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The Effects of Deleting LRB E3 Ligase Region 2 on Red Light Responses in Arabidopsis thaliana

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The signatories of the committee members below indicate that they have read and approved the thesis of Sabina Liskey in partial fulfillment of the requirements for the degree of Master of Science.

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DEDICATIONS

To my mom for always being proud of me, pushing me to be the best I can be, and telling everyone how cool her daughter is (even when you do not fully understand the crazy scientific jargon I use). I would not be the woman I am today if it was not for her. She would hang this on the fridge in a heartbeat if she had a magnet strong enough to hold all these pages.

To my partner for his encouragement to reach my goals and for being someone to lean on when those goals feel unattainable. I thank him for loving me as the nerdy scientist I am.

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ABSTRACT

The ability of plants to absorb light through photoreceptors is essential for successful plant development. Phytochromes are important plant photoreceptors that detect and absorb red and far-red light and must function properly for plant survival. The Light Response BTB proteins (LRB) play an important role in a plant's response to red light by degrading phytochrome B (PhyB) via the ubiquitin proteasome system (UPS). Although there has been a successful investigation into understanding the function of the LRBs, the function of a few regions within LRB proteins remains unknown, including a portion located on the N-terminal end of the protein, termed Region 2. This study assessed the function of Region 2 by studying the red-light response that occurs when Region 2 is genetically mutated in *Arabidopsis thaliana*. A mutant gene that has deleted portions of Region 2 (LRB-R2D) was created using site directed mutagenesis techniques and transformed into *A. thaliana lrb1-1 lrb2-1 mutants*. The function of the LRB-R2D proteins were assessed via plant growth when exposed to a red light environment. It was found that *lrb1-1 lrb2-1* plants containing LRB-R2D reverted back to the wild type phenotype. These results suggest that the function of Region 2 may not play a role in some red light responses. Although the function of Region 2 has yet to be determined, future studies searching for interacting factors with Region 2 may reveal possible functions. This study has ruled out red light responses being important for Region 2, but the methodology can be applied to other regions within the LRB protein such as Region 1 or Region 3. Further understanding of Region 2 and other regions of LRB can enhance the comprehension of this protein and its effects on plant development, which could impact agricultural practices in the future.

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List of Abbreviations

BTB - bric-a-brac, tamtrack, broad complex

CUL3 - Cullin3

DK - darkness/no light

E1 - ubiquitin activating enzyme

E2 - ubiquitin conjugating enzyme

E3 - ubiquitin protein ligase

GFP - green fluorescent protein

GFP-LRB1 - GFP attached to full length LRB1 protein

LRB - light response BTB

LRB-R2D - LRB protein with Region 2 flanking NLS removed

MS+Suc - Murashige + Skoog plates containing 20% sucrose

NLS - nuclear localization sequence

Pfr - active phytochrome

Phy - phytochrome

PIF - phytochrome interacting factor

Pr - inactive phytochrome

RBX - RING-box protein

RL - red light

SDM - site directed mutagenesis

SDM1 - product of first site directed mutagenesis event

SDM1/2 - product of second site directed mutagenesis event

T0 - *lrb1-1 lrb2-1 A. thaliana* plants

T1 - product of transformed T0 plants with hygromycin resistance

T2 - product of self crossed T1 plants

Ub - ubiquitin

UPS - ubiquitin proteasome system

CHAPTER 1: INTRODUCTION

Introduction

It is vital that plants have the ability to absorb light through several different types of photoreceptors in order for successful plant development to occur. Specific photoreceptors called phytochromes play an important role in detecting and absorbing red and far-red light wavelengths. This absorption is crucial for the survival of the plant as it allows for important light responses to occur. One essential light response is the ability to degrade phytochrome B (PhyB) when red light is detected. The Light Response BTB proteins (LRB) act by degrading PhyB as a response to red light wavelength detection. This occurs through a signaling cascade that involves the ubiquitin proteasome system (UPS). Understanding the function of LRBs is important as it allows for further understanding of red light responses and phytochrome degradation. Previous studies have investigated the function of LRBs, but the function of a few regions within LRB proteins remains unknown. One of these unknown regions is Region 2, located on the N-terminal end of the LRB protein.

