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Functional Characterization of a Jacalin-like Lectin Domain-Containing Protein in *Arabidopsis thaliana*

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FUNCTIONAL CHARACTERIZATION OF A JACALIN-LIKE
LECTIN DOMAIN-CONTAINING PROTEIN IN
ARABIDOPSIS THALIANA

A Thesis Presented
to the Graduate School
of Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Plant and Environmental Sciences

by
Shane Reighard
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Accepted by:
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ABSTRACT

Jacalin-related lectins (JRLs) are proteins that bind carbohydrates. Functionally, JRLs are thought to be involved in a diverse array of functions including biotic stress defense, intracellular storage, abiotic stress response, and plant development. Recent *Arabidopsis thaliana* microarray data indicate that a Jacalin-like domain-containing protein (*JLL1*) exhibits highly root specific expression. Previous transcriptomic and proteomic studies indicate that *JLL1* may have a dual role *in planta* as a biotic stress defense protein and in plant development. In order to better understand the physiological function of *JLL1* in *Arabidopsis*, several different analyses were conducted examining its regulatory sequences, spatial expression, responsiveness to abiotic stress, and its impact on seed germination. The results of these investigations reveal that *JLL1* exhibits high sequence similarity with two adjacent jacalin domain-containing proteins. The *cis*-regulatory elements within *JLL1*'s promoter region are largely associated with plant development and metabolism. The spatial expression of *JLL1* was localized in the vascular-associated regions of the plant roots, leaf vasculature, and root tip (cap). RTPCR data indicate that *JLL1* is negatively regulated during abiotic stress, and *JLL1* mutant seeds exhibited delayed germination under abiotic stress conditions. Our data supports the assertion that *JLL1* has a dual role *in planta* as a protein involved in hormone-mediated early plant development and as a secreted non-specific defense protein.

DEDICATION

I would like to dedicate this work to my father, Gregory, and my mother, Angela, who have been very supportive to me throughout my life.

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CHAPTER 1: LITERATURE REVIEW

IA. INTRODUCTION

Demographic, environmental, and social issues continue to influence the food security of billions of people. Global agriculture will encounter both old and new problems in the 21st century requiring a coordinated response by governments, industry, and the scientific community to develop effective strategies to combat each problem.

A broad spectrum of research (macroscale to the molecular) is required to develop comprehensive solutions to these emergent issues. Consequently, my research is focused on understanding the molecular physiology of the model genetic organism *Arabidopsis thaliana*. Specifically, I hope to characterize a putative stress response gene coding for a jacalin-like lectin domain-containing protein (annotated *JLL1*). Microarray data indicate that *JLL1* exhibits strong root-specific expression suggesting that this gene's function is connected to activities found exclusively in the roots. Since many forms of stress response (abiotic and biotic stress), rhizosphere interactions, and water uptake are activities performed by root tissues, *JLL1* may be an element in one of these molecular processes.

I will focus on abiotic stress during this review-- to the exclusion of other potential processes-- because abiotic stress is ubiquitous, has a large impact on

global crop systems, and there is a possibility that *JLL1* may have a role in a plant's response to this type of stress.

In this review, I will briefly introduce a few of the current and future challenges facing agriculture, discuss the molecular basis of plant responses to abiotic stress, highlight current genetic engineering strategies to combat abiotic stress, provide an overview of the lectin protein family with an emphasis on the Jacalin-related lectins, cover previous research concerning *JLL1*, and conclude by stating my hypothesis which forms the basis of my research.

IB. AGRICULTURAL CHALLENGES IN THE PRESENT AND FUTURE

A variety of factors act together to create food insecurity. Due to the complicated nature of this problem, I will focus primarily on three issues that will be preeminent during the 21st century: demographic pressure, climate change and abiotic stress.

Demographic Issues

The world population doubled between 1950 and 1995.⁵ Current estimates project the world population to reach eight billion by 2020, nine billion by 2050, and eventually stabilizing at eleven billion by 2150.^[5,14] This growth in population will strain inefficient food distribution systems, and contribute to the scarcity of freshwater in many regions of the world. With approximately one in seven people undernourished today, strained food distribution systems would have a profound impact on the health and development of poorer communities.¹⁴ Expanding

agricultural land usage in many regions is financially unfeasible due to the economic incentives towards other industries, while many acres of existing arable land have been lost to urbanization, desertification, biofuel usage, salinization and soil erosion.¹⁴ Additionally, the growing affluence of the world feeds into this problem through increased consumption.

The aforementioned problems present a “threefold challenge” to global food security. To meet this challenge, the agricultural industry must satisfy three criteria: food production must be environmentally friendly and sustainable, the poorest citizens must be adequately fed, and the industry must increase its production to satisfy an increasingly affluent population.¹⁴ With these challenges, researchers predict that food production must increase by 70-100% by 2050.^[14,42] Other studies suggest that yearly levels of food production must increase by 44 million metric tons.³⁶ One solution may involve reducing the “Yield Gap”, which is the disparity between the actual yield of a hectare of land and the best possible yield.¹⁴ Increased fertilizer use, genetically engineered crops, and better land management will help to decrease this disparity.

Climate Change

In addition to demographic pressures, the changes in climate brought on by global warming will have a substantial impact on regional weather patterns.^{[15,}

^{21]} Global temperatures are predicted to increase by 2.5-4.3 °C by 2100. The impact of these increased temperatures has already been observed in the \$5

billion per year reduced yield of cereal crops in the 1980s and 90s.⁴² Climate scientists predict that global warming will increase the incidence of drought, heatwaves, tropical cyclones and flooding, while the increased atmospheric CO₂ levels will have a mixed impact on the relative survival of C3 and C4 plants.²¹

Climate change will also impact the availability and quality of water resources in specific global regions. Mid and low latitudes will experience a decrease in available water, while higher latitudes will have increased water availability.¹⁵ The warmer climate will also alter the pH and ionic content of the available freshwater. Regions already experiencing demographic problems (south Asia, sub-Saharan Africa, and small islands) will be more severely impacted by these climatic changes.^[15, 42]

Abiotic Stress

Abiotic stress encompasses the non-living factors that negatively impact the growth and development of living organisms. This type of stress includes salinity, drought, heat, oxidative conditions, cold temperatures, and high wind. Abiotic stress is the primary cause of crop loss worldwide, reducing the yields of many major crops by over 50%.⁴³ Salinization alone has caused the loss of 30% of arable land the last twenty-five years.⁴³

Due to their sessile nature, plants have evolved a diverse array of mechanisms to combat abiotic stress. These stress response mechanisms (physiological and genetic) are of particular interest to plant molecular biologists

because the information derived from these systems impacts other fields of research while providing practical tools to engineer and breed crops for abiotic stress tolerance. Elucidating these mechanisms allow scientists to: understand how plants integrate environmental cues into its molecular regulation and development, discover how gene regulation changes in response to the environment, determine the systemic roles of gene families, and decode plant evolutionary history through the comparison of analogous systems. These studies also provide the foundation for applied technologies including genetically engineered crops, marker assisted breeding, and analytical or diagnostic field-tests to assess crop vitality.

The next section examines the mechanisms plants use to survive and thrive during abiotic stress. The integration of this knowledge into genetically engineered crop systems is then covered in section four.

IC. PLANT MOLECULAR PHYSIOLOGY AND ABIOTIC STRESS

Abiotic stress disrupts systemic functions causing the loss or disruption of important processes. For example, drought or saline stress alters the water potential which eventually disrupts plant homeostasis. Oxidative stress promotes the denaturation of protein through the generation of excess reactive oxygen species (ROS). Plants utilize three main protein categories to maintain systemic integrity. These include: signal cascades and transcription factors, defensive

proteins (i.e. heat shock proteins, free-radical scavengers), and water/ion transporters (aquaporins, ion channels).⁴³

The initiation of these molecular networks requires an initial sensing of the stress. In some species, a sensor molecule may change confirmation indicating that there has been an environmental change. In this case, an integral membrane protein detects changes in membrane fluidity or the separation of the membrane from the cell wall.²¹ Plant species also can detect environmental stress through the accumulation of metabolite indicators, ROS or signals from the mitochondria.⁴⁸ These indicator molecules may correspond with reduced energy levels or an ATP release generating a signal that stimulates the initiation of nuclear transcription.⁴⁷ The ubiquitous protein kinase family SnRK1 (*Arabidopsis thaliana*) is an example of signaling molecules that respond to metabolic cues.⁴⁶

Since stressors (salt, heat, drought, osmotic) are interconnected, many common biochemical pathways are activated during the plant's response. Additionally, the presence of multiple, simultaneous stressors in the field environment complicate our understanding of plant physiology by making it difficult to control and replicate treatments in the laboratory. This issue is briefly discussed at the end of this section.

Plant Response Mechanisms

Transcriptional networks that respond to abiotic stress have been organized into regulons that respond to upstream signaling molecules including

histidine kinases (HKs) and mitogen-activated protein kinases (MAPKs).²³ These regulons include *CBF/DREB* (cold-stress response), *AREB/ABF* (salinity, drought), and *MYC/MYB* (ABA-dependent).^[21, 43, 49, 50] There is significant overlap in the genes activated by each regulon, thus stress responses converge at different regulatory levels.

Plants also utilize defensive molecules to protect against stress. Heat shock proteins (HSP) are up-regulated under heat, salinity and water stress. HSPs are activated by Heat Shock Factors that bind to *cis*-acting heat shock elements. HSPs act as chaperones protecting the endogenous cellular machinery by maintaining their native conformation.⁵¹ Under oxidative, salt, drought and high light stress, ROS (OH^- , H_2O_2) are generated, which damage endogenous macromolecules and membranes. Plants combat these species through the induction of antioxidant molecules (catalase, superoxide dismutase) that convert the ROS to non-reactive products.^[43,52]

Compatible solutes are molecules that are over-expressed in response to osmotic stress. These solutes, or osmolytes, help the cell maintain turgor and drive water uptake. Proline, quaternary amines (glycine betaine) and sugars (mannitol) all serve as compatible solutes.⁴³ Ion transporters also play an important role in protecting the plant from osmotic and saline stress. Antiporters (Na^+/H^+) control ion concentrations across the cell membrane while also maintaining cytoplasmic pH and cell turgor.⁴³

Abiotic Stress and the Field Environment

Abiotic stress treatments in the lab are often dissimilar to what the plants experience in the field environment.^[21,27] In the field, multiple-simultaneous stressors may impact a plant. For example, drought stricken regions experience the combined stresses of drought, heat and salinity stress. Additionally, large fluctuations in the stress intensity and duration occur over a short period of time, as opposed to the more constant conditions found in laboratory treatments. The developmental stage of the plant also determines its vulnerability to the stress (e.g. flowering period increases vulnerability) and the molecular mechanisms with which the plant can respond.^[21,27]

Since the plant has a unique acclimation response to each abiotic stress, a combination of stresses may also elicit a response that is unique to that combination. This is most apparent when two simultaneous stressors cause antagonistic physiological responses. The heat/drought stress combination is an example of a situation where the plant's physiological responses to the individual stresses are fundamentally antagonistic. Under heat stress, the plant opens its stomata to cool, however, drought conditions causes the plant to close its stomata to preserve internal water.²⁷

ID. GENETICALLY ENGINEERED CROPS AND MODERN AGRICULTURE

Genetic engineering of crop species for enhanced abiotic stress tolerance is a powerful way to mitigate many of the current and future agricultural problems

while decreasing environmental and financial costs. However, unlike the monogenic solutions to biotic stress tolerance, the complexity of abiotic stress complicates efforts to engineer the plant's physiology to effectively respond and thrive in harsh environments.^[7,43] Current efforts have focused on modifying plant defense mechanisms to increase their effectiveness.

Methods to Enhance Plant Abiotic Stress Tolerance

The modification of regulatory networks through the overexpression of component transcription factors is one method researchers have used to increase abiotic stress tolerance. The overexpression of the cold-response (CRT/DRE) transcription factor CBF1 has demonstrated increased cold-stress tolerance in *Arabidopsis*.⁵³ Altering the solute accumulation pathways is another approach to enhance plant defense. The rate-limiting enzyme in proline biosynthesis (P5CS) is subject to feedback inhibition. A study in 2000 demonstrated that an induced mutation in the P5CS that eliminated enzymatic inhibition increased proline accumulation two-fold.⁵⁴

Other studies have focused on overexpressing ion transport proteins found in the cellular membranes. As described previously, these proteins enable the plant to maintain homeostasis and ion concentrations across the membrane. Researchers found that the over-expression of the vacuolar ion antiporter AVP1 (a H⁺-pump protein) increased the salt and drought tolerance of *Arabidopsis thaliana* plants.⁵⁵ Another study found that tomato plants over-expressing the

vacuolar Na⁺/H⁺ antiporter protein *AtNHX1* accumulated higher amounts of sodium in their leaf tissues, but fruit content and yield were not impacted.⁵⁶

Genes involved in detoxification and ROS scavenging can provide engineered plant species oxidative, salt, heat, and drought tolerance. Transgenic tobacco plants over-expressing iron(Fe)-superoxide dismutase, an ROS scavenger, demonstrated increased oxidative stress tolerance when the plants were exposed to ozone.⁵⁷ Other studies have shown that increasing the production of the compounds in the xanthophyll cycle (through the overexpression of a gene involved in zeaxanthin biosynthesis) reduced the susceptibility of *Arabidopsis* to high light and high temperature damage.⁵⁸

Future engineering strategies could utilize new developments in plant genetics and genomics. Between 20%-40% of eukaryotic genes are uncharacterized or poorly understood.⁵⁹ Additionally, most of these genes are species specific, thus these uncharacterized proteins may have novel functions related to the species' environment including abiotic stress tolerance. Genes specific for stress tolerant species (i.e. halotolerant plants or cold-tolerant fish) could be introduced into crop varieties to improve their stress resistance through improved or novel stress pathways.²¹

Accelerated flowering and senescence are two barriers to crop yield in a stressful environment. Studies have shown that expressing cytokinin pathway enzymes under a drought responsive promoter delayed leaf senescence and increased plant productivity.^[60,61] Controlling the epigenetic changes required for

a plant to move from the vegetative to reproductive stages of its growth may also improve crop yield.²¹

The previous two sections provided an overview of plant abiotic stress response, and the current (and future) strategies utilized to improve plant stress tolerance. The next two sections will examine our current knowledge of lectins with a specific emphasis on jacalin-related lectins (or jacalin-like lectins) and *JLL1*.

1E. THE PHYSIOLOGICAL AND BIOCHEMICAL ROLE OF LECTINS

Lectins are described as “glycoproteins that bind reversibly to specific mono- or oligosaccharides without altering the structure of the bound ligand”.²⁴ These proteins have four structural classifications based on the number and type of carbohydrate binding domains (CBD). The four classes include: merolectins (one CBD), hololectins (at least two identical CBDs), superlectins (at least two non-identical CBDs), and chimerlectins (a fusion of a CBD in tandem with an unrelated domain). Carbohydrate specificity of lectins is varied; however, they have a higher affinity for oligosaccharides than simple sugars. Lectins with structurally different CBDs can also recognize and bind the same sugars.²⁴

As a group, lectin proteins are considered very heterogeneous exhibiting a broad range of biochemical and physiochemical properties. These proteins are also ubiquitous, as they are found in a diversity of organisms, from viruses to humans.^[16,38] Lectin proteins have been classified into seven families based on

their structural properties, binding specificity and the organisms from which they were isolated.²⁴ With the advent of new sequencing technologies¹⁹, refined sequence comparisons, and evolutionary/serological relationships have expanded the number of lectin families to 12.³⁷

The interaction between the lectin protein and a specific glycoconjugate (or small hydrophobic molecule⁶²) is the molecular basis of a lectin's physiological role.²⁴ From this interaction, plant lectins are thought to have a role in biotic stress response (plant defense)^[3,16,24, 28, 32, 39], abiotic stress response^[3,16, 39, 64], intracellular functions (nitrogen storage, direct glycoprotein traffic)^[24, 32, 39, 58, 63], mediating the association between leguminous plants and nitrogen-fixing bacteria.^[32, 63], the recognition of molecules on cell surfaces or fluids³², and plant development.²⁸ With such a diverse set of functions, many plant lectins are also thought to have dual roles, one extracellular and one intracellular.^[24, 28] For example, certain seed and vegetative tissue lectins may act as nitrogen storage proteins during plant development; however, when the plant is subjected to biotic stress, the lectins act as defense proteins.

Biotic Stress Response

Due to their specificity in binding glycoconjugates found on microorganisms and the gut-cell surfaces of arthropods, lectins are thought to be involved in plant biotic defense.²⁴ Plant lectins may be elements in the two main biotic defense mechanisms inherent to plants: passive and active. Passive

defense involves the establishment of physical barriers, biochemical or morphological adaptations that hinder pathogen/herbivore attack. The toxicity of lectins to insects and herbivores is an example of the lectin's role in passive defense.^[24, 28] The active defense mechanism involves the specific synthesis and localization of defense related molecules at the region of attack.⁶² The accumulation of barley lectin and wheat germ agglutinin in nematode infested roots, and the localization of these lectins to the nematode feeding sites (these proteins were not induced by a separate nematode species) are examples supporting the role of lectins in active plant defense.⁶⁵

Abiotic Stress Response

Due to their specificity to foreign glycans, lectins were originally considered to be defense proteins against herbivores or pathogens²⁴. Increasing evidence now suggests that many lectins have an endogenous role in the cell³⁹. The first study that verified this interaction focused on the jacalin-related lectin, *saIT*, which was expressed in the roots and sheaths of rice after salt, drought, ABA and biotic stress treatments.⁶⁶ Later studies have found several lectins that are responsive to abiotic stress including abscisic acid (ABA), which is a plant hormone associated with abiotic stress^[67, 68].

Wheat germ agglutinin (a generalized term for wheat lectin) was induced by ABA treatment in the roots of wheat seedlings^[2,30]. This same group of lectins accumulated in wheat seedlings during salt³¹ and heat²⁶ treatments suggesting

that these proteins are involved in general stress response.^[7, 26] Under cold stress, researchers found that lectin-like proteins in cold-adapted winter wheat seedlings can “control membrane functional activities during the course of cold adaptation”.¹⁸ Additionally, lectins in mistletoe and cabbage have demonstrated cryoprotective properties⁶⁴.

Jacalin-related Lectins

Jacalin-related lectins (JRLs), also called jacalin-like lectins, contain domains similar (or identical) to the jacalin domain, which was originally isolated from the jackfruit (*Artocarpus integrifolia*). The jacalin-domain can bind mannose, maltose, and galactose.¹² Physiologically, jacalin-related proteins are involved in a diverse array of functions. Early studies discovered that Moraceae jacalin-like lectins were abundant in seed tissues and demonstrated anti-insect activity. Researchers speculated that these lectins are storage proteins with an accessory defensive application. In the family *Convolvulaceae*, another group of lectins are jacalin-like. This group of proteins contains members that are rhizome-specific, cytoplasmic, and members exhibiting mitogenic activity.^[12,23,35]

Jacalin-related proteins are also active in biotic defense response with some studies suggesting that defense related JRLs share a common ancestry.²⁰ These inducible JRLs have between 2-6 tandem jacalin domains.³⁹ The protein *RTM1* is a constitutively expressed JRL found in vascular-associated cells. *RTM1* (along with *RTM2*) are JRLs that function in the phloem and sieve

elements to restrict the movement of the tobacco etch virus (TEV) in *Arabidopsis*. It is speculated that *RTM1* is involved in the “generation, perception, or transport of a systemic signal” used to restrict TEV. [8, 9] Additionally, other studies have found that JRLs are components of the salicylic acid and jasmonic acid defense pathways^[41, 44]

Many lectins are developmentally regulated in a similar manner to storage proteins thus they may have a role in nitrogen storage during germination.⁵⁸ JRLs in black mulberry tree bark were shown to be involved in cellular storage with a galactose-specific and a mannose-specific lectin exhibiting vacuolar and cytoplasmic accumulation respectively.^[25, 40] Additionally, JRLs are involved in plant cellular and morphological development. Two antagonistic JRLs regulated the size of the ER body-type b-glucosidase complexes in *Arabidopsis*.²² A recent study reported a jacalin-related lectin in *Eichhornia crassipes* that promoted root-elongation during sulfur-deficient conditions⁶⁹.

JRLs also exhibit responsiveness to abiotic stress conditions. Water-deficit, mechanical wounding, and ABA treatments induced the up-regulation of two JRLs, *Hfr-1* and *Wci-1*.³³ In another study, a mannose-binding JRL in rice was isolated in salt-stressed rice suggesting the “importance of protein-carbohydrate interactions” in plant stress response and the role of JRLs as stress-responsive genes.⁴⁵ Further research is required to understand the specific role jacalin-related lectins may have in a plant’s physiological response to environmental stress.

1F. PREVIOUS RESEARCH ON JLL1

As a member of a poorly categorized protein family, *JLL1* has not been explicitly studied. However, transcriptomic, proteomic, and yeast-two hybrid studies provide some important insights into the regulation of *JLL1*. *Jacalin-like lectin 1*'s genomic sequence is found on chromosome 1 in *Arabidopsis thaliana* (AT1G52070). This 315 aa protein contains two tandem jacalin (or mannose-binding) domains making it a hololectin. The TAIR database (<http://www.arabidopsis.org/index.jsp>) indicates *JLL1* is expressed in root tissue, and is localized in the endomembrane system.

