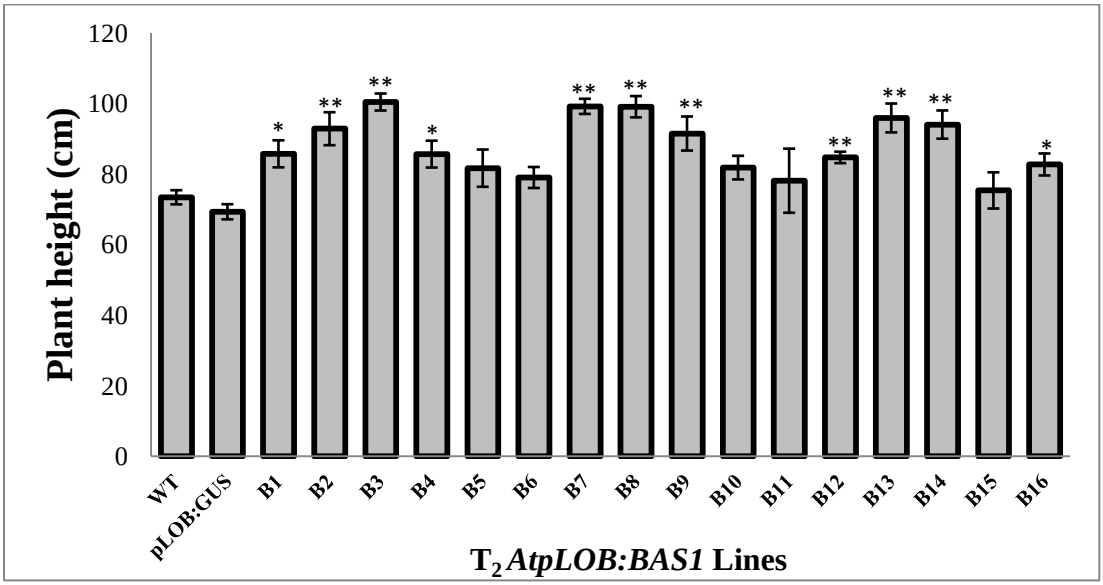
C. Quantification of the lamina joint angle of the lowest leaf and flag leaf. Angles were measured from vertical using ImageJ.

Values are means ± SE (n=2-8 plants, 3 tillers/plant). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

**A.**


T2 AtpLOB:BAS1 Lines
WT
pLOB:GUS
B1
B3
B4
B5
B8
B9
B10
B11
B12
B13
B14
B15
B16
BAS1[1]
ACT1
700 bp
200 bp

**B.**


Plant height (cm)
120
100
80
60
40
20
0
WT
pLOB:GUS
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
B13
B14
B15
B16
T2 AtpLOB:BAS1 Lines

**C.**

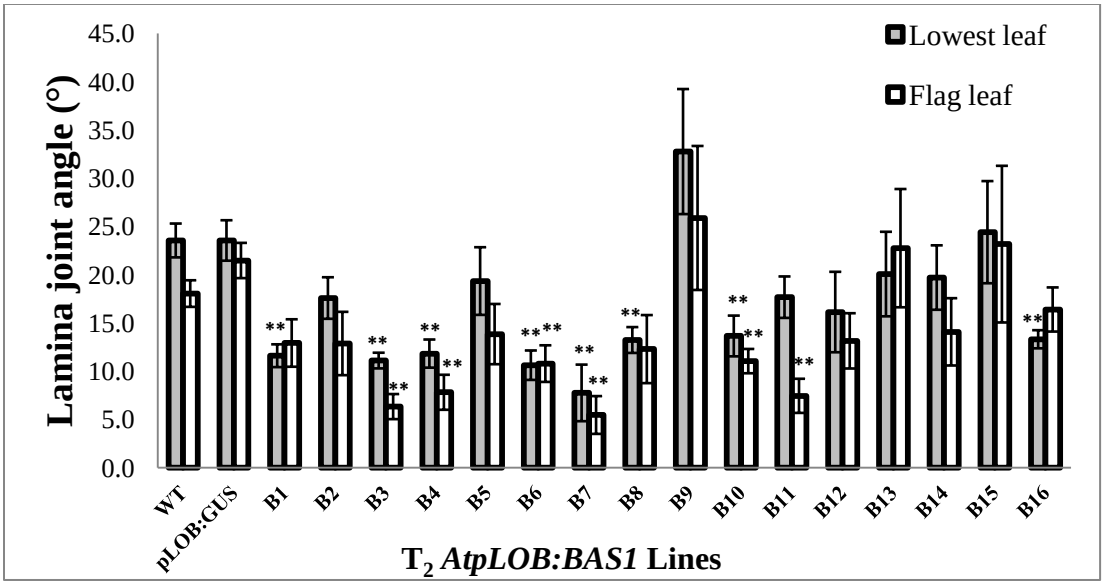

Lamina joint angle (°)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Lowest leaf
Flag leaf
WT
pLOB:GUS
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
B13
B14
B15
B16
T2 AtpLOB:BAS1 Lines

**Figure 2.3. Phenotypic characterization of $T_3$ homozygous *AtpLOB:BAS1* transgenic plants during the fourth leaf stage.**

A. Quantitative measurement of plant height.

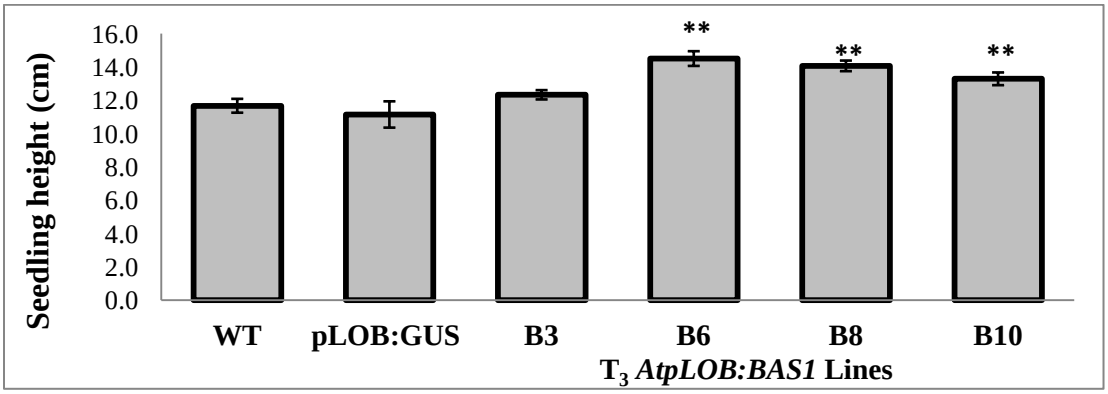
B. Quantitative measurement of the lamina joint angle of the third leaf. Close up images of the lamina joint were taken and the lamina joint angle was measured using ImageJ.

C. Root lengths. Seedlings were grown vertically on half strength MS medium and roots were measured when the second leaf began to unroll.

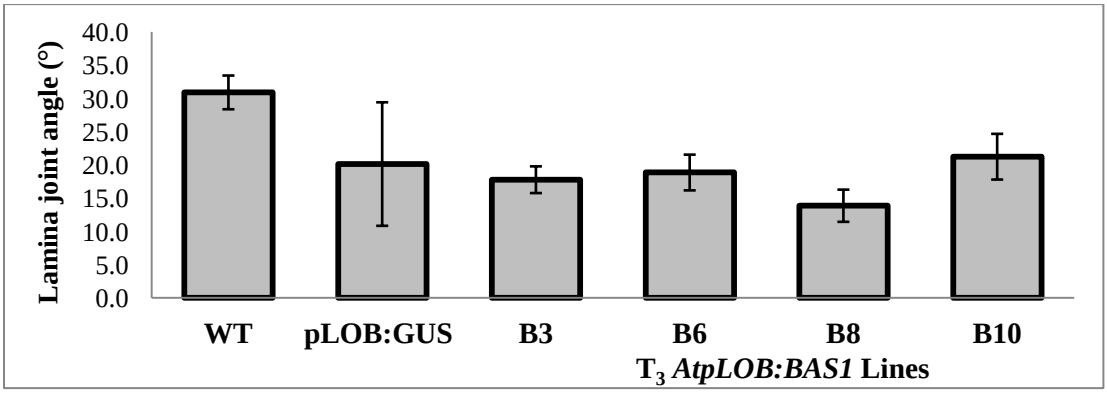
Values are means ± SE (n>15 plants). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

D. RT-PCR analysis of *AtBAS1* transcripts in dissected fourth leaves. *Act1* was used as a control. S=Sheath (1 cm below the lamina joint); LJ=Lamina joint; B= Blade (1 cm above the lamina joint). Black bars represent intervening lanes that were excised.

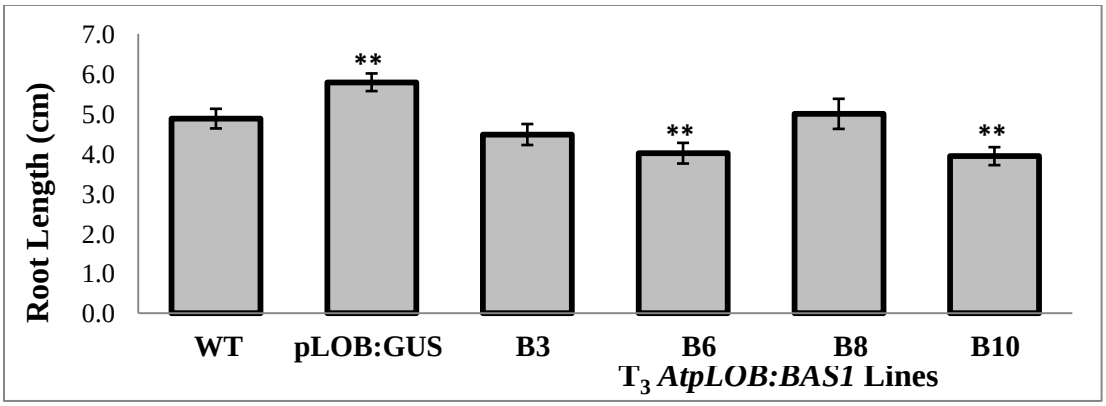
**A.**


Seedling height (cm)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
**
**
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**B.**


Lamina joint angle (°)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**C.**


Root Length (cm)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
**
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**D.**

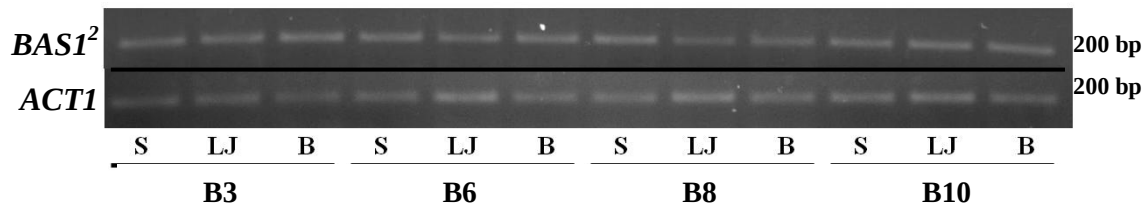

BAS1²
ACT1
200 bp
200 bp
S
LJ
B
S
LJ
B
S
LJ
B
S
LJ
B
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**Figure 2.4. Expression profile of *AtBAS1* in $T_3$ homozygous *AtpLOB:BAS1* transgenic plants during the heading stage.**

RT-PCR analysis of *AtBAS1*expression in various tissues in wild-type and *AtpLOB:BAS1* plants. *Act1* was used as a control. Black bars represent intervening lanes that were excised.

LS=Lowest leaf sheath (1cm below the lamina joint); LLJ=Lowest leaf lamina joint; LB=Lowest leaf blade (1cm above the lamina joint); FS=Flag leaf sheath (1cm below the lamina joint); FLJ=Lowest leaf lamina joint; FB=Flag leaf blade (1cm above the lamina joint); N= Uppermost node; IN=Uppermost internode; and P=Immature inflorescence.

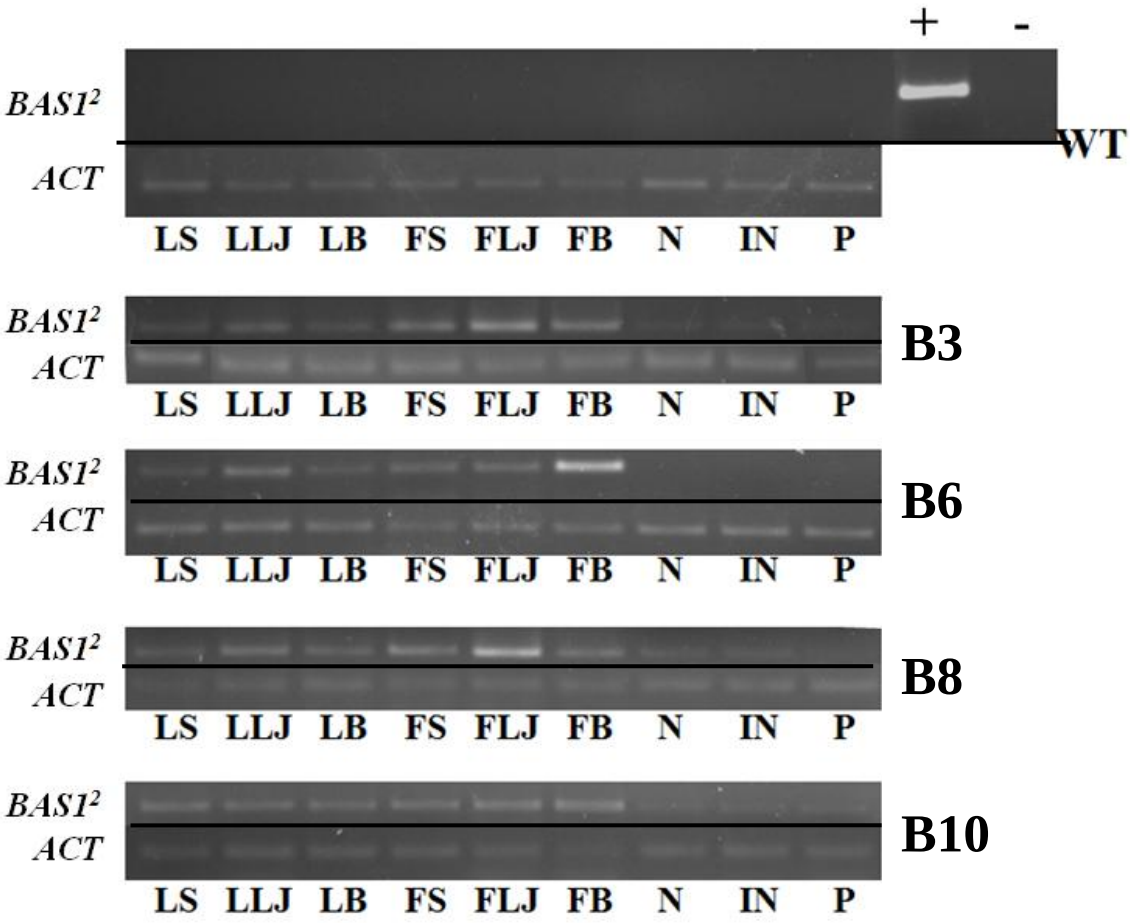

+
-
BAS1²
ACT
WT
LS LLJ LB FS FLJ FB N IN P
BAS1²
ACT
B3
LS LLJ LB FS FLJ FB N IN P
BAS1²
ACT
B6
LS LLJ LB FS FLJ FB N IN P
BAS1²
ACT
B8
LS LLJ LB FS FLJ FB N IN P
BAS1²
ACT
B10
LS LLJ LB FS FLJ FB N IN P

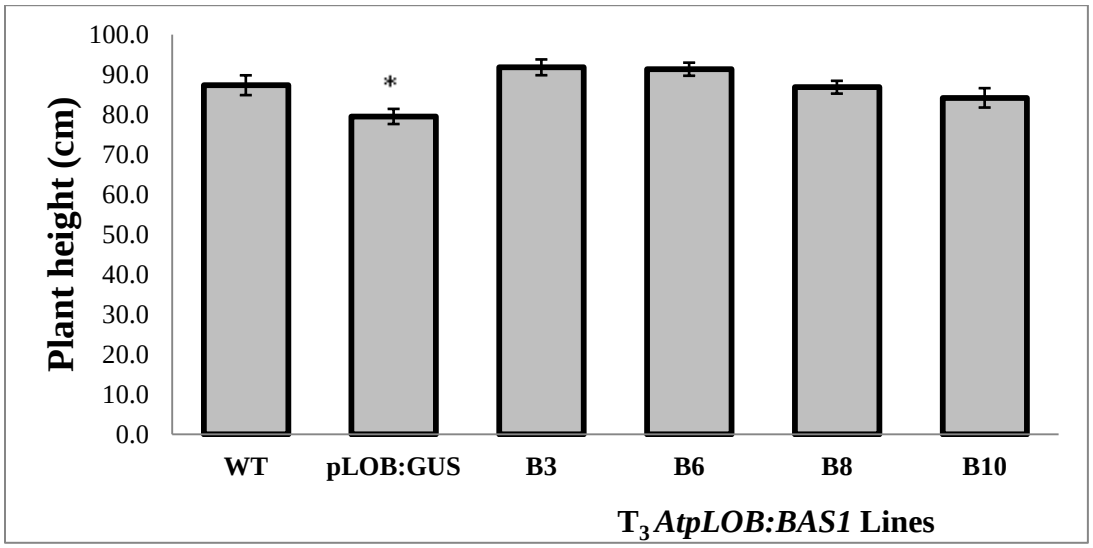
**Figure 2.5. Overall phenotypic characterization of homozygous $T_3$ *AtpLOB:BAS1* transgenic plants during the heading stage.**

A. Quantitative measurements of plant height.

Values are means ± SE (n=10-12 plants). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

B. Gross morphology of wild-type and *AtpLOB:BAS1* transgenic lines.

**A.**


Plant height (cm)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
*
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**B.**

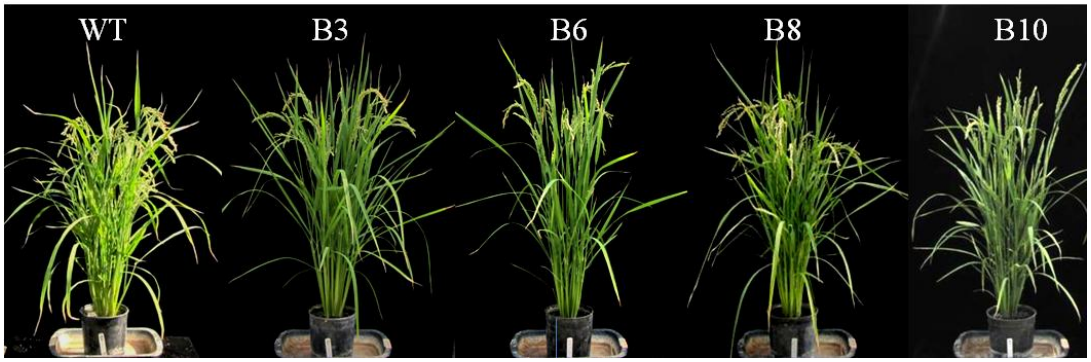

WT
B3
B6
B8
B10

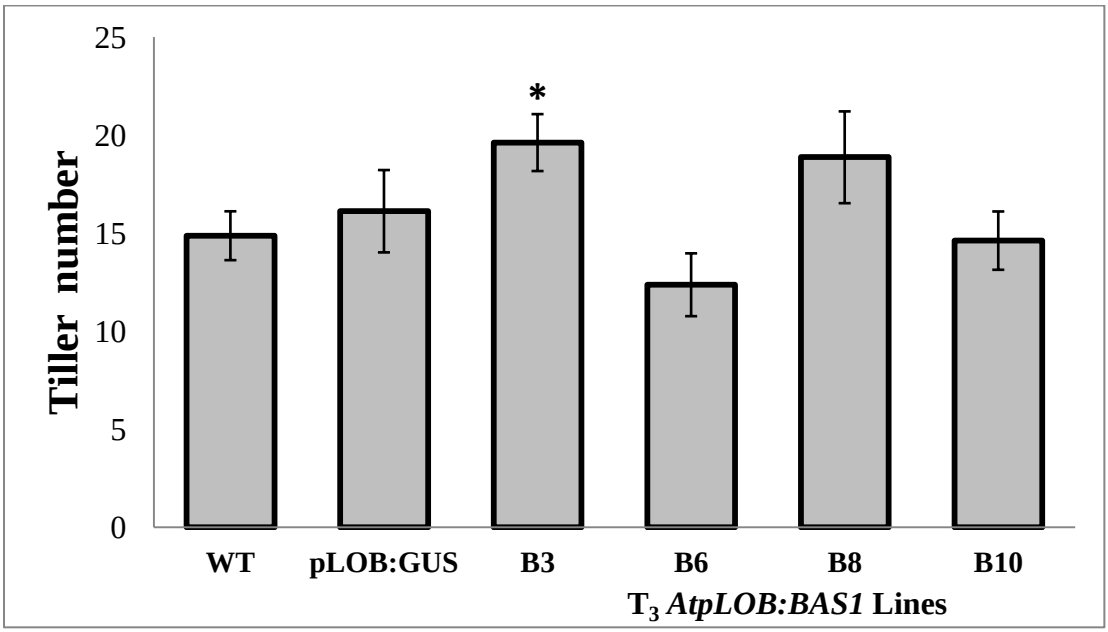
**Figure 2.6. Quantitative analysis of tiller number and tiller-to-tiller angle in homozygous $T_3$ *AtpLOB:BAS1* transgenic plants during the heading stage.**

A. Number of tillers number per plant. Values are means ± SE (n=8 plants).

B. Tiller to tiller angle. Tiller angle was measured between the outermost tiller on the left side and the outermost tiller on the right.