The overall objective of this study was to analyze the relationship between LRB Region 2 and red light responses. This was completed by creating a mutant LRB protein which lacked Region 2 (LRB-R2D), transforming it into *A. thaliana*, and exposing LRB-R2D seedlings to red light conditions for observation of their growth patterns in comparison to wild type *A. thaliana* also exposed to red light conditions. This would determine if red light has a role in the overall function of LRB Region 2. This study analyzed two different LRB-R2D lines, where the LRB-R2D sequence has been randomly inserted into the *A. thaliana* sequence. This allowed for phenotypes from gene interruption to be easily identified as different results amongst LRB-R2D lines would be visible after red light exposure. It was important to have two experimental lines to

obtain a reliable and accurate representation of the effects that red light exposure had when lacking Region 2. Throughout the study, these experimental lines were compared to positive and negative controls. The positive control used contained a non-mutant functional LRB protein, whereas the negative control contained a mutant non-functional LRB protein.

This study is aiming to answer the following question: Is there a relationship between LRB Region 2 and red light signaling? Region 2 is highly conserved amongst other plant species, indicating a vital function. Within the highly conserved sequence, a nuclear localization sequence lies. Outside of this, there are sequences with high conservation that have an unknown function. The sequence for Region 1 is also highly conserved and has been previously studied. It was shown that there may be a relationship with red light signaling, which alludes to a possible relationship with red light signaling and Region 2. It was originally hypothesized that Region 2 of the LRB protein would show a significant role regarding red light signaling. This was expected to be shown via red light exposure causing photomorphogenesis to occur within LRB-R2D plants resulting in the hypocotyls being shorter than wild type plants that have a non-mutant Region 2.

This study plays an important role in both the scientific community as well as the agricultural community. Further understanding of the LRB protein will contribute more knowledge to the scientific community regarding light responses including red light signaling, which in turn can provide more knowledge on plant growth and development in relation to its environment. These findings are impactful as they can benefit the agricultural community by contributing to the ideology of novel agricultural techniques that could be developed, aiding in producing better plant products for consumers.

CHAPTER 2: LITERATURE REVIEW

Responses to Light in Plants

The ability for plants to absorb light is important for energy production using photosynthesis. Exposure to sunlight allows plants to absorb wavelengths of light by chlorophyll where the light can then be converted into energy and stored for use in the photosynthetic processes (Leivar et al., 2012). In order to use light most effectively, plants are able to detect different wavelengths and intensities of light through the use of photoreceptors. Photoreceptors allow plants to monitor and respond to different light environments to grow optimally. Blue-light and ultraviolet-light wavelengths are sensed by cryptochromes, phototropins and UVR8 respectively (Casal, 2007). Blue light wavelength absorption is important for allowing plants to adapt to their environment by impacting chlorophyll production, chloroplast movement, phototropism, and producing shorter stems (Lazzarin et al., 2021).

Red and far-red light wavelengths are sensed by the phytochromes (Phy). Aside from being absorbed by chlorophyll for photosynthesis, red and far-red light play a role in plant growth and development. High red/far-red ratios result in the reduction of stem growth and decreased cell expansion, whereas high far-red/red ratios contribute to enhancing cellular expansion and allowing for stem growth (Lazzarin et al., 2021). Red and far-red light wavelengths are also responsible for controlling crucial developmental processes such as seedling germination, flowering, and resistance against pathogens (Franklin & Quail, 2009). Plants can detect minute changes in their light environment by the ratios of light passing through or reflecting off of other plants and/or structures. Shade avoidance occurs when there are high far-red/red ratios, which can cause stem elongation and/or decreased leaf expansion. This response allows for plants to outgrow neighboring plants in order to avoid restricted sunlight

absorption from occurring (Franklin & Whitelam, 2005). Photomorphogenesis is a developmental mechanism where plants grown in the dark undergo rapid and extreme changes in morphology when exposed to light for the first time. This includes chlorophyll synthesis, leaf expansion, and hypocotyl growth (Fig. 1).