A transcriptome study in 2004 conducted on *Arabidopsis* tricarboxylic acid cycle mutants *mls-2* and *icl-2*, demonstrated a 3.3 and a 9.3 fold reduction, respectively, in *JLL1* transcripts compared to wild-type controls. The proximal locus, AT1G52060 also showed a reduced expression profile in *mls-2* mutants. These tricarboxylic acid cycle mutants exhibit reduced growth and slow establishment on media, with *icl-2* having the most severe phenotype. According to the authors, the *icl-2* mutants grew slowly (compared to wild-type) and were unable to convert lipids into carbohydrates creating a phenotype that is akin to "carbohydrate starvation".¹⁰

Another transcriptomics study in 2005 examined the changes in gene expression during germination caused by the establishment of facultative heterochromatin. Trichostatin A (TSA), an inhibitor to histone deacetylase (HDAC), was applied to *Arabidopsis* seeds during germination. *JLL1* exhibited a

3.89 fold decrease in expression when TSA was applied to the germinating seeds³⁴ (This was the highest fold reduction reported). Since epigenetic changes are utilized to change the expression profile of cells during seed germination, the inability of HDAC to form heterochromatin would impact the expression of genes activated after the establishment of heterochromatin. This data suggests that *JLL1* may have a role in seed germination or is involved in a process that supports early seedling growth.

Four F-box proteins (VFB) were characterized in a 2007 study. These proteins belong to a family known to regulate auxin and ethylene response. VFB mutants exhibited delayed growth and reduced lateral root formation. Microarray data from this study indicates that *JLL1* exhibits a 1.59 fold repression in the VFB mutants. This reduction was concurrent with several enzymes involved in cell wall metabolism. Since these enzymes are not responsive to auxin, the authors speculated that the misregulation of this gene set is due to the interrupted development of VFB mutants.²⁹

A yeast two-hybrid study published in 2007 determined that *JLL1* interacts with the *Arabidopsis Response Regulator 5* (ARR5), which is a nuclear response regulator involved in the two-component signaling pathway.¹² ARR5 has been found to mediate the cross-talk between auxin and cytokinin during plant development¹⁷. It also exhibited elevated expression in response to cytokinin with localized expression in the apical root meristem and the vascular associated regions of mature roots.¹ These studies suggest that *JLL1* may have a role in

plant growth, development, or metabolism. However, other studies suggest *JLL1* is a biotic-defense protein.

A proteomics study from 2010 indicates that *JLL1* (along with AT1G52060 and AT1G52050) demonstrates more than a 3-fold increase in rhizosphere secretion before flowering than after flowering. Protein secretions have been implicated in offensive, defensive, and symbiotic interactions with soil organisms. Defense-related proteins exhibited the highest secretion before flowering. Additionally, the biotic defense enhanced mutant *cpr5-2*, which accumulates larger amounts of salicylic acid than wild-type, also secreted higher levels of *JLL1*.^{70, 11} Researchers investigating the transcriptional changes in *Brassica oleracea* during insect (*Pieris rapae*) feeding found that *JLL1* expression increased during caterpillar attack.⁴ This study utilized an *Arabidopsis* microarray, thus the experimental expression levels may not be reflective of the actual genes expressed in *Brassica oleracea* especially if *JLL1* is *Arabidopsis* specific.

IG. RESEARCH OVERVIEW AND HYPOTHESIS

Lectins (and especially jacalin-related lectins) remain a poorly understood class of proteins. Previous research indicates that *JLL1* may have a dual role *in planta*. This lectin's endogenous role may be as a hormone-mediated early plant growth and metabolism protein, while its exogenous activity (as a secreted protein) may be biotic stress defense. However, all of these previous studies

were large-scale “-omics” projects that provided the response of this gene under certain conditions or establish an *in vitro* interaction (yeast-two hybrid). To our knowledge, there have been no *JLL1* specific studies seeking to characterize the function of this gene.

My characterization of *JLL1* will begin with an *in silico* analysis to determine structurally related jacalin-like lectins in *Arabidopsis thaliana*. The promoter region of *JLL1* will also be assessed for *cis*-regulatory elements, which provide insight into the regulation of this gene. Tissue based semi-quantitative RTPCR analysis and transgenic *Arabidopsis* containing a promoter-GUS fusion construct will provide information on the tissue-specificity of *JLL1*. *JLL1*'s promoter-GUS reporter construct will also be introduced into turfgrass (a monocot) and tobacco (a dicot) to determine if the root-specificity of *JLL1*'s promoter is conserved between species. If it is, this promoter may be useful as a root-specific biotechnology tool, and if it does not exhibit similar expression, it suggests that this sequence is regulated in a manner that is specific to *Arabidopsis* (and species closely related to it).

A semi-quantitative RTPCR examining the expression of *JLL1* under the abiotic stress conditions of salt, drought, and ABA will be conducted. Previous studies did not examine *JLL1*'s expression under abiotic stimuli thus these studies will supplement the current data, and determine if this gene may be involved in abiotic stress response. This focus on abiotic stress is based on the microarray data-- which demonstrated strong root specificity-- because roots are

the first tissues to sense and respond to many abiotic stressors. Additionally, if *JLL1* has a role in plant growth or development, it may be down-regulated under abiotic stress conditions. Previous studies have indicated that genes involved in growth, development or metabolism exhibit down-regulation under abiotic stress due to growth inhibition. [71, 72, 73]

Homozygous T-DNA insertional mutants and overexpression lines will be generated to assess the phenotypic impact of *JLL1* on *Arabidopsis* physiology. These experiments will provide clues to the function of *JLL1* including the processes that this gene is active in.

In summary, this project aims to characterize the root-specific lectin *JLL1* in *Arabidopsis thaliana* through the combination of bioinformatics, *in vitro* and *in vivo* expression studies, and mutant/over-expression analyses. Based on the literature and our initial microarray data, my hypothesis is:

JLL1 is a root-specific jacalin-like lectin that is negatively responsive to abiotic stress due to its role in plant growth, development and/or biotic stress response.

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CHAPTER TWO

BIOINFORMATIC ANALYSIS OF *JLL1*

IIA. INTRODUCTION

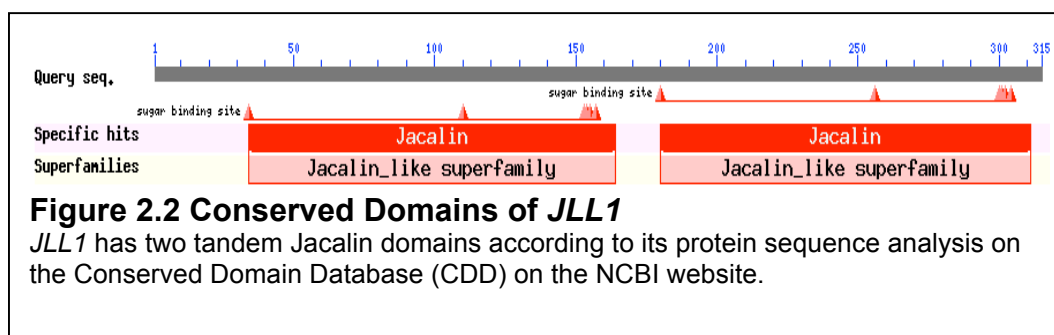
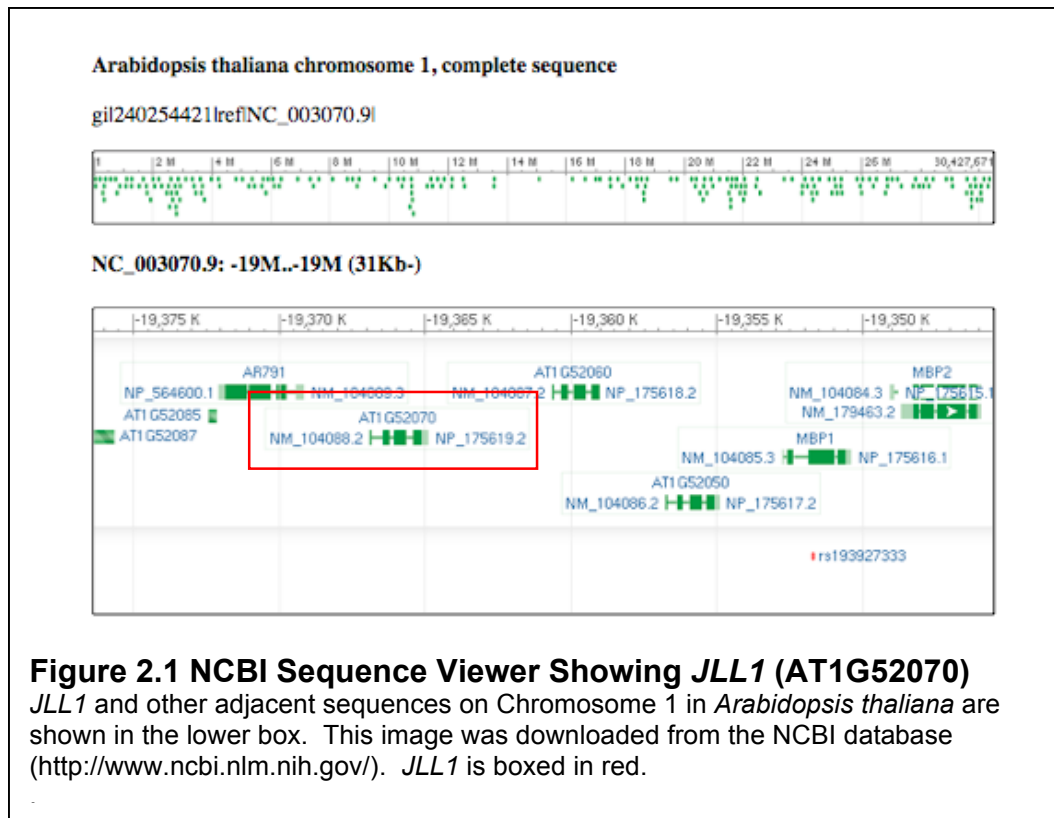
Bioinformatic techniques are able to elucidate the possible physiological and biochemical functions of *JLL1*. Since structure defines function at the molecular level, the structural similarity between two proteins suggests that these two molecular species share similar functions. Additionally, the regulatory sequences of *JLL1* can be assessed for *cis*-acting regulatory elements that relate to specific physiological processes. The presence (or even abundance) of certain families of regulatory elements within the promoter region suggests that this gene may be expressed during those processes.

This *in silico* analysis has two main goals. The first goal is aimed at determining the protein sequence similarity between *JLL1* and other Jacalin-related lectins found in *Arabidopsis thaliana* along with other closely related species. The second goal was to assess *JLL1*'s promoter region for the presence of *cis*-regulatory elements that may provide greater insight into how *JLL1* is regulated *in planta*.

An Overview of *JLL1*

Jacalin-like lectin 1 (JLL1) is a 315 aa (2,037 bp) protein found on chromosome 1 in *Arabidopsis thaliana* (Figure 2.1) containing two tandem jacalin-like superfamily domains (also known as mannose-binding domains)

(Figure 2.2). *JLL1* also contains four exons and three introns. Gene Ontology (or GO) annotations (downloaded from the NCBI website) for *JLL1* and two jacalin-related lectins that are adjacent on Chromosome 1 are also shown (Table 2.1). *JLL1* demonstrated greater sequence similarity with AT1G52060 than with AT1G52050 (Table 2.2).



GO Annotation					
Gene ID	Biological Process	Cellular Component	Molecular Function	Development stage	Expressed In
AT1G52060	*	*	*	*	root
<i>JLL1</i>	*	endomembrane system	*	*	root
AT1G52050	*	endomembrane system	*	4 anthesis, E expanded cotyledon stage	carpel, hypocotyl, pollen, root, seed, sepal, stem

Table 2.1 GO Annotations for *JLL1* and Two Adjacent Jacalin-like Lectins

The two Jacalin-like genes located adjacent to *JLL1* on Chromosome 1 did not have complete functional annotations. *JLL1* is predicted to localize in the endomembrane system and expressed in the root tissues. An asterisk (*) indicates there was no data. GO Annotations were found on the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

Sequence Level Comparisons to <i>JLL1</i>			
Gene ID	Length	Identity (%)	Gaps (%)
AT1G52060	969 (n)	86	1
	314 (p)	82	1
At1G52050	1145 (n)	78	2
	313 (p)	71	2

Table 2.2 Sequence Similarity between *JLL1* and Adjacent Jacalin-like Lectins

AT1G52060 is more similar to *JLL1* at the sequence level than AT1G52050. Both the nucleotide (n) and the protein (p) sequences of AT1G52060 demonstrated over 80% identity with *JLL1*. These sequences were compared using the sequence alignment setting (bl2seq) in the BLAST algorithm. Default settings were used. (n) and (p) indicate the identity of the sequence compared with (n) indicating the cDNA sequence and (p) indicating the protein sequence.

Protein Sequence Comparisons between *JLL1* and other Jacalin-related Lectins

It is widely thought that highly similar protein sequences produce similar tertiary structures. Since structure defines protein function, the transitive relationship between sequence, structure, and function may be largely upheld. There are significant problems with this relationship at the structural and functional level. Many studies have addressed the situations where this relationship breaks down leaving the sequence based comparison approach poorly predictive.^[74, 75, 76] However, in this study, closely related proteins in the same organism are compared to gain insight into the potential functions of *JLL1* thus the comparisons are still valid, at least, at the sequence level.

The primary objective of this experiment is to understand which Jacalin-related lectins are similar to *JLL1* at the sequence level and have been functionally characterized. We can then speculate on which JRLs share a similar function to *JLL1* through the transitive relationship between sequence, structure, and function. In order to broaden the number of comparative query sequences, and to build a more comprehensive picture of the relationships between intraspecific and interspecific proteins, two different sequence analyses were completed.

Analysis of Predicted *cis*-Regulatory Elements in *JLL1*'s Promoter Region

Clues to the regulation of *JLL1* can be found through the prediction and analysis of promoter-based *cis*-regulatory elements. These elements are

sequences generally located upstream of the transcriptional start site (TSS) where transcription factors assemble to drive the expression of the gene. The prediction of promoters and *cis*-regulatory elements *in silico* has been demonstrated to be reliable.⁸³ For this analysis, the PLACE database^[84,85] <<http://www.dna.affrc.go.jp/PLACE/signalscan.html>> was used to analyze *JLL1*'s promoter region to predict the presence of *cis*-regulatory elements.

IIB. MATERIALS AND METHODS

Protein Sequence Comparison #1- Gclust

Twenty-one sequences extracted from Gene Cluster 4271 on the Gclust Server version 2007-10 (<http://gclust.c.u-tokyo.ac.jp/>).^[77, 78] were compared using the multiple sequence alignment software ClustalX Version 2.0.12 (downloaded from www.clustal.org/clustal2/)⁸⁰. These sequences are composed of homologous loci (including orthologs and paralogs) found in *Arabidopsis thaliana*. Default settings were used for the ClustalX alignment. GO annotations from the Gene Ontology website⁸¹ (www.geneontology.org) have also been provided (Table 2.2). A phylogenetic tree was generated using the ClustalX software (Figure 2.3).

Protein Sequence Comparison #2- Homologene Database and BLASTp

This analysis expands the number of compared sequences to seventy-two, and the candidate loci were compiled using two separate methods: the homologene database on NCBI (<http://www.ncbi.nlm.nih.gov/homologene>) and a BLASTp search.⁷⁹ Forty-two (of the 73) protein sequences were extracted from the Homologene database (Query: AT1G52070) on the NCBI website.

The remaining thirty sequences were generated from a BLASTp search (query: NP_175619.2). Non-hypothetical sequences (excluding those from the Homologene database) demonstrating greater than 40% similarity to *JLL1* were added to a master list (.txt file). These seventy-three sequences were then input into ClustalX, and a phylogenetic tree (Figure 2.4) was generated from this list. Twenty-two of the compared sequences were from other species (Appendix A: Table A-1).

JLL1 Promoter Sequence Selection

The sequence upstream of *JLL1*'s transcriptional start site (TSS) was downloaded (2.8 kb) from the NCBI database <<http://www.ncbi.nlm.nih.gov/>>. The 2.8 kb promoter sequence was truncated to around 1 kb which corresponds with the size and distances utilized in previous studies.⁸⁶ The annotated promoter sequence (~1kb) is shown in Figure A-2 (Appendix A). Eukaryotic promoter elements including the TSS (red), TATA box (green) and CAAT box

(blue) were annotated onto the sequence using the plantpromoter db version 2.1 software < <http://133.66.216.33/ppdb/cgi-bin/index.cgi>>. ⁸⁷

PLACE Database Analysis of *JLL1*'s Promoter Region

Previous studies found that many of the most significant associations between a *cis*-regulatory element in a promoter and the regulation of a gene were within 200 bp of the TSS. ⁸⁶ Due to this, elements residing within 300 bp of *JLL1*'s TSS were the focus of this analysis. The elements found within this range were compiled into an Excel (Microsoft® Office 2008) spreadsheet and annotated using information from the database. Table A-2 (in Appendix A) contains the identities of elements found within this 300 bp window. These elements were also mapped to *JLL1*'s promoter sequence (Figure 2.5).

IIC. RESULTS

Protein Sequence Comparison #1: Gclust

From the phylogenetic tree (Figure 2.3) it is apparent that *JLL1* has higher sequence similarity to loci AT1G52050 and AT1G52060 when compared to other Jacalin-related lectins found within *Arabidopsis thaliana*. Unfortunately, these two sequences, along with most of the other sequences, have not been experimentally categorized thus they have unknown biological functions (Table 2.3).