Values are means ± SE (n=10-12 plants). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

**A.**


25
20
15
10
5
0
Tiller number
*
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**B.**

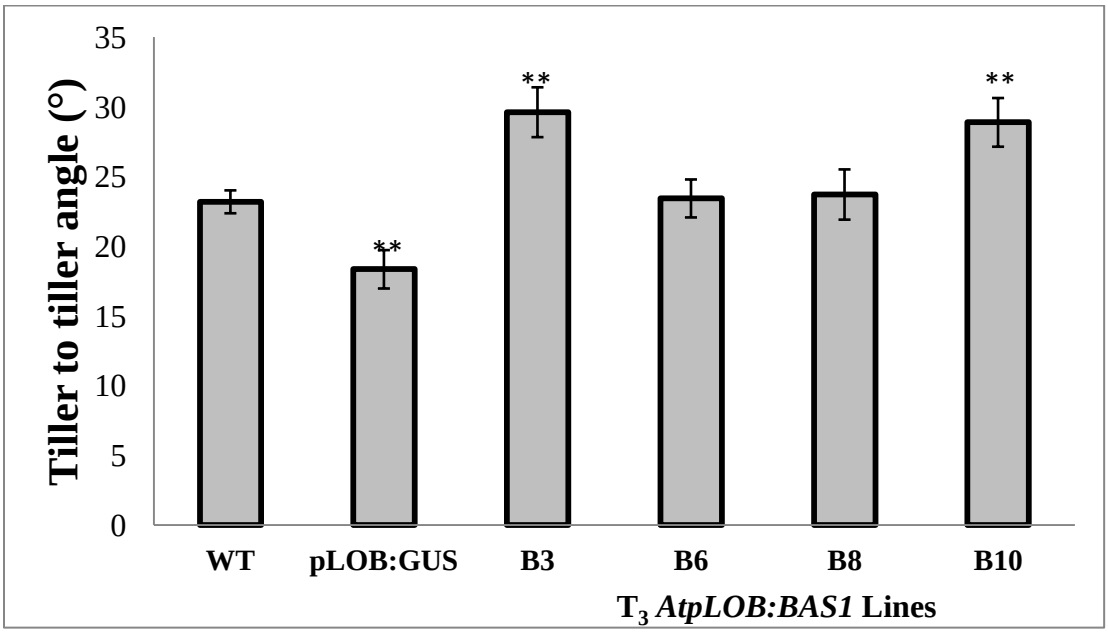
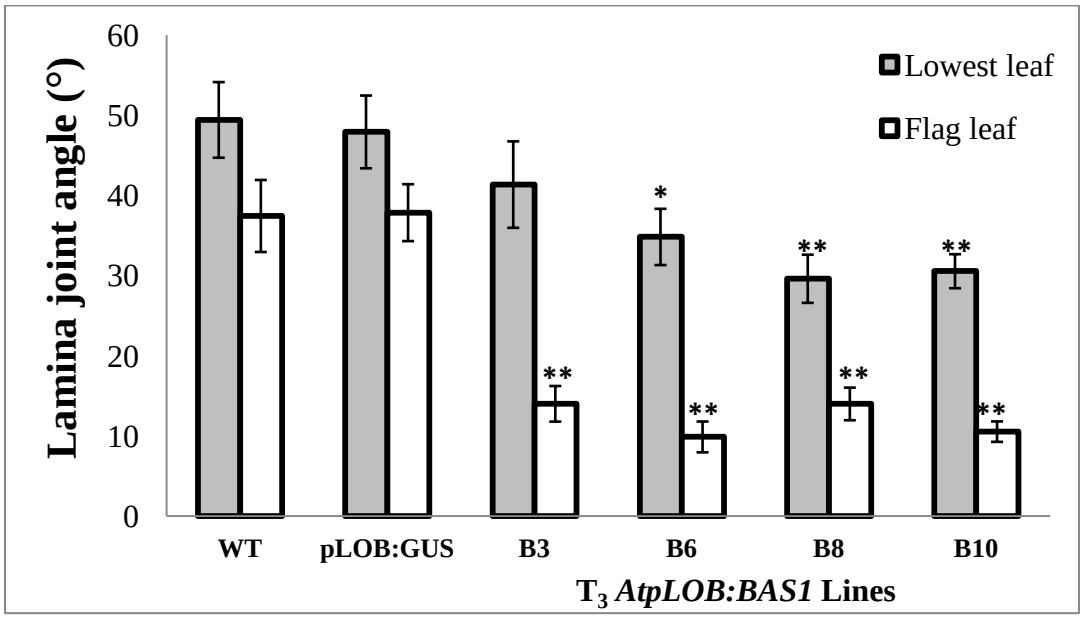

35
30
25
20
15
10
5
0
Tiller to tiller angle (°)
**
**
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**Figure 2.7. Characterization of the lamina joint in homozygous $T_3$ *AtpLOB:BAS1* transgenic plants during the heading stage.**

A. Quantification of the lamina joint angle of the lowest leaf and flag leaf on three random tillers per plant. Angles were measured from vertical using ImageJ. Values are means ± SE (n=10-12 plants, 3 tillers/plant). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

B-K. Close up view of the lowest lamina joint (B-F) and the flag leaf lamina joint (G-K) of wild-type and *AtpLOB:BAS1* transgenic lines.

**A.**


Lamina joint angle (°)
60
50
40
30
20
10
0
Lowest leaf
Flag leaf
*
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

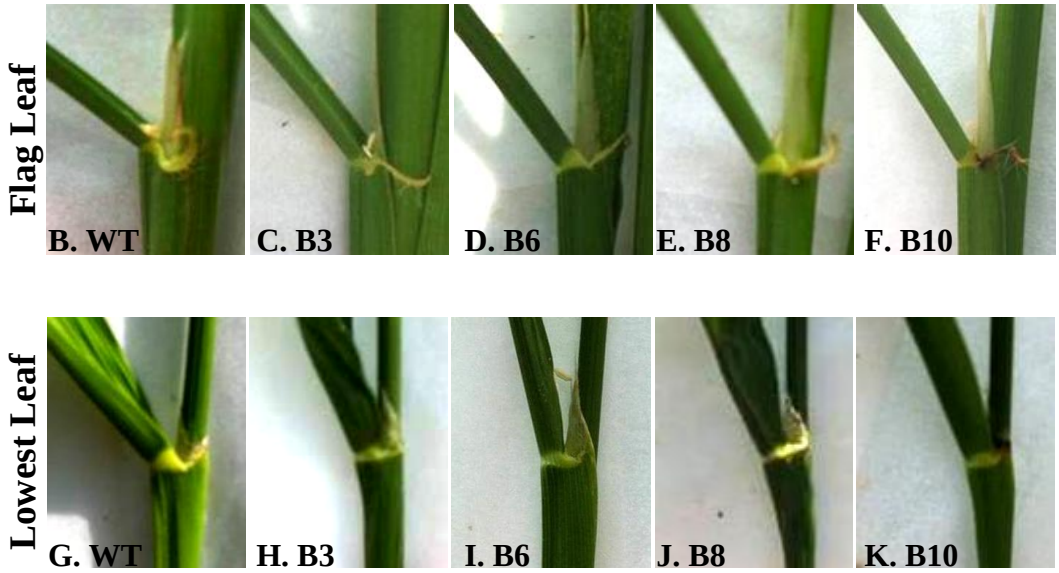

Flag Leaf
B. WT
C. B3
D. B6
E. B8
F. B10
Lowest Leaf
G. WT
H. B3
I. B6
J. B8
K. B10

**Figure 2.8. Quantitative characterization of the lowest leaves and flag leaves in homozygous $T_3$ *AtpLOB:BAS1* transgenic plants during the heading stage.**

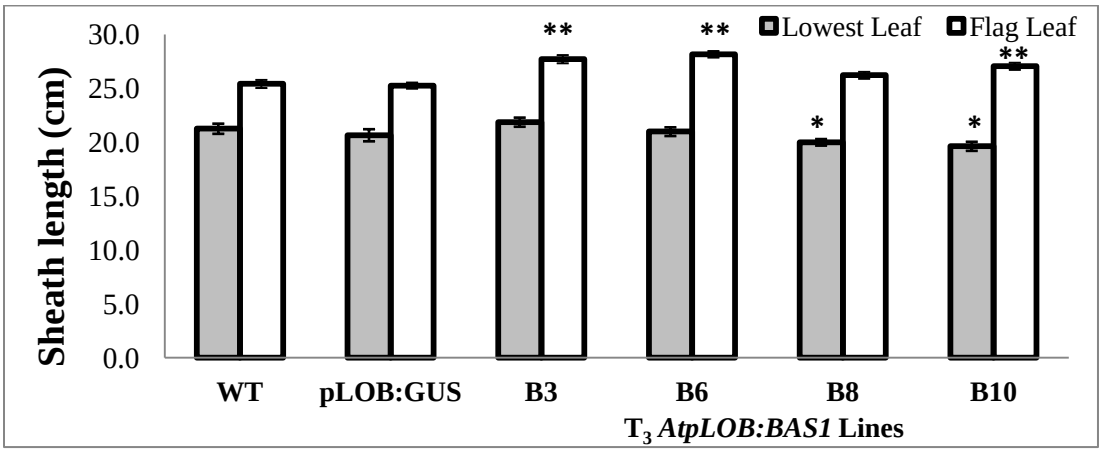
A. Leaf sheath lengths measured from the base of the *sheath* to the lamina joint.

B. Leaf blade lengths measured from the lamina joint to the tip of the blade.

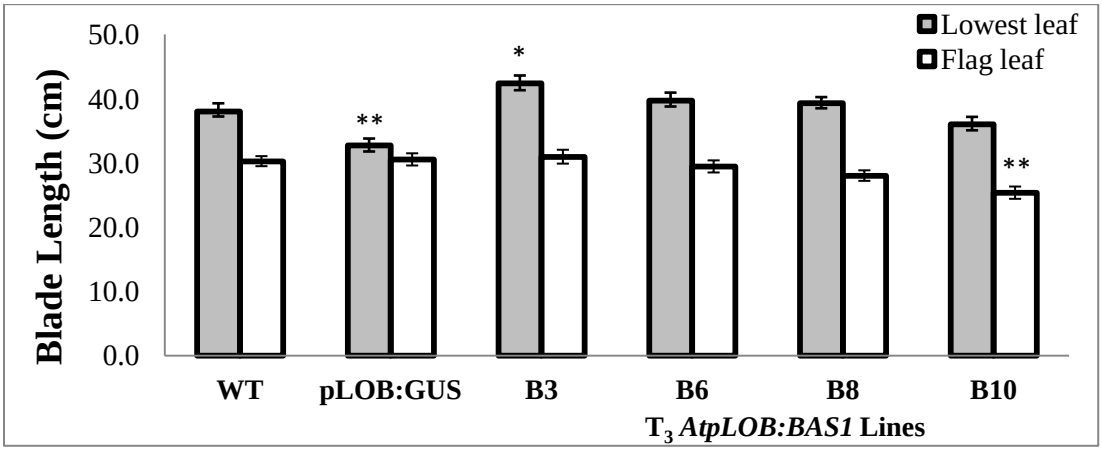
C. Leaf blade width measured from the widest part of the blade.

Values are means ± SE (n=10-12 plants, 3 tillers/plant). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

**A.**


Sheath length (cm)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Lowest Leaf
Flag Leaf
**
**
*
*
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**B.**


Blade Length (cm)
50.0
40.0
30.0
20.0
10.0
0.0
Lowest leaf
Flag leaf
**
*
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**C.**

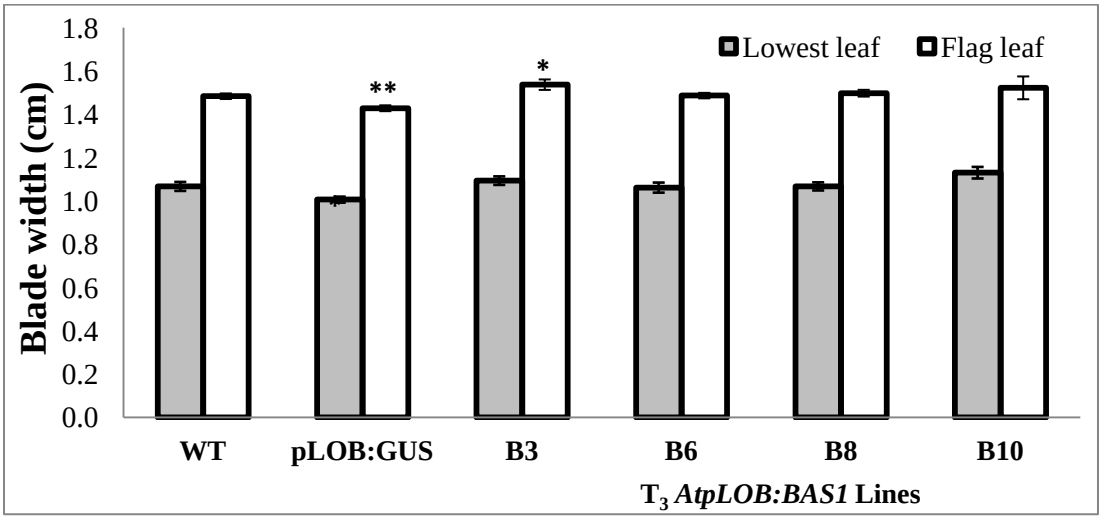

Blade width (cm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Lowest leaf
Flag leaf
**
*
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

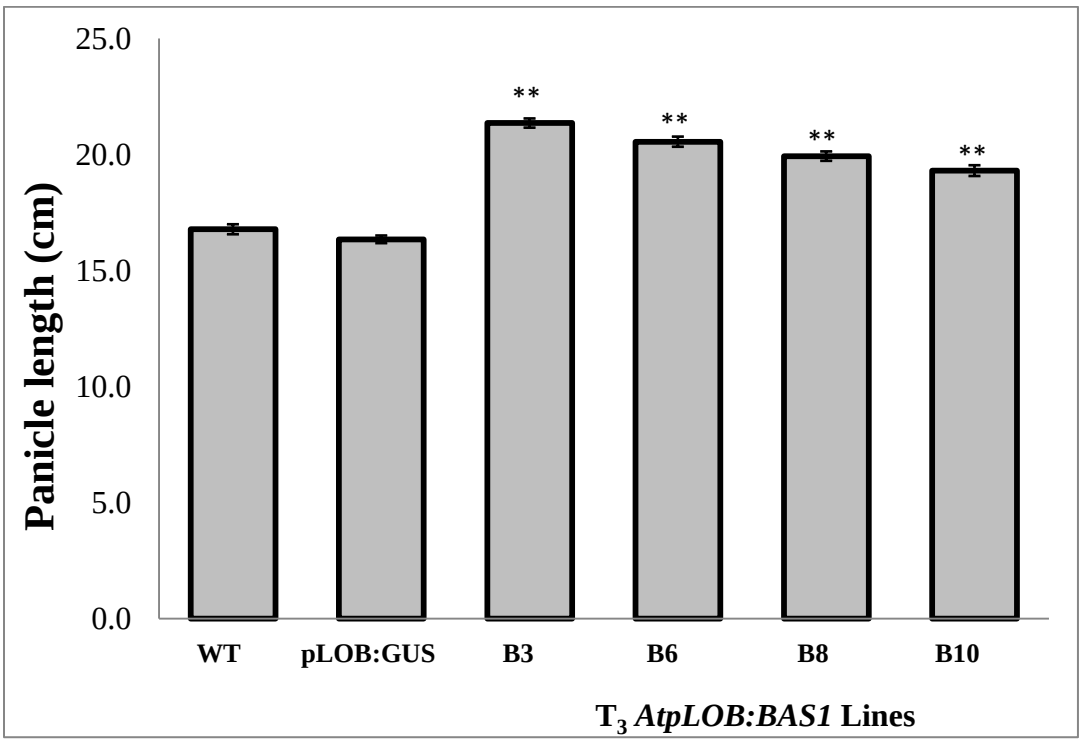
**Figure 2.9. Characterization of inflorescences in homozygous $T_3$ *AtpLOB:BAS1* transgenic plants during the heading stage.**

A. Quantitative measurement of panicle height measured from the node to the terminal spikelet of the degenerated tip of the rachis.