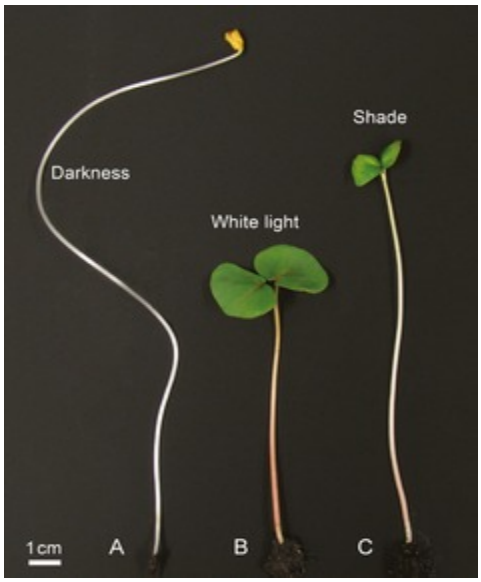

Figure 1: Photomorphogenesis in *Fallopia convolvulus*
(Kutschera & Briggs, 2013)

The growth of *Fallopia convolvulus* (wild buckwheat) in dark conditions (left) is altered when compared to red light conditions (right) as photomorphogenesis occurs.

Light and Agriculture

As light plays an important role in plant growth and development, there are some agricultural issues associated with light responses in plants. A common agricultural complication is wind damage to crops. One such example is corn; corn is generally planted 8-10 inches apart to maximize yield per acre which can result in continuous stem elongation in order to compete for sunlight. Strong winds can affect crops that grow to staggering heights due to shade avoidance in two common ways: green snap and root lodging (Thelen, 2018). Green snap occurs when the stem of the crop experiences an overbearing amount of pressure from wind which

results in the stem to break. Root lodging occurs when plants are flattened or tipped to the extent of root exposure occurring. This disrupts the root system of the crop, which is difficult to overcome. Both green snap and root lodging can largely affect the overall yield of crops. In many cases, the overall impact of wind cannot be determined until harvest (Thelen, 2020). This economically impacts farmers as they are unable to prevent or prepare for this tragedy to crops and can suffer immensely from a poor harvest. Understanding the impact of light responses on cellular processes within plants could assist in agricultural practices to allow for more efficient crop production, harvesting, and maintenance.

Red Light Signaling in Plants

Phytochromes (Phy) are a type of photoreceptor that allows for the regulation of plant development, including seed germination, chlorophyll synthesis, and flowering by processing the red and far red light environment (Li et al., 2011). There are two photoconvertible conformations of Phy in plants: Pr and Pfr (Chen et al., 2004) (Fig. 2). Inactive Phy (Pr) can be transformed to active Phy (Pfr) when exposed to red light. Upon red light exposure, Pfr is relocated from the cytosol to the nucleus where it acts together with a set of transcription factors called phytochrome interacting factors (PIF) to regulate different genes involved in red light responses (Chen et al., 2004). Pfr can be reverted back into the inactive Pr form when exposed to far-red light (Casal, 2007) (Fig. 2). *A. thaliana* has five Phy proteins: PhyA-PhyE. PhyA has been shown to modify plant responses to far-red light. PhyB, along with the less dominant photoreceptors Phy-C, -D, and -E, control red light responses in *A. thaliana* (Casal, 2007). The ratio of PfrB and PrB is crucial for physiological responses to the environment (Franklin & Quail, 2009). When red/far red ratios are high, more PhyB is located into the nucleus. This leads to the plant having the red light response of stem elongation restriction. When red/far-red ratios are low, more PhyB

remains in the Pr form and is localized into the cytosol. This leads to increased stem elongation, decreased cotyledon opening, and reduced chlorophyll synthesis (Franklin & Quail, 2010) (Boylan & Quail, 1991). PIFs are able to be associated with PhyB when activated in red light conditions, but they dissociate from PhyB when deactivated in far-red light conditions (Toettcher et al., 2011).