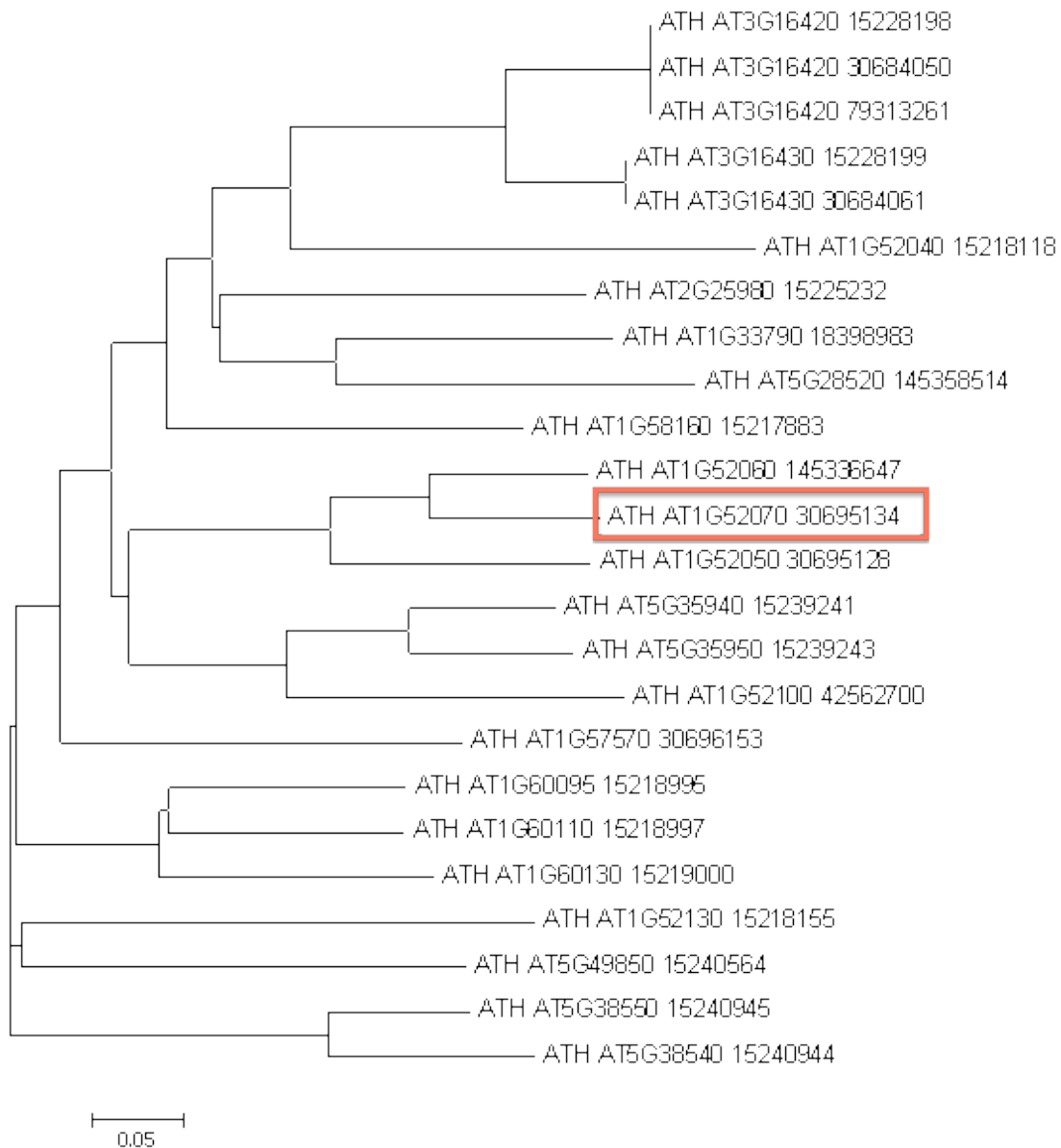


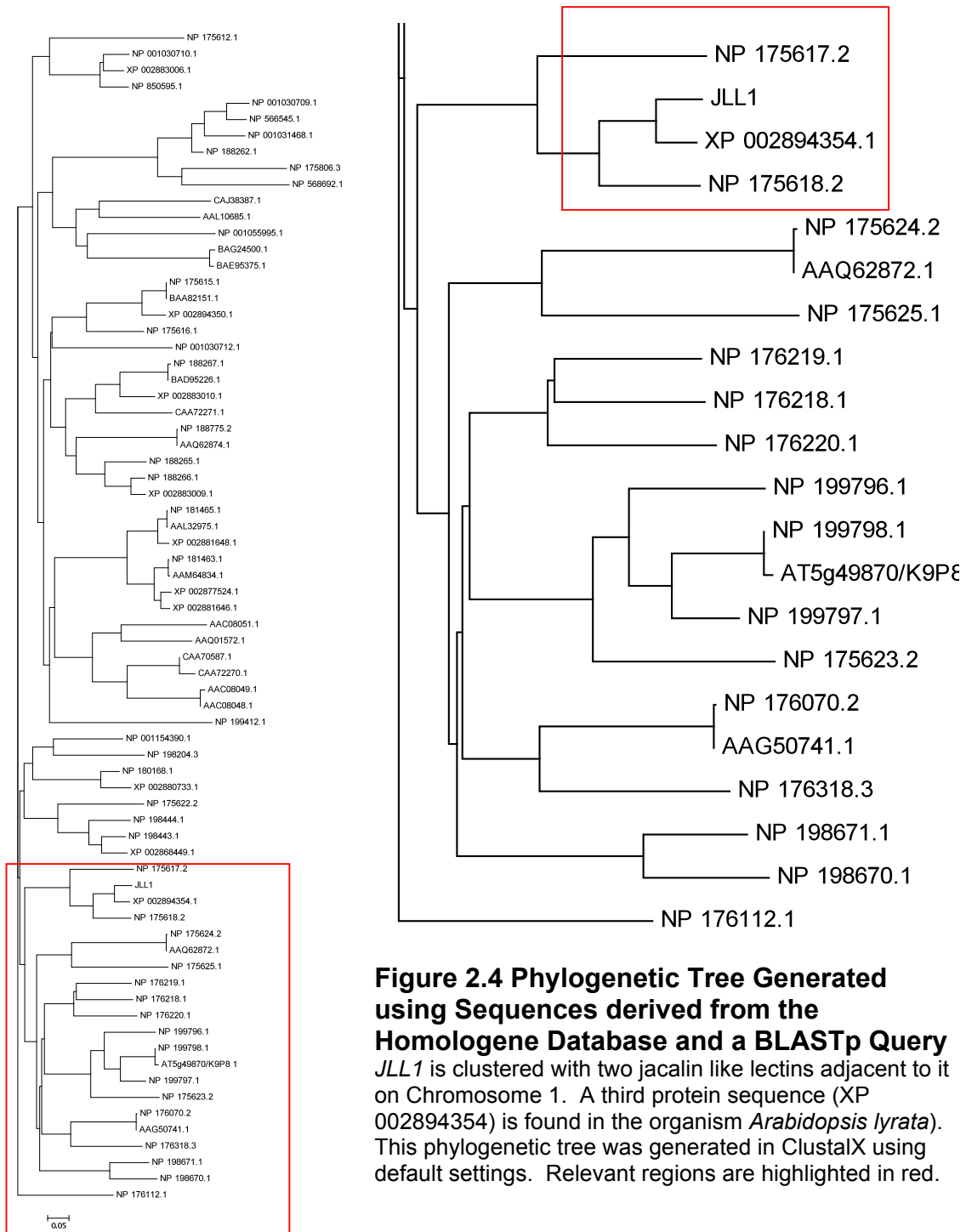
Figure 2.3 Phylogenetic Tree Generated from Gene Cluster 4271 on the Gclust Database

JLL1 demonstrates high protein sequence similarity with the two Jacalin-like lectins adjacent to it on Chromosome 1. Both of the adjacent sequences (AT1G52060 and AT1G52050) are located in the same group with *JLL1* highlighted in red. All three of these genes contain two predicted jacalin domains. ClustalX using the default settings generated this phylogenetic tree.

Gene ID	GO Annotation			
	Biological Process	Cellular Component	Molecular Function	Development stage
AT3G16420	Protein folding	cytosol, nucleus,	Copper-ion binding	seedling development
AT3G16430	*	*	Copper-ion binding	*
AT1G52040	Defense response	nucleus, vacuole	*	seed development stages, seedling development stages
AT2G25980	*	plasmodesma	*	*
AT1G33790	*	chloroplast	*	Bilateral stage, expanded cotyledon stage, mature embryo stage, petal
AT5G28520	*	*	*	*
AT1G58160	*	*	*	anthesis, leaf senescence stage, petal differentiation
AT1G52060	*	*	*	*
<i>JLL1</i>	*	endomembrane system	*	*
AT1G52050	*	endomembrane system	*	anthesis, expanded cotyledon stage
AT5G35940	*	plasmodesma	*	*
AT5G35950	*	*	*	*
AT1G52100	*	membrane	molecular function	*
AT1G57570	*	*	*	*
AT1G60095	*	*	*	*
AT1G60110	*	*	*	*
AT1G60130	*	*	*	*
AT1G52130	*	*	*	*
AT5G49850	*	*	*	*
AT5G38550	*	*	*	*
AT5G38540	*	*	*	*

Table 2.3: GO Annotations of the genes compared in the phylogenetic tree generated using information from the Gclust Database

Few of the jacalin-related lectins in *Arabidopsis* have been characterized experimentally. Only two of the sequences examined in this analysis have a corresponding biological function (AT3G16420 and AT1G52040). Asterisks (*) indicate there was no information available.



Protein Sequence Comparison #2- Homologene Database and BLASTp

Three proteins demonstrate close sequence similarity to *JLL1* (Figure 2.4). NP_175617 (AT1G52050) and NP_175618 (AT1G52060) have already been shown to be very similar to *JLL1* from the first sequence analysis. The other protein in this cluster, XP_002894354.1, is a jacalin lectin family protein found in *Arabidopsis lyrata*. This 315 aa protein, according to the Conserved Domain Database⁸⁵ (accessed through NCBI), contains two tandem jacalin-like superfamily domains.

PLACE Database Analysis of *cis*-Regulatory Elements

Figure 2.5 and Table A-2 demonstrate that *JLL1* has a diverse array of potentially active promoter-based *cis*-regulatory elements. However, not all of these regulatory elements are active *in planta* because the activity of an element is contingent on many factors including tissue identity, DNA access, and the arrangement of adjacent regulatory proteins. This data does illuminate potential processes that *JLL1* may be involved in. Interestingly, many of these elements are associated with processes that are known to utilize jacalin-related lectins including storage and plant development.

300 bp Upstream of *JLL1*'s TSS

TGTAAGCCTGTATATAAAAAAAAAACACAACCTTGTAATTAATTTGATATCCAAGCATTATAAATCCATGTTTTTAAAGAAATAGTT
TCTTTCTATATCGCTTGAATCGACGTTATTTTAAAATTAATGCATGCGGTGTAAGTGTAGCTAAATACTTTTAAAAGGCGAAAATAA
GAACTGATAAACATTTTTCTATAATGCCTCATAGGCCACTAGTTATAAACTAGTAATTTCCATATGTGAAAGACCCAGAACTGTGT
GTATAAATAAGAATCGTCAGCCATGGCTTCTTACCAATCACCAAGCAGCACAGCGATC

Pollen	Hormone	Light Regulation
Abiotic Stress	Photosynthesis	Storage/ Amylase

Figure 2.5: *JLL1*'s Promoter Sequence Annotated with PLACE Database Predicted Regulatory Elements

JLL1's promoter sequence has a variety of regulatory elements with growth and development related elements being predominant. The different colors represent the biological processes each element is associated with. There are repeated elements in this sequence (refer to Table A-2 for more information).

IID. CONCLUSIONS

Several conclusions may be drawn from the *in silico* analysis of *JLL1*.

First, *JLL1* appears to have high sequence similarity with two proximally located jacalin-related lectins therefore these genes may have similar tertiary structure and biological function. These three genes may be paralogs, however, the analysis lacks the complexity (and comprehensiveness) to state this conclusively. Co-regulation of these genes due to their close proximity on the chromosome is also conceivable. Second, *JLL1* may have an ortholog in the species *Arabidopsis lyrata* due to the demonstrated sequence similarity between these two proteins. Since natural selection acts to conserve protein sequence, the speciation event separating *Arabidopsis thaliana* from *lyrata* would not change these two sequences dramatically.

Finally, the predicted *cis*-regulatory element composition of *JLL1*'s promoter region generated a diverse array of potential elements. While many of the predicted elements may be due to the random assortment of base pairs (i.e. $\frac{1}{4}^n$ probability of finding certain elements randomly in the sequence), the condensed analysis window (only 300 bps) and the location of the elements (within 300 bp of TSS) would constrict sequence randomness. The promoter region of a gene is under selective pressure due to the high density of transcription factors that must assemble in the region thus errant mutations may not accumulate readily. The predicted elements were quite diverse, however, many of them were involved in processes related to growth and metabolism (light regulation, photosynthesis, storage, and hormonal control). This supports previous studies (see section 1F), which found reduced expression of *JLL1* in growth and metabolic mutants. Together, these factors support the assertion that *JLL1* has a role in plant growth, development or carbohydrate metabolism.

IIE. REFERENCES

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CHAPTER THREE

JLL1 EXPRESSION ANALYSIS

IIIA. INTRODUCTION

A gene's function is revealed by its spatial and temporal expression profile. Previous studies (Section 1F) have shown that *JLL1* demonstrates dramatic down-regulation in metabolic and growth mutants, and increased extracellular secretion in defense enhanced mutants. The implications of these results point to *JLL1* having a dual role in *Arabidopsis*, one intracellular as a storage or metabolism protein (potentially in carbohydrate metabolism or glycoprotein transport), the other role is extracellular as a non-specific biotic defense protein.^[24, 28]

Other studies have already established (through transcriptomic and proteomic investigations) how *JLL1* responds in growth/development mutants, and in defense enhanced mutants. There have been no studies examining how *JLL1* responds to abiotic stress conditions. An investigation into the abiotic stress responsiveness of *JLL1* would be illuminating for two reasons. First, the microarray data indicates that *JLL1* exhibits strong root specific expression. The roots are the plant tissue that experiences many abiotic stressors first (salt, xenobiotic compounds, drought, mechanical disruptions), thus it is not outside the realm of possibility that highly root specific proteins would be responsive to this type of stress.

The second reason *JLL1* should be tested under abiotic stress conditions is to investigate whether or not *JLL1*'s expression is consistent with it being a growth, metabolism or biotic stress gene. Recent studies demonstrate that growth and metabolism genes are down-regulated under abiotic stress conditions.^[71, 72, 73] Furthermore, the cross-talk between abiotic and biotic stress response pathways elicit antagonistic responses between ABA (abiotic stress responsive) pathways and jasmonic acid-ethylene (biotic stress responsive) pathways.^[88, 89] Thus, abiotic stress conditions cause the plant to reduce the expression of many biotic stress related genes. Due to these interactions, *JLL1* may exhibit reduced expression under abiotic conditions if it is a gene involved in plant growth, development or biotic stress response.

In order to comprehensively characterize *JLL1*, three experiments were conducted to elucidate its expression profile. Tissue specific RTPCR was performed to verify the tissue-specificity of *JLL1*. A promoter-reporter construct was introduced into wild-type *Arabidopsis thaliana* to determine the specific tissues *JLL1* demonstrates expression in. The same construct was also introduced into turfgrass (a monocot) and tobacco (a dicot) to assess the viability of *JLL1*'s promoter as a tool in biotechnology to drive root specific expression in commercial and research plant species. The expression of this promoter in dissimilar species also provides a clue on the uniqueness of *JLL1*'s root specific expression in *Arabidopsis*. Dissimilar expression in turfgrass and tobacco would suggest *JLL1*'s regulatory regions act in a unique manner in *Arabidopsis* thus

JLL1 may be a unique gene within the *Brassicaceae* family. Finally, a semi-quantitative RTPCR was performed on wild-type *Arabidopsis thaliana* plants after they were exposed to drought, salt and ABA treatments to determine the change in expression of *JLL1* in response to these conditions.

Spatial Expression Analysis of *JLL1*

A β -glucuronidase (GUS) promoter-reporter expression system enables an investigator to visualize the specific tissues where a gene is expressed through the accumulation of the intense blue stain, chloro-bromoindigo. A gene's promoter region is fused upstream of the GUS gene. When the staining buffer is applied to a transgenic plant containing the promoter-GUS fusion construct, the substrate, X-gluc, is cleaved *in vivo* by GUS producing the colorless glucuronic acid and an intense blue stain (chloro-bromoindigo).⁹¹ Tissues containing an active promoter will accumulate stain, while tissues and cells not expressing GUS will remain unstained. This promoter-GUS reporter system was employed to visualize the expression of *JLL1 in vivo*.

IIIB. MATERIALS AND METHODS

Tissue-specific RTPCR

A tissue specific RTPCR reaction was employed to amplify cDNA from three-week old *Arabidopsis thaliana* (ecotype Columbia) seedlings grown on half-strength Murashige & Skoog ($\frac{1}{2}$ MS) media plates.⁹⁰ The *Arabidopsis* tissues

were separated based on their identity, and the shoot, roots, and whole-plant (without root) tissues were powderized separately using liquid N₂. Total RNA from the tissues was isolated using the TRIzol® LS Reagent (Invitrogen™) followed by additional extraction steps. The isolated RNA was subjected to DNaseI treatment (Invitrogen™ commercial kit) and cDNA synthesis was performed using the SuperScript® III First-Strand Synthesis System from Invitrogen™.

The cDNA was amplified using two primers ordered from Integrated DNA Technologies, JL_RT_F (5'-CACACAGCACAGCGATCAT-3') and JL_RT_R (5'-AGTCTCGAATTACGAAGGA-3'). The cDNA sequence of *JLL1* and the corresponding primers used for all *JLL1* RTPCR reactions is shown in Figure B-1. The amplification size is 975 bp while the primer annealing temperature used was 60°C. A constitutively expressed actin gene (AT3G18780) was used to normalize cDNA concentrations across the three samples. The number of cycles used for the RTPCR reaction was 26. Two separate tissue-specific RTPCR reactions were run (Figure 3.1).

Binary Vector Construction

JLL1's promoter region (2.5 kb) was amplified using polymerase chain reaction (Reaction conditions: 98C for 180s, 32 cycles of 98C 10s, 58C 30s, 72C 90s;) and the primers JL_P_F 5'-TGAAAAATCGCTTAAAGATTTGGG-3' (T_m=51.5 °C and JL_P_R 5'-CGGATCCTCGAGATCGCTGTGCTGTGGTGATT

GG-3' ($T_m=60^\circ\text{C}$). The amplicon was cloned into the pGEM-T Easy vector (Promega) using Promega's TA cloning kit (Figure 3.3). A large, 2.5kb fragment was amplified for two reasons. First, enhancer sites far upstream of the core promoter may be active in regulating *JLL1*'s expression, thus our promoter must capture as many of these as possible. Second, the protein AR791's (hypothetical protein in the actin-binding protein family) promoter region could be incorporated into the completed vector providing clues on where this protein is expressed through the examination of fluorescence in the plant tissues resulting from the expression of the Green Fluorescent Protein (GFP).

Primers were designed to amplify several base-pairs from the 5' UTR (3' of *JLL1*'s TSS). (Figure 3.2) The pGEM plasmid (pHL204), containing *JLL1*'s promoter, was transformed into *E. coli* made artificially competent through the addition of divalent cations (CaCl_2) to cold bacterial culture. The *E. coli* was plated onto Luria Broth containing Ampicillin that was seeded with IPTG and X-gal for blue-white colony screening. A polymerase chain reaction was run on individual white colonies to determine if they contained the proper insert in the pGEM vector (Figure 3.4). Verified colonies were then cultured in L.B. Amp media at 37°C overnight. The pHL204 plasmid was extracted from the *E. coli* culture using the Quantum Prep® Plasmid Mini-prep kit (Bio-Rad).

A BamH1-Xho1 fragment was released from pHL204, and ligated into the backbone HBT-sGFP95. This construct was transformed into competent cell *E. coli*, and the bacteria were plated onto selection media (Amp) for colony PCR

verification of the construct (pHL205), and the verified colonies were cultured overnight for plasmid isolation. (Figures 3.5 and 3.6) A 3.6 kb fragment, *Eco*R1 (blunted)-*Xho*1(blunted), was ligated into the pSBbar#5-GUS-nos binary vector which was cut with *Hind*III (blunted) to form pHL206 (Figure 3.7). Verification digests and a PCR reaction confirmed the orientation of *JLL1*'s promoter sequence in pHL206 (Figure 3.8). The binary vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 via electroporation (2500V), and the culture was plated on L.B Tetracycline (selects for *Agrobacterium*) and spectinomycin (selects for the vector) media. The plated bacteria was incubated 2 days at 28°C. The presence of pHL206 in *Agrobacterium* was confirmed by PCR (Figure 3.9).

Transformation of *Arabidopsis thaliana*

The binary vector pHL206 was transformed into wild-type *Arabidopsis thaliana* plants via the flower dip method.⁹² T0 seeds were sown on soil and the seedlings were treated with the herbicide Finale® (Bayer) to select for transformation events. Seedlings that survived the treatment were allowed to grow to maturity, genomic DNA was isolated and a PCR reaction was run to verify the insertion of pHL206 into the plant's genome (Figure 3.10). Seeds from positive events were harvested, and sown on half-strength MS media containing Finale® (Bayer) for GUS staining.

Transformation of *Agrostis stolonifera* and *Nicotiana tabacum*

Creeping bentgrass (*Agrostis stolonifera* L.) and Tobacco (*Nicotiana tabacum*) were transformed with pHL206. The detailed transformation procedures of creeping bentgrass¹⁰⁹ and tobacco¹¹⁰ can be found in the corresponding literature. The general process of turfgrass transformation involves five sequential steps: agro-infection, co-cultivation, antibiotic treatment, selection and plant regeneration.¹⁰⁹ The generation of embryonic callus is accomplished by placing surface sterilized seeds on callus-induction media enriched with 6-benzylaminopurine (BAP), a synthetic cytokinin, at a concentration of 0.5 mg/l.

One day prior to *Agrobacterium* transformation, small (1-2 mm) pieces of callus are placed on infection media containing 100µM acetosyringone, which aids in *Agrobacterial* infection. Transformation of the callus involves 10µl aliquots of *Agrobacterial* culture onto the callus followed by 3 days of co-cultivation in the dark. Infected callus are transferred to callus induction media containing the antibiotics cefotaxime and carbenicillin to suppress the growth of *Agrobacterium*. After 2 weeks, the callus are transferred to selection media containing phosphinothricin (PPT) for approximately 2 months. Resistant callus is then transferred to regeneration media containing BAP and myo-inositol. Regenerated plants are transferred to a growth chamber for propagation.

Tobacco transformation involves *Agrobacterium* infection of leaf discs¹¹⁰. Leaf discs from 4-week-old tobacco tissue (~0.5cm squares) were transferred

into a petri dish containing the *Agrobacterium* culture (OD 600 0.9-1.0) for 5 minutes. The discs were blotted dry and placed with abaxial side of the leaf in contact with callus-inducing selection media for 2-3 weeks. Once shoots appear, the discs are transferred to new media that does not contain callus induction hormones, until root growth is established. These plants are then placed in the greenhouse. PCR verification of the presence of pHL206 in regenerated tobacco and creeping bentgrass was performed after regeneration (Figures 3.11 and 3.12).

GUS Staining of Transgenic *Arabidopsis thaliana*

Arabidopsis containing the promoter-GUS construct were grown on half-strength (1/2) MS media containing Finale® (Bayer). The seeds were grown for 3-4 weeks on the ½ MS media, and then transferred to a microcentrifuge tube containing the GUS staining solution (50mM 0.1M PO₄, 0.2% TritonX, 2mM Ferrocyanide, 2mM Ferricyanide, ddH₂O, 2mM X-Gluc substrate). The tubes were placed under vacuum for 1hr, and then incubated for 24 hours at 37°C. The plants were destained in 75% ethanol until no chlorophyll remained in the tissues. The stained *Arabidopsis* plants were imaged using a Meiji Techno Biological Microscope and a Canon Rebel T1i camera. Stained plants are shown in Figure 3.13, 3.14, and 3.15. One-week-old seedlings were stained to determine if *JLL1*'s expression changes during early development. (Figure 3.13)

Three to four week old *Arabidopsis thaliana* plants were also stained (Figure 3.14).

GUS Staining of Transgenic Creeping Bentgrass and Tobacco

Methodologies employed to stain and image creeping bentgrass, tobacco and *Arabidopsis thaliana* are identical, however, due to lower expression, the creeping bentgrass and tobacco remained in the staining solution for up to 1 week until a stain could be visualized. The plants were then de-stained using 75% ethanol.

Abiotic Stress Test *Arabidopsis thaliana* Growth Conditions

Wild-type *A. thaliana* seeds were grown in a hydroponic system (Figure 3.17) that was constructed (and run) in accordance with a previous study.⁹³ The hydroponic system was placed in a Percival- Intellus™ environmental growth chamber set to 23°C/20°C Day/night with a 12 hour photoperiod.

Abiotic Stress Treatments

At 3-4 weeks of growth, *A. thaliana* plants were inspected to ensure there was no disease or tissue damage. The hydroponic systems were then moved from the growth chamber to the laboratory for the abiotic stress treatments. The three treatments were 100µM ABA⁹⁷, drought (3MM Whatman Paper)⁹⁴, and 200 mM of NaCl^[95, 96]. Each treatment had three replicates with the ABA/NaCl

treatments applied directly to the hydroponic system's growth media. The drought treatment involved drying out the root system of *A. thaliana* through the use of Whatman paper (Figure 3.18). The ABA treatment lasted 2 hours with tissue being isolated before the treatment, at one hour, and at two hours. The NaCl and drought treatments lasted 4 hours with tissue being isolated at 0, 0.5 hr, 2 hr, and 4 hrs.