B. Number of grains per panicle.

Values are means ± SE (n=10-12 plants, 3 panicles/plant). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

**A.**


25.0
20.0
15.0
10.0
5.0
0.0
Panicle length (cm)
**
**
**
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**B.**

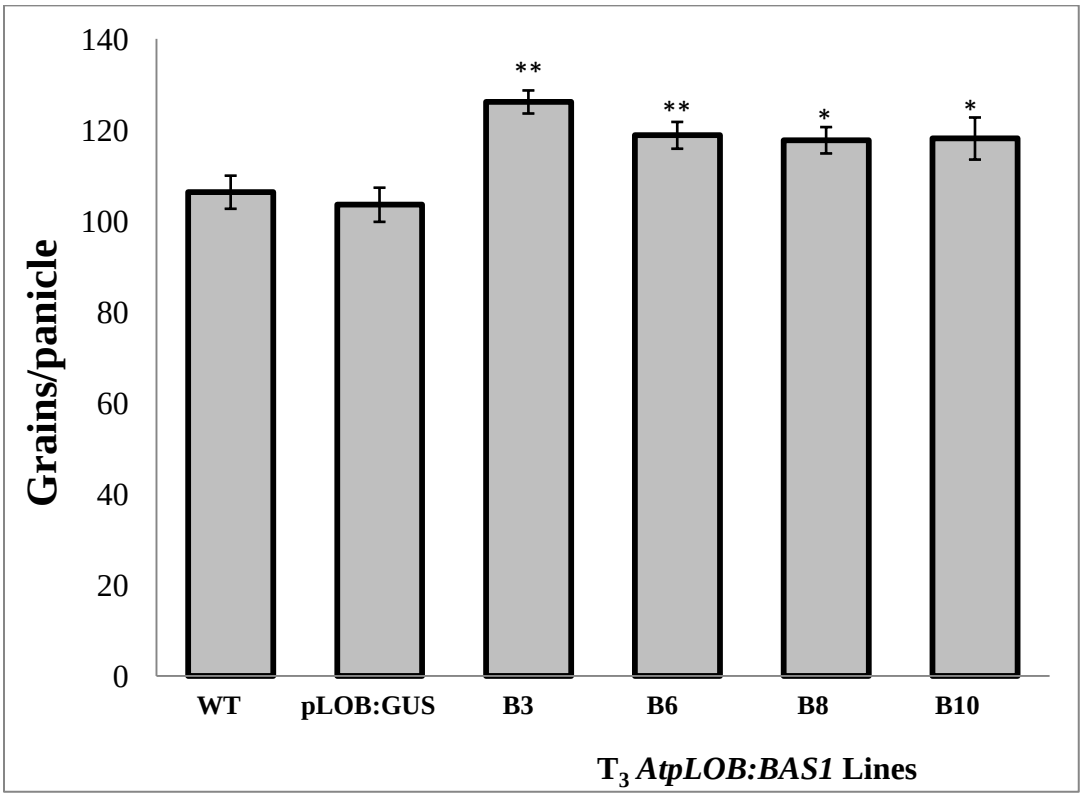

140
120
100
80
60
40
20
0
Grains/panicle
**
**
*
*
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**Figure 2.10. Grain characterization of homozygous $T_3$ *AtpLOB:BAS1* transgenic plants.**

A. Weight of 1,000 grains per plant.

B. Grain morphology.

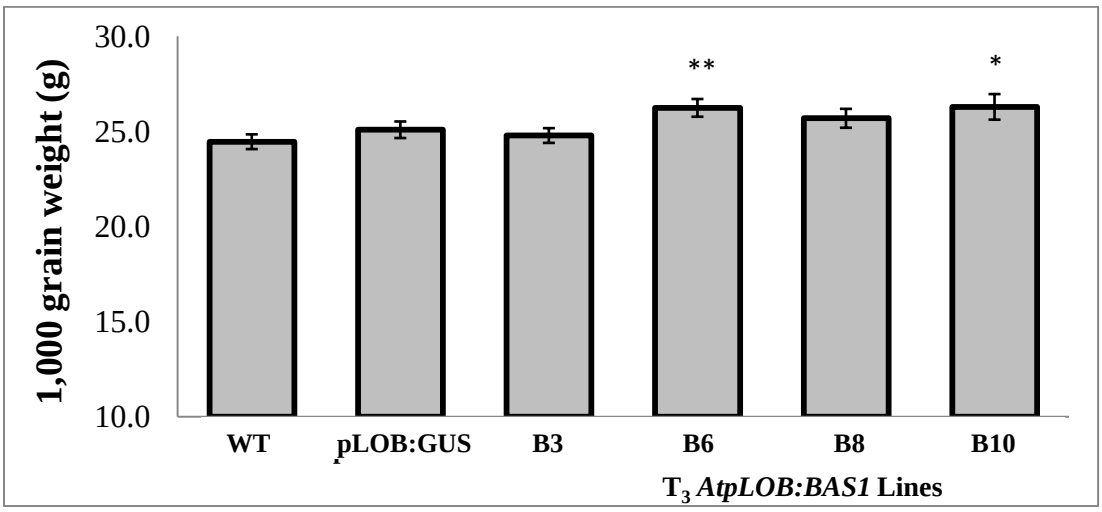
C. Grain length.

D. Grain width.

E. Length to width grain ratio

For A, values are means ± SE (n=10-12 plants). For C-E, values are means ± SE (n=10-12 plants, 30 grains/plant). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test. Scale bar = 2mm.

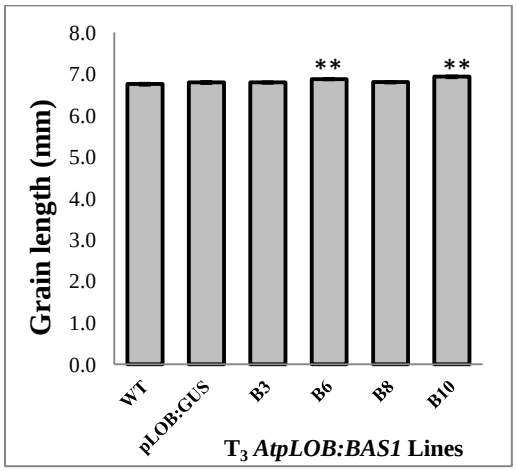
**A.**


1,000 grain weight (g)
30.0
25.0
20.0
15.0
10.0
**
*
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

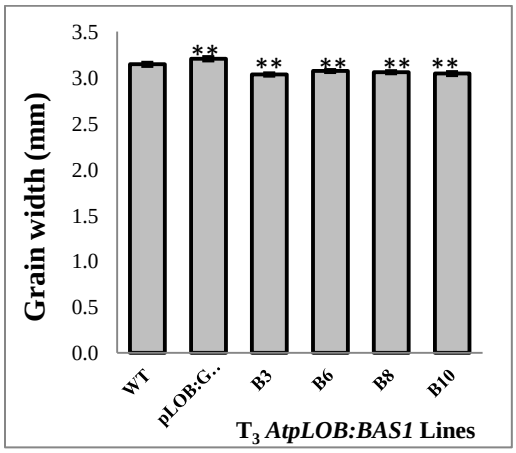
**B.**


WT
pLOB:GUS
B3
B6
B8
B10

**C.**


Grain length (mm)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**D.**


Grain width (mm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
**
**
WT
pLOB:G..
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**E.**

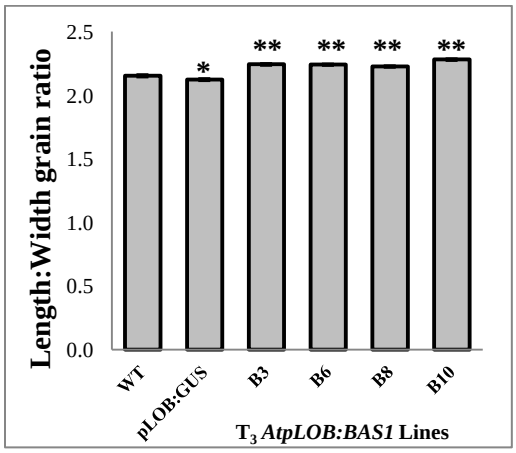

Length:Width grain ratio
2.5
2.0
1.5
1.0
0.5
0.0
*
**
**
**
**
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**Figure 2.11. Characterization of the main shoot during in homozygous $T_3$ *AtpLOB:BAS1* transgenic plants the heading stage.**

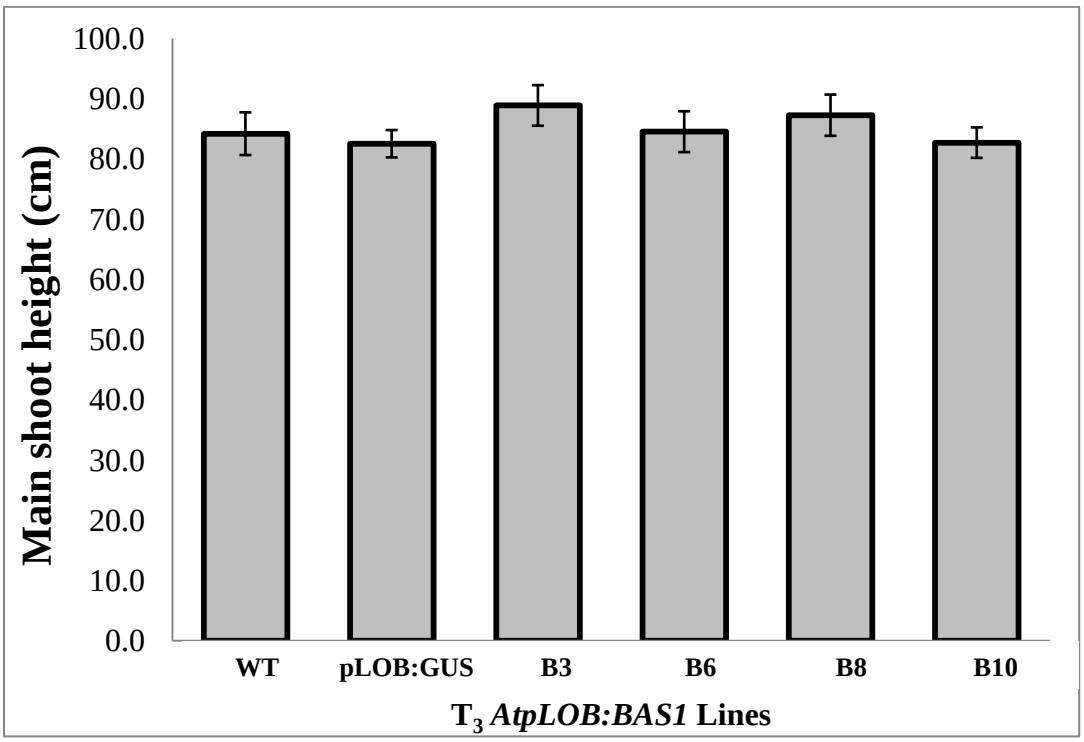
A. Main shoot height measured from the base of the shoot to the terminal spikelet of the degenerated tip of the rachis.

B. Internode elongation pattern of the main shoot (leaves have been removed). I to V indicated the five uppermost internodes.

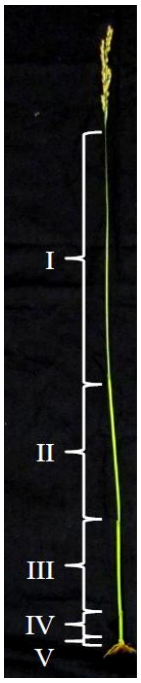
C. Internode elongation patterns as a percentage of the internodes on the main shoot. I (purple), II (green), III (red), IV (blue) and V (light blue) indicates the first, the second, the third, fourth and fifth internode, respectively.

Values are means ± SE (n=8 plants). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

**A.**


Main shoot height (cm)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**B.**


I
II
III
IV
V

**C.**

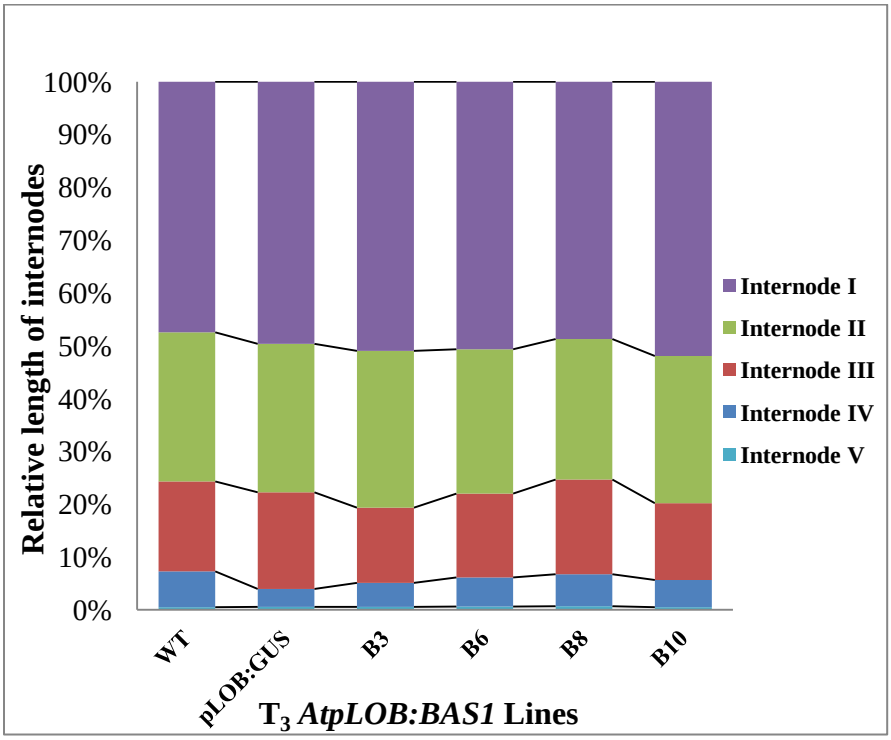

Relative length of internodes
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines
Internode I
Internode II
Internode III
Internode IV
Internode V

**Figure 2.12. Characterization of the main shoot inflorescence in homozygous $T_3$ *AtpLOB:BAS1* transgenic plants.**

A. Schematic representation of the inflorescence.

B. Quantitative measurement of the panicle height.

C. Length of primary rachis branches.

D. Internode lengths between eight primary branches (counting acroptetally) were measured and grouped into 5mm intervals

E. Number of lateral organs in the main inflorescence.

F. Length of pedicels on the lateral spikelets of the lowest two primary rachis branches.

G. Number of grains per main panicle.

P=Panicle; PB=Primary rachis branch; SB=Secondary rachis branch; LS=Lateral spikelet; TS=Terminal spikelet; PD=Pedicel; and DTP=Degenerative tip of the rachis. Values are means ± SE (n=8 plants). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

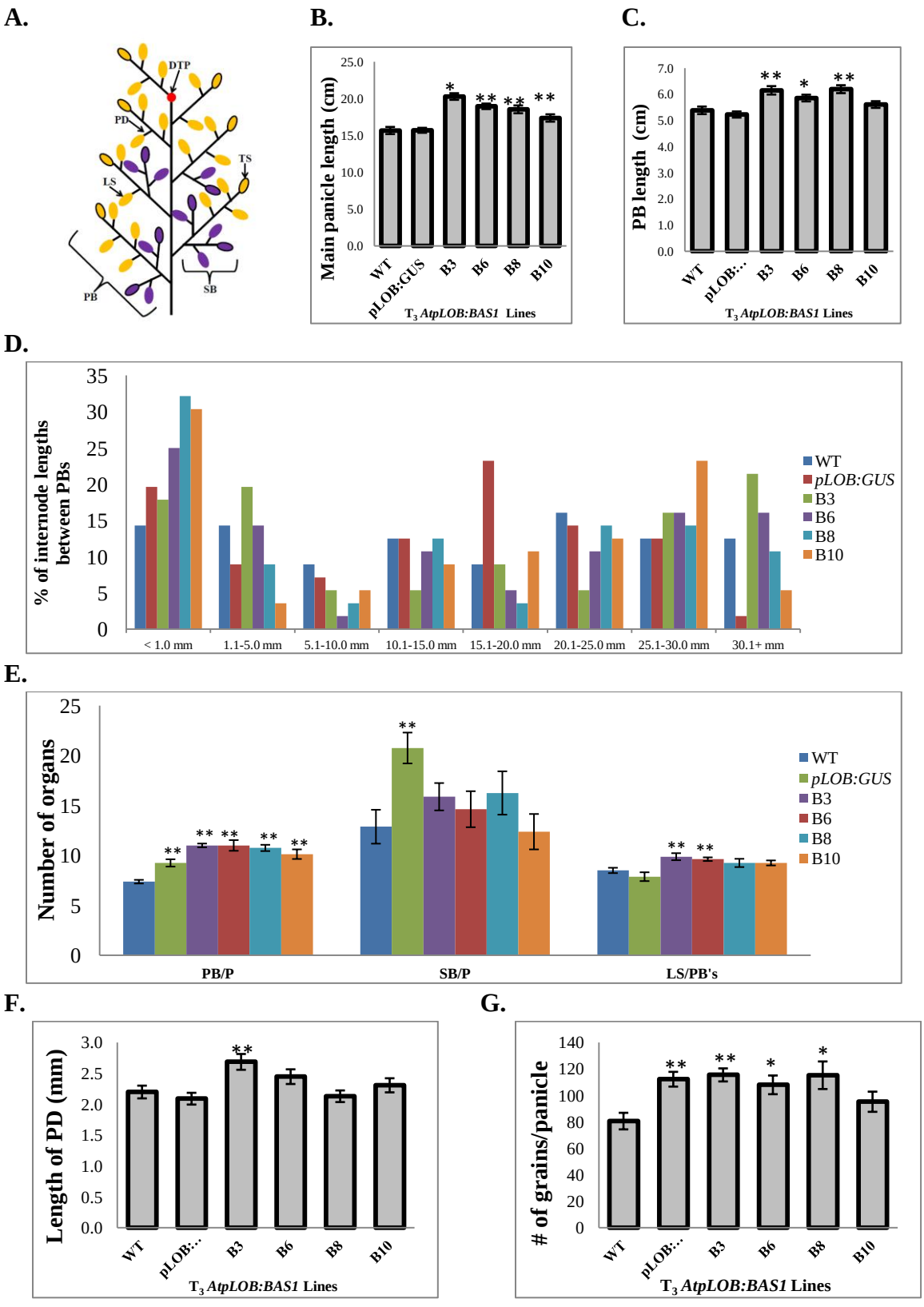

A.
DTP
PD
TS
LS
PB
SB
B.
Main panicle length (cm)
25.0
20.0
15.0
10.0
5.0
0.0
WT
pLOB:GUS
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines
C.
PB length (cm)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
WT
pLOB:…
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines
D.
% of internode lengths between PBs
35
30
25
20
15
10
5
0
< 1.0 mm
1.1-5.0 mm
5.1-10.0 mm
10.1-15.0 mm
15.1-20.0 mm
20.1-25.0 mm
25.1-30.0 mm
30.1+ mm
WT
pLOB:GUS
B3
B6
B8
B10
E.
Number of organs
25
20
15
10
5
0
PB/P
SB/P
LS/PB's
WT
pLOB:GUS
B3
B6
B8
B10
F.
Length of PD (mm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT
pLOB:…
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines
G.
# of grains/panicle
140
120
100
80
60
40
20
0
WT
pLOB:…
B3
B6
B8
B10
T3 AtpLOB:BAS1 Lines

**Figure 2.13. Comparison of 24-epiBL effect on etiolated lamina inclination in wild-type and in homozygous $T_3$ *AtpLOB:BAS1* transgenic plants.**

A dose response to 24-epiBL of the second lamina joint. Angles were measured from vertical using ImageJ.