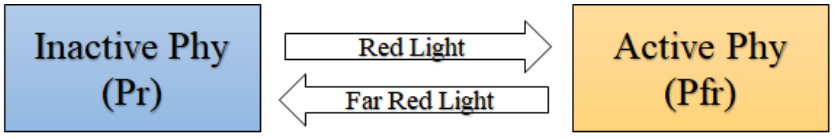

Figure 2: Activation and Inactivation of Phy

In red light, Pr (inactive) undergoes a conformational change resulting in activated Phy, known as Pfr (active). Pfr can be reverted to Pr when exposed to far-red light or dark conditions.

Ubiquitin Proteasome System

Shortly after the Phy localization into the nucleus, the active Pfr form is degraded through the Ubiquitin/26S Proteasome System (UPS) (Fig. 3), where PhyB interacts with several different components to become ubiquitinated (Pham, 2017). The UPS works to degrade proteins by first attaching a small ubiquitin protein to the targeted protein. This ubiquitin is activated and ligated to the target protein via an enzyme cascade consisting of a ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and a ubiquitin protein ligase (E3) (Nandi et al., 2006) (Fig. 3). Recognition and specificity of the targeted protein is possible through the E3 ligase. The E3 enzyme family is one of the most populated gene families in *A. thaliana* as it is composed of over 1,500 different E3 genes, whereas there are only two E1 genes and 37 E2 genes (Hua & Vierstra, 2011) (Smalle & Vierstra, 2004). The extensiveness of the E3 enzyme family indicates

that protein degradation is highly regulated and has a broad range of specificity for targets. Once one ubiquitin is attached to the phytochrome on the target protein, ubiquitination of ubiquitins can further occur resulting in a polyubiquitin chain. This polyubiquitin chain then dictates how fast the protein will be degraded. (Nandi et al., 2006).

The 26S proteasome is able to recognize poly-ubiquitinated proteins. Once recognized, ubiquitins are cleaved from the target protein to be reused. The target protein is denatured and enters into the proteasome on one end. Inside, proteolytic cleavage happens and the protein pieces exit the other end where they can be further degraded into individual amino acids the cell can reuse (Al-Sandy et al., 2006) (Nandi et al., 2006) (Smalle & Vierstra, 2004).

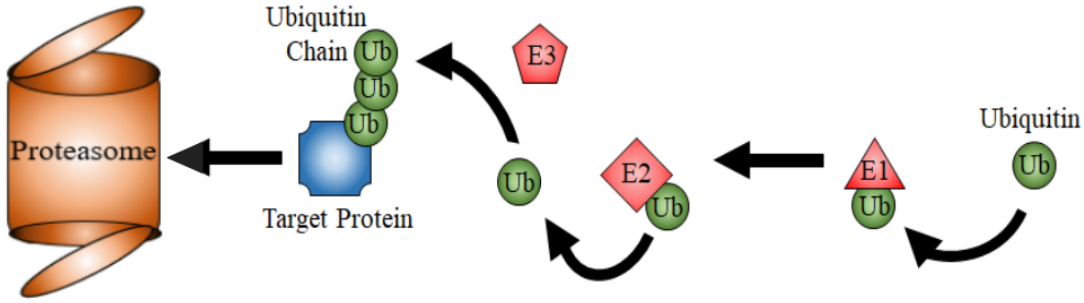

Figure 3: Ubiquitin Proteasome System

The ubiquitin proteasome system (UPS) involves attaching ubiquitin (Ub) onto proteins that are targeted for degradation through the proteasome. Ubiquitin is passed from E1 to E2 and finally to E3 enzymes. The E3 enzyme is able to transfer the ubiquitin to the targeted protein. This process can occur several times before full degradation occurs, resulting in a polyubiquitin chain. The proteasome can recognize this chain and degrade the targeted protein. The ubiquitins are then recycled.