Total RNA Isolation and cDNA Synthesis for Abiotic Stress Tests

Refer to "Tissue Specific RTPCR" Section.

Semi-quantitative RTPCR Analysis of *JLL1* Expression

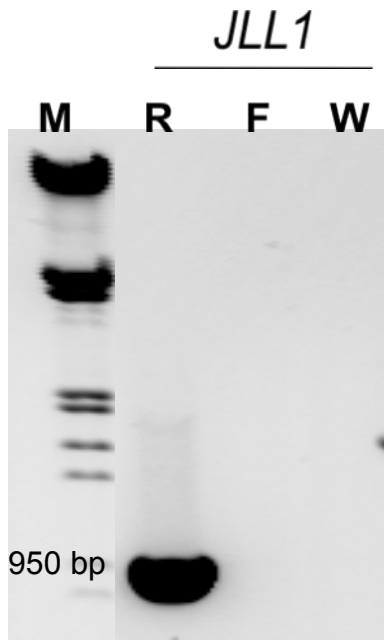
The results from the abiotic stress treatments are shown in Figures 3.19 and 3.20. *JLL1* cDNA was amplified at a higher PCR cycle number (33 or 32) and at a normal cycle number (26 or 24) to determine if *JLL1* is expressed at low levels in the leaf tissues.

IIIC. RESULTS

Tissue Specific RTPCR

JLL1 demonstrates root specific expression. Two separate RTPCR experiments confirmed that *JLL1* is expressed in the root tissues, with no detectable expression in the rest of the *Arabidopsis* tissues.

A. Experiment #1



B. Experiment #2:

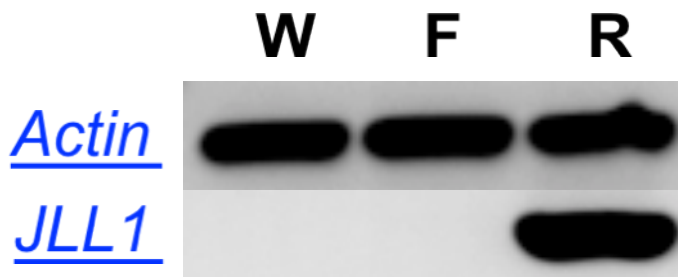


Figure 3.1: Tissue-specific RTPCR Analysis of *JLL1*

JLL1 demonstrates root specific expression. Two separate RTPCR reactions were run. Experiment 1 (A) and Experiment 2 (B) generated amplicons of 975 bp, which corresponds to *JLL1*'s cDNA sequence. Whole plant without root tissue (W), Flowers (F) and Root (R) tissues were included in each analysis. The Actin control in Experiment 2 is the gene AT3G18780.

Binary Vector Construction

JLL1's promoter region was successfully cloned into the vector pHL204 (Figure 3.2 and 3.3). pHL204 was introduced into *E. coli* (Figure 3.4) and the intermediary vector pHL205 was constructed and verified (Figures 3.5 and 3.6).

taattccatatgtgaaagaccagaac**TGTGTGATAAATA**agaatcgtag
 ccatggcttcttc**ACCAATCACCACAGCACAGCGATC**

Figure 3.2: JL_PR1 Primer Orientation For *JLL1* Promoter Amplification

Putative TSS for *JLL1* (Red), 5' UTR of *JLL1* (Green), JL_PR1 Annealing Site (Underlined text), TATA Box (Blue) The amplified promoter regions incorporated the transcriptional start site of *JLL1* and the important promoter elements including the TATA box.

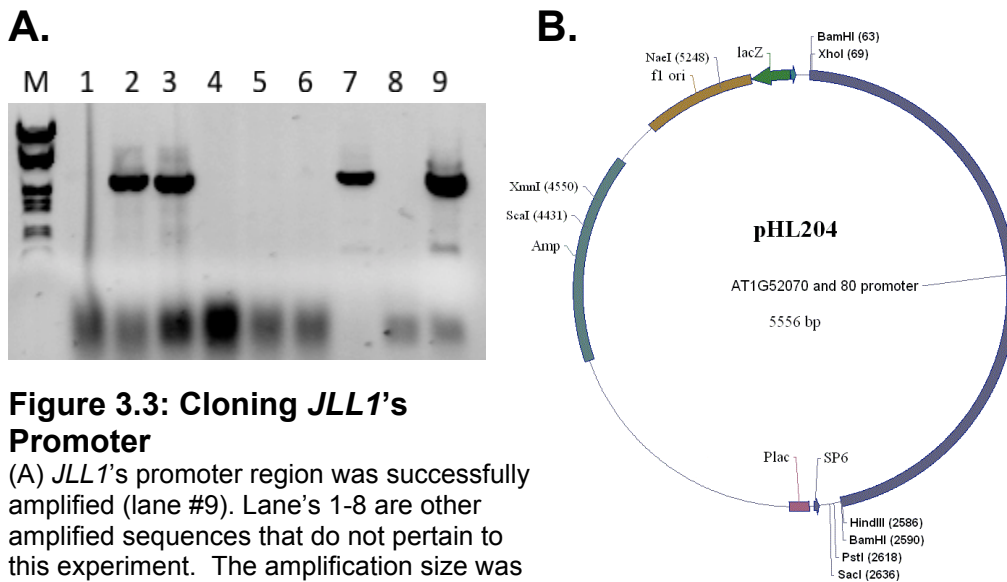


Figure 3.3: Cloning *JLL1*'s Promoter

(A) *JLL1*'s promoter region was successfully amplified (lane #9). Lane's 1-8 are other amplified sequences that do not pertain to this experiment. The amplification size was approximately 2.5kb, which is the predicted size. (B) Plasmid Map of pHL204- the pGEM Vector Containing *JLL1*'s promoter.

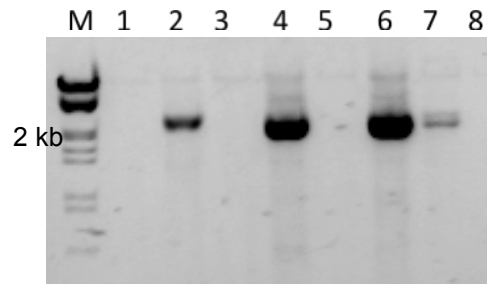


Figure 3.4: PCR Result for pHL204 after Introduction into *E. coli*

The amplified promoter was successfully cloned into the pGEM vector and into *E. coli*. The expected amplicon is 2.5kb. Colonies 2, 4, and 6 were successfully transformed. Lanes 1-6: Amplified Promoter, Positive Control (lane 7), Negative control (lane 8)

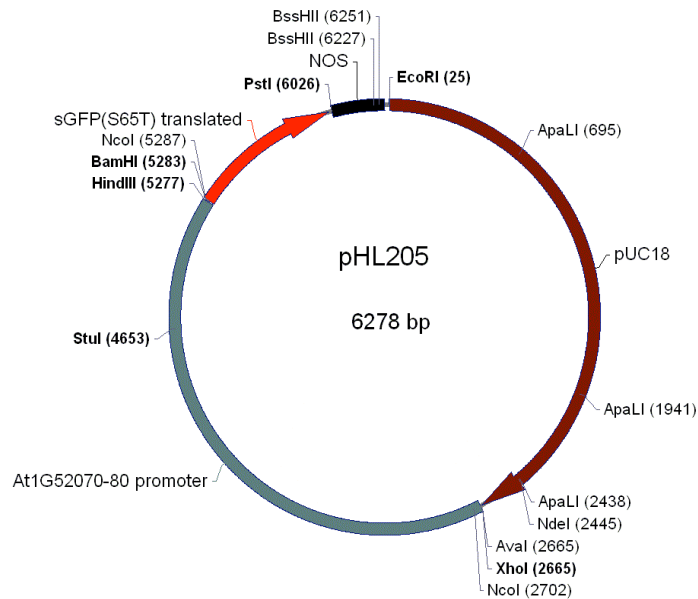


Figure 3.5: pHL205 Plasmid Construct

JLL1's promoter sequence (*BamH1-Xho1*) was ligated upstream of the sGFP reporter sequence with terminator (NOS) sequence forming pHL205. The introduction of pHL205 into *E. coli* amplified of the promoter-sGFP sequence through bacterial DNA and cellular replication.

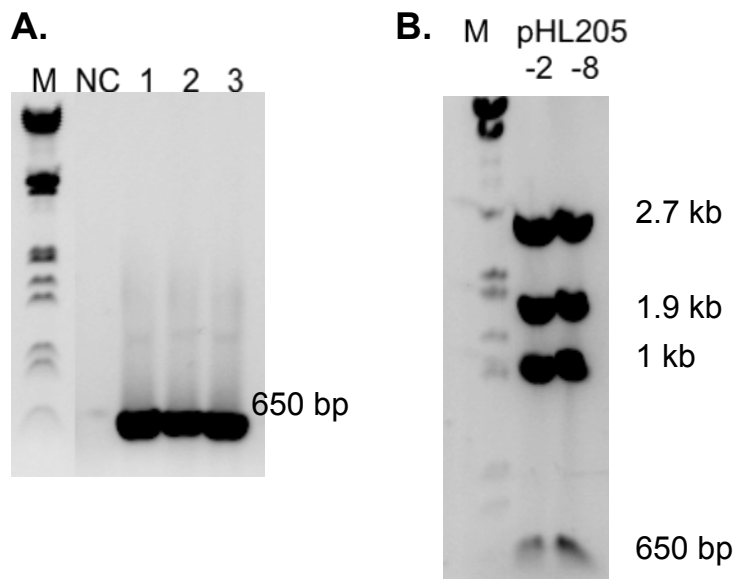


Figure 3.6: Verification of pHL205's orientation and presence in *E. coli*.

(A) PCR using sGFP primers on *E. coli* colonies to verify the presence of pHL205. Amplicon size is approximately 600bp. Lanes: Negative control (NC), 1-3 independent colonies.
 (B) Verification Digest of pHL205 using *EcoR*1, *Stu*I, and *Nco*I. Predicted sizes after digest are 654 bp, 1 kb, 1.9 kb, and 2.7 kb. Lanes -2 and -8 are pHL205 plasmids isolated from different *E. coli* colonies.

pHL206 was constructed and verified using PCR and restriction digest (Figure 3.7 and 3.8). *Agrobacterium tumefaciens* was successfully transformed with pHL206 using the floral dip method (Figure 3.9). The presence of the binary vector in the *Agrobacterium* was verified using PCR.

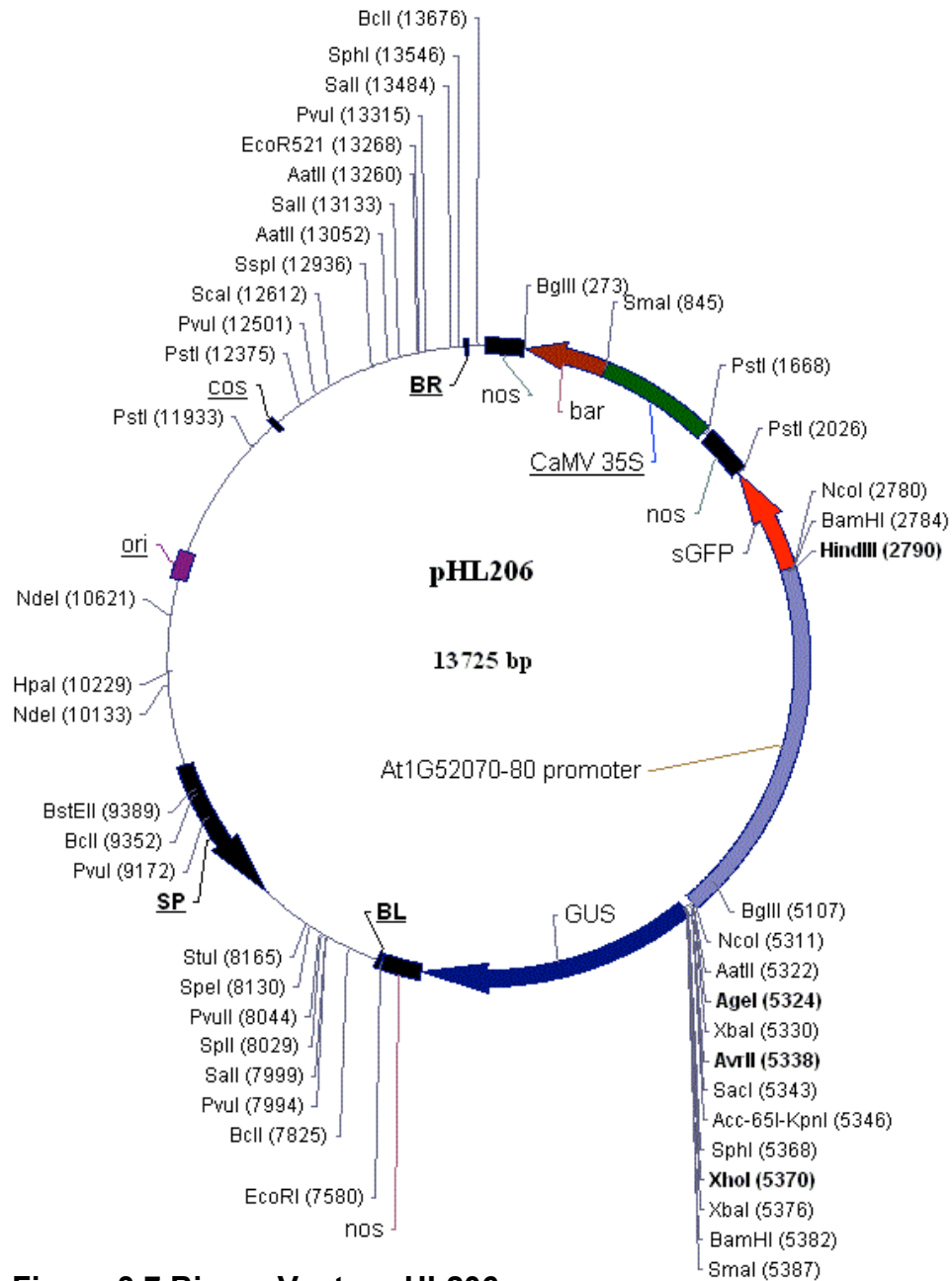


Figure 3.7 Binary Vector pHL206

JLL1's promoter sequence is between two reporter genes, GUS and GFP. The promoter for *JLL1* will drive the expression of GUS while the putative promoter for AR791 will drive the expression of sGFP. This vector also contains an antibiotic resistance gene, spectinomycin, and the bar herbicide resistance gene.

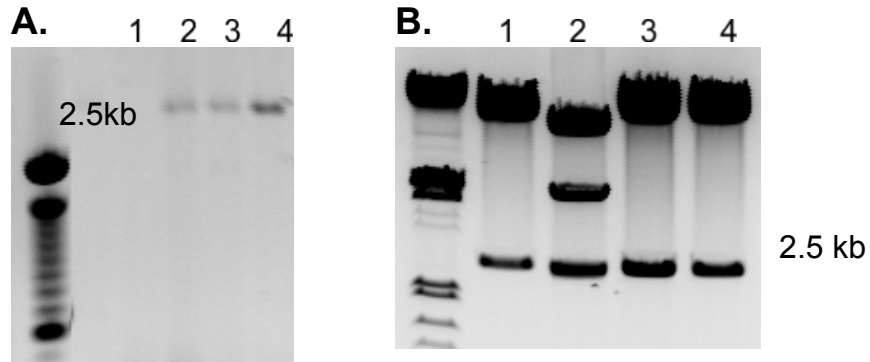


Figure 3.8: Verification of pHL206's Promoter Orientation

(A) Colony PCR verifying the presence of pHL206 in *E. coli*, and the correct orientation of the 3.6 kb fragment from pHL205. The BarR and sGFPF primer set were used to amplify the entire promoter region. (B) Verification digest to ensure the proper orientation of *JLL1*'s promoter into pHL206. The restriction enzymes *Hind*III and *Xho*I were used to cut pHL206 with an expected fragment size of ~2.5 kb. Binary vectors 1, 3, and 4 have the proper orientation.

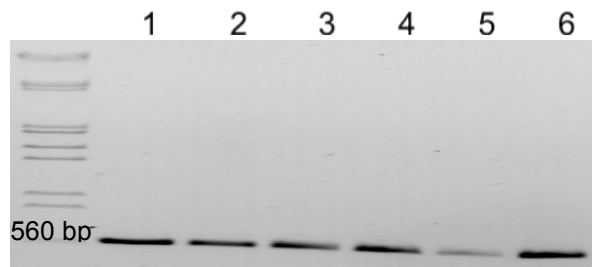


Figure 3.9: Colony PCR of pHL206 in *Agrobacterium tumefaciens*

Agrobacterium were successfully transformed with pHL206. The sGFP F&R primers amplify fragments around 550 bp. Each lane represents an independent colony on the selection media (L.B. Tet + Spe). All six colonies contain the binary vector.

Arabidopsis thaliana, *Agrostis stolonifera* and *Nicotiana tabacum*

Transformation

Arabidopsis thaliana, *Nicotiana tabacum*, and *Agrostis stolonifera*

were successfully transformed with pHL206. PCR was used to verify the presence of the binary vector in the plant genome (Figures 3.10, 3.11 and 3.12).

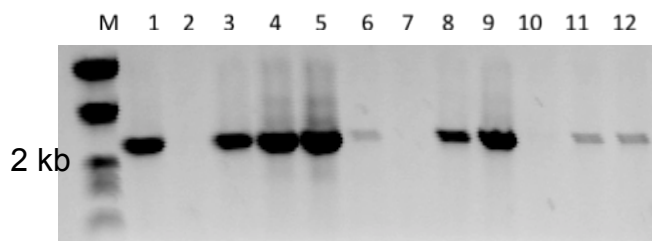


Figure 3.10: PCR to Identify Transformation Events in *A. thaliana*

JLL1 promoter forward and GUS reverse primers were used to amplify the T-DNA insertion(s) in *A. thaliana*. Nine transgenic events were confirmed. Each lane represents a separate transgenic event. Predicted amplicon is approximately 2.5kb.

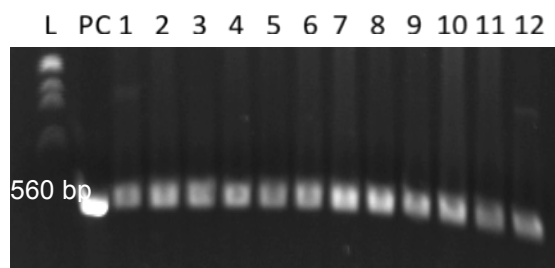


Figure 3.11: Verification of pHL206 Transformation of Tobacco (*Nicotiana tabacum*)

Twelve separate transgenic tobacco events were verified. sGFP forward and reverse primers were utilized in a PCR reaction using tobacco genomic DNA. Lanes 1-12 are separate transformation events. A positive control (PC) is also provided. Predicted amplicon is approximately 500 bp.

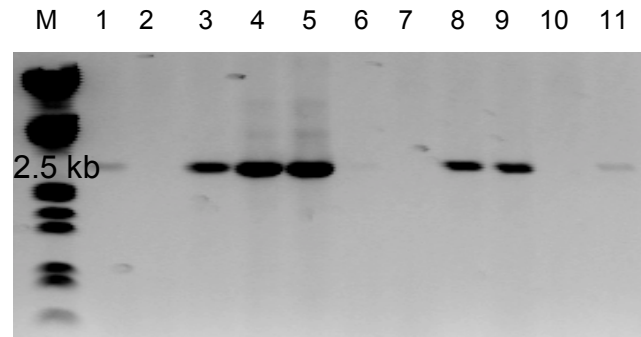


Figure 3.12: Verification of pHL206 Transformation of Creeping Bentgrass (*Agrostis stolonifera*)

Seven transgenic creeping bentgrass events were confirmed by PCR (primers JL promoter F and GUS reverse) of creeping bentgrass genomic DNA. Predicted size was 2.5kb.

Arabidopsis thaliana GUS Stain

One and three week old *A. thaliana* exhibited staining in the root tissues and leaf vasculature (Figure 3.13 and 3.14). From the staining data (Figure 3.14), it appears that *JLL1*'s expression is largely root specific with expression localizing in the root tips, central portions of the roots, and the leaf vasculature. Furthermore, it appears that there is an absence of expression in the cellular regions immediately behind the root cap. This region may include the zones of cell division, elongation, and/or the zone of differentiation. Out of nine transgenic *Arabidopsis thaliana* events only three exhibited staining in the plant tissues, one of which showed the strongest GUS staining. The absence and low GUS expression in some of the transgenic lines is most likely due to “position effect”, which is not an uncommon phenomenon for transgene expression in transgenic plants. Figure 3.15 illustrates the difference between the high and low

expression transgenic events. The GFP assessment of the localization of AR791 was unsuccessful. No GFP expression was found in *Arabidopsis* tissues.

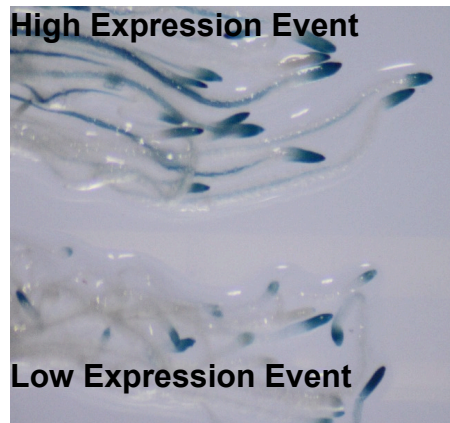


Figure 3.15: Comparison of the low and high GUS expression Transgenic Events

The low-expression events lacked staining in the leaf tissues, while the high expression event demonstrated heavy staining in the root tissues and the leaf vasculature.