Values are means ± SE (n>6 plants/concentration). Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

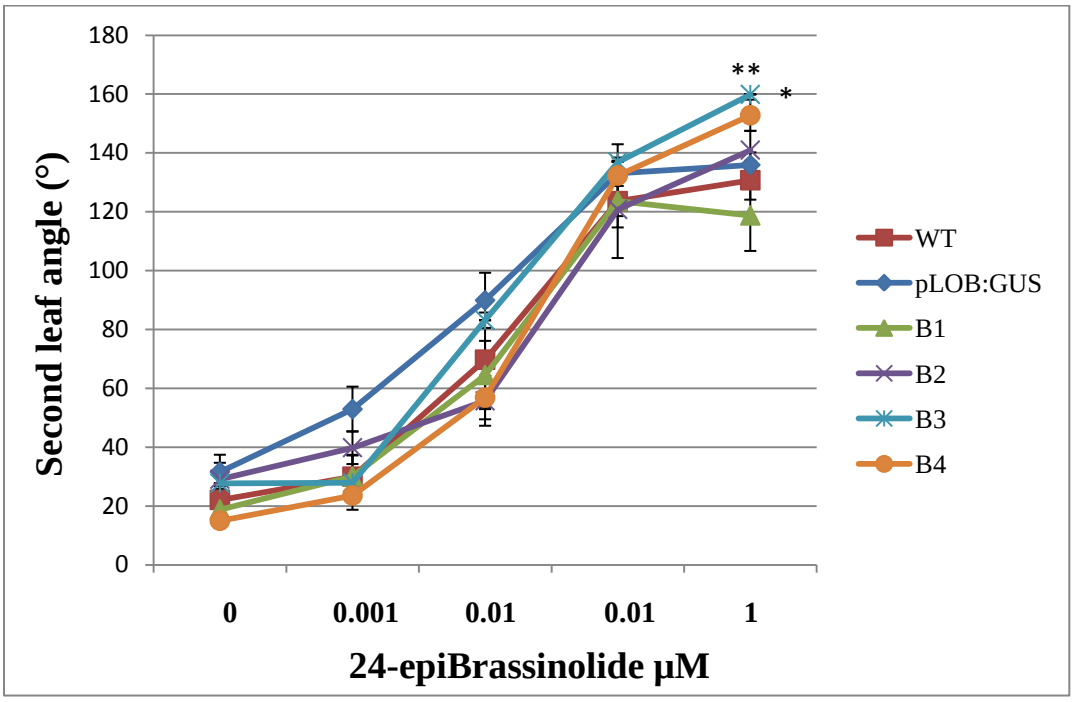

Second leaf angle (°)
180
160
140
120
100
80
60
40
20
0
**
*
0
0.001
0.01
0.01
1
24-epiBrassinolide µM
WT
pLOB:GUS
B1
B2
B3
B4

**Table 2.1. Oligonucleotide sequences**

| Purpose | Gene | Primer Name | Sequence (5'-3') |
|---|---|---|---|
| GENOTYPE | *HYG* | HYG FWD | GCCATGTAGTGTATTGACCGATT |
| GENOTYPE | *HYG* | HYG REV | AGTTTAGCGAGAGCCTGACCTAT |
| RT-PCR | *ACTIN* | ACT1 FWD | GCCGTCCTCTCTCTGTATGC |
| RT-PCR | *ACTIN* | ACT1 REV | GCCGTTGTGGTGAATGAGTA |
| RT-PCR | *BAS1*[1] | BAS1 FWD | GGCGGAGACAAAACGCTAT |
| RT-PCR | *BAS1*[1] | BAS1 REV | CGGTAGGTGCATGCTGATAA |
| RT-PCR | *BAS1*[2] | qBAS1 FWD | CAATCATAGCGGTCCATCAT |
| RT-PCR | *BAS1*[2] | qBAS1 REV | GGAGCCAAGTGAAAGGTGAA |

**Table 2.2. Quantitative measurements of WT, *pLOB:GUS*, and 4 independent $T_3$ homozygous *pLOB:BAS1* transgenic lines**

| | | | | | *AtpLOB:BAS1* independent lines | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **WT** | ***pLOB:GUS*** | **B3** | **B6** | **B8** | **B10** |
| **Seedling Stage** | | **Height (cm)** | 11.7 ± 0.4 | 11.1 ± 0.3 | 12.3 ± 0.5 | 14.5 ± 0.3** | 14.1 ± 0.4** | 13.3 ± 0.3** |
| | | **3rd Leaf Angle (°)** | 30.9 ± 3.4 | 20.1 ± 1.7 | 17.8 ± 3.6 | 18.9 ± 2.9 | 13.9 ± 2.4 | 21.3 ± 2.7 |
| | | **Root Length (cm)** | 4.9 ± 0.2 | 5.8 ± 0.2** | 4.5 ± 0.2 | 4.0 ± 0.1** | 5.0 ± 0.3 | 3.9 ± 0.1** |
| **Overall Heading Stage** | | **Height (cm)** | 87.3 ± 2.5 | 79.5 ± 1.9* | 91.8 ± 2.0 | 91.3 ± 1.6 | 86.8 ± 1.6 | 84.2 ± 2.4 |
| | | **Tiller Number** | 15 ± 1.2 | 16 ± 2.1 | 20 ± 1.5* | 12 ± 1.6 | 19 ± 2.3 | 15 ± 1.5 |
| | | **Tiller to Tiller (°)** | 23.2 ± 0.8 | 18.3 ± 1.4** | 29.6 ± 1.8** | 23.4 ± 1.4 | 23.7 ± 1.8 | 28.9 ± 1.7** |
| | **Lowest Leaf** | **Leaf Angle (°)** | 49.4 ± 4.7 | 47.9 ± 4.5 | 41.4 ± 5.4 | 34.8 ± 3.5* | 29.6 ± 3.0** | 30.6 ± 2.1** |
| | | **Sh Length (cm)** | 21.3 ± 0.5 | 20.6 ± 0.6 | 21.9 ± 0.4 | 21.0 ± 0.4 | 20.0 ± 0.3* | 19.6 ± 0.4* |
| | | **Bl Length (cm)** | 38.0 ± 1.3 | 32.7 ± 1.1** | 42.4 ± 1.2* | 39.7 ± 1.2 | 39.3 ± 0.9 | 36.0 ± 1.1 |
| | | **Bl Width (cm)** | 1.1 ± 0.02 | 1.0 ± 0.01* | 1.1 ± 0.02 | 1.1 ± 0.02 | 1.1 ± 0.02 | 1.1 ± 0.03 |
| | **Flag Leaf** | **Leaf Angle (°)** | 37.5 ± 4.5 | 37.9 ± 3.5 | 14.0 ± 2.2** | 9.9 ± 1.9** | 14.0 ± 2.0** | 10.6 ± 1.3** |
| | | **Sh Length (cm)** | 25.4 ± 0.4 | 25.3 ± 0.3 | 27.7 ± 0.4** | 28.2 ± 0.3** | 26.2 ± 0.3 | 27.0 ± 0.3** |
| | | **Bl Length (cm)** | 30.3 ± 0.8 | 30.5 ± 0.9 | 31.0 ± 1.1 | 29.4 ± 0.9 | 28.0 ± 0.8 | 25.3 ± 1.0** |
| | | **Bl Width (cm)** | 1.5 ± 0.01 | 1.4 ± 0.01** | 1.5 ± 0.02* | 1.5 ± 0.01 | 1.5 ± 0.02 | 1.5 ± 0.05 |
| | | **P Length (cm)** | 16.8 ± 0.2 | 16.3 ± 0.2 | 21.4 ± 0.2** | 20.6 ± 0.2** | 19.9 ± 0.2** | 19.3 ± 0.2** |
| | | **# of Grains/P** | 106.3 ± 3.6 | 103.6 ± 3.8 | 126.2 ± 2.5** | 118.8 ± 2.9** | 117.8 ± 2.9* | 118.1 ± 4.6* |
| | | **1,000 Grains (g)** | 24.5 ± 0.4 | 25.1 ± 0.4 | 24.8 ± 0.4 | 26.2 ± 0.5** | 25.7 ± 0.5 | 26.3 ± 0.7* |
| | | **Grain Length (mm)** | 6.75 ± 0.02 | 6.79 ± 0.03 | 6.79 ± 0.02 | 6.87 ± 0.02** | 6.80 ± 0.02 | 6.93 ± 0.02** |
| | | **Grain Width (mm)** | 3.14 ± 0.01 | 3.21 ± 0.01** | 3.03 ± 0.01** | 3.07 ± 0.01** | 3.06 ± 0.01** | 3.04 ± 0.01** |
| | | **Grain L:W** | 2.15 ± 0.01 | 2.12 ± 0.01* | 2.24 ± 0.01** | 2.24 ± 0.01** | 2.23 ± 0.01** | 2.80 ± 0.01** |
| **Main Shoot** | | **Height (cm)** | 84.2 ± 3.5 | 82.5 ± 2.3 | 88.9 ± 3.4 | 84.5 ± 3.4 | 87.3 ± 3.4 | 82.7 ± 2.5 |
| | | **P Length (cm)** | 15.7 ± 0.5 | 15.7 ± 0.3 | 20.3 ± 0.4** | 19.0 ± 0.4** | 18.5 ± 0.5** | 17.4 ± 0.5** |
| | | **PB Length (cm)** | 5.39 ± 0.1 | 5.23 ± 0.1 | 6.15 ± 0.2** | 5.86 ± 0.1* | 6.19 ± 0.2** | 5.61 ± 0.1 |
| | | **# of PB/P** | 7.4 ± 0.2 | 9.3 ± 0.4** | 11.0 ± 0.2** | 11.0 ± 0.5** | 10.8 ± 0.3** | 10.1 ± 0.5** |
| | | **# of SB/P** | 12.9 ± 1.7 | 20.8 ± 1.5* | 15.9 ± 1.4 | 14.6 ± 1.8 | 16.3 ± 2.2 | 12.4 ± 1.8 |
| | | **# of LS/PB** | 8.5 ± 0.3 | 7.9 ± 0.4 | 9.9 ± 0.4** | 9.6 ± 0.2** | 9.3 ± 0.4 | 9.3 ± 0.3 |
| | | **Length of PD** | 2.2 ± 0.10 | 2.1 ± 0.10 | 2.7 ± 0.13** | 2.4 ± 0.12 | 2.1 ± 0.09 | 2.3 ± 0.11 |
| | | **# of Grains/P** | 80.6 ± 6.3 | 112.3 ± 5.6** | 115.5 ± 4.9** | 108.0 ± 7.1* | 115.3 ± 10.4* | 95.3 ± 7.6 |

Sh=Sheath; Bl=Blade; P=Panicle; PB=Primary rachis branch; SB=Secondary rachis branch; LS=Lateral spikelet; and PD=Pedicel.

Values are means ± SE. Significant differences were identified at the 5% (*) and 1% (**) probability levels using Student's *t*-test.

## Chapter 3

## Genome-Wide Analysis of the *Lateral Organ Boundaries* Domain Gene Family and Characterization of *LOB* Orthologs in *Oryza sativa*


### Abstract

Previous studies have identified the rice *LATERAL ORGAN BOUNDARIES* domain (*LBD*) gene family, which comprises 35 members encoding transcription factors that share a conserved DNA-binding domain located at the N-terminal region. *LOB*, the founding member of this gene family in *Arabidopsis*, is expressed in the organ boundaries and regulates organ separation, in part, by modulating the accumulation of Brassinosteroids (BR). In this study, an independent genome-wide analysis was conducted on the rice *LBD* gene family. Experimental evidence from publicly available expression data was gathered to validate the reported annotation and identify additional *OsLBD* genes. Through phylogenetic analysis, class Ia *OsLBD* genes, *OsRAMOSA2* (*OsRa2*), *OsINDETERMINATE GAMETOPHYTE1* (*OsIG1*), *OsLOB4*, and *IG1/AS2-LIKE1* (*OsIAL1*) were further analyzed based on their high sequence similarity to AtLOB protein sequence. This analysis can in part determine which OsLBD proteins are most likely to be orthologous to AtLOB. Given that only the LOB-domain is conserved among the LBD proteins, phylogenetic criteria may be insufficient to identify rice LOB orthologs. Because *AtLOB* is expressed in organ boundaries and is BR regulated, class Ia *OsLBD*s were examined for both pattern of expression and possible BR regulation using publicly available expression data. Class Ia *OsLBDs* were found to be expressed in

lamina joints, inflorescences, and floral organs, suggesting that these class Ia *OsLBD*s may have similar functions. Using BR-hormone response data, class Ia *OsLBD*s do not appear to be regulated by BR, suggesting that this feature may not be conserved among these genes. Characterization of *OsRA2* and other closely related *OsLBD* genes may allow us to identify new genes involved in the control of shoot architecture.

**Introduction**

Proper meristem maintenance and the formation of lateral organs require the coordination of various processes in order for patterning and proliferation to happen correctly (3). To maintain proper meristem fate, *KNOX* (*KNOTTED1-like homeobox*) genes, such as *SHOOTMERISTEMLESS* (*STM*), are expressed in the shoot apical meristem, (SAM) and are down-regulated in the lateral organ primordia (17). The boundary between the SAM and organ primordia plays a significant role in the regulation of plant architecture (26). This boundary serves as a barrier to isolate population of cells within their distinct domains. It is a region of limited growth, containing slowly dividing cells that are smaller than cells in adjacent regions and that have distinct gene expression (26). Several boundary-specific genes have been identified and shown to play a role in organ separation and meristem maintenance (26).

The *LATERAL ORGAN BOUNDARIES* (*LOB*) domain (*LBD*) genes encode a family of plant-specific transcription factors that share a conserved 100 amino-acid domain located at the N-terminal region responsible for DNA-binding (Fig. 3.1A) (29). Within this domain, the conserved region contains a zinc finger-motif and a Leucine

zipper-like coiled-coil motif that functions in protein-protein interactions (10; 14; 29). In *Arabidopsis*, the *LBD* gene family is comprised of 43 members that can be separated into two classes according to the structure of the LOB-domain (29). Class I LBD proteins include 37 *Arabidopsis* genes, which contain a leucine-zipper-like domain, whereas class II LBD proteins are composed of six *Arabidopsis* genes, which have an incomplete leucine-zipper-like domain (14; 20; 29). This difference between the two classes suggests that while class I LBD proteins may function in protein-protein interactions, class II proteins, which lack the coiled-coil motif, may have functions that are distinct from class I (29).

In *Arabidopsis*, *LOB* is expressed in the boundary between the SAM and initiating lateral organs (29). It regulates organ separation in the boundary, in part, by modulating the accumulation of BRs, a group of plant steroid hormones that regulate cell division and expansion (1; 5). *lob* mutants have defects in organ separation causing a subtle fusion between organs (1). Phylogenetic analyses positioned *LOB* in a clade that included *ASYMMETRIC LEAVES2 (AS2)*, *LBD10*, *LBD25/DOWN IN DARK AND AUXIN1 (DDA1)*, and *LBD36/ASYMMETRIC LEAVES2-LIKE1 (ASL1)* (20; 22). Despite their close phylogenetic relationship, these genes exhibit different patterns of expression and have largely distinct functions in plant development (20; 22). *AS2* functions in the specification of leaf organ polarity and floral development (12; 15; 32; 33). Mis-expression of *AS2* results in adaxialization of the abaxial tissue and *as2* mutants have downward curled leaves with asymmetric leaf lobes as well as altered floral organs (15; 25; 28; 33). *LBD10* was recently characterized and found to regulate pollen development

(13). *lbd10* mutants displayed defects in male gametophytes, which lead to an increase in aborted pollen and reduced seed set (13). *DDA1* functions in auxin response and photomorphogenesis (21). *dda1* mutants exhibited weak phenotypes and had a diminished response to auxin and when grown in the dark (21). *AtASL1* functions in the control of cell fate determination in petal development (4). It has been shown that *AtASL1* and *AS2* act redundantly to establish floral organ boundaries (4). In *asl1* loss-of-function mutants, there were no visible changes in phenotype compared to wild-type (4). In contrast, *asl1 as2* double mutants had defects in proximal–distal patterning in *Arabidopsis* petals and sepals, causing them to curl outward (4). In *asl1 as2* double mutants, sepals were narrower exposing the inner floral organs in contrast to wild-type sepals, which completely enclose the buds (4).

The maize genome encodes 43 LOB-domain proteins (38). Based on phylogenetic studies, the putative *LOB* ortholog in maize is *Ramosa2* (*ZmRA2*), which controls inflorescence architecture (2). *ra2* mutants have defects in tassel branching with short determinate branches replaced by an increased number of long, indeterminate, upright branches (2). Based on high amino acid sequence similarity with the N-terminal region of AtAS2, its maize ortholog is *INDETERMINATE GAMETOPHYTE1* (*ZmIG1*) (6). Similar to the *Arabidopsis as2* mutants, the maize *ig1* mutants have abnormal leaves with defects in organ polarity as well as abnormal floral development (6).

Previous studies identified 35 LOB-domain genes in the rice genome (36). Phylogenetic analysis of OsLBD amino acid sequences classified the *LBD* genes into three classes (36). The Class I *LBD* group contains 29 members and is characterized by

the presence of a Leucine-zipper-like domain, while class II members have no Leucine-zipper-like domain (14; 36). Class III, which have so far only been reported in rice, has only one member containing two LOB-domains (36). While the members of the *OsLBD* gene family have been identified through the presence of the LOB-domain, few *OsLBD* genes have been functionally characterized. The first rice *LBD* to be characterized is *Crownrootless1* (*CRL1*)/*Adventitious rootless1* (*ARL1*), which is a target of auxin signaling and involved in the formation of crown roots (11; 16). *crl1/arl1* mutants have defects in the formation of crown roots and a reduced number of lateral roots on seminal roots (11; 16). The orthologs of *CRL1* are *ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS (RTCS)* in maize and *LBD29* in *Arabidopsis*, and both are involved in root formation (7; 9; 29; 30; 34). The maize *RTCS* also plays a role in crown root and lateral root development and *rtcs* mutants completely lack formation of crown and lateral seminal roots (9). *LBD29* is expressed in roots and involved in lateral root formation. *LBD29* overexpression results in an increase in lateral roots whereas *lbd29* mutants have a decrease in the number of lateral roots (7; 29).

*OsIG1/OsAS2*, the ortholog of *AtAS2* and *ZmIG1*, is required for shoot differentiation, lateral growth of leaf development, and floral development, functions that are conserved with *AtAS2* and *ZmIG1* (18; 37). *OsIG1*-RNAi lines are dwarfed with wider-angled leaves that had wrinkled blades caused by alterations in bulliform cell division and differentiation, and defects in floral organ development (37). Overexpression of *OsIG1* results in inhibited shoot differentiation and abnormal leaves that twist upward and lack auricles (18). *Arabidopsis* plants mis-expressing *AtAS2*

exhibited altered leaf morphology, while *Atas2* mutants have downward curled leaves as well as altered floral organs (15; 25; 28; 33). The characterization of *OsIG1* suggests that the functions are conserved in the establishment of leaf morphology and floral development. While both *Atas2* and *Osig1*mutants have abnormal leaf development, *OsIG1* is expressed throughout the leaf and does not alter adaxial/abaxial polarity, whereas *AtAS2* expression is only expressed on the adaxial region (12; 15; 19; 32; 33). This suggests divergent roles between *AtAS2* and *OsIG1* in leaf development. Based on phylogenetic analyses, *OsRA2* is thought to be orthologous to *LOB* and *ZmRA2* (2). *AtLOB* functions in the organ boundaries and regulates BR accumulation and *ZmRA2* functions in controlling inflorescence architecture, which affects tassel branch angles. The function of *OsRA2* is unknown (1; 2; 29). Based on functional conservation of other *LBD* family members in different plant species, it is reasonable to hypothesize that *LOB* and *OsRA2* will have conserved functions.