LRB Complex

The E3 ligase that has been shown to degrade PhyB is the Light Response BTB (LRB) E3 complex (Fig. 4). There are three *LRB* genes in Arabidopsis (*LRB1* and *LRB2* and *LRB3*), however, *LRB3* is most likely a pseudogene and does not play a role in red light regulation (Christians et al, 2012). The LRB proteins signal for ubiquitin addition by binding directly to

PhyB and transferring ubiquitin onto it by working in complex with other proteins including Cullin3 (CUL3), the RING-box protein (RBX), and the E2 conjugated to Ubiquitin. In *lrb1-1 lrb2-1* Arabidopsis mutants, degradation of PhyB does not occur and the buildup of phytochrome in the nucleus can result in hypersensitivity to the red light (Shi, 2016). This red light hypersensitivity can result in restricted stem elongation, shorter hypocotyls, increased chlorophyll production, circadian rhythm, and flowering time disturbances (Christians et al, 2012).

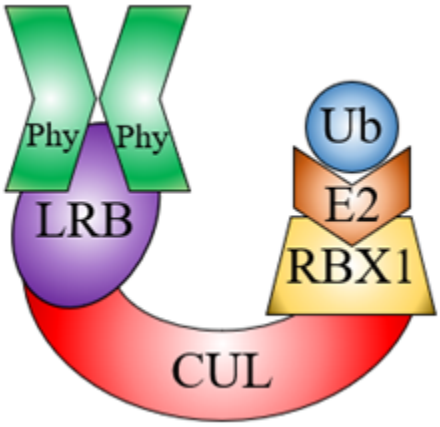

Figure 4: Light Response BTB Complex

The Light Response BTB complex acts to ubiquitinate PhyB. The LRB, CUL3 and RBX1 position the Phy protein (dimer) for ubiquitination by bringing Phy in close proximity to the Ub and catalyzing the transfer of ubiquitin to it.

The light response BTB (LRB) protein consists of several domains, which have been conserved in many plant species. Researchers have been able to uncover the function of the majority of these conserved regions. The conserved Phy/PIF region on the LRB protein interacts with both PhyB and PIFs. This allows both PhyB and the PIFs to be ubiquitinated at the same time (Pham, 2012) (Fig. 5). It is the association of PhyB with the PIFs that leads to their mutual ubiquitination and subsequent degradation. The BACK and BTB regions of the LRB protein interact with CUL3, which acts as a scaffolding protein to bring in various components together

to facilitate ubiquitination of the target protein (Orosa et al., 2017). In red light conditions, the BTB domains also facilitate dimerization of the LRB proteins (Qu et al., 2010). On the other end of CUL3, RBX is bound and can recruit the Ub-E2 complex (Ahmed et al., 1998) (Hartmann et al., 2011). When LRB and the CUL3-RBX ligase complex interact with the LRB, correct positioning is achieved for efficient transfer of ubiquitin to PhyB and PIFs is possible (Hartmann et al., 2011) (Fig. 4).

Figure 5: LRB Protein

From N-Terminal End: Region 1, 2, and 3 have unknown functions. BTB and BACK regions together allow for interactions with CUL3 and E3 ligase function. The Phy/PIF region allows for PhyB to interact with LRB. The focus of this study is the function of Region 2 (in red). Note: The regions are not drawn to scale.

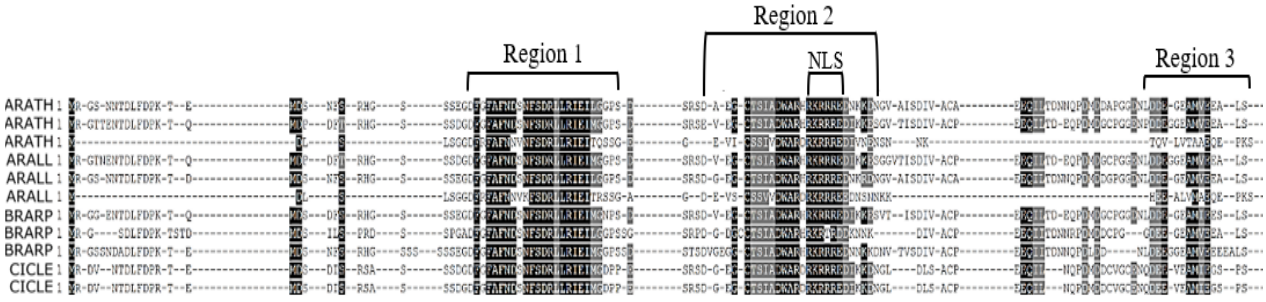

Figure 6: Highly Conserved Regions of LRB2

Amino acid alignment of region 1, 2, and 3 in the LRB region of various plant species (i.e ARATH: A. thaliana, ARALL: A. Lyrata, BRAPB: B. dystochyon, CICLE: C. clementina). Gray areas represent more than 50% regions conserved and black areas represent more than 90% regions conserved (Christians et al., 2012).