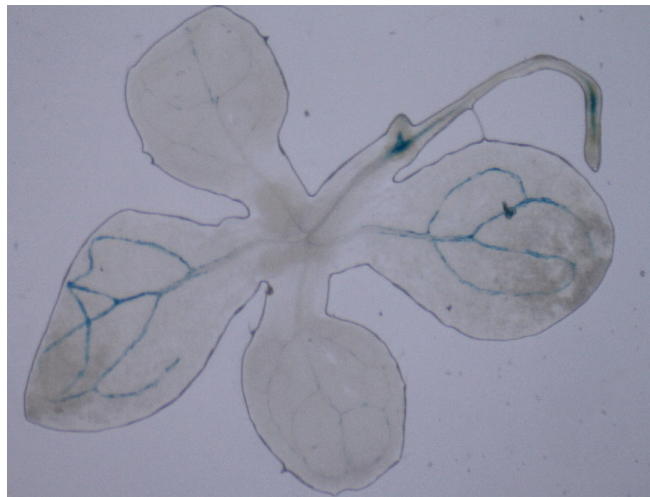


Figure 3.13: *JLL1* Expression in *A. thaliana* One-week after Germination

JLL1 appears to be expressed early on in plant development in both the root and leaf tissues. GUS staining of the one-week old seedling was found in the vasculature of the true leaves and the root tissues.

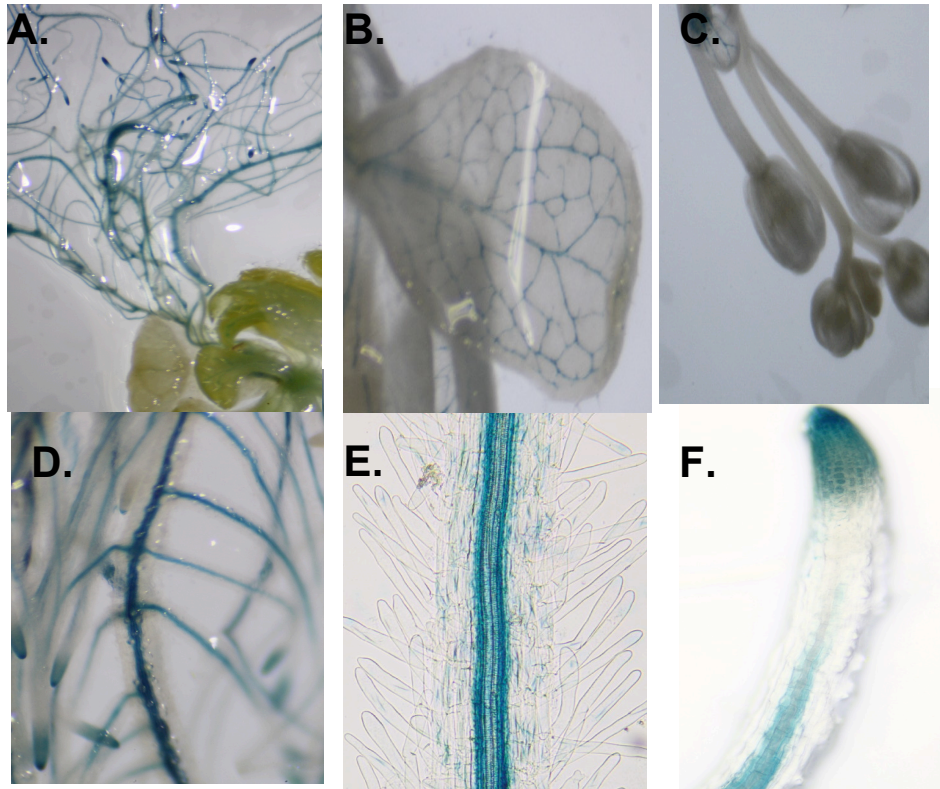


Figure 3.14: *Arabidopsis thaliana* GUS-stain

JLL1 exhibits high levels of expression in the root tissues including the vascular associated tissues and the root tip. The leaf tissues also have vascular expression. No expression was found in the stem or flowering tissues. Whole (3 wk old) plant (A), True leaf (B), Flowers with a newly formed leaf (C), Root system with lateral roots and root tips (D), enhanced image of a mature root (E), representative root tip and zone of elongation (F)

Creeping Bentgrass and Tobacco GUS Stains

The staining data for Creeping Bentgrass and Tobacco (Figure 3.16) demonstrates that *JLL1*'s promoter is not active in the root tissues because no stain was localized to the roots. However, staining was found in the sheath in Creeping Bentgrass, and the central stem (possibly vasculature) of the Tobacco plants. Two conclusions may be drawn from these stains. First, the *JLL1*

promoter is active in the central region (stem) in both the monocot and dicot species suggesting that it may display similar regional expression in other species. This would be an important attribute for a promoter used in biotechnology. Second, the dissimilar staining pattern compared to *Arabidopsis thaliana* suggests that *JLL1* is uniquely regulated in *Arabidopsis*.

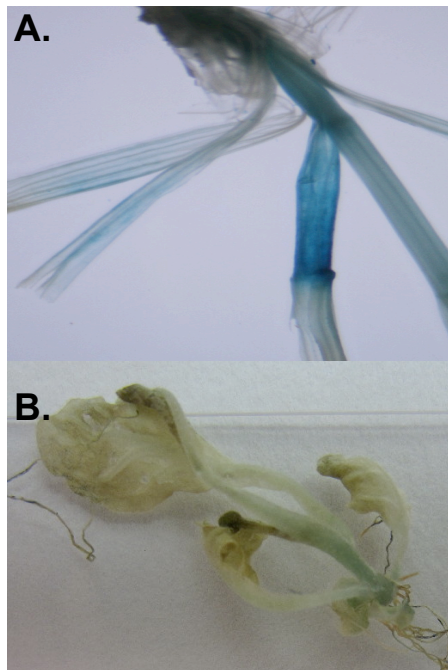


Figure 3.16: GUS Stained Creeping Bentgrass (*Agrostis stolonifera*) and Tobacco (*Nicotiana tabacum*)

(A) Creeping bentgrass exhibited GUS staining in the sheath tissues of the plant.
(B) Tobacco had light staining throughout the central stem region. The stain was diffuse and could not be localized.

Abiotic Stress Treatments

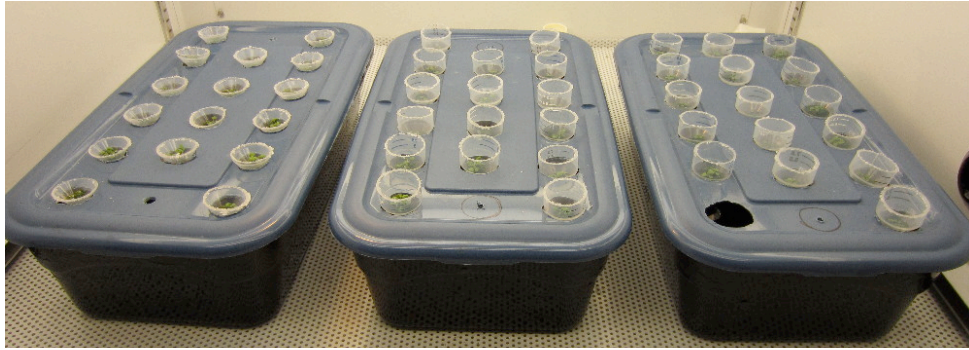


Figure 3.17: Hydroponic System in the Environmental Growth Chamber

Plant materials were grown in the hydroponic growth system before abiotic stress treatments.



Figure 3.18: Experimental Set-up for Abiotic Stress Treatments

(A) NaCl and ABA treatments were conducted directly in the hydroponic system in order to reduce additional stress on the plant.

(B) 3MM Whatman paper dried out the root tissues before total RNA isolation.

Whatman paper was placed on top of the root tissues ensure they were completely dehydrated.

The results from the semi-quantitative RTPCR analysis suggest that *JLL1* is down regulated under abiotic stress. The fold-reduction appears to be more significant in the sodium chloride treatments (Figure 3.19) than in either the drought or ABA treatments. It is also necessary to note that during the ABA treatment (Figure 3.20), *JLL1* expression significantly decreases at one hour but the expression level is partially restored at two hours. Expression of *JLL1* was also found in the leaf tissues in both RTPCR reactions, however, the level of RNA was very low restricting relevant comparisons between different leaf treatment times due to the high variability in isolating low quantity mRNA. The expression in the leaf tissues verifies the staining found in the leaf vasculature during the promoter-GUS analysis of *JLL1*.

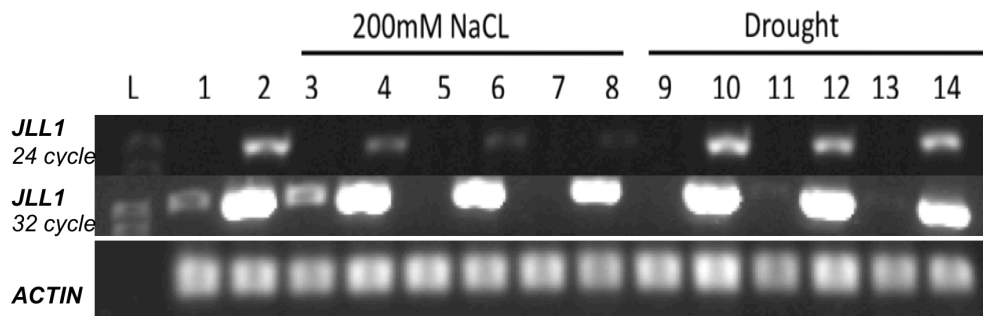


Figure 3.19: Expression Profile of *JLL1* during NaCl and Drought Treatments

JLL1 is down regulated under NaCl and drought treatments. *JLL1* is also expressed at low levels in the leaf tissues. The longer the salt treatment, the greater the down regulation of *JLL1* compared to the non-treated control. Lanes: 1 (0hr Leaf), 2 (0hr Root), 3 (0.5 hr Leaf-NaCl), 4 (0.5 hr Root-NaCl), 5 (2 hr Leaf-NaCl), 6 (2 hr Root-NaCl), 7 (4hr Leaf-NaCl), 8 (4 hr Root- NaCl), 9 (0.5 hr Leaf-Drought), 10 (0.5hr Root-Drought), 11 (2 hr Leaf-Drought), 12 (2 hr Root-Drought), 13 (4 hr Leaf-Drought), 14 (4 hr Root- Drought)

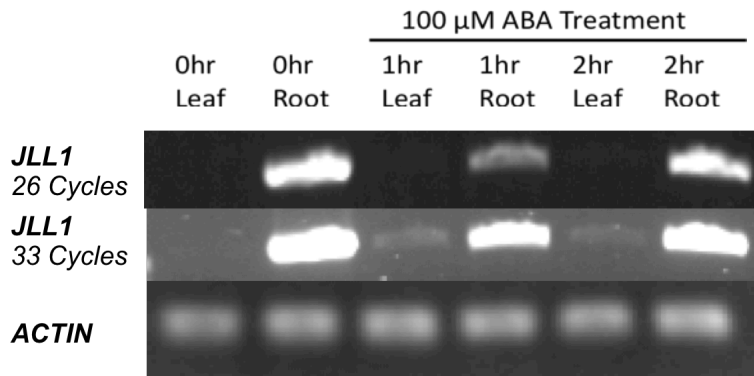


Figure 3.20 Expression Profile of *JLL1* during ABA Treatment

JLL1 is down regulated after 1 hour of ABA treatment, and at 2 hours of treatment the expression level is partially restored. Low levels of *JLL1* expression were confirmed in the leaf tissues. Actin normalization ensured consistent quantities of cDNA was used during PCR amplification.

IIID. CONCLUSIONS

JLL1 demonstrates localized expression in the root cap, vascular portion of mature roots and the leaf vasculature. It is also expressed early in plant development (see Figure 3.13). *JLL1*'s promoter exhibited poor levels of expression in Creeping Bentgrass and Tobacco, and its expression was dissimilar from that found in *Arabidopsis*. No GFP expression to support an additional promoter region driving AR791, was found in *Arabidopsis* tissues; however, an improved UV microscopy set-up is required before fully ruling out expression.

Under abiotic stress, *JLL1*'s expression level is reduced especially during the sodium chloride treatment. A less dramatic reduction was observed during the drought treatment. The ABA treatment caused *JLL1*'s expression level to fluctuate. At one hour of treatment, *JLL1* exhibited much lower expression than the untreated sample, however, its expression was partially restored at two hours. This may be due to the fact that ABA signals in response to environmental stress.^[97, 98] Since the plants treated with 100 μ M of ABA were not under abiotic stress (they were treated with ABA directly on their growth media), the initial hormonal signal may have induced physiological changes in the plant to adjust to perceived stress, but once the signal dissipated (i.e. the environment did not change) the plant may have returned to its normal physiological state.

IIIE. REFERENCES

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CHAPTER FOUR

MUTANT ANALYSIS

IVA. INTRODUCTION

Comparing the phenotypic characteristics of a single gene mutant (knock-down or knock-out) to wild-type is a logical way to derive information on the functional characteristics of that gene. In order to better understand the *in planta* role of *JLL1*, we subjected wild-type and *JLL1* T-DNA insertional mutants to abiotic stress treatments on ½ MS plates over a period of half a month. The germination and greening rates (cotyledon development) were recorded.

Previous studies utilized germination rate and cotyledon formation comparisons between *Arabidopsis* wild-type, mutant and over-expression lines to help elucidate the function of genes *in planta*.^[100, 101] *JLL1* mutant seeds and wild-type seeds were compared in a similar manner. Since the expression profile of *JLL1* was examined under abiotic stress conditions, it is logical to start preliminary germination studies using the same abiotic stress treatments (although with different intensities) so that comparisons may be drawn between the *JLL1* expression profiles (see Figures 3.19 and 3.20), and the germination phenotype.

IVB. MATERIALS AND METHODS

Origin of the Mutant Line

A T-DNA insertional mutant was ordered from the TAIR website <<http://www.arabidopsis.org/index.jsp>> and prepared by the Arabidopsis Biological Resource Center at The Ohio State University. The mutant, SALK_134751, had a verified insertion in an exon of *JLL1*. The seeds were planted on soil and allowed to grow under normal conditions.

Verifying Homozygous Insertional Mutants

The segregating progenies initiated from initial mutant seeds were first screened for homozygous individuals by PCR analysis of genomic DNA. Three sets of primers were designed on the Salk Institute Genomic Analysis Laboratory website (<http://signal.salk.edu/tdnaprimers.2.html>). These primers target different sequences around the T-DNA insertion site (Figure C-1). The T-DNA border primer LB (annotated BP) is the internal primer designed to anneal to the T-DNA insert and amplify out towards the right primer (RP). RP and the left primer (LP) are primers that are targeted to the flanking sequences of the T-DNA insert.

When these three primers are run together, three outcomes are possible. If the plant is homozygous for the T-DNA insertion at a particular locus, BP and RP will amplify a fragment between 410 – 710 bp long. If the plant is wild-type, RP and LP will amplify a single band around 900-1000 bp. Finally, if the plant is hemizygous for the T-DNA insertion, two bands will be amplified, one around

900-1000, another band would be 410-710 bp. A graphical representation of this analysis is provided in Figure 4.1. RTPCR analysis after mutants are grown was used to determine if *JLL1* expression was eliminated.

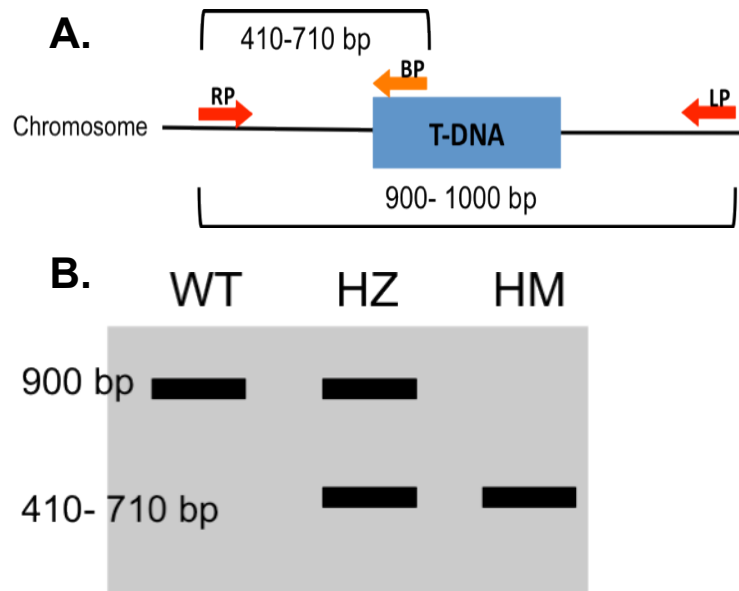


Figure 4.1: Diagram of T-DNA Insertion Verification Analysis

(A) Model of *JLL1* with T-DNA insertion and primers annotated. The distance between the right (RP) and left (LP) primers is between 900-1000bp without the T-DNA insertion. (B) Gel Electrophoresis profile of the three different PCR amplification patterns from variable T-DNA insertion events. (WT= wild-type, HZ= heterozygous, HM= Homozygous)

Assessing the Transcriptional Activity of *JLL1* in T-DNA Insertional Mutants

A study from 2008 determined that T-DNA insertions are effective at knocking out a gene's function 90% of the time⁹⁹. However, there are still many insertional mutants that still retain some level of expression. To verify whether *JLL1* expression was eliminated in the T-DNA insertional mutants, an RTPCR

was performed on cDNA isolated from homozygous mutant *Arabidopsis thaliana* plants.

Seed and Media Preparation

All seeds applied to the media were surface sterilized with 70% ethanol (1 min), and then two 20% Chlorox (20 min) treatments on a shaker. The sterilized seeds were then washed with ddH₂O five times before they were placed on the media, half strength Murashige and Skoog (½ MS). The salt and mannitol media treatments were applied to the media solution before autoclave. ABA was added after the media solution was autoclaved.

Treatment Conditions

Three treatments were applied to the wild-type and mutant seeds. Sodium chloride, mannitol (to simulate drought conditions), and Abscisic acid (ABA) were added to (separate) autoclaved media (NaCl and mannitol added before autoclave, ABA after autoclave). The concentrations of each treatment varied. The sodium chloride treatments were 100 mM, 150 mM, 175 mM, and 200 mM. Mannitol treatments were 50 mM, 100 mM, 150 mM, 200 mM, and 250 mM. ABA treatments were 1µM and 3µM. Each treatment set had a non-treated control (1/2 MS only) containing the same batch of sterilized seeds to ensure that the sterilization process is not responsible for changes in germination or cotyledon development (Figure 4.9). Three replicates were used for each individual treatment. The finished plates containing the surface sterilized seeds

were placed in the Percival- Intellus™ environmental growth chamber set to 23°C/20°C Day/night with a 12 hour photoperiod. (Figure 4.8)

Synthesis of a *JLL1* Overexpression Construct

In order to adequately understand *JLL1*'s function, *Arabidopsis* lines over-expressing *JLL1* must be generated. First a binary vector containing a constitutive promoter (CaMV35S) driving the expression of *JLL1* was synthesized. The coding sequence was amplified from genomic DNA using the two genomic primers found in Figure C-1 (Appendix C). The genomic DNA was used to amplify the coding sequence because the sequence would be inserted back into the *Arabidopsis thaliana* genome, thus the mRNA will be processed the same as the endogenous *JLL1* transcript.

IVC. RESULTS

Verifying Homozygous T-DNA Insertional Mutants

The primer combination BP, RP and LP was run on genomic DNA isolated from the candidate plants. (Figure 4.2) From the gel image, candidate #7 appears to be hemizygous, however, its “wild-type” band is not the same size as the bands in other plants. The two bands (~650 bp and ~875 bp) fit outside the expected range for a hemizygous insertional events. Two separate PCR reactions (BP+RP) and (RP+LP) were performed to better understand the identity of this mutant (Figure 4.3).

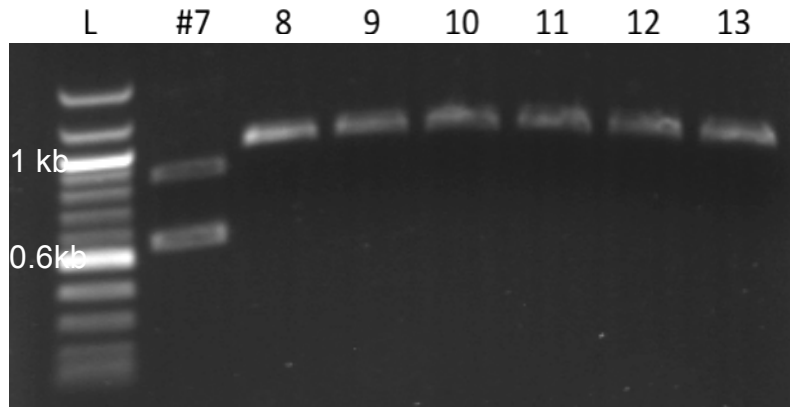


Figure 4.2: PCR Analysis of Putative *JLL1* Mutants Using the Primers RP, LP, and BP

Plant #7 has a dissimilar amplification pattern from verified wild-type plants (lanes 8-13). These two bands appear to be indicative of a hemizygous insertional mutant, however, the size of 875 bp is different from the predicted value of 1kb. Further analysis using separate primer pairs is required to fully understand the identity of this mutant.