To date, genome-wide analyses have been conducted for maize, *Arabidopsis*, apple, and rice *LBD* gene families and their evolutionary comparisons have been examined (29; 31; 36; 38). In this study, an independent genome-wide analysis of class I *OsLBD* genes was conducted. This independent analysis of rice *LBD* genes allowed identification of all 35 of the previously reported LBD proteins and 3 additional class I LBD proteins that were not previously reported. Each class I LBD annotation was confirmed and edited using available experimental evidence from publicly available expression data. Phylogenetic analyses of class I *LBD* genes confirmed four *OsLBD* genes to be the most closely related to *AtLOB*: *OsRAMOSA2 (OsRA2)*,

*OsINDETERMINATE GAMETOPHYTE1 (OsIG1)*, *OsLOB4*, and *IG1/AS2-LIKE1 (OsIAL1)*. While the OsLBD proteins share a conserved DNA-binding domain at the N-terminal region, the variable C-terminal region can give these genes diverse functions making it difficult to identify *AtLOB* orthologs solely on the basis of phylogenetic analyses. The expression patterns of *OsRA2*, *OsIG1*, *OsLOB4*, and *OsIAL1* and possible regulation by BR were also examined using publicly available expression data. Class Ia *OsLBDs* were expressed in lamina joints, inflorescences, and floral organs. Using BR hormone response data from seedlings treated with BL, none of the class Ia genes were regulated in response to exogenous BR suggesting BR regulation is not conserved among *AtLOB* and class Ia *OsLBD*s.

## Material and Methods

### Identification of *OsLBD* genes in Rice

Japonica rice (Nipponbare) *Oryza sativa LBD* genes were identified by using the *Arabidopsis* LOB amino acid sequence in a protein search query using the protein Basic Local Alignment Search Tool (BLASTP) on two rice genomic sequence databases: NCBI (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MSU Rice Genome Annotation Project (TIGR, http://rice.plantbiology.msu.edu/) with an e-value cutoff of $4e^{-04}$. NCBI and MSU TIGR had a different locus identifier for each *LBD* gene, so protein sequences between the two databases were matched using protein pairwise alignment bl2seq (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Experimental

evidence was gathered from expressed sequence tags (ESTs) and full-length cDNA (FL-cDNAs) using NCBI's UNIGENE program (http://www.ncbi.nlm.nih.gov/unigene/) and MSU Rice Genome Browser (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/) to confirm the protein sequence for every identified *OsLBD* gene.

**Sequence alignment and phylogenetic analysis**

Class I OsLBD amino acid sequences were aligned using CLUSTALW in GeneDoc (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html) with a gap-opening penalty 10 and a gap extension penalty 0.2. The alignment was hand edited and shaded using the GeneDoc Program (http://genedoc.software.informer.com/). A phylogeny was constructed using the LOB-domain of the class I OsLBD proteins using the neighbor-joining (NJ) method in MEGA6 (molecular evolutionary genetics analysis, (http://www.megasoftware.net/). The reliability of the tree was tested using a bootstrapping method with 1000 replicates.

**Expression profile analysis of class I *OsLBD* genes.**

Microarray expression and RNA-seq data for class I *OsLBD* genes were gathered from multiple datasets using the MSU TIGR gene ID as a query search. Data from GENEVESTIGATOR (https://genevestigator.com/gv/plant.jsp), Rice Oligonucleotide Array Database (http://www.ricearray.org/), Bio-Analytic Resource Electronic Fluorescent Pictographs (BAR eFP, http://bar.utoronto.ca/welcome.htm), RNA-seq data

(http://rice.plantbiology.msu.edu/expression.shtml), and Rice Expression Profile Database (RiceXPro, http://ricexpro.dna.affrc.go.jp/) programs were collected.

## Results

### *In silico* analysis of the *OsLBD* gene family.

In a previous study, 35 *LOB-DOMAIN* (*LBD*) genes in the rice genome were identified based on the presence of the conserved LOB-domain (Fig. 3.1) (36). In this study, an independent analysis of *OsLBD* genes was conducted to validate the reported annotation and determine if additional *OsLBD* genes might be identified. Using two *Oryza sativa* genome databases, NCBI (National Center for Biotechnology Information), http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MSU Rice Genome Annotation Project (TIGR, http://rice.plantbiology.msu.edu/), a pBLAST search using the *Arabidopsis* LOB protein sequence as a query was conducted to identify proteins containing a LOB-domain (Fig. 3.1B). All 35 of the previously reported *OsLBD* genes and three new *OsLBD* genes (*LBD1-13*, *LBD2-2*, and *LBD4-1*) that were not previously reported were identified.

The rice *LBD* genes are separated into three classes by the presence (class I) or absence (class II) of the Leucine zipper-like coiled-coil motif, or presence of two LOB-domains (Fig. 3.1) (29; 36). A database of the 29 class I, 5 class II, 1 class III, and the 3 new *OsLBD* genes (*LBD1-13*, *LBD2-2*, and *LBD4-1*) was created containing their genomic, coding, and protein sequences (Table 3.2). Sequence analysis of *LBD1-13*, *LBD2-2*, and *LBD4-1* reveals that they have an incomplete LOB-domain (data not

shown). *LBD1-13* contains both the C-block motif and the Leucine zipper-like coiled-coil motif, but lacks the GAS block motif. *LBD2-2* has a missing C-block motif but contains a partial GAS block motif and the full Leucine zipper-like coiled-coil motif. *LBD4-1* contains a partial LOB-domain, missing the zinc finger-motif but contains the Leucine zipper-like coiled-coil motif. Based on the classification of class I and II groups, *LBD1-13*, *LBD2-2*, and *LBD4-1* are categorized as class I due to the presence of the Leucine zipper motif. Expression data gathered from various databases indicates that *LBD1-13*, *LBD2-2*, and *LBD4-1* are expressed throughout the plant at very low levels suggesting they are not likely to be pseudogenes.

**Sequence alignment and phylogenetic analysis of class I *OsLBD* genes.**

To validate the reported annotation for class I *OsLBD* genes, experimental evidence from publicly available expression data was collected (Table 3.2). Experimental evidence of transcripts, ESTs and full-length cDNA sequences was gathered from various databases. Using this information, an alignment using amino acid sequences for AtLOB and class I OsLBD proteins was carried out to identify the three motifs within the LOB-domain (Fig. 3.1B and 3.2). The first motif in the LOB-domain consists of the C-block containing four conserved Cys residues $CX_2CX_6CX_2C$. *LBD1-3*, *LBD1-6*, and *LBD8-1* deviated from this motif slightly by containing seven amino acids between the second and third Cys residue, a configuration that was not found among *Arabidopsis*, maize or *Malus domestica* LBD proteins (31; 36; 38). The second motif, the GAS block, was identified starting with a $FX_6FG$ motif and ending with a $DPX_2G$

motif. The Leucine zipper-like coiled-coil motif, $LX_6LX_3LX_6L$, was identified at the end of the LOB-domain in all 32 Class I LBDs. LBD1-7 was the only protein with 16 amino acids between the C-block and GAS block, while other genes had 1 to 5 amino acids between them. LBD8-1 contained five more amino acids within the GAS block while LBD8-3 had a 25 amino-acid deletion within this region. Due to the incomplete LOB-domain of *LBD1-13*, *LBD2-2*, and *LBD4-1*, these genes were omitted from the sequence alignment.

To determine which OsLBD class I proteins are most likely to be orthologous to *AtLOB*, a phylogeny was constructed using the LOB-domain amino acid sequence of the class I OsLBDs (Fig. 3.3). LBD1-13, LBD2-2, and LBD4-1 were not included in the analysis due the lack of the C-block motif. Based on this analysis, the class I OsLBD proteins can be divided into 5 subclasses (classes Ia-e), similar to what was previously found in rice (36). Four OsLBD proteins fell within the same clade as AtLOB and were identified as OsRAMOSA2 (OsRA2), OsINDETERMINATE GAMETOPHYTE1 (OsIG1), OsLOB4, and IG1/AS2-LIKE1 (OsIAL1) (2; 6). Previous studies have shown these four genes, *OsRA2*, *OsIG1*,*OsLOB4*, and *OsIAL1*, to have high sequence similarity to *AtAS2* and its closest homologs, *AtLOB*, *AtASL1*, *AtLBD10*, and *AtDDA1*, as well as *ZmRA2, ZmIG1, ZmIAL1, ZmIAL2,* and *ZmIAL3* in maize (2; 6; 12; 29). Phylogenetic analysis of class Ia proteins with their closest homologs in *Arabidopsis* and maize was performed (Fig. 3.4). Within the class Ia phylogeny further subdivision was possible. ZmRA2 and OsRA2 are in the same clade as AtLOB, which confirms previous analysis that OsRA2 is most closely related to ZmRA2 by the high degree of sequence identity

within the LOB-domain (Fig. 3.5) (2). In the clade with AtAS2, four maize proteins ZmIG1, ZmIAL1, ZmIAL2, and ZmIAL3 and two rice proteins, OsIG1 and OsIAL1, closely align, suggesting possible conserved function between the proteins (Fig. 3.6). *AtASL1,* which functions in the control of cell fate determination in petal development, and *OsLOB4,* whose function is unknown, are closely related by sequence, suggesting the possibility that *OsLOB4* may play a role in floral development in rice (4).

**Expression profiles of *RA2, IG1, LOB4,* and *IAL1*.**

Functional conservation between other *LBD* genes in different plant species has been confirmed. Examining the expression pattern of *RA2*, *IG1*, *LOB4*, and *IAL1*should allow us to identify which *OsLBD* genes are the best candidate *LOB* ortholog(s) in rice and give insight to their functions. The spatial expression patterns of *RA2*, *IG1*, *LOB4*, and *IAL1* under normal growth conditions were analyzed using publicly available expression data (Fig. 3.7). *AtLOB* is expressed in the boundary between the SAM and initiating lateral organs and *ZmRA2*, the putative *LOB* ortholog in maize, is found to be expressed in the axillary meristems of the inflorescences, but is not expressed in vegetative leaves (1; 2; 29). *OsRA2* showed high expression in the inflorescence, SAM, shoot, and root (Fig. 6). Examining the level of expression in the lamina joints (collar) in mature leaves, *OsRA2* is moderately expressed in the lamina joints of lower leaves but is expressed at a lower level in the lamina joints of the flag leaf. *OsIG1* is moderately expressed throughout the entire plant but is highly expressed in the inflorescences and SAM (Fig. 6) In the lamina joints of the lower and flag leaves, *OsIG1* had moderate

expression. Throughout the plant, *OsLOB4* is low to moderately expressed, and highly expressed in the pollen grains. *OsLOB4* is moderately expressed in the lower and flag leaf lamina joints. *OsIAL1* is moderately expressed throughout the plant but highly expressed in floral organs, panicle, mature leaf, SAM and in the lamina joints of both the lower and flag leaves. The similarity in expression patterns in the inflorescences and floral organs amongst these four *OsLBD* genes, suggests that the class Ia *OsLBD*s may have similar functions.

***RA2, IG1, LOB4,* and *IAL1* are not regulated by BR during the seedling stage.**

Many *AtLBD* genes play a role in hormone regulation and response, which is important for proper development (8; 20; 23; 24; 27). *AtLOB*, in particular, regulates organ separation in the boundary, in part, by modulating the accumulation of BRs (1). *ZmRA2* is found to be expressed in the axillary meristems of the inflorescences and *ra2* mutants have upright tassels but its relationship with BR is still unknown (2). To determine if *OsRA2*, *OsIG1*, *OsLOB4*, and *OsIAL1* are BR regulated similar to *AtLOB*, microarray data from seedlings treated with 1 µM BL in a time course series of 3, 6, 8, and 12 hrs, was gathered from RiceXPro microarray database. *RA2*, *IG1*, *LOB4*, and *IAL1* were examined for up or down regulation of transcript levels (Fig. 3.8). None of the four genes exhibited a change in expression in response to the BL at any time point, which provides evidence that BR regulation is not conserved.

**Discussion**

The *LBD* gene family is a plant-specific transcription factor family that shares a 100 amino-acid domain with three conserved motifs located at the N-terminal region (20). The *LBD* family has been studied in monocots and dicots and has been shown to play a role in various developmental processes (20; 31; 36; 38). Previous rice genome-wide analyses predicted the presence of 35 LBD proteins (36). In this study, an independent genome-wide analysis of rice *LBD* genes was performed to confirm the reported annotation and identify any new *LBD* genes. Using the *Arabidopsis* LOB protein sequence as a query and the BLAST algorithm, all 35 of the previously reported LOB-domain proteins and three new LBD proteins that were not previously reported were identified. Using updated experimental evidence of transcripts from various rice genome databases, each amino acid sequence for each *OsLBD* gene was hand edited and confirmed.

To identify the conserved motifs within the LOB-domain, an amino acid alignment was conducted using class I OsLBD amino acid sequences. Class II and III *LBD*s were not included in the analysis due to incomplete leucine-zipper-like domain and duplicated LOB-domain, which may have functions that are distinct from class I (29). In all class I *OsLBD*s, the C-block and GAS block were conserved similar to *Arabidopsis*, maize, and *Malus domestica LBD*s (31; 36; 38). When looking closely at the motifs in detail, the C-block motif varied slightly from the C-block motif found in *Arabidopsis*, maize, and *Malus domestica* LBDs. *LBD1-3*, *LBD1-6*, and *LBD8-1* contained a seventh amino acid between the second and third Cys residue, which was not found among any of

the *Arabidopsis*, maize or *Malus domestica LBD*s. Among the four plant species, the GAS block had two completely conserved residues, (G) in the $FX_6FG$ motif and (P) in the $DPX_2G$ motif. The leucine zipper-like motif was more variable, a feature found in all four plant species.

For phylogenetic analysis, an alignment using the protein sequence of the rice class I LOB-domains including the *Arabidopsis* LOB protein sequence was done to determine which *OsLBD*s are most likely to be orthologous to *AtLOB* based on sequence similarity. Phylogeny based on alignment of the LOB-domain shows that class I *OsLBD* genes are divided into five subclasses (class Ia-e), which is similar to what was previously reported (36). OsRA2, OsIG1, OsLOB4, and OsIAL1 fall within the same clade as AtLOB (class Ia). Phylogenetic studies between plant species, such as *Arabidopsis* and maize, show that *LBD* genes within the same subclass have similar functions. *ZmIG1* and *AtAS2,* which are clustered together, have similar function in leaf polarity and floral development and ZmIG1 interacts with ROUGH SHEATH2 (RS2), the AtAS1 ortholog, a MYB transcription factor (35). In *Malus domestica*, sequence alignment and phylogenetic analysis revealed *MdLBD11* was highly similar to *AtAS2* (31). When functionally characterized in transgenic *Arabidopsis*, MdLBD11 interacted with AtAS1, like the interaction between AtAS2 and AtAS1 (35). The conservation between these orthologous *LBD* genes suggests conserved functions among monocots and dicots. Closer examination of the *OsLBD*s in class Ia and their orthologs in *Arabidopsis* and maize may give insight into the functions of genes that have yet to be analyzed. While the function of *OsRA2* is unknown, its protein sequence is highly

similar to ZmRA2 and AtLOB, suggesting a putative role in organ boundaries and controlling inflorescence architecture, in particular affecting the angles of the panicle branches (2).

Examining rice genes most closely related to *LOB* for pattern of expression in the organ boundaries and possible regulation by BR and will give better insight into whether *OsRA2* and *AtLOB* functions are conserved. Utilizing publicly available microarray data, spatial expression profiles of *RA2*, *IG1*, *LOB4*, and *IAL1* in various plant tissues confirmed analysis previously reported (36). To determine if any of the genes are expressed in organ boundaries similar to *AtLOB*, the expression pattern of these genes in the blade/sheath junction (collar) was examined. *IG1* and *LOB4* had moderate expression while *IAL1* had high expression at both lower and flag leaf position. *OsRA2* is moderately expressed in the lower lamina joints but is expressed at a low level in the lamina joints of the flag leaf. The expression of *OsRA2* in the lamina joint suggests that perhaps *LOB* and *OsRA2* have conserved function in the boundaries.

Many *AtLBD* genes play a role in hormone regulation and response, which is important for proper development (20). *AtLOB* regulates organ separation in the boundary, in part, by modulating the accumulation of BRs (1). To determine if the selected *OsLBD* genes most closely related to *AtLOB* are regulated by BR, microarray data from experiments of rice seedlings treated with exogenous 24-epi-brassinolide in a time course of 0, 1, 3, 6, and 12 hr were analyzed (Fig. 3.8). Total RNA extracted from the shoots treated with 24-epi-BL was examined for up- or down- regulation of transcript. *OsRA2*, *OsIG1*, *OsLOB4*, and *OsIAL1* showed no response to exogenous BL at all time

points. While this data was taken from whole shoots, it is possible that these genes are expressed in precise tissues, in particular the lamina joint, and have very low transcript abundance throughout the shoot. An independent experiment of seedlings treated with exogenous 24-epi-brassinolide in a time course was conducted and RNA was isolated from dissected second and third leaf lamina joints (data not shown). Results were inconsistent between biological replicates, perhaps due the difficulty of excising the 1-mm lamina joint, which included blade and sheath tissues. Including more blade/sheath tissues can reduce the overall transcript abundance that is found specifically in the lamina joint. To alleviate this problem, doing a BR treatment on older plants where the lamina joints are more pronounced and easier to dissect may help. A similar experiment is currently being conducted by another graduate student in the lab using an aeroponic system. This system allows the plants to be grown to an older developmental stage and exogenous BL can be added as the plants are growing.

Previous genome-wide analysis of the maize, *Arabidopsis*, apple, and rice *LBD* gene families and their evolutionary comparisons have been examined showing that the function of some *LBD* genes are conserved between the different plant species (29; 31; 36; 38). This study provided an updated independent genome-wide study of rice *LBD* genes and identified *LOB* orthologs in rice. Since there is functional conservation between other *LBD* genes in different plant species, *OsRA2* is predicted have similar function to *AtLOB*. Characterization of *LBD* genes in rice may allow us to identify new genes involved in the control of shoot architecture.

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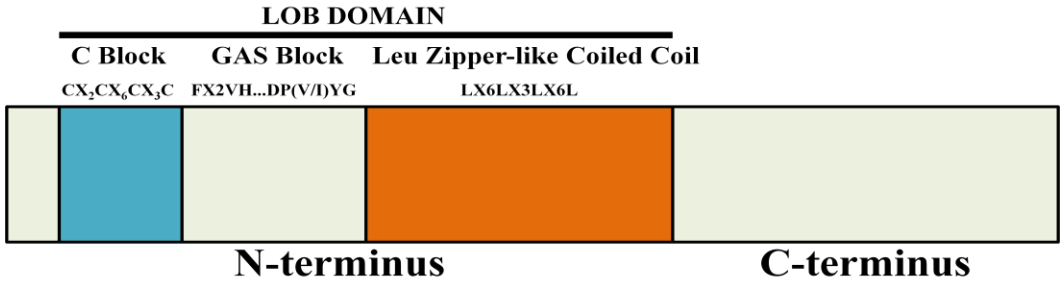
38. Zhang Y-M, Zhang S-Z, Zheng C-C. 2014. Genomewide analysis of LATERAL ORGAN BOUNDARIES Domain gene family in *Zea mays*. *J. Genet.* 93:79-91

**Figure 3.1. The general structure of the *LATERAL ORGAN BOUNDARIES* (*LOB*) domain and protein sequence of AtLOB.**

A. Schematic drawing of the general structure of a LOB-domain protein with the C block motif, GAS block motif, and Leucine Zipper-like Coiled-coil motif in the N-terminal region and variable C-terminal region.

B. Amino acid sequence of AtLOB, the founding member of the LOB-domain gene family. The LOB-domain is highlighted in gray, C-block and GAS block are underlined with solid and dashed lines, respectively. The Leucine zipper-like coiled-coil motif is marked by the double underline.