Although many of the conserved domain (Phy/Pif, BTB, and BACK) functions are known, there are still some regions whose functions are not known. Region 1, 2, and 3 of LRBs

are highly conserved amongst several plant species, however, their function is still partly unknown. Region 1 was recently investigated and seems to be necessary for red light responses (unpublished, Christians Lab). However, the function of region 2 still has not been investigated to date. Region 2 contains the NLS which allows for LRB proteins to be transported into the nucleus where it can ubiquitinate the Phy proteins. The canonical NLS found in the LRBs is RKRRE (Salanoubut et al., 2000). However, the NLS makes up only part of Region 2. Flanking the NLS, there are portions of Region 2 that are also highly conserved that have unknown significance to the function of LRB. Previously, a BLAST search was performed on Region 2 that uncovered some similarity of Region 2 to the RBX protein, which is part of the E3 ligase complex that binds directly to CUL (Fig. 7).

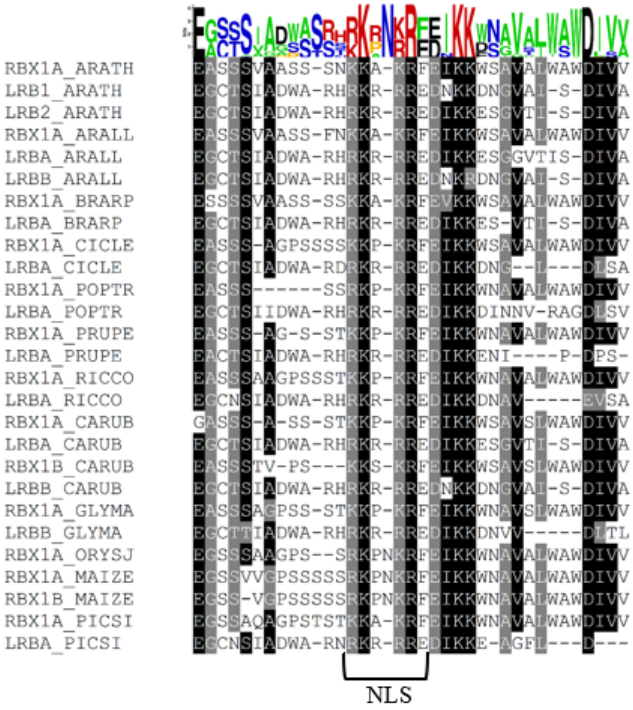

Figure 7: Similarities Between Regions of RBX1 and Region 2 of the LRBs

Amino acid alignment of RBX1 and LRB Region 2 amongst various plant species (ARATH: A. thaliana, ARALL: A. Lyrata, BRAPB: B. dystochyon, CICLE: C. clementina, POPTR: P. tremula, PRUPE: O. nobilis, RICCO: R. communis, CARUB: C. rubella, GLYMA: G. hispida). Gray areas represent more than 50% regions conserved

and black areas represent more than 90% regions conserved (Unpublished, Christians lab).