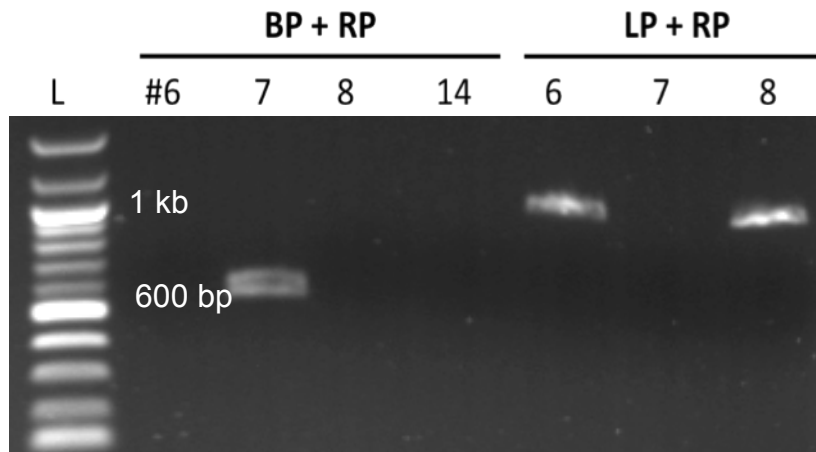


Figure 4.3: PCR Analysis the *JLL1* mutant using Two Separate Primer Reactions (RP+BP) and (LP+RP)

The band at #7 in the BP+RP reaction and the lack of a band in the same sample in the LP+RP reaction indicates that this mutant has a T-DNA insertion in both *JLL1* alleles. Further analysis using forward and reverse genomic primers combined with the BP primer will determine if any of the T-DNA insertions was head-to-head.

The results in Figure 4.3 indicate that there is an insertion in both *JLL1* alleles because the LP+RP primer reaction failed to amplify the insertional site. However, this does not account for the two bands found in the first PCR reaction. Another series of PCR tests was performed to determine if the T-DNA was a head-to-head insertion, which would account for the double band because the BP primer would direct amplification towards both the RP and LP primers.

Four separate reactions were run using sample 7's genomic DNA to determine if the T-DNA insertion was head-to-head in at least one allele. The forward and reverse genomic primers JLF and JLR (which are specific to *JLL1*) were each amplified with BP, the two genomic primers were added together in the same reaction (without BP), and a negative control --where the genomic DNA was replaced by water—was tested in a separate reaction (Figure 4.4).

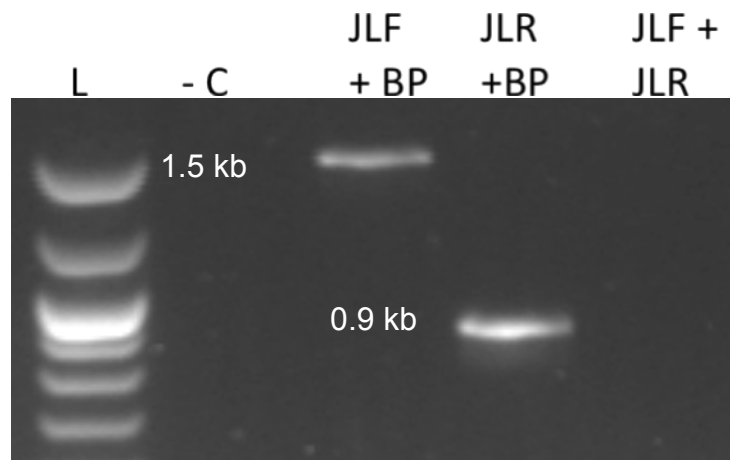


Figure 4.4: PCR Analysis of Salk_134751 using *JLL1* Genomic and T-DNA Border Primers

At least one of the T-DNA insertions in mutant #7 is head-to-head. The genomic forward (JLF) and reverse (JLR) primers were run with the border primer (BP). The amplification with the JLR indicates there is a head-to-head insertion due to there being a BP site in the opposite orientation.

The amplified fragment sizes observed in Figure 4.3 indicate that plant #7 has at least one head-to-head T-DNA insertion in *JLL1* because both primer pairs, JLF+BP and JLR+BP, had amplification. Additionally, JLR+BP produced an amplicon around 900bp, which was one of the band sizes that correspond to the first PCR reaction (Figure 4.2). This size is comparable to the first PCR reaction because JLR's annealing site is adjacent to the primer LP's annealing site (see Figure C-1). A final PCR reaction was run to verify that the two genomic primers could not amplify *JLL1*'s genomic region in candidate plant #7 (Figure 4.5). The expected size of the positive control is 1.9kb. Additionally, the progeny of the original mutant were tested for the T-DNA insert using two reactions, LP+RP+BP and the genomic primers (JLF+JLR). All progeny were confirmed to have the T-DNA insertions (image not shown).

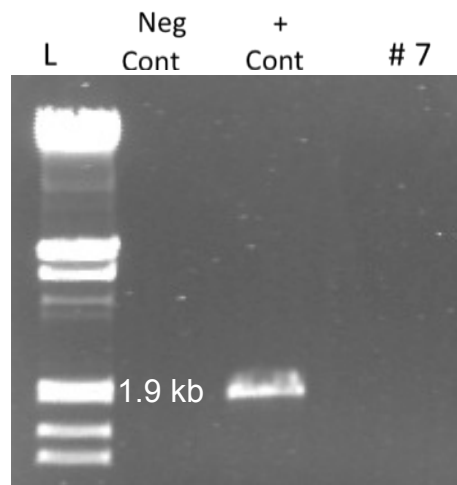


Figure 4.5 Analysis of Salk_134751 Using Only *JLL1* Genomic Primers

JLL1 is homozygous for the T-DNA insertion. The lack of a band in lane #7 indicates that the genomic primers were unable to amplify over the T-DNA insertions found in both copies of *JLL1*. However, this lack of amplification cannot be attributed to a problem with the reaction because the positive control (+ Cont.) was successful in amplifying *JLL1*.

Based on the PCR amplification data, a model of the head-to-head T-DNA insertion into one (or both) *JLL1* locus of the mutant candidate #7 was created (Figure 4.6).

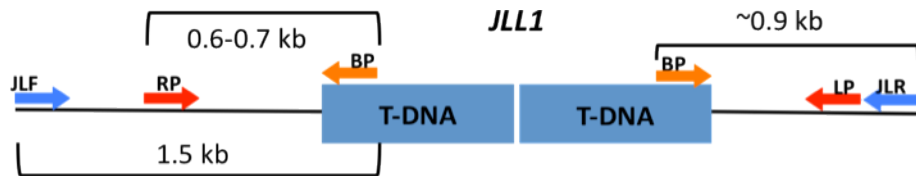


Figure 4.6: Model of the Head-to-Head T-DNA Insertion into *JLL1*

Assessing the Transcriptional Activity of *JLL1* in T-DNA Insertional Mutants

An RTPCR comparing wild-type and mutant cDNA did not detect any *JLL1* expression in the mutant, indicating that in *JLL1* homozygous T-DNA insertional mutants, the expression of *JLL1* is completely knocked out (Figure 4.7).

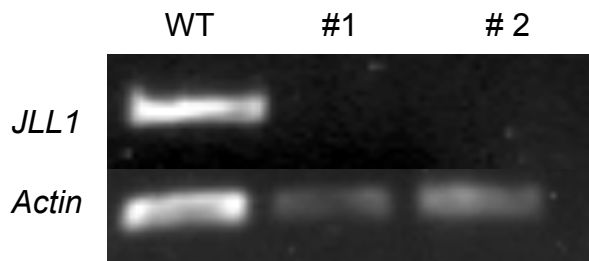


Figure 4.7: RTPCR to Analyze Expression of *JLL1* in Mutant *Arabidopsis*

JLL1 homozygous insertional mutants did not exhibit expression of *JLL1*. Lanes #1 and #2 are from two separate mutant plants. A wild-type (WT) control was also performed to verify the fidelity of the RTPCR reaction. The *JLL1* and actin (control) RTPCR primers were used to amplify the cDNA.

Germination Analysis of *JLL1* Mutants

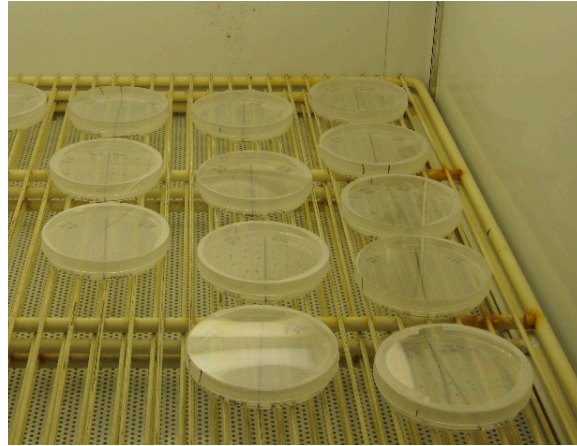


Figure 4.8: Percival- Intellus™ Growth Chamber Containing Germination Plates

Germination test plates were placed in a Percival-Intellus™ environmental growth chamber set to 23°C/20°C Day/night with a 12 hour photoperiod.

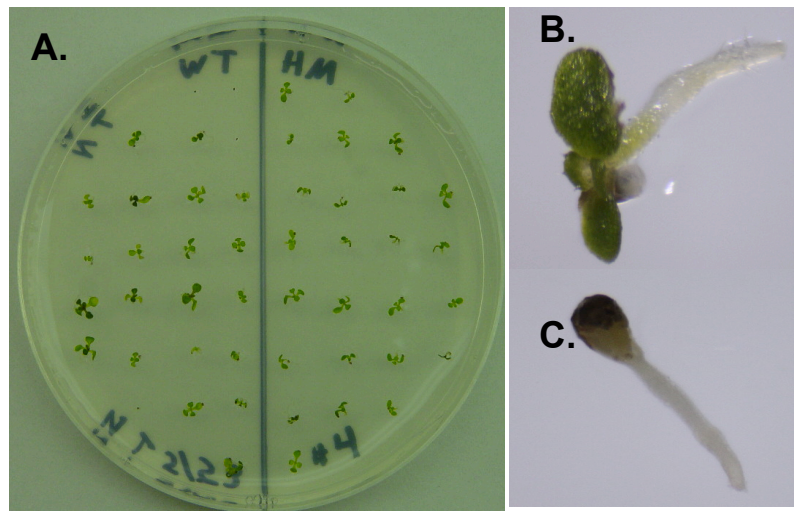


Figure 4.9: Germination Plate Experimental Set-up

(A) Non-treated control plate (8 days), these plates are used to verify the seed sterilization treatment and natural germination rates are the same between the WT and mutant seeds. (B) An example of cotyledon development in the growth media. (C) An example of a germinated seed

The results from the five most significant treatments are shown in Figures 4.10 and 4.11 (175 mM NaCl), 4.12 and 4.13 (200 mM NaCl), 4.14 and 4.15 (250 mM Mannitol), 4.16 and 4.17 (1 μ M ABA), and 4.18 and 4.19 (3 μ M ABA).

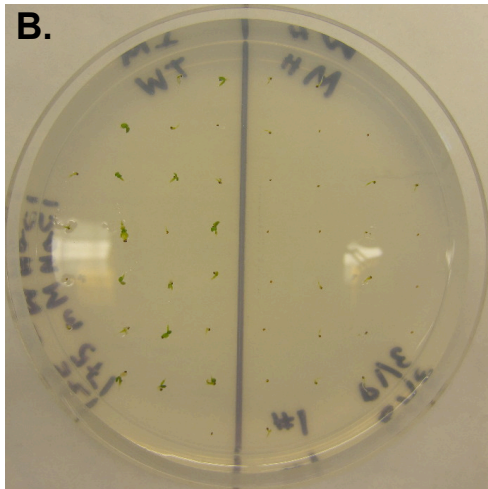
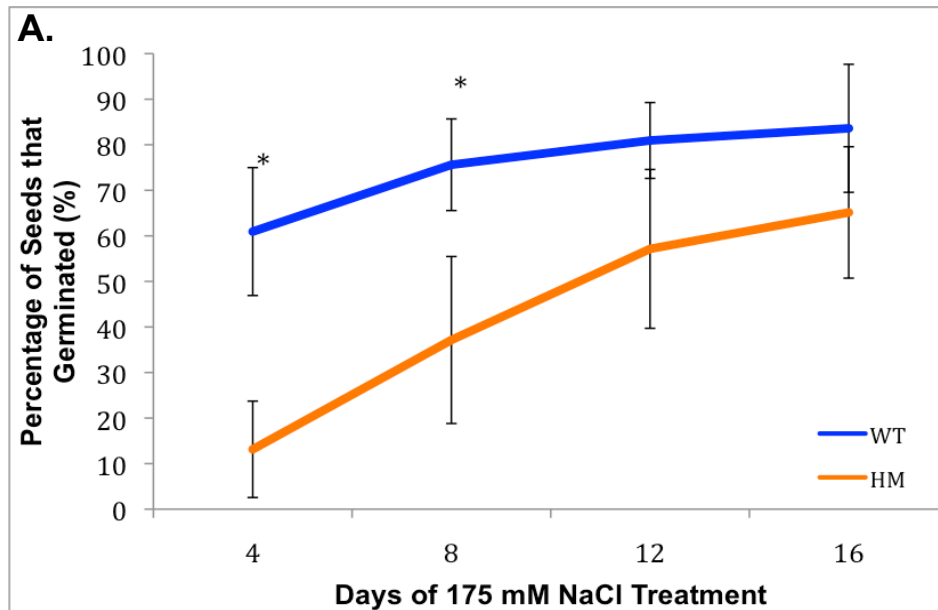


Figure 4.10: Germination Performance of WT and *JLL1* Mutant Seeds under 175 mM NaCl

Wild-type seeds germinated earlier than the homozygous mutant seeds under 175 mM of NaCl. (A) Percentage of seeds that germinated over 16 days. Wild-type (WT) and the homozygous mutant (HM) seeds. (“*” indicates statistical significance of $P < 0.05$) (B) A representative plate (1 replicate) from this treatment.

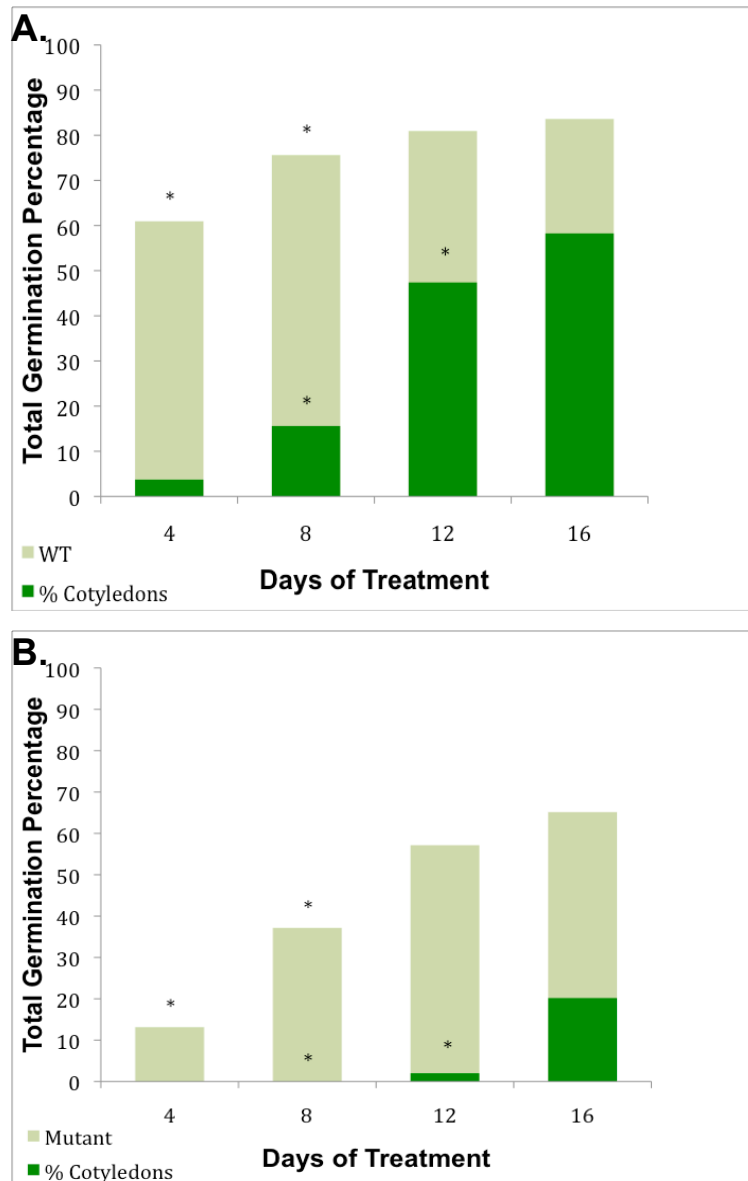


Figure 4.11: Germination Percentages of WT and Mutant Seeds with Percentage of Seeds forming Cotyledons at 175 mM NaCl

Wild-type seeds germinated earlier than mutant seeds. The earlier germination of the wild-type seeds is most likely responsible for the higher cotyledon development during all four time-periods.

(* indicates a statistically significant difference $P < 0.05$)

(A) Wild-type Seeds (green bars indicate the percentage of germinated seeds forming cotyledons)

(B) Mutant Seed germination and cotyledon development.

Figures 4.10 and 4.11 suggest that 175 mM salt treated wild-type seeds germinate earlier than *JLL1* mutant seeds. The earlier germination (most apparent at 4 and 8 days) corresponds with a higher percentage of wild-type seeds forming cotyledons (measurements at day 8 and 12).

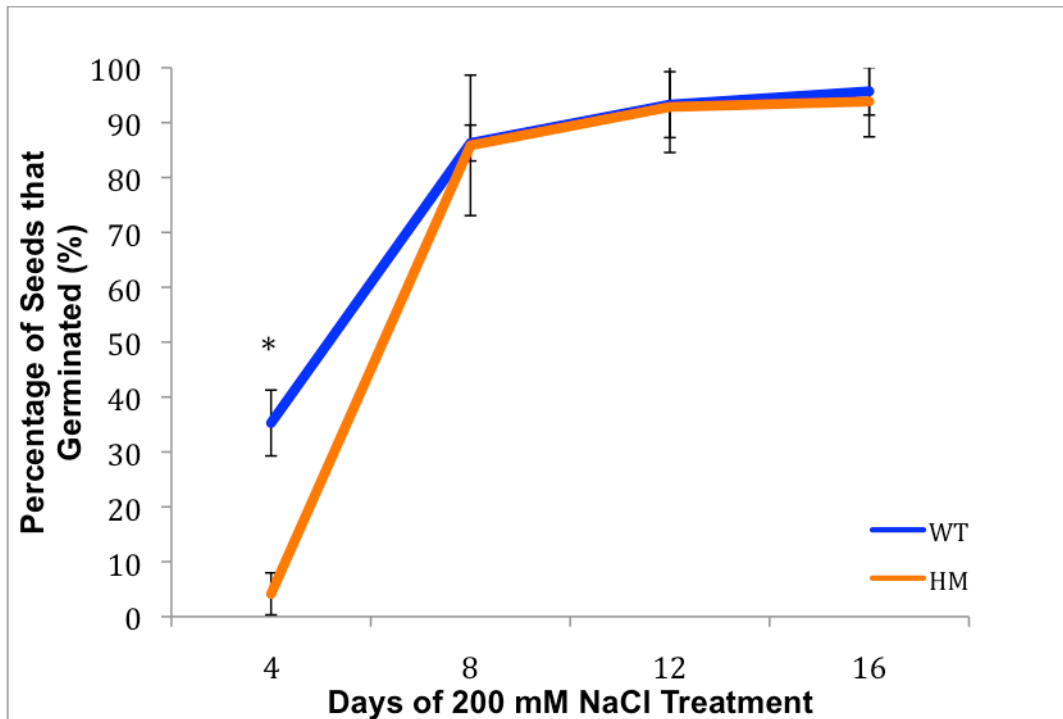


Figure 4.12: Germination Performance of Wt and Mutant Seeds under 200 mM NaCl Treatment

The wild-type (WT) and *JLL1* homozygous mutants (HM) had dissimilar germination percentages at four days when subjected to 200 mM NaCl treatment. The differences in germination percentages are negligible starting at eight days. (“*” indicates a statistically significant difference at $P < 0.05$)

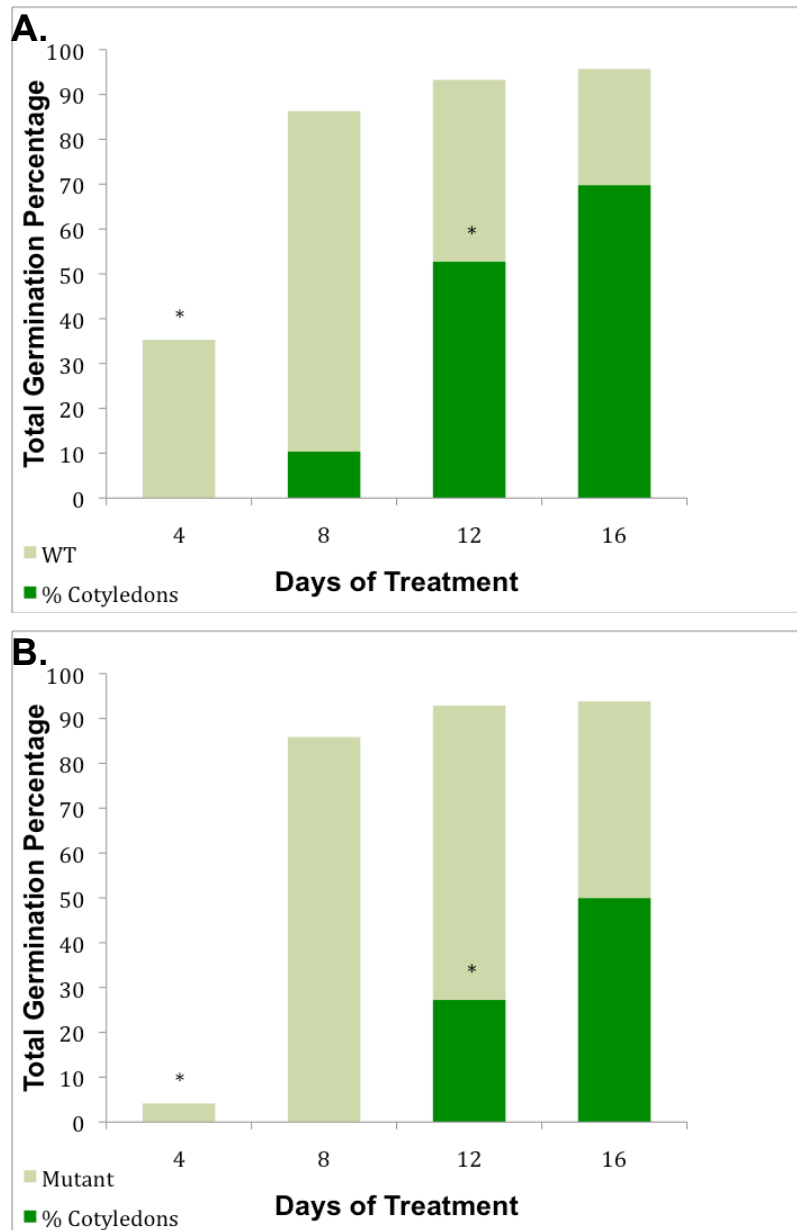


Figure 4.13: Germination Percentages and Cotyledon Development of Wild-type and Mutant Seeds under 200 mM NaCl Treatment.