**A.**


LOB DOMAIN
C Block
GAS Block
Leu Zipper-like Coiled Coil
CX2CX6CX3C
FX2VH...DP(V/I)YG
LX6LX3LX6L
N-terminus
C-terminus

**B.**

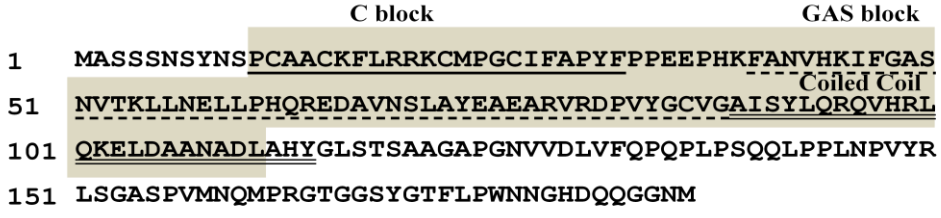

C block
GAS block
Coiled Coil
1 MASSSNSYNSPCAACKFLRRKCMPGCIFAPYFPPEEPHKFANVHKIFGAS
51 NVTKLLNELLPHQREDAVNSLAYEAEARVRDPVYGCVGAISYLQRQVHRL
101 QKELDAANADLAHYGLSTSAAGAPGNVVDLVFQPQPLPSQQLPPLNPVYR
151 LSGASPVMNQMPRGTGGSYGTFLPWNNGHDQQGGNM

**Figure 3.2. Class I OsLBD protein alignment.**

Protein sequence alignment of the rice class I LOB-domains including the *Arabidopsis* LOB protein sequence. Alignment was produced using CLUSTALW in the GeneDoc Program. 100% conserved amino acids are highlight in black, 80% conserved amino acids are highlighted in dark gray, and 60% conserved amino acids are in light gray.

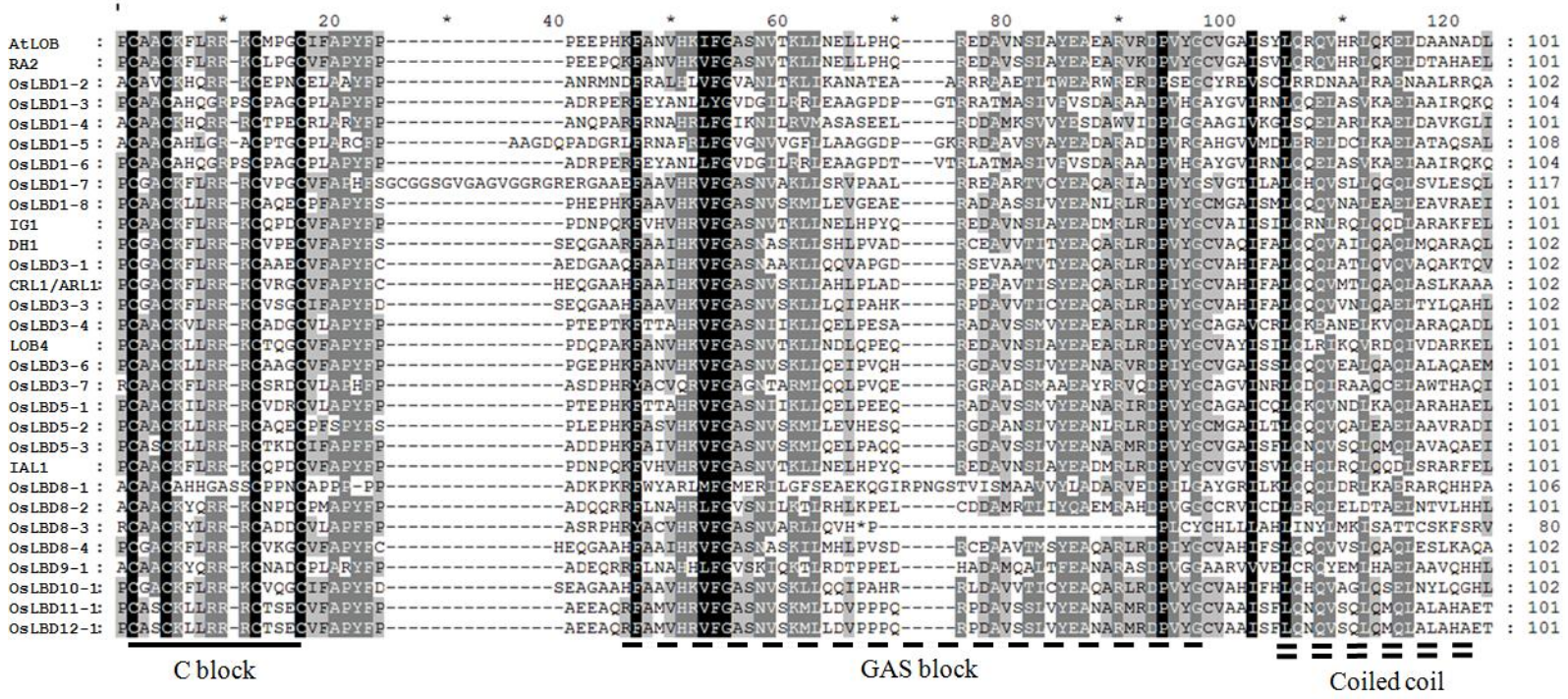

C block
GAS block
Coiled coil

**Figure 3.3. Phylogenetic tree of OsLBD proteins.**

Phylogeny based on alignment of the LOB-domain of class I OsLBD proteins including AtLOB. The amino acid sequences of the LOB-domains were aligned using CLUSTALW and hand edited. The phylogenetic tree was constructed using the NJ method in MEGA6. The reliability of the tree was tested using a bootstrapping method with 1000 replicates. OsLBD subclasses are indicated with vertical lines (Classes Ia–e).

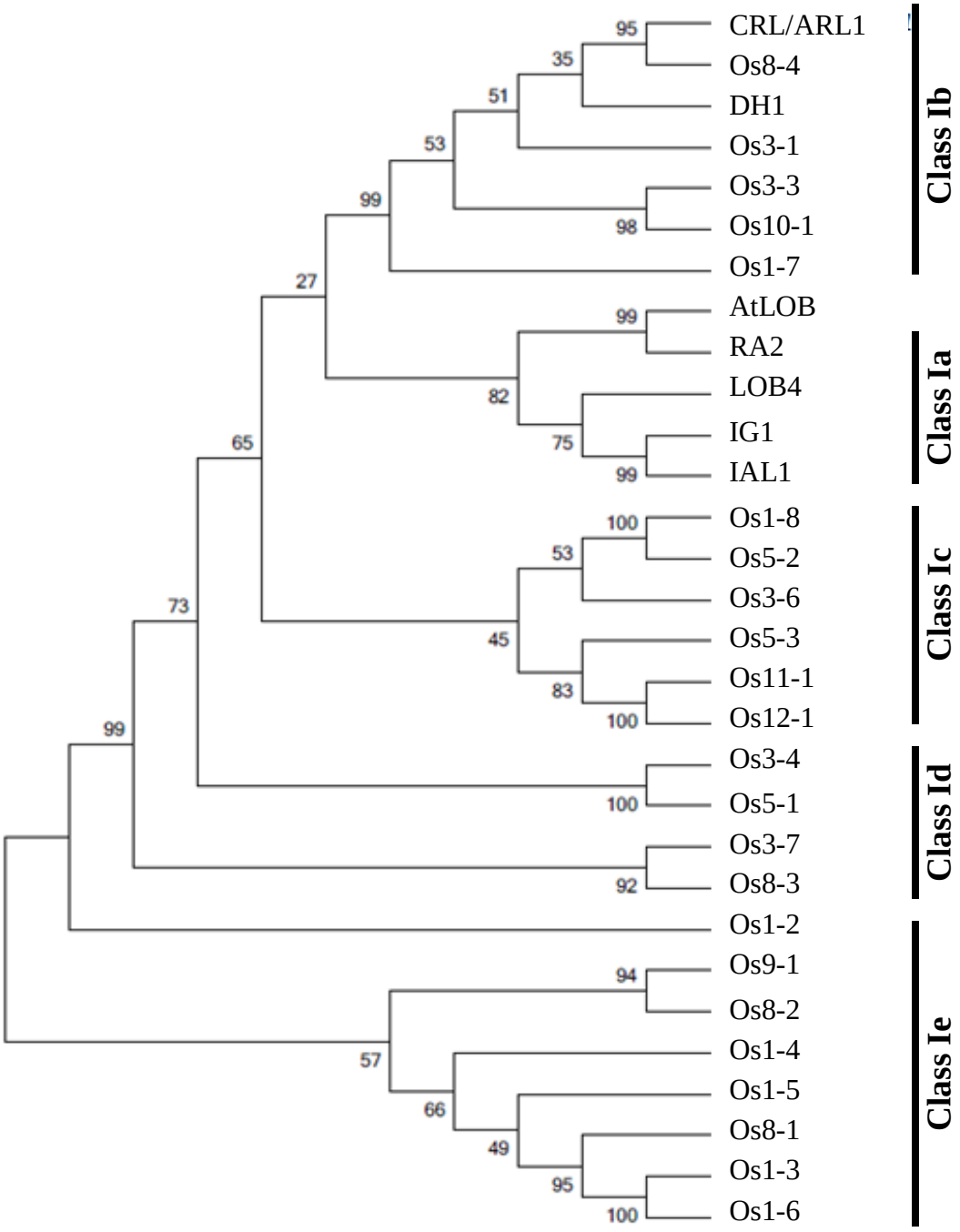

CRL/ARL1
Os8-4
DH1
Os3-1
Os3-3
Os10-1
Os1-7
AtLOB
RA2
LOB4
IG1
IAL1
Os1-8
Os5-2
Os3-6
Os5-3
Os11-1
Os12-1
Os3-4
Os5-1
Os3-7
Os8-3
Os1-2
Os9-1
Os8-2
Os1-4
Os1-5
Os8-1
Os1-3
Os1-6
Class Ib
Class Ia
Class Ic
Class Id
Class Ie

**Figure 3.4. Phylogenetic analysis of class Ia subgroup and their putative orthologs in maize and *Arabidopsis*.**

Phylogenetic tree of *OsRA2* and other closely related LOB-domain proteins, *OsIG1*, *OsLOB4*, and *OsIAL1* and the putative orthologs in *Arabidopsis* and maize.

.

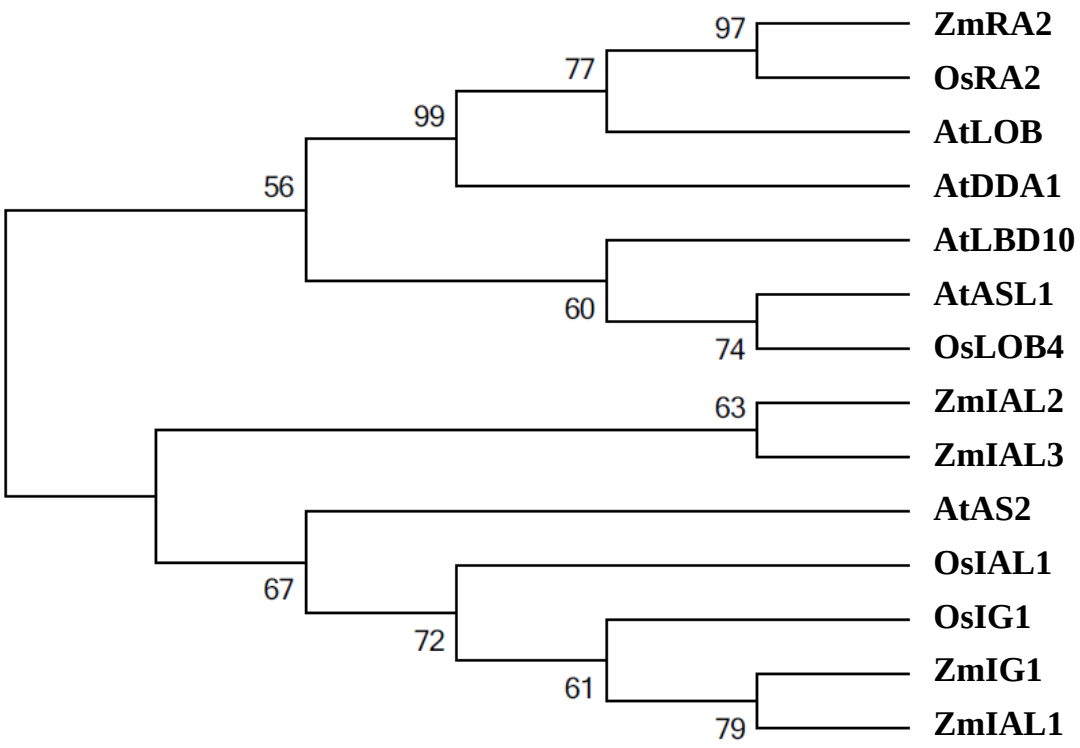

97
77
99
56
60
74
63
67
72
61
79
ZmRA2
OsRA2
AtLOB
AtDDA1
AtLBD10
AtASL1
OsLOB4
ZmIAL2
ZmIAL3
AtAS2
OsIAL1
OsIG1
ZmIG1
ZmIAL1

**Figure 3.5. Protein sequence alignment of AtLOB and the putative orthologs in maize and rice.**

Alignment of the LOB-domain of AtLOB and its orthologs in maize and rice, ZmRA2 and OsRA2. Conserved amino acids are highlighted in black and similar amino acids are highlighted in gray. Consensus sequence is shown below alignment.


C block
GAS block
AtLOB 11 PCAACKFLRRKCMPGCIFAPYFPPEEPHKFANVHKIFGASNVTKLLNELLPH
OsRA2 30 PCAACKFLRRKCLPGCVFAPYFPPEEPQKFANVHKVFGASNVTKLLNELLPH
ZmRA2 30 PCAACKFLRRKCLPGCVFAPYFPPEEPQKFANVHKVFGASNVTKLLNELLPH
PCAACKFLRRKCLPGCVFAPYFPPEEPqKFANVHKVFGASNVTKLLNELLPH
GAS block
Coiled coil
AtLOB 63 QREDAVNSLAYEAEARVRDPVYGCVGAISYLQRQVHRLQKELDAANADL
OsRA2 82 QREDAVSSLAYEAEARVKDPVYGCVGAISVLQRQVHRLQKELDTAHAEL
ZmRA2 82 QREDAVSSLAYEAEARVKDPVYGCVGAISVLQRQVHRLQKELDAAHAEL
QREDAVsSLAYEAEARVKDPVYGCVGAISvLQRQVHRLQKELDvAhAeL

**Figure 3.6. Protein sequence alignment of AtAS2 and the putative orthologs in maize and rice.**

Alignment of the LOB-domain of AtAS2 and its orthologs in maize and rice, ZmIG1, ZmIAL1, ZmIAL2, ZmIAL3, OsIG1 and OsIAL1. Conserved amino acids are highlighted in black and similar amino acids are highlighted in gray. Consensus sequence is shown below alignment.

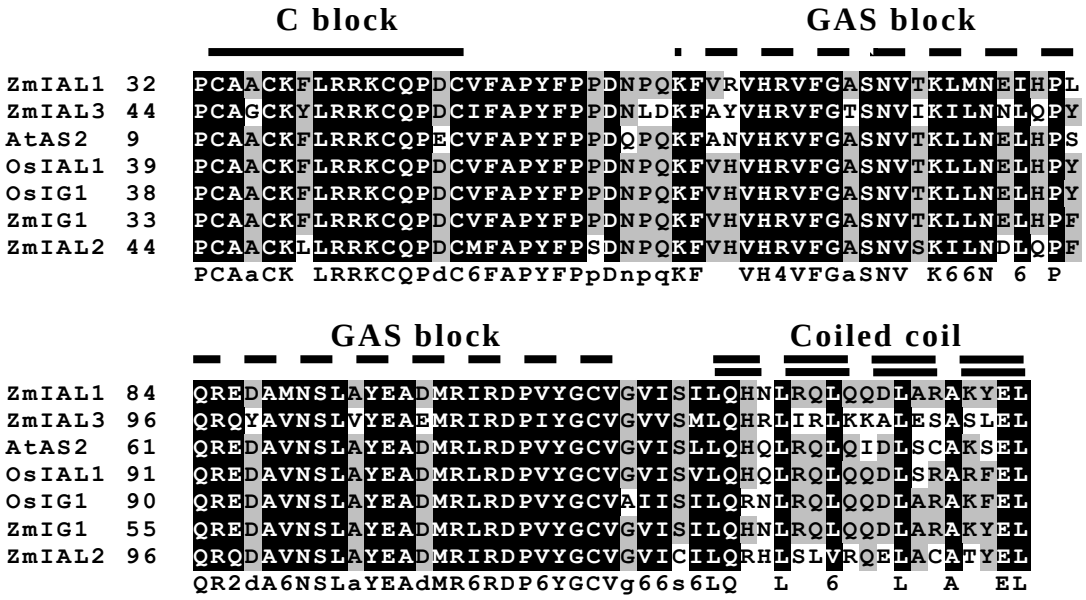
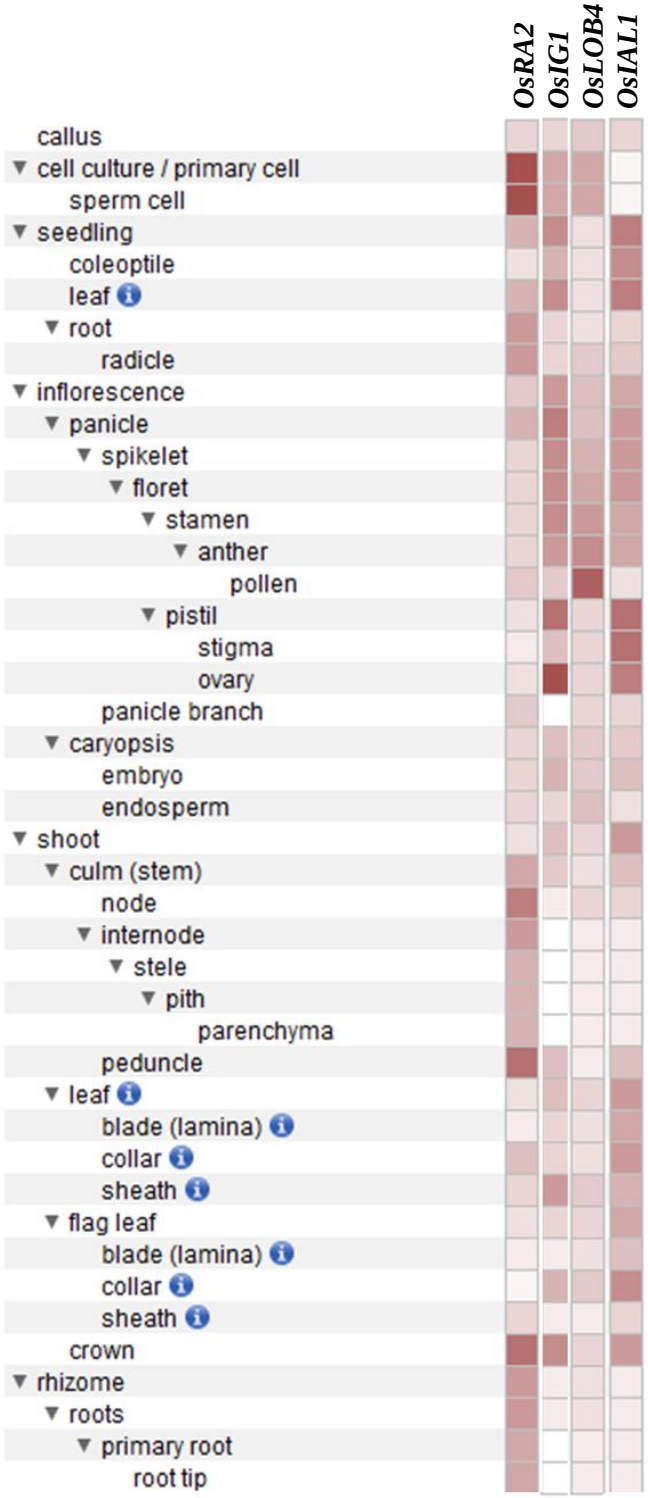

C block
GAS block
ZmIAL1 32 PCAACKFLRRKCQPDCVFAPYFPPDNPQKFVRVHRVFGASNVTKLMNEIHPL
ZmIAL3 44 PCAGCKYLRRKCQPDCIFAPYFPPDNLDKFAYVHRVFGTSNVIKILNNLQPY
AtAS2 9 PCAACKFLRRKCQPECVFAPYFPPDQPQKFANVHKVFGASNVTKLLNELHPS
OsIAL1 39 PCAACKFLRRKCQPDCVFAPYFPPDNPQKFVHVHRVFGASNVTKLLNELHPY
OsIG1 38 PCAACKFLRRKCQPDCVFAPYFPPDNPQKFVHVHRVFGASNVTKLLNELHPY
ZmIG1 33 PCAACKFLRRKCQPDCVFAPYFPPDNPQKFVHVHRVFGASNVTKLLNELHPF
ZmIAL2 44 PCAACKLLRRKCQPDCMFAPYFPSDNPQKFVHVHRVFGASNVSKILNDLQPF
PCAaCK LRRKCQPdC6FAPYFPpDnpqKF VH4VFGaSNV K66N 6 P
GAS block
Coiled coil
ZmIAL1 84 QREDAMNSLAYEADMRIRDPVYGCVGVISILQHNLRQLQQDLARAKYEL
ZmIAL3 96 QRQYAVNSLVYEAEMRIRDPIYGCVGVVSMLQHRLIRLKKALESASLEL
AtAS2 61 QREDAVNSLAYEADMRLRDPVYGCVGVISLLQHQLRQLQIDLSCAKSEL
OsIAL1 91 QREDAVNSLAYEADMRLRDPVYGCVGVISVLQHQLRQLQQDLSRARFEL
OsIG1 90 QREDAVNSLAYEADMRLRDPVYGCVAIISILQRNLRQLQQDLARAKFEL
ZmIG1 55 QREDAVNSLAYEADMRLRDPVYGCVGVISILQHNLRQLQQDLARAKYEL
ZmIAL2 96 QRQDAVNSLAYEADMRIRDPVYGCVGVICILQRHLSLVRQELACATYEL
QR2dA6NSLaYEAdMR6RDP6YGCVg66s6LQ L 6 L A EL

**Figure 3.7. *OsRA2, OsIG1, OsLOB4,* and *OsIAL1* expression data gathered from various databases.**

**A.** Temporal and spatial expression profiles gathered from the Genevestigator database (Affymetrix Rice Genome Array). Images were modified from Genevestigator.