As the function of the unknown portions of Region 2 flanking the NLS are not currently being investigated, the overall goal of this project was to determine the role of these portions of Region 2 in the LRB protein in regards to red light signaling. To do this, LRB proteins with parts of Region 2 deleted (LRB-R2D), were designed. A full deletion of Region 2 would most likely inhibit nuclear localization of the LRBs, and render them unable to ubiquitinate PhyB in the nucleus. To prevent this, a more targeted approach was taken regarding the flanking regions of Region 2. An *A. thaliana* plant was constructed to delete the regions flanking the NLS, but not the NLS itself. This allowed for the functional significance of the highly conserved Region 2 to be determined through its absence. The LRB-R2D gene was transformed into *A. thaliana* plants lacking functional LRB proteins (*lrb1-1 lrb2-1* double mutants), to determine the functional complementation abilities of the transgene. If LRB-R2D was fully functional and is able to properly respond to red light, the transgenic plants containing LRB-R2D would rescue the phenotype of the *lrb1-1 lrb2-1* double mutants. This would indicate that the function of Region 2 does not involve red light signaling. If LRB-R2D containing plants were unable to rescue the *lrb1-1 lrb2-1* phenotype, it may be that the LRB-R2D protein can not degrade phytochromes which would result in the accumulation of phytochromes in the nucleus, resulting in the *lrb1-1 lrb2-1* background phenotype to show. This would indicate that the flanking regions in Region 2 are involved with PhyB degradation when exposed to red light.

CHAPTER 3: MATERIALS AND METHODS

Purification of the pENTR LRB1 Coding Sequence

E. coli containing the pENTR LRB1 coding sequence (LRB1/cds) were grown on LB/Kan_{50} plates overnight in 37℃. Single colonies were chosen and grown in LB/Kan_{50} broth overnight in 37℃ with agitation. DNA from these overnight cultures was then extracted using the Promega Wizard DNA Extraction Kit. Briefly, a pellet was formed from the overnight cultures and resuspended using the resuspension buffer provided in the kit. The lysis solution was then used followed by the alkaline protease solution and allowed to incubate for 5 minutes. This was neutralized using the neutralization solution and centrifuged. The clear lysate was decanted into the provided spin column and centrifuged. The spin column was then washed twice using the provided washing solution. The DNA was then eluted using nuclease free water and the purified pENTR LRB1/cds DNA was used for further experimentation.

Designing/cloning the LRB-R2D Construct via Site Directed Mutagenesis

In order to investigate if the flanking regions of the NLS found in Region 2 play a role in red light responses, LRB proteins lacking Region 2 (LRB-R2D) were designed and placed back into the *lrb1-1 lrb2-1* mutant.

Site Directed Mutagenesis reaction: The LRB-R2D DNA construct was created by deleting the regions flanking the NLS while preserving the nuclear localization sequence (NLS). The purified pENTR LRB1/cds DNA was used as the template for the site-directed mutagenesis (SDM). The SMD1 primer pair was designed using NEBaseChanger and was used along with the Q5 Site Directed Mutagenesis kit (New England Biolabs) to delete portions of Region 2 that were present downstream of the NLS. Following this, a second site directed mutagenesis was performed to remove the remaining flanking region using the SDM2 primer pair that was also

designed using NEBaseChanger. For each, a site directed mutagenesis reaction was prepared using 1X Q5 Hot Start High Fidelity Master Mix supplied in the NEB Q5 Site Directed Mutagenesis kit, 50 μM of the forward and reverse primers, 25 ng/μL pENTR LRB/cds DNA, and nuclease free water. This site directed mutagenesis reaction was amplified at the following conditions: initial denaturation at 98°C for 30 seconds, 25 cycles of: denaturation at 98℃ for 10 seconds, melting temperature at 60℃ for 30 seconds (for both SDM1 and SDM2 primers), renaturation at 72℃ for 5.5 minutes (30 seconds/kb), and final extension at 72℃ for 2 minutes. The resulting construct lacking the flanking regions of the NLS in Region 2 is referred to as LRB-R2D.

KDL Treatment: Following amplification, a kinase, ligase, and Dpn1 (KLD) treatment occurred on the SDM reaction. The KDL treatment allowed for phosphorylation, intramolecular ligation/circularization, and template removal to occur. The KDL treatment was completed as followed according to the manufacture protocol. The reactions consisted of SDM reaction, KDL buffer, and KDL Enzyme Mix in proportions according to the manufacturer's protocol. This mixture was incubated at room temperature for 5 minutes prior to being transformed into *E. coli*.