(A) Wild-type (WT) seeds demonstrate a greater germination total at 4 days, and higher cotyledon development at 12 days (green bars indicate the percentage of germinated seeds forming cotyledons).

(B) Homozygous mutant seeds had similar germination totals after 4 days, and the cotyledon development was not significantly different at 16 days.

(* indicates a statistically significant difference $P < 0.05$)

Figures 4.12 and 4.13 demonstrate results similar to the 175 mM salt treatments; however, the differences between the wild-type and mutant seeds are not as dramatic. The wild-type seeds begin to germinate earlier than the mutant seeds (4 days) however; the mutant seeds quickly catch up to the wild-type seeds' germination total. The differences in the percentage of cotyledons are also not as significant. The total number of seeds germinating is overall greater in the 200 mM salt treatment than the 175 mM salt treatment (See figures 4.10 and 4.12). This is unexpected since a lower germination rate is predicted, as the abiotic stress treatments get more intense.

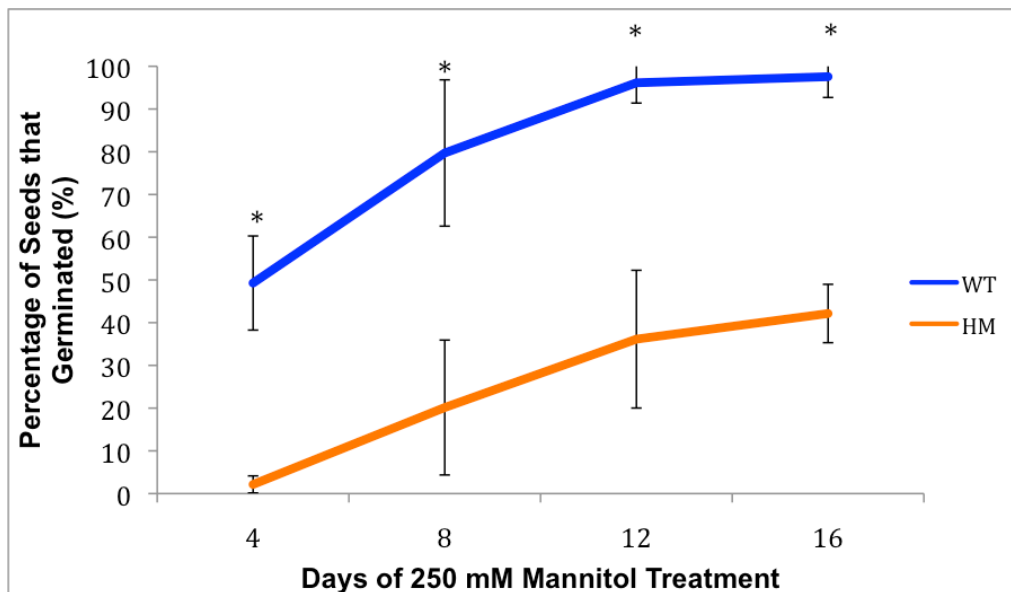


Figure 4.14: Germination Performance of Wild-type and Mutant Seeds under 250 mM Mannitol Treatment

Throughout the 250 mM Mannitol treatment, the wild-type (WT) seeds had a significantly higher germination percentage when compared to the *JLL1* homozygous mutants (HM). (“*” indicates statistical significance at $P < 0.05$)

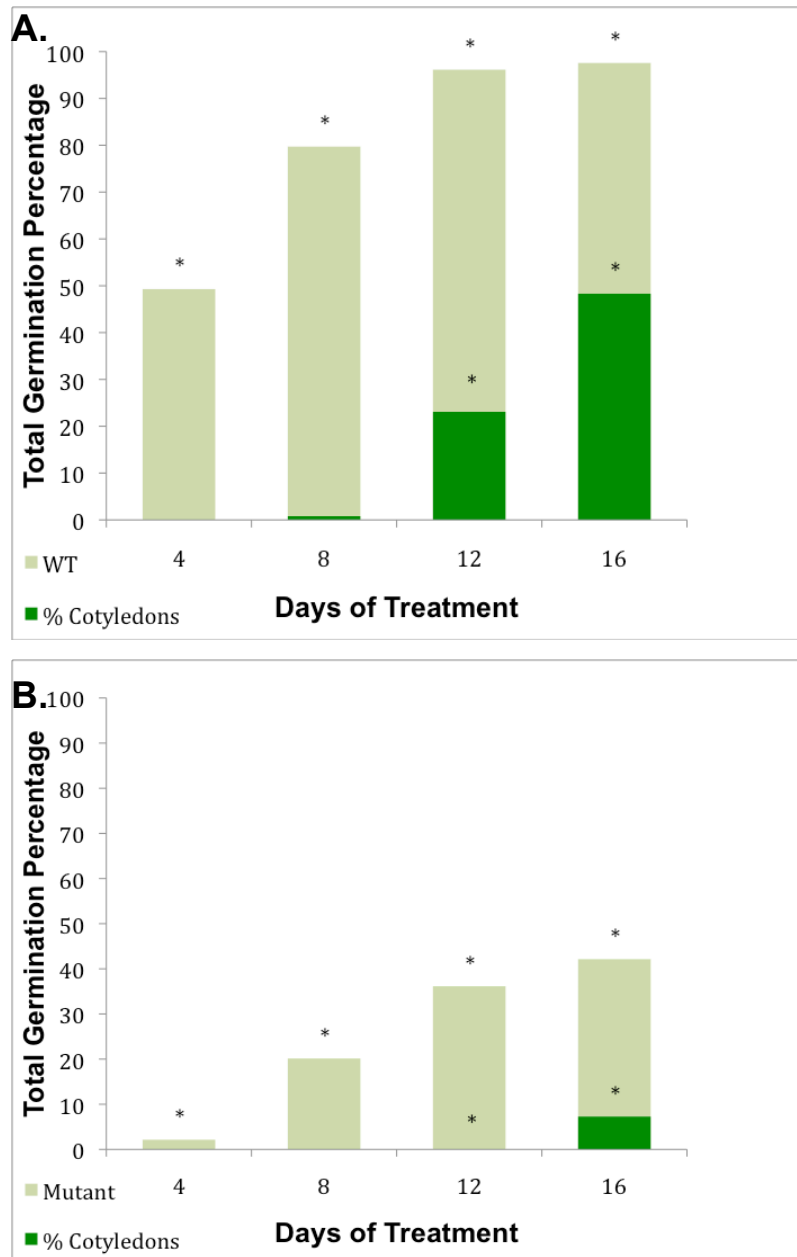


Figure 4.15: Germination Percentages of WT and Mutant Seeds with Percentage of Seeds forming Cotyledons at 250 mM Mannitol

(A) Wild-type Seeds (WT) demonstrate higher germination totals throughout the treatment duration. (green bars indicate the percentage of germinated seeds forming cotyledons)

(B) Mutant Seeds demonstrated significantly lower germination percentages and cotyledon development.

(* indicates a statistically significant difference $P < 0.05$)

Figures 4.14 and 4.15 show significant differences between the wild-type and mutant seeds. The mutant seeds demonstrated a significantly lower germination total. In addition, the percentage of germinated seeds forming cotyledons is also significantly different at 12 and 16 days.

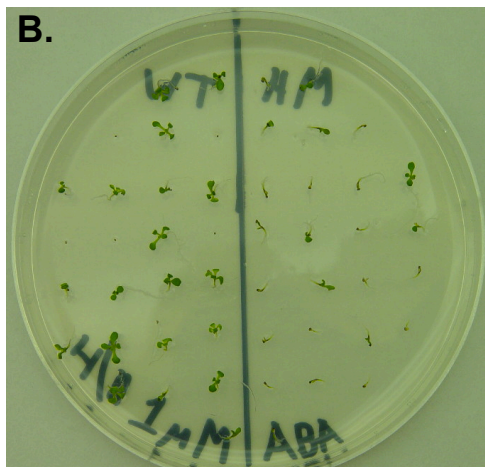
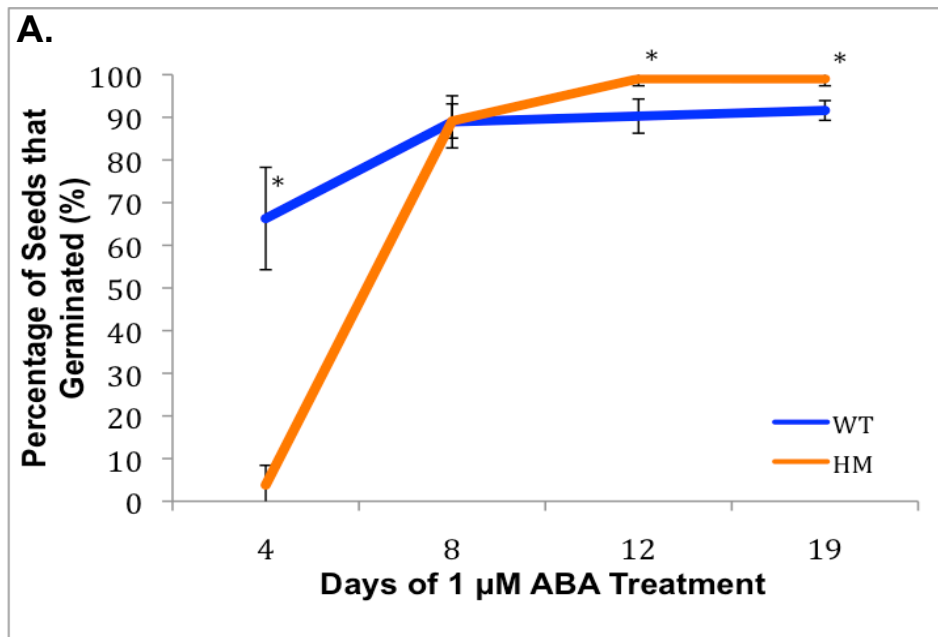


Figure 4.16: Germination Performance of WT and *JLL1* Mutant Seeds under 1 μM ABA.

(A) Percentage of wild-type (WT) and homozygous mutant (HM) seeds that germinated under ABA treatment over 19 days. Wild-type seeds demonstrated a greater germination percentage at 4 days, (B) One replicate from this treatment (8 days). (“*” indicates statistical significance at $P < 0.05$)

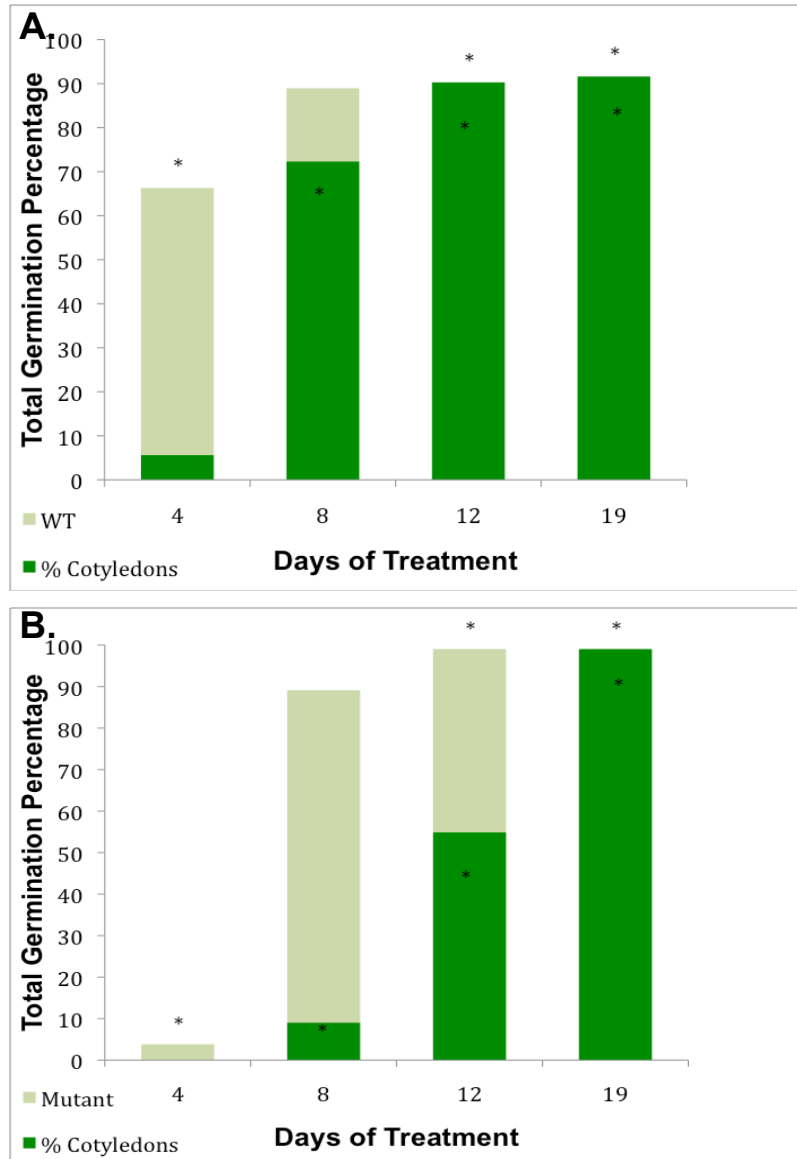


Figure 4.17: Germination Percentages of WT and Mutant Seeds with the Percentage of Seeds forming Cotyledons at 1µM ABA

(A) Wild-type Seeds (WT) had a greater germination percentage at four days of treatment. Wt seeds also had a more significant cotyledon formation at 8 and 12 days. (green bars indicate the percentage of germinated seeds forming cotyledons) (B) Mutant seeds demonstrated delayed germination and cotyledon formation. (“*” indicates a statistically significant difference P<0.05)

Figures 4.16 and 4.17 suggest that the wild-type seeds germinate earlier than the mutant seeds. However, the mutant seeds exhibit very similar germination and cotyledon formation percentages towards the middle and end of the analysis. Figure 4.18 displays the effect of the 1 μ M ABA treatment.

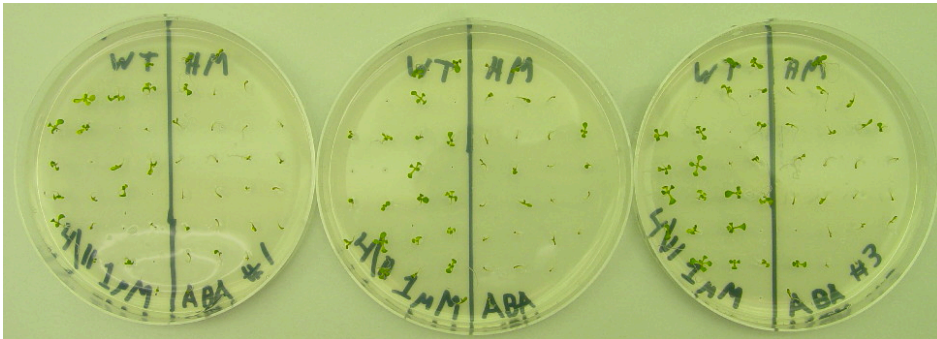


Figure 4.18: 1 μ M ABA Treatment (12 days)

JLL1 mutants (HM) exhibited decreased germination and cotyledon development under 1 μ M abscisic acid (ABA) treatment when compared to wild-type (WT) seeds.

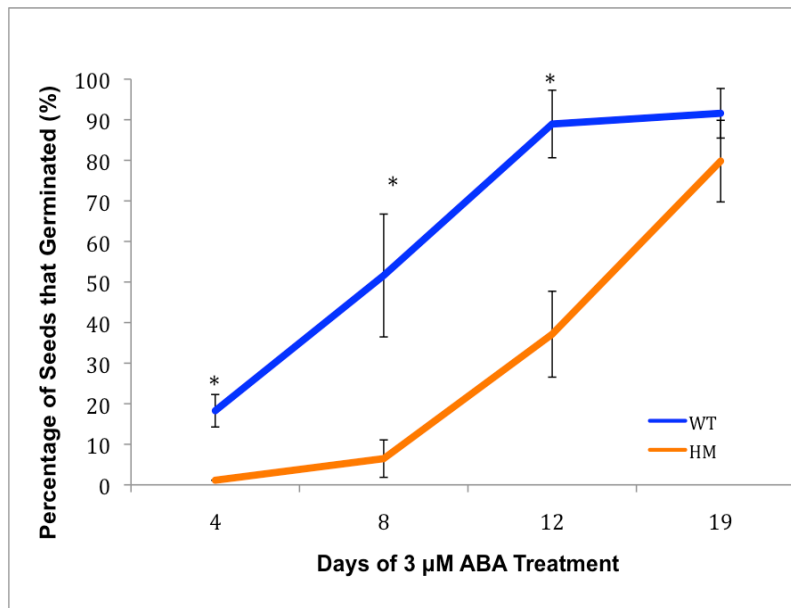


Figure 4.19: Germination Performance of Wild-type and Mutant Seeds under 3 μ M of ABA Treatment

Wild-type (WT) seeds demonstrated a higher germination percentage compared to the homozygous mutant (HM) seeds during the first 12 days of ABA treatment. (“*” indicates a statistically significant difference at $P < 0.05$)

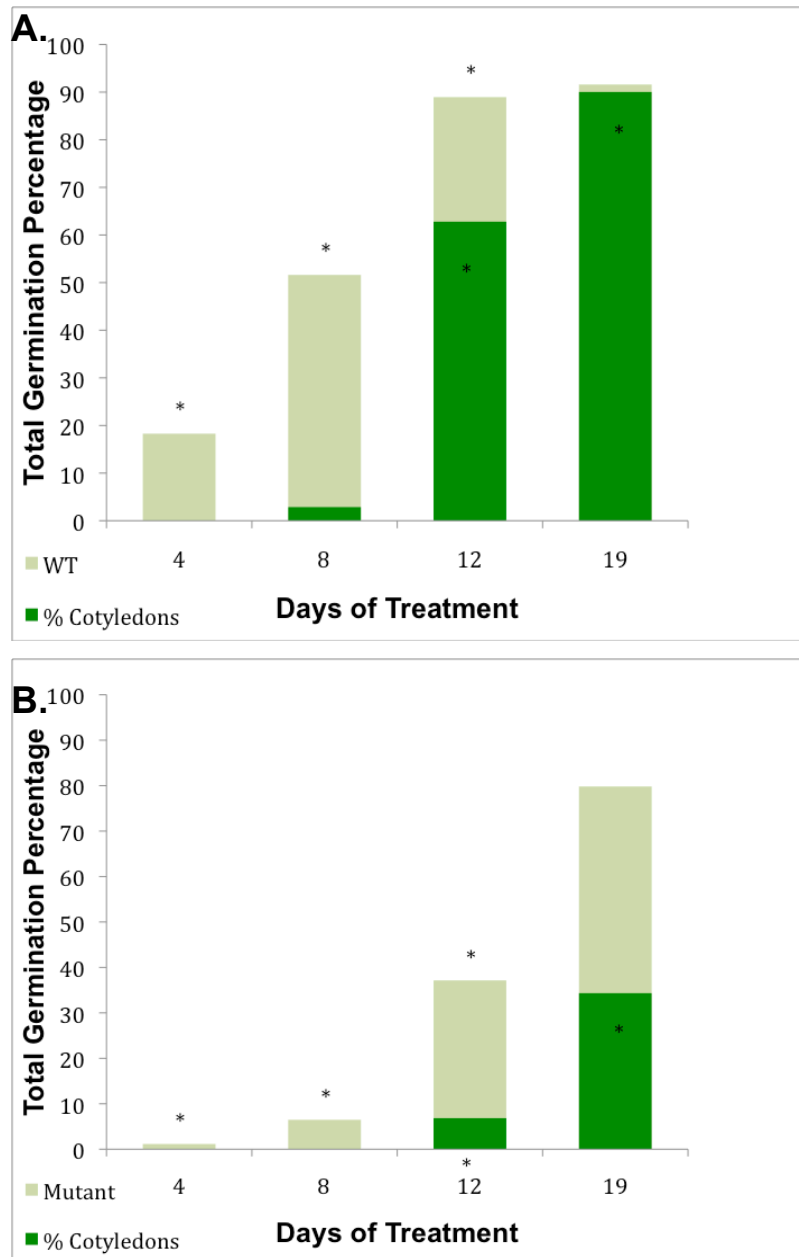


Figure 4.20: Germination Percentages of WT and Mutant Seeds with Percentage of Seeds forming Cotyledons at 3 μ M ABA

(A) Wild-type Seeds (WT) had significantly greater germination and cotyledon formation rates throughout the treatment period. (green bars indicate the percentage of germinated seeds forming cotyledons)

(B) Mutant seed data.