**B.** Expression profiles of individual *LBD*s during various developmental processes gathered from Bio-Analytic Resource Electronic Fluorescent Pictographs (BAR eFP) microarray database. Images were modified from BAR eFP. Expression values are means ± SD. (Kapoor S, et al. 20014)

**A.**


OsRA2
OsIG1
OsLOB4
OsIAL1
callus
cell culture / primary cell
sperm cell
seedling
coleoptile
leaf
root
radicle
inflorescence
panicle
spikelet
floret
stamen
anther
pollen
pistil
stigma
ovary
panicle branch
caryopsis
embryo
endosperm
shoot
culm (stem)
node
internode
stele
pith
parenchyma
peduncle
leaf
blade (lamina)
collar
sheath
flag leaf
blade (lamina)
collar
sheath
crown
rhizome
roots
primary root
root tip

**B.**

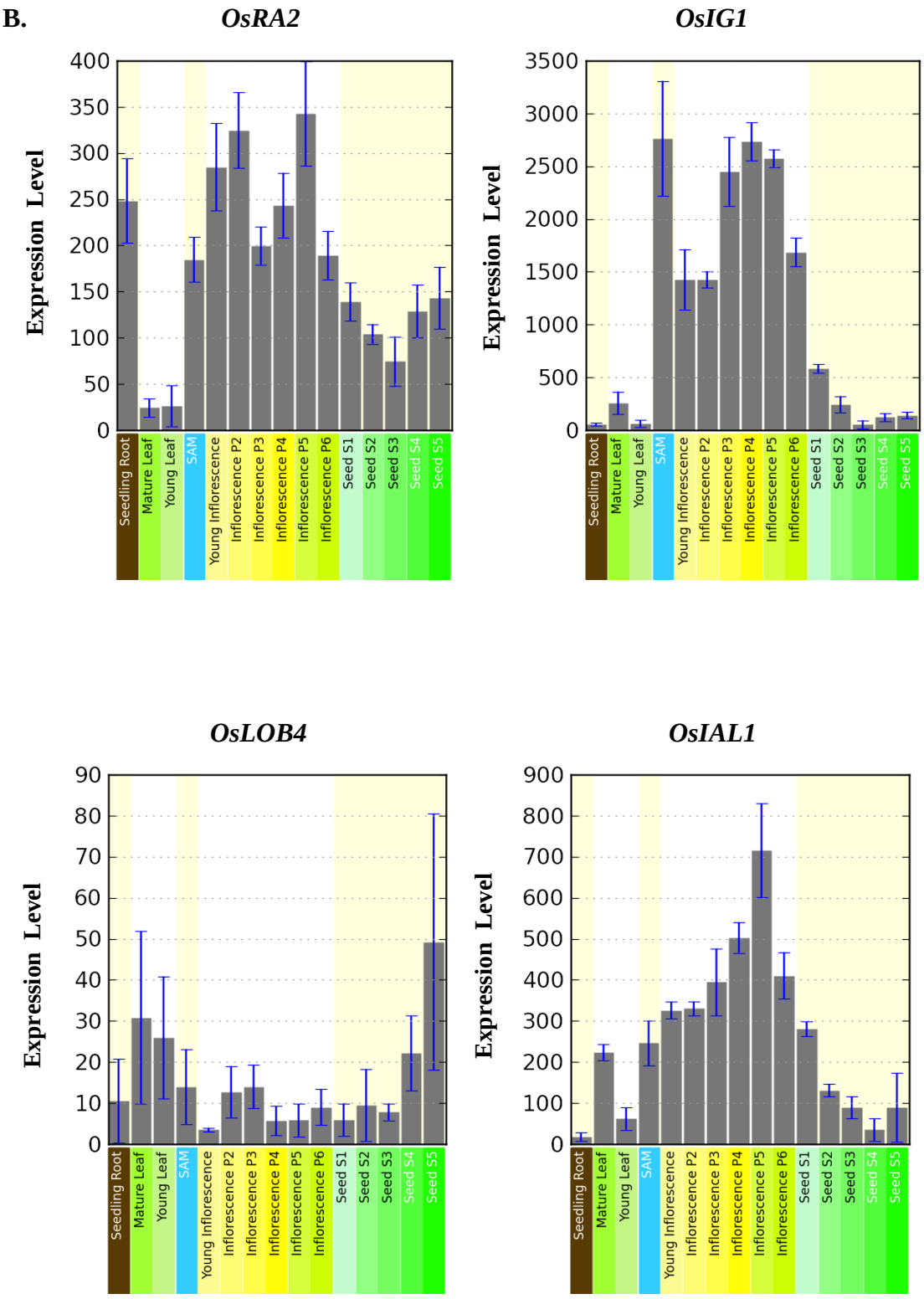
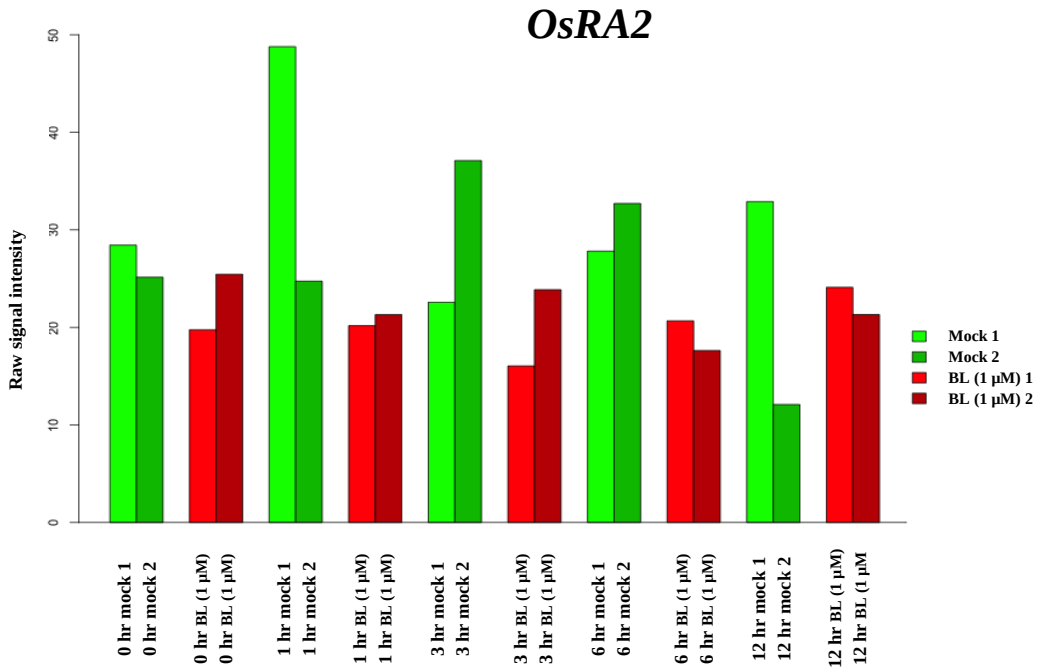
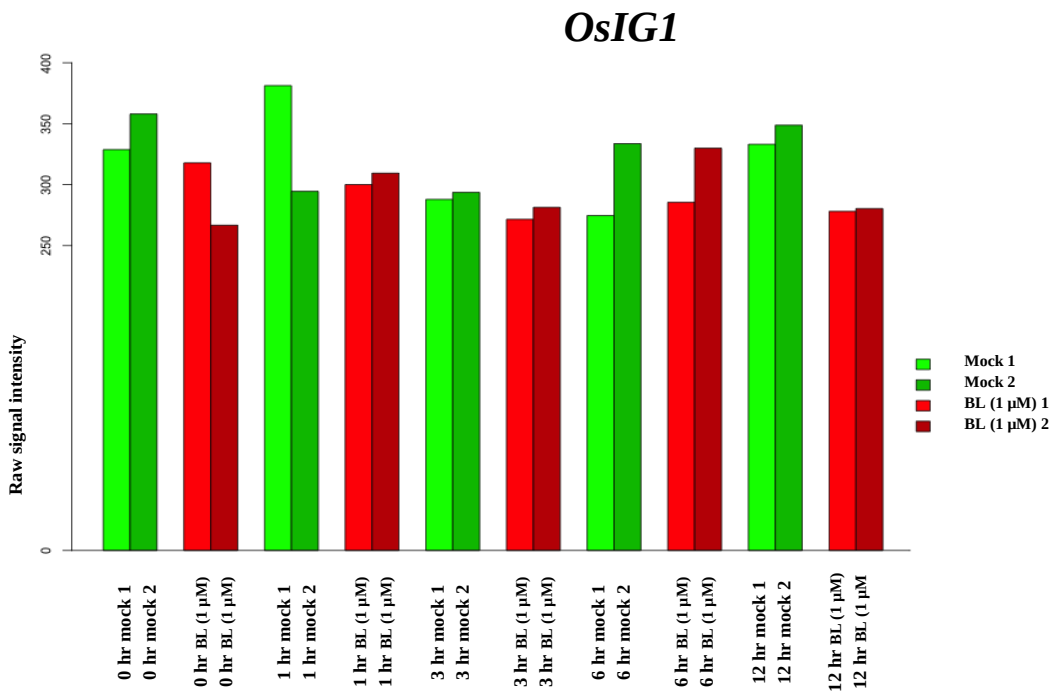
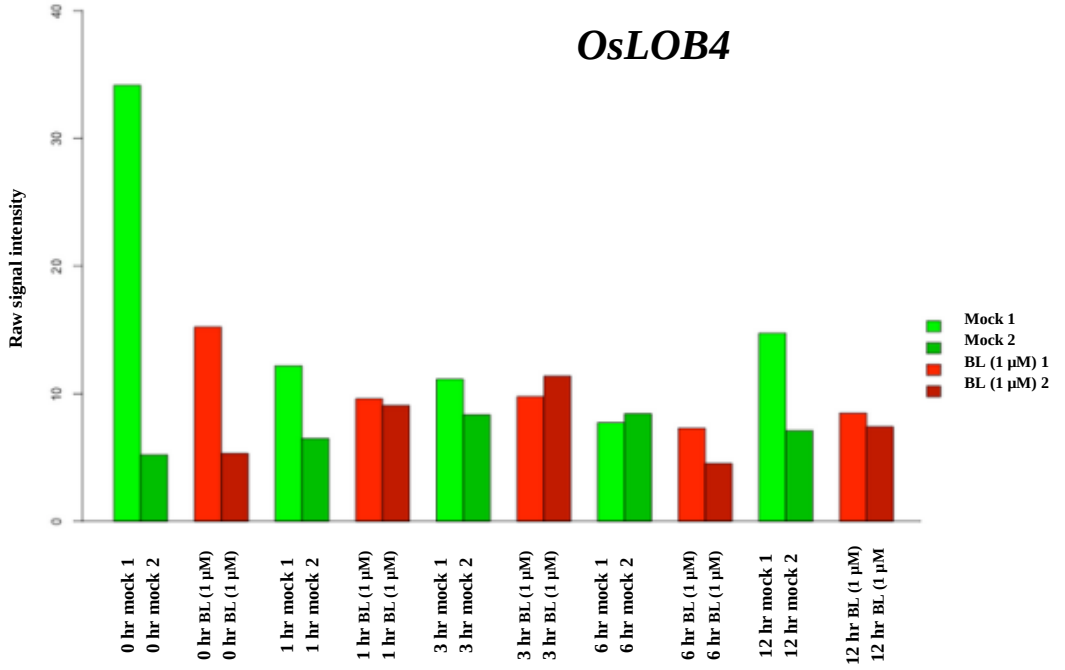

OsRA2
OsIG1
OsLOB4
OsIAL1
Expression Level
Seedling Root
Mature Leaf
Young Leaf
SAM
Young Inflorescence
Inflorescence P2
Inflorescence P3
Inflorescence P4
Inflorescence P5
Inflorescence P6
Seed S1
Seed S2
Seed S3
Seed S4
Seed S5

**Figure 3.8. *OsRA2*, *OsIG1*, *OsLOB4*, and *OsIAL1* are not induced by exogenous BR.** Agilent two-color microarray analysis from RiceXPro was used to analyze the up/down regulation of the four *LBD* genes. Seven-day old seedlings were treated with 1 μM BL and shoots were collected at 0, 1, 3, 6, and 12 h incubation. Images were modified from RiceXPro.


OsRA2
Raw signal intensity
0
10
20
30
40
50
0 hr mock 1
0 hr mock 2
0 hr BL (1 µM)
0 hr BL (1 µM)
1 hr mock 1
1 hr mock 2
1 hr BL (1 µM)
1 hr BL (1 µM)
3 hr mock 1
3 hr mock 2
3 hr BL (1 µM)
3 hr BL (1 µM)
6 hr mock 1
6 hr mock 2
6 hr BL (1 µM)
6 hr BL (1 µM)
12 hr mock 1
12 hr mock 2
12 hr BL (1 µM)
12 hr BL (1 µM
Mock 1
Mock 2
BL (1 µM) 1
BL (1 µM) 2


OsIG1
Raw signal intensity
0
250
300
350
400
0 hr mock 1
0 hr mock 2
0 hr BL (1 µM)
0 hr BL (1 µM)
1 hr mock 1
1 hr mock 2
1 hr BL (1 µM)
1 hr BL (1 µM)
3 hr mock 1
3 hr mock 2
3 hr BL (1 µM)
3 hr BL (1 µM)
6 hr mock 1
6 hr mock 2
6 hr BL (1 µM)
6 hr BL (1 µM)
12 hr mock 1
12 hr mock 2
12 hr BL (1 µM)
12 hr BL (1 µM
Mock 1
Mock 2
BL (1 µM) 1
BL (1 µM) 2


OsLOB4
Raw signal intensity
0
10
20
30
40
Mock 1
Mock 2
BL (1 µM) 1
BL (1 µM) 2
0 hr mock 1
0 hr mock 2
0 hr BL (1 µM)
0 hr BL (1 µM)
1 hr mock 1
1 hr mock 2
1 hr BL (1 µM)
1 hr BL (1 µM)
3 hr mock 1
3 hr mock 2
3 hr BL (1 µM)
3 hr BL (1 µM)
6 hr mock 1
6 hr mock 2
6 hr BL (1 µM)
6 hr BL (1 µM)
12 hr mock 1
12 hr mock 2
12 hr BL (1 µM)
12 hr BL (1 µM

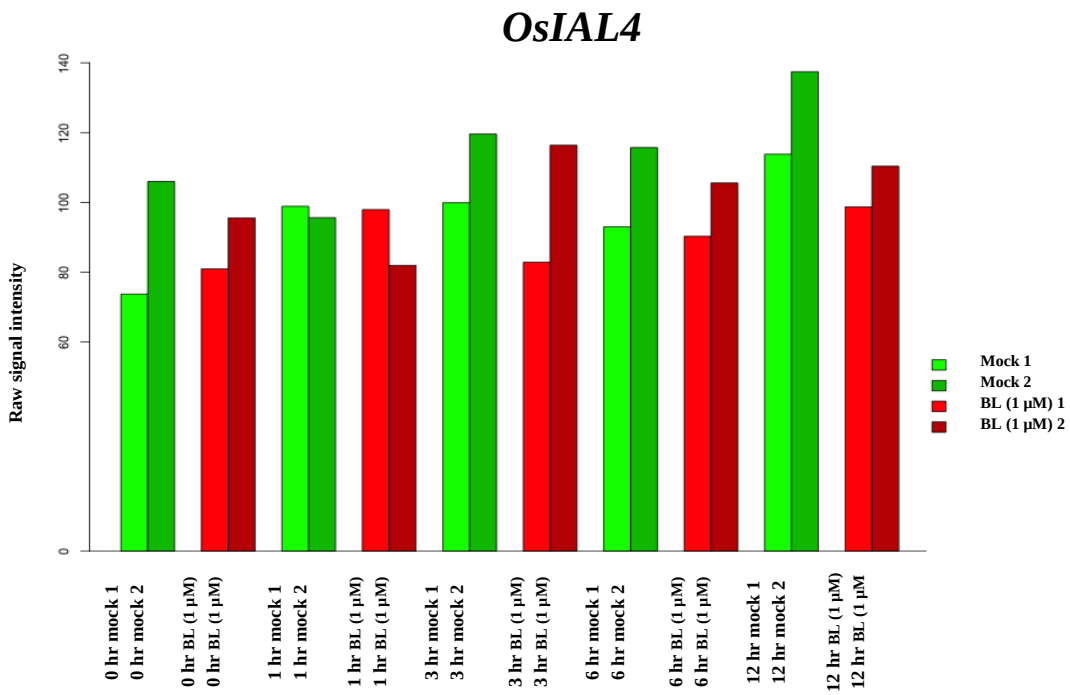

OsIAL4
Raw signal intensity
0
60
80
100
120
140
Mock 1
Mock 2
BL (1 µM) 1
BL (1 µM) 2
0 hr mock 1
0 hr mock 2
0 hr BL (1 µM)
0 hr BL (1 µM)
1 hr mock 1
1 hr mock 2
1 hr BL (1 µM)
1 hr BL (1 µM)
3 hr mock 1
3 hr mock 2
3 hr BL (1 µM)
3 hr BL (1 µM)
6 hr mock 1
6 hr mock 2
6 hr BL (1 µM)
6 hr BL (1 µM)
12 hr mock 1
12 hr mock 2
12 hr BL (1 µM)
12 hr BL (1 µM

**Table 3.1. Oligonucleotide sequences**

| Purpose | Gene | Primer Name | Sequence (5'-3') |
|---|---|---|---|
| RT-PCR | *ACTIN* | ACT1 FWD | GCCGTCCTCTCTCTGTATGC |
| RT-PCR | *ACTIN* | ACT1 REV | GCCGTTGTGGTGAATGAGTA |
| RT-PCR | *OsRA2* | OsRA2 FWD | AGTGAACGCCATTAGCACCA |
| RT-PCR | *OsRA2* | OsRA2 REV | AACCTGGGATGAATGGGCTG |
| RT-PCR | *OsIG1* | OsIG1 FWD | TTGATTCGGCGGTTGTTGTG |
| RT-PCR | *OsIG1* | OsIG1 REV | AGCATGCACACAGTACAGCA |
| RT-PCR | *OsIAL4* | OsIAL4 FWD | GAGGGCTTGTTTACCACCAC |
| RT-PCR | *OsIAL4* | OsIAL4 REV | AAATGCAGCAAGCCACACTA |
| RT-PCR | *OsLOB4* | OsLOB4 FWD | ATGCCTCCCTGAAAGCATCC |
| RT-PCR | *OsLOB4* | OsLOB4 REV | GCTCCATGATGAGGGTCTGT |

**Table 3.2. Rice genes encoding LOB-domain proteins**

| Rice Gene Name | Gene | MSU Locus | NCBI Locus (Accession number[a]) | Experimental Evidence (EST sequence clone[1] FL-cDNA clone[2]) |
|---|---|---|---|---|
| | **Class I** | | | |
| RA2 | OsLBD1-1 | Os01g07480 | Os01g0169400 | CI277719.1[1], CI131539.1[1], C98868.1[1], C98869.1[1], CI116935.1[1], BE041023.1[1], CK008680.1[1], CK042518.1[1], AK105527[2], EF540767[2], AB200238[2] |
| | OsLBD1-2 | Os01g14030 | Os01g0242400 | CI177145.1[1], AK108724[2] |
| | OsLBD1-3 | Os01g39070 | Os01g0571800 | CI095526.1[1] |
| | OsLBD1-4 | Os01g39150 | Os01g0572800 | |
| | OsLBD1-5 | Os01g39160 | BAB91895.1[a] | |
| | OsLBD1-6 | Os01g39220 | BAD45187.1[a] | CI095526[1] |
| | OsLBD1-7 | Os01g56530 | Os01g0772100 | CI513116.1[1], CI463833.1[1], CI512945.1[1], CI514400.1[1], CI731998[1], AK242468[2] |
| | OsLBD1-8 | Os01g60960 | Os01g0825000 | CI224720.1[1], CI566537.1[1], CI226301.1[1], CI383188.1[1], CI137996.1[1], CT863295.1[1], AK121919[2] |
| IG1 | OsLBD1-9 | Os01g66590 | Os01g0889400 | ESTs refer to NCBI UNIGENE, AK071407[2], AK119575[2], EF540766[2], AB200237[2] |
| DH1 | OsLBD2-1 | Os02g57490 | Os02g0820500 | CI74812.1[1], CI310491.1[1], CI170920.1[1], CI073073.1[1], CI621486.1[1], C26871.1[1], CI187212.1[1], CI184302.1[1], AK064187[2], AK103441[2], AU100784 1 OR 2, CX099534 1 OR 2 |
| | OsLBD3-1 | Os03g05500 | Os03g0149000 | CA758794[1], CI267794[1], CI264518[1], CI73655[1], CI237102[1], CI264509[1], CI185185[1], CI560822[1], CI85260[1], AU101687[1], CI428937[1], AU070675.1[1], AU071262.1[1], CI776424.1[1], CI776424.1[1], AU162639.1[1], CX104056.1[1], AK107949.1[2] |
| CRL1/ ARL1 | OsLBD3-2 | Os03g05510 | Os03g0149100 | CI310491[1], CI621486[1], AB200234.1[2], AY736375.1[2] |
| | OsLBD3-3 | Os03g14270 | Os03g0246900 | |
| | OsLBD3-4 | Os03g17810 | Os03g0287400 | CI262359[1], CI188393[1], CI361727.1[1], CT851601.1[1], CT832143.1[2], AK072515.1[2] |
| LOB4 | OsLBD3-5 | Os03g41600 | Os03g0612400 | CI590403.1[1] |
| | OsLBD3-6 | Os03g45750 | Os03g0659700 | CI028626.1[1], CI665030.1[1], CI357754.1[1], CI295889.1[1], CI406301.1[1], CI441867.1[1], AK071550.1[2] |
| | OsLBD3-7 | Os03g57670 | ABF99280[a] | CI040611.1[1], AK063229.1[2] |
| | OsLBD5-1 | Os05g03160 | Os05g0123000 | CK011554[2] |
| | OsLBD5-2 | Os05g07270 | Os05g0165450 | CI124219[2] |
| | OsLBD5-3 | Os05g27980 | Os05g0346800 | AU070665.2[1] |
| IAL1 | OsLBD5-4 | Os05g34450 | Os05g0417000 | CB673235.1[1], CK010166.1[1] |
| | OsLBD8-1 | Os08g06659 | Os08g0163800 | CI762341.1[1], CI758561.1[1], CI758372.1[1], CI758778.1[1], CI101891.1[1], CI545603.1[1], AK107012.1[2], AK318588.1[2] |
| | OsLBD8-2 | Os08g31080 | Os08g0402100 | CI257087.1[1], CI598868.1[1], CI102012.1[1], CI347861.1[1], CI598621.1[1], CI354873.1[1], CI356809.1[1], CX109756.1[1], AK240652.1[2] |
| | OsLBD8-3 | Os08g40520 | Os08g0517100 | CI063461.1[1], CI307957.1[1], AK112071.1[2], AK318594.1[2] |
| | OsLBD8-4 | Os08g44940 | Os08g0563400 | EE591430.1[1], AU070666.1[1] |
| | OsLBD9-1 | Os09g19950 | Os09g0364100 | CI278703.1[1], CI595626.1[1], AK071233.1[2] |
| | OsLBD10-1 | Os10g07510 | Os10g0162600 | AB200236.1[2] |
| | OsLBD11-1 | Os11g01550 | Os11g0106900 | CI211964.1[1], CF337420.1[1], CI281004.1[1], CI118625.1[1], CI445507.1[1], EG710695.1[1], CI668088.1[1], CI407256.1[1], CI636811.1[1], AU070665.2[1], BX898263.1[1], AK241761.1[2], CT828113.1[2], AK109211.1[2] |
| | OsLBD12-1 | Os12g01550 | Os12g0106200 | CI211964.1[1], CF337420.1[1], CI281004.1[1], CI118625.1[1], CI445507.1[1], EG710695.1[1], CI668088.1[1], CI407256.1[1], CI636811.1[1], AU070665.2[1], BX898263.1[1], AK241761.1[2], CT828113.1[2], AK109211.1[2] |

**Table 3.2 continued**

| | | | |
|---|---|---|---|
| **Class II** | | | |
| OsLBD1-10 | Os01g03890 | Os01g0129600 | |
| OsLBD1-11 | Os01g32770 | Os01g0511000 | |
| OsLBD3-8 | Os03g33090 | Os03g0445700 | |
| OsLBD3-9 | Os03g41330 | Os03g0609500 | |
| OsLBD7-1 | Os07g40000 | Os07g0589000 | |
| **Class III** | | | |
| OsLBD1-12 | Os01g39040 | AP003308.3 | CT834991.1[2] |
| **New LBDs** | | | |
| OsLBD1-13 | Os01g39180 | | |
| OsLBD2-2 | Os02g48270 | | AU070665.2[1] |
| OsLBD4-1 | Os04g33250 | | |

**Conclusion**

The majority of the world relies on cereal crops as an important source of nutrition for human and animal consumption (15). With the world population estimated to exceed 9.5 billion people by the year 2050, global food shortages and food security will continue to be serious global problems if crop production does not meet food demands (4; 14; 15; 21). With approximately 50% of the human population relying on rice as their major source of nutrition, especially in developing countries, scientists must find ways to increase rice grain production (12; 15; 21; 29). Manipulation of rice architecture to create high yielding rice varieties has been the target of many rice breeding programs (20). Rice traits that have lead to the growth in rice production include the production of multiple grain-bearing tillers, dark, wide, and erect leaves, and vigorous, deep root systems (1; 13; 20).

Hormone functions in crop plants have been studied for their important roles in altering plant architecture for agricultural improvement (1; 28). In rice, the plant steroid hormone BR plays an essential role for normal growth and development (17). BRs affect various physiological and developmental processes such as stem elongation, cell expansion and division, contributing to tiller and leaf erectness, and plant height (5; 22; 26). Rice plants with reduced BR accumulation are short in stature, have decreased fertility, smaller grains, compact tillering, and upright leaves compared to wild-type (1; 5; 26). Leaf erectness is an important agronomic trait in rice that is beneficial to increasing biomass and grain yield (22). Rice plants with erect leaves require less growth space, allowing for higher density planting, which contributes to increased grain yields per acre

(22). While many traits affected by reduced BR are beneficial to increasing yield, other traits such as reduced height and fertility diminish the benefits (5; 13). Altering BR accumulation in specific tissues can be an effective approach for directing phenotypic changes that are beneficial for agriculture. Modifying rice plants to have site specific reduction of BR accumulation specifically at the lamina joint, should lead to increased leaf erectness without the detrimental effects on plant architecture caused by BR deficiency (8; 22).

In *Arabidopsis*, *LOB* encodes a plant-specific transcription factor expressed in all organ boundaries and functions in organ separation and meristem maintenance (2; 9; 23). LOB contains a conserved 100 amino-acid domain (the LOB domain) that is found in 42 other LOB Domain (LBD) proteins (23). Loss-of-function *lob* mutants exhibit defects in organ separation causing a subtle fusion between organs (2; 23). Treatment of *Arabidopsis* plants with exogenous BR increased the accumulation of *LOB* transcripts, revealing that LOB is BR regulated (2). Experiments to identify downstream targets of LOB identified *BAS1*, an enzyme that inactivates BR, as a target of LOB transcriptional regulation (19; 27). These interactions result in a negative feedback loop where LOB is positively regulated by BR and LOB activates expression of *BAS1* (2). This LOB-BR feedback loop limits BR accumulation in organ boundaries resulting in the boundary cells being smaller and having limited cell division (2; 10).

*LOB* has been shown to play an important role in modulating the accumulation of BRs to regulate organ separation (2). The work in this dissertation is extending studies on LOB function to rice, an agronomically important crop plant. Understanding the role

of BR signaling in rice and the ability to alter BR response in specific tissues is a promising approach for crop improvement. A major trait affected by altered BR levels is leaf erectness (1; 8; 17). Producing rice plants with an erect shoot architecture could allow for higher density planting that will result in an increase in biomass and grain yield that can benefit farmers.

One aim of this research is determine if the LOB-BR feedback loop is conserved in rice. The goal was to test whether expressing *LOB* and *BAS1* specifically in the lamina joint, which is predicted to reduce BR levels, could alter leaf angles without affecting other aspects of plant architecture. An *AtpLOB:GUS* fusion construct, which drives boundary expression in *Arabidopsis*, was introduced into rice to determine whether this promoter could also drive expression in rice organ boundaries. The *AtLOB* promoter drove expression in a pattern confined to the shoot/root base and the blade/sheath junctions suggesting that *AtpLOB* is regulated in a similar manner in rice. In addition, the expression pattern of *AtpLOB:GUS* in the blade/sheath junction suggests that this region is also a boundary similar to the meristem/leaf boundary. The *AtLOB* promoter was then used to drive the expression of *AtLOB* and *AtBAS1* to manipulate local BR accumulation in the rice lamina joint, with a goal of creating plants that have a more erect stature than wild-type.

In chapter one, transgenic plants were generated expressing *AtLOB* under the *AtLOB* promoter (*AtpLOB:LOB*). If *AtLOB* function is conserved in rice, then this transgene might activate the rice *BAS1* ortholog and repress BR signaling, resulting in erect leaves. While there were no alterations in plant architecture during the seedling

stage, mature *AtpLOB:LOB* plants exhibited traits associated with BR deficiency suggesting the effect of AtLOB on plant architecture occurs later in development. Mature *AtpLOB:LOB* plants had erect leaves and wider blades compared to wild-type, both traits that are associated with a reduction in BR. Closer examination of the lamina joint and blade of BR-deficient mutants show there is a disruption of cell elongation and division on the adaxial surface causing the leaf to be more vertical and the blade to be wider (8; 24). Alterations in leaf morphology in *AtpLOB:LOB* plants suggest there may be site-specific reduction of BR accumulation. Overall, these results indicate that *AtLOB* expression in a distinct domain alters leaf angle and shape without other detrimental effects on fertility and plant height, which is caused by overall BR deficiency.

In chapter two, transgenic rice plants expressing the *AtBAS1* under the *AtLOB* promoter (*AtpLOB:BAS1*) were created with the goal of reducing BR accumulation in boundaries resulting in increased leaf erectness without other changes in plant architecture. Previous studies broadly misexpressed the rice *BAS1* homologs, *CYP734A*s, resulting in a BR-deficient, dwarf phenotype (22; 25). These results lead to the prediction that expressing *CYP734A*s in a restricted pattern should site specifically reduce BR levels, resulting in erect leaves. Detailed phenotypic characterization of *AtpLOB:BAS1* plants revealed characteristics associated with both reduced BR and enhanced BR signaling. In the seedling stage, *AtpLOB:BAS1* plants were taller with shorter roots compared to wild-type. During the heading stage, *AtpLOB:BAS1* plants exhibited erect leaves, which is a trait associated with BR deficiency, but they also had alterations in the inflorescences typically seen as a result of increased BR signaling.

*AtpLOB:BAS1* inflorescences were elongated with increased branching and contained longer, slender grains. These results indicate that the restricted expression pattern of *AtBAS1* in rice alters leaf inclination as well as inflorescence architecture. The unexpected changes in the inflorescence may be due to a compensatory growth response in response to reduced BR in the boundaries.

In chapter three, an independent genome-wide analysis of the rice *LBD* gene family was conducted to identify and characterize putative *LOB* orthologs in rice. *Arabidopsis AS2* and its orthologs in maize and rice, *ZmIG1* and *OsIG1*, have been shown to have conserved functions (6; 11; 16; 31). This suggests that the functions of *LOB* and BR regulation may be conserved between *Arabidopsis* and rice. Based on phylogenetic studies, *OsRa2*, which has an unknown function, is thought to be orthologous to *LOB* based on its similarity to the putative LOB ortholog in maize *RA2* (3). This study found the rice genome to encode 38 *LOB*-domain proteins, three more than previously reported, which share a conserved DNA binding domain at the N-terminal region (23; 30). Phylogenetic analysis confirmed four class Ia *OsLBD* genes, *OsRA2*, *OsIG1*, *OsLOB4*, and *OsIAL1*, to be the most closely related to *AtLOB*. Given that only the LOB domain is conserved between LBD proteins, the variable C-terminus can give these genes diverse functions making it difficult to identify *AtLOB* orthologs only on the basis of phylogenetic analysis (23).

Since *LOB* is expressed in organ boundaries and is positively regulated by BR, class Ia *OsLBD* genes were examined for pattern of expression and BR regulation. These criteria should allow us to identify the best candidate *LOB* ortholog(s) in rice. All class

Ia *OsLBD*s were expressed in lamina joints, inflorescences, and floral organs, similar to *AtLOB*, suggesting that class Ia *OsLBD*s may have similar functions to *AtLOB* (2; 23). To determine if class Ia *OsLBD*s function in BR signaling, BR hormone response data, was gathered. None of the class Ia *LBD*s exhibited a change in expression in response to BR, suggesting BR regulation is not conserved among *AtLOB* and rice orthologs.

To further understand the relationship between *LOB, BAS1*, and BR feedback loop in rice, future studies should be carried out as follows:

1) Both *AtpLOB:LOB* and *AtpLOB:BAS1* transgenic plants exhibited altered plant architecture, erect leaves, elongated inflorescences with increased branching, and longer, slender grains. Examining the lamina joint in closer detail in *AtpLOB:LOB* and *AtpLOB:BAS1* transgenic lines is necessary to determine if the cause of altered leaf inclination is due to defects in cell morphology similar to BR mutants (18; 32; 33). Misexpression of *IBH1*, a transcription factor that mediates BR regulation, results in rice plants with erect leaves caused by the reduction in cell elongation or division on the adaxial surface of the lamina joint (32). This suggests that the altered leaf inclination in *AtpLOB:BAS1* plants is perhaps caused by the disruption of cell elongation/division due to the inactivation of endogenous BRs on the adaxial surface of the lamina joint. Alterations in grain size may also be the result of altered cell division/elongation in the longitudinal plane and reduced cell division/elongation in the transverse plane. Observing cross and longitudinal sections and SEM images of the lamina joint and grains can uncover the cause of the erect leaves and altered grains.

2) The altered plant architecture in both *AtpLOB:LOB* and *AtpLOB:BAS1* transgenic plants suggest endogenous levels of BR may be reduced in the lamina joint. To determine if this is true, comparative analysis of endogenous BR intermediates in wild-type, *AtpLOB:LOB,* and *AtpLOB:BAS1* lamina joints can be done using gas chromatography mass spectrometry (7). The concentration of brassinolide, the most bioactive BR in rice, should be reduced compared to wild-type. If this holds true in both *AtpLOB:LOB* and *AtpLOB:BAS1* plants, it would suggest that the LOB-BR feedback loop is conserved in rice.

3) While the expression patterns of class Ia *OsLBD*s, *OsRA2*, *OsIG1*, *OsLOB4*, and *OsIAL1* were examined using publicly available expression data, other methods can be used to get an in depth analysis of the expression patterns. *in situ* hybridization can give a detailed analysis of the expression pattern in organ boundaries. Using *in situ* hybridization to examine the expression pattern of class Ia *OsLBD*s in rice shoot apices and young leaf tissues can determine which putative *LOB* orthologs are expressed in organ boundaries or lamina joints. Observing the pattern of transcript accumulation in rice tissues will determine if any of these genes are expressed in a similar pattern to *LOB* and *ra2*. If the transcript levels are too low to be detected through *in situ,* qRT-PCR on dissected lamina joints can be performed. Promoter:*GUS* constructs can also be created to give a more detailed expression analyses.

4) To determine if class Ia *OsLBD*s are BR regulated, I conducted an independent experiment in which rice seedlings were treated with exogenous BR in a time-course. RNA was isolated from dissected second and third leaf lamina joints, which were about

1mm in width. To determine if the BR-induction worked, the transcript accumulation of a known gene to be up regulated by BR was used. Unfortunately, there were variable results and inconsistency between biological replicates in three independent experiments. A solution to this problem may be to treat older rice plants with exogenous BL. Older plants will have lamina joints that are more prominent, making it easier to excise only the lamina joint without including any blade and sheath tissue. Using only the lamina joint tissue can better determine if these genes are regulated by BR similar to *AtLOB.*

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