(* indicates a statistical significance of $P < 0.05$)

The 3 μM ABA treatment further delayed the development of mutant and wild-type seeds (compared to the 1 μM treatment). The mutant still demonstrated lower total germination (days 4 through 12) compared to the wild-type plants. In addition, the percentage of seeds forming cotyledons was much lower in the mutant seeds throughout the experiment.

Synthesis of a *JLL1* Overexpression Construct

The over-expression binary vector was synthesized (Figure 4.21). The presence of *JLL1*'s genomic sequence in the binary vector was verified by PCR (Figure 2.22).

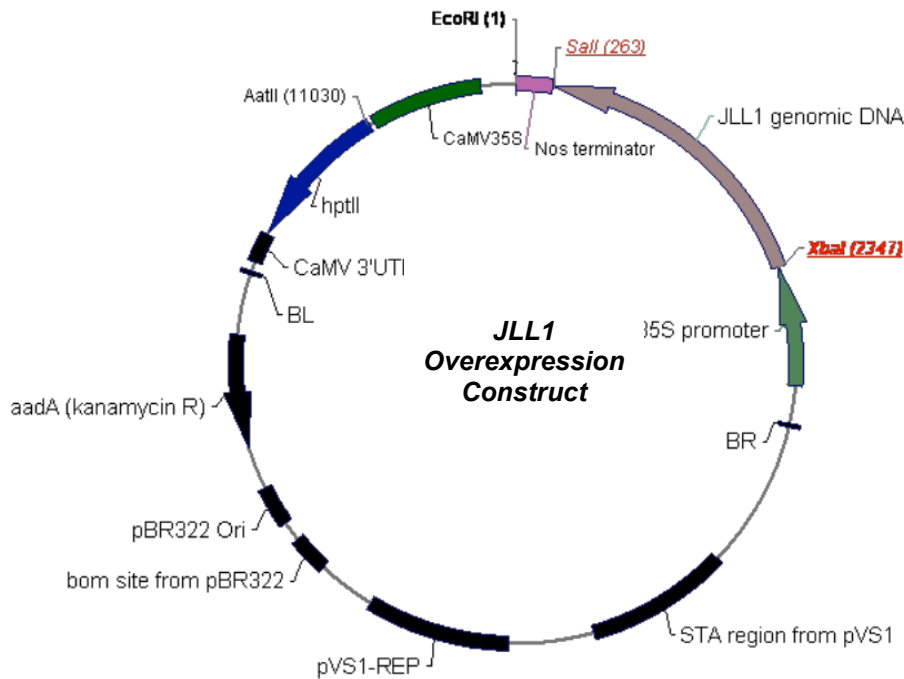


Figure 4.21: *JLL1* Overexpression Vector

The constructed vector contains the *JLL1* genomic sequence driven by the Cauliflower mosaic virus 35 S promoter. The *hptII* resistance gene and the *Agrobacterium* selectable marker, kanamycin, are also on the binary vector.

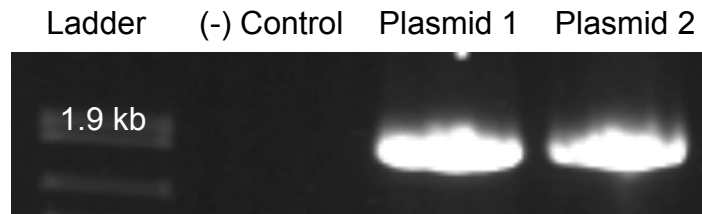


Figure 4.22: Verification of *JLL1*'s Genomic Sequence in the Overexpression Construct

Genomic primers were run to verify the presence of the *JLL1* genomic sequence in the binary vector. Both of the generated plasmids were verified to contain the sequence. Predicted size of the amplicon is 1.9 kb.

The overexpression construct was introduced into *Agrobacterium*, and then transformed into *Arabidopsis thaliana* via the flower dip method.

IVD. CONCLUSIONS

JLL1 mutants exhibit delayed germination on the abiotic stress treatment plates. This delay is most apparent in the first four to eight days during germination. Additionally, this germination delay probably contributes to the lower number of expanded cotyledons because the mutant plants are behind in development. The *JLL1* mutants did not exhibit morphology or development that differs from wild-type *Arabidopsis*.

Overall, these preliminary results suggest that *JLL1* mutants demonstrate delayed germination under abiotic stress conditions. A recent study has shown that phytohormone pathways interact with sugars during seed germination and early plant development.¹⁰⁴ The cause of this delay is unknown, however, since *JLL1* contains two sugar-binding domains and is negatively-regulated by ABA

(see Figure 3.18), it is plausible to speculate that *JLL1* may be involved in this early developmental cross-talk.

IVE. REFERENCES

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- ¹⁰⁰ Lee, Sangmin, Pil Joon Seo, *et al.* "A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in *Arabidopsis*." *Plant Journal*. (2012).
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CHAPTER 5

FUTURE WORK AND CONCLUSIONS

VA. CONCLUSIONS

This investigation provides some insight into *JLL1*'s physiological role in *Arabidopsis thaliana*. The *in silico* analysis established that *JLL1* has high sequence similarity with two, proximally located jacalin-like lectin domain-containing proteins on chromosome 1. Due to their close proximity and high sequence level identity, it is probable that these genes share a common function and may even be paralogs. Additionally, a putative ortholog (XP_002894354.1) was found in *Arabidopsis lyrata*. The analysis of *cis*-regulatory elements in *JLL1*'s promoter region suggests that *JLL1* may have a role in plant growth and development due to the relative abundance of elements that are associated with metabolism, hormone response, and storage.

The promoter-reporter analysis demonstrated expression in the root cap, vascular associated tissues in the root system, and the vasculature of the leaves. Interestingly, the stain is absent from the zones immediately behind the root cap region. These regions may include the zone of elongation and/or the zone of differentiation. The staining in the leaf vasculature is consistent with the localization of the Jacalin-like lectin RTM2, which is known to restrict the movement of Tobacco Etch Virus in *Arabidopsis*.⁸ This suggests that *JLL1* may have an analogous role *in planta*.

The expression in the root cap is intriguing for several reasons. First, the root cap is known to secrete macromolecules and glycoproteins in a mucilage that aids in root movement, regulates the soil-microbial community, defends against herbivores, inhibits the growth of competing plant species, and encourages symbiotic interactions.¹⁰⁵ *JLL1* expression in this secretory zone corresponds with previous studies that found *JLL1* protein in the rhizosphere (Section 1F). Additionally, several of the functions of the mucilage could be attributed to lectins that bind and inhibit the utilization of cell surface glycoproteins or soil carbohydrates.

The root cap has also been shown to be involved in root growth-rate maintenance and root-architecture.¹⁰⁶ Since these processes are known to be hormone mediated including auxin and cytokinins,^[107, 108] *JLL1* may have a role in the growth or development of roots through an interaction with *Arabidopsis Response Regulator 5*. (See section 1F)

The expression profile of *JLL1* corresponds with the original hypothesis that *JLL1* would be down-regulated under abiotic stress conditions due, potentially, to its role in growth or biotic stress response. Interestingly, *JLL1* transcripts did not significantly decrease under drought treatment, but did exhibit dramatic down-regulation under NaCl treatments suggesting that salt-responsive pathways have a greater antagonistic effect to *JLL1* expression than the drought response pathways. The potential reasons behind the fluctuations in *JLL1* expression during ABA treatment are covered in section IIID. Finally, the

germination studies, even though they are preliminary, demonstrate that *JLL1* mutants exhibit delayed germination under abiotic stress conditions. This delay could be attributed to *JLL1*'s role in plant growth and metabolism

In summary, *JLL1* is a Jacalin-like domain-containing protein that is expressed in *Arabidopsis* vasculature and root tips. It is negatively responsive to the abiotic stress conditions NaCl, drought, and ABA, and may serve a dual role *in planta* as a protein involved in hormone mediated early plant development and as an exudated biotic defense protein.

VB. FUTURE WORK

This paper details the preliminary studies focused on understanding the function of *JLL1*. Many more experiments are required to elucidate the role of this lectin. A qRT-PCR will be used to quantitate the levels of *JLL1* under abiotic and biotic stress conditions. Overexpression lines are currently being generated, and their phenotypes will be compared with mutant and wild-type lines in extensive germination experiments. Pathogen treatments will also need to be applied to determine the impact of biotic stress. A sub-cellular localization construct is also under construction to determine where *JLL1* is localized in the cell. Finally, mutants of the two proximal jacalin-like lectin domain containing proteins will need to be characterized and compared to the *JLL1* mutant phenotype.

VC. REFERENCES

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APPENDICIES

APPENDIX A

PHYLOGENETIC ANALYSIS OF *JLL1*

Table A-1: Interspecies sequences incorporated into the ClustalX Protein Sequence Comparison

Species	Number of Sequences
<i>Arabidopsis lyrata</i>	10
<i>Brassica napus</i>	6
<i>Brassica rapa</i> subs. <i>Pekinensis</i>	1
<i>Plantago major</i>	1
<i>Oryza sativa</i> <i>Japonica</i>	1
<i>Morus nigra</i>	1
<i>Cycas rumphii</i>	1
<i>Cycas revoluta</i>	1

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Atagaggatgggtgaagtctctggttgatctcgtcttagatggccttattga
cttaagtcacatcttgtagcttttagtatcaaatgatcttaaatctattcttagctt
actttaccaatctaattgatattccccatctagtccctcgaaatagttttcatgc
cgaattttcatatagatatcacattatccaaagaaaaacttgcgaaccaac
cctaaaagcatctataccaaaacagattccccatgagccatgagaaactatgt
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gtaattaaactaattaagtggtataaaactcatcatgaggatttgtaaagtgtttc
agaacatgcatgtgtgcatatagaaaatctaaaaaacatataagtccacgtac
gatattaataagtttaattaaatgttacaCAATTaatcaaaacatattcattgt
ttcaagctcaaaaacgtttggttaaataagcctgtatataaaaaaaaaaac
acaactgtaaattaattgatatccaagcattataaatccatgtttttaagaat
agtttcttctatatcgctgaatcgacggtattttaaattaatgcatgctgtaagt
gtagctaaatacttttaaaaggcgaaaataagaactgataaacattttctataa
tgccatagggccactagttataaaactagtaatttccatagtgaagaccag
aactgTGTGATAAATAAgaatcgtcagccatggcttctcACCAat
caccacagcacagcgatc
```

Figure A-2: *JLL1* Promoter Sequence Used in the *cis*-regulatory Analysis

TSS (red), TATA Box (green), and CAAT Box (Blue)

Table A-3: Cis-regulatory Elements found within 300 bp Upstream of *JLL1*'s Transcriptional Start Site

Factor (Sites within - 300 bp)	Number of sites within 1000bp	Sequence	Tissue	Putative Physiological Functions
ACGTATERD1 (x2)	4	ACGT		Abiotic Stress
ARR1AT (x4)	16	NGATT		Hormone Responsive
ASF1MOTIFCAMV	1	TGACG	root, leaf	Abiotic/Biotic Stress
BOXIINTPATPB (x2)	3	ATAGAA	plastids	
CACTFTPPCA1 (x4)	17	YACT	mesophyll	Photosynthesis
CARGCW8GAT (x2)	1			
CATATGGMSAUR (x2)	1	CATATG		Hormone Responsive
CBFHV	2	RYCGAC		Abiotic Stress
CAATBOX1	4	CAAT	seed	
CCAATBOX1 (x2)	2	CCAAT		
CGACGOSAMY3	1	CGACG		Amylase
DOFCOREZM (x4)	16	AAAG	leaf, shoot	
EBOXBNNAPA (x2)	8	CANNTG	seed	Storage
GATABOX (x3)	11	GATA	leaf, shoot	Light Regulation
GT1CONSENSUS (x4)	13	GRWAAW	leaf, shoot	Light Regulation
GT1CORE	1	GGTTAA	leaf, shoot	
GT1GMSCAM4	5	GAAAAA		Abiotic/Biotic Stress
GTGANTG10 (x3)	11	GTGA	pollen	Pollen
IBOXCORE	2	GATAA	leaf, shoot	Light Regulation
L1BOXATPDF1	1	TAAATGYA	shoot apical meristem	
MARTBOX	1	TTWTWTTWTT		
MYB1AT	2	WAACCA	leaf, seed	Abiotic Stress
MYBST1	2	GGATA		
MYCCONSENSUSAT (x2)	4	CANNTG	leaf, seed	Abiotic Stress
POLLEN1LELAT52 (x4)	8	AGAAA	pollen	Pollen
PYRIMIDINEBOXOSRAMY1A	1	CCTTTT	embryo, seed	Amylase
REALPHALGLHCB21	3	AACCAA		Light Regulation
RYREPEATBNNAPA	2	CATGCA	seed	Seeds/Storage
RYREPEATGMGY2		CATGCAT	seed	
RYREPEATLEGUMINBOX		CATGCAY	seed	
SORLIP1AT	2	GCCAC	root	Light Regulation
TATABOX2 (x2)	2	TATAAAT		
TATABOX4	3	TATATAA		
TATABOX5 (x3)	3	TTATTT		
TATAPVTRNALEU	1	TTTATATA		
WRKY71OS	5	TGAC		Hormone Responsive

APPENDIX B
SPATIAL EXPRESSION OF *JLL1*

ACCAAT**CACCACAGCACAGCGATCATG**GTTATCATCTATATATTTCTTTTTCTCTCCTCAG
 CCATTATAGATTCAACTGGGTTGGCAAAGGCCCAAAGTTGGATGCAATTGGTGGGAAG
 GGAGGCAAGCAGTGGGACGATGGAGCTGACCATGACAATGTAGCAAAGGTTTATATAAG
 AGGTGGTCTTGAAGGCATACAATACATCAAATTTGATTATGTCAAAGATGGAAAACTATA
 GATGCATCTATCCATGGTGTTCGGGTAGCGGTTTCACACAGACGTTTGAGATTGATTAT
 CAAAACAGTGAATATATTGTATCTGTTGATGGCTACTACGACAAATCTGGTACGATGCAAG
 CACTTGAATTCAAACCAACCTGAAGACTTCTGAAGTGATTGGATATCCAAAGGGTACTA
 CAAAGTTTTCACTCGGTGGAGTCAATGGCAAGATGGTGATTGGCTTCCATGGATCTGCTG
 GGAAAGTCCTAAACTCCATTGGAGCATATTTAACAACAGCTCCTCCTACTAAGTCACAACCT
 TGTAGGTGGTCTAACCGGAGGCGAACCTTGGGATGATGGCTCTAATTATGATGGCGTGA
 AAAAGATATCTGTCACTTACATTAGCACTCTTATAAGGAGTATCAATGTGGACTATGAAAA
 GGACGGCCAAGTTGTAACACGTTACCACGGGATGAAGAATGGAGATACAGAGGAGTTTG
 TGATAGACTATCCAAATGAGTATTTGATATCAGTGGAGGGAACCTACAACATACTCCCG
 ATGATAACGTTTTGGTCATTAGGTCGTTGATTTTCAAACATCAAAGGGGAGAATCTCTCC
 CACATATGGGTTTTGTGTCAGGTACCAAATTTGTGTTGGAGAGCCAAGGTAATGCTATTGT
 TGGATTCTATGGGCGGGATGGTGGTGCTTTGACGCTATCGGAGTTTACTTCTCTCCAAT
TCCTTCGTAATTCGAGACTATAAAGGCTATAAAACCATATGGTTAGATGGAAATATAGTCA
 CGAACTTCATCTTGTTTAAGGCTCTCATATCTACAATGATTTACTACCTACTCCGATGTTT
 CTTAATCAGTAATTTCTTTTCGAGTTTACAATCTCTTGTAATAAAACAAGGTTTAATTATGA
 AACCTGTATTTAGTATTTAAAAATAAAATATTATTATAATTCTGAATTAATGCAAACAAA
 ACTTTTAAAGCTCC

JL_RT_F - aggatc**CACCACAGCACAGCGATCAT**

JL_RT_R- actgc**AGTCTCGAATTACGAAGGA**

TM= 60°C, amplification size **975 bp**

Figure B-1: *JLL1*'s cDNA Sequence with RTPCR Primers

Blue letters signify the location of forward or reverse primers. Red letters indicate the start and stop codons.

APPENDIX C
MUTANT ANALYSIS OF *JLL1*

(JLGen-F)

ACCAATCACCACAGCACAGCGATC**ATG**GTTATCATCTATATATTTCTTTTTCTCTCCTCAGCC
ATTATAGGTAACCTAAGCTCTCCTTATCCACACTTTTTATTACACCACTGATGTTAATACTAA
GGAGATCAGCTTCCCTTATGATCCACTATTTACACACGCCAATAGCTTATTCCCTCTTCTTCAC
TAAAATATATAACACTATCACATAATGTTATAATGAGATAATAATTACATATGAACATAACACTA
TATATATAGTAAGTTTTGAATATGGTATGAATTTATTTGTTGTTTTGGCAACATCAAATATA
GGTTTGTTATAAATTTGCATAAAATTTCCAGGCAAGCCATTCTTCTCTTTAAAAACAATTG
GTGGTGTCTTGGTATATAATATTTATTTTCGAGACAGAAAAGTATATTGAATATAATTAGAAAACA
CAATACCCTAATTTAAATTTTATGTTGAAAGATTCAACTGGGTTGGCAAAGGCCAAAAGTTG
GATGCAATTGGTGGGAAGGGAGGCAAGCAGTGGGACGATGGAGCTGACCATGACAATGTA
GCAAAGGTTTATATAAGAGGTGGTCTTGAAGGCATACAATACATCAAATTTGATTATGTCAAAG
ATGGAAAACTATAGATGCATCTATCCATGGTGTTCGGGTAGCGGTTTCACACAGACGGTAT
GTTTTAATGTATAAATGTTAAGTCTAGTGACCAATGAAACTCAAACCTGGATCTTATTATGTCG
TGGTTCCCATATCACATATATTAGGTTATTGGCTACTGTGTACAAAGTCTTATTAATAACAAT
CACCATTTTTGTTGCAGTTTGAGATTGATTATCAAACAGTGAATATATTGTATCTGTTGATG
GCTACT**ACGACAAATCTGGTACGATGC**AAGCACTTGAATTCAAAACCAACCTGAAGACTTCT
GAAGTGATTGGATATCCAAAGGGTACTACAAGTTTTCACTCGGTGGAGTCAATGGCAAGAT
GGTGATTGGCTCCATGGATCTGCTGGGAAAGTCCAAACTCCATTGGAGCATATTTAACAA
CAGCTCCTCCTACTAAGTCACAACCTTGAGGTGGTCTAACC GGAGGCGAACCTTGGGATGA
TGGCTCTAATTATGATGGCGTGAAAAAGATATCTGTCACTTACATTAGCACTCTTATAAGGAGT
ATCAATGTGGACTATGAAAAGGACGGCCAAGTTGTAACACGTTACCACGGGATGAAGAATGG
AGATACAGAGGAGGTAAGTGAATCTTATCTCTTAAACATAACATTCTTAAATCTTAATTAGTCCA
TCGATATATTTAACTTGTATATATAAAAACCAATTACACTCACTAGGCTTTGATTAATTTACGC
GTTTTTTATTACAAATTTATTGACAATAAGTCAAACAGTCAATGTATCACTTAAATACTATATCAG
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TGATAGACTATCCAAATGAGTATTTGATATCAGTGGAGGGAACCTACAACATACTCCCCGATG
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ATGGGCTTTGTGTCAGGTACCAAATTTGTGTTGGAGAGCCAAGGTAATGCTATTGTTGGATTCT
ATGGGCGGGATGGTGGTCTTTTCGACGCTATCGGAGTTTACTTCTCTCCAATTCCTTCGTAA
TTCGAGACTATAAAGGCTATAAAACCATATGGTTAGATGGAAATATAGTCA**CGAACTTCATCTT**
GTTTTAAGGC TCTACAATG**ATTACTACCTACTCCGAT**(JLGen-R)
GTTTCTTTAATCAGTAATTTCTTTTCGAGTTTACAATCTCTTGTAATAAAAACAAGGTTTAATTATG
AAACCTGTATTTTCAGTATTTAAAAATAAAAATATTATTATAATTCTGAATTAATGCAAACAAAAC
TTTTAAAGCTCC

SALK_134751 Verification Primers:

LP 5'-GCCTTAAACAAGATGAAGTTCG 3' TM 52.6

RP 5'-ACGACAAATCTGGTACGATGC-3' TM 55.2

BP 5'-ATTTTGCCGATTTCCGGAAC – 3' TM 51.5

Figure C-1 : JLL1's Genomic Sequence Annotated with the Mutant Analysis Primers

- Orange- Genomic Primers
- Blue- RP (top) and LP (bottom) Primers
- Green- Region of T-DNA insertion
- Red- Translational Start Codon