E.coli Transformation: 50 μL of NEB 5-alpha Competent E. coli cells was thawed on ice. After thawing, 5 μL of the prepared KDL mix was added and the cells were carefully agitated to mix. The cells were incubated on ice for 30 minutes and then heat shocked at 42℃ for 30 seconds. The cells were returned to ice for 5 minutes. 950 μL of SOC (provided in NEB Q5 Site Directed Mutagenesis kit) was added to the cells. The cells were incubated at 37℃ for 1 hour with agitation. Cells were plated onto an LB/Kan_{50} selection plate and incubated overnight at 37℃. The KLD treatment and transformation into NEB 5-alpha Competent *E. coli* cells were performed after each site directed mutagenesis.

Confirmation of pENTR LRB/cds-SDM1/2

The confirmation of pENTR LRB/cds-SDM1/2 was completed via colony PCR using the uniquely designed SDM1/2 Genotype primers (see Primer Information table, Table 1). These primers were designed to amplify the entirety of Region 2 including the NLS. Once amplified, the PCR products were run on a 1% agarose gel. The SDM1/2 sequence was ~1200 base pairs. Once the SDM1/2 sequence was identified, the plasmid was sequenced using M13 forward and reverse primers. The sequencing reports were then analyzed using EMBOSS Needle pairwise comparison program (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) with manual base confirmation to ensure the site directed mutagenesis was complete and no other mutations were present in the entire gene.

Insertion of LRB/cds-SDM1/2 into pMDC99

LR Reaction: LR reaction was performed to transfer the LRB/cds SDM1/2 from the pENTR cloning vector, into the pMDC99 plant expression destination vector. The Invitrogen LR Clonase II enzyme mix was used and the protocol provided from the manufacturer was followed. Briefly, the pMDC99 destination vector was added to the LRB/cds-SDM1/2 pENTR entry vector, then TE buffer (pH 8.0) and LR Clonase II enzyme mix was added to the reaction and was mixed well, according to the manufacturer's protocol. The reaction was incubated for 1 hour at 25°C prior to adding the provided Proteinase K solution to terminate the reaction. The sample was then incubated at 37°C for 10 minutes.

Transformation: The LR reaction was transformed into competent *E. coli* cells using the heat shock method that occurred by adding 1 µL of the LR reaction into 20 µL of *E. coli* cells. This was allowed to incubate on ice for 30 minutes prior to being heat shocked at 42°C for 30 seconds. The cells were then transferred back to ice for 2 minutes. 100 µL of S.O.C. media was

added to the cells and they were incubated for 1 hour at 37°C with agitation. They were then plated on LB/Kan_{50} and grown overnight.

The successful LR reaction and transformation was confirmed through PCR. The DNA was extracted from the *E. coli* using the Promega Wizard DNA Extraction Kit. The pMDC99 primers (see Primer Information table) were used and amplification occurred for 25 cycles under the following conditions: initial denaturation at 98℃ for 30 seconds, denaturation at 98℃ for 10 seconds, melting temperature at 52℃ for 30 seconds, renaturation at 72℃ for 2 minutes, and final extension at 72℃ for 2 minutes. The PCR product was run out on a 1% agarose gel and a band of 1250 bp, as well as sequencing, confirmed the presence of LRB/cds-SDM1/2 in pMDC99 (data not shown).

Transformation of LRB-R2D into *Agrobacterium tumefaciens*

pMDC99 containing the LRB/cds-SDM1/2 (referred to as LRB-R2D following LR reaction) was transformed into *A. tumefaciens* using the heat shock transformation method. Briefly, a 50 µL sample of competent *A. tumefaciens* cells were thawed on ice prior to adding 5 µL of 100 ng/µL LR pMDC99 DNA. This was thoroughly mixed and flash frozen via liquid nitrogen for 30 seconds. The *A. tumefaciens* was immediately heat shocked at 37℃ for 5 minutes. After, 500 µL of SOC media was added and the *A. tumefaciens* culture was incubated at 28℃ for 2-4 hours with agitation. This culture of *A. tumefaciens* was plated on LB+Kan (100 μg/mL) plates for selection and incubated at 28℃ for 48 hours.

LRB-R2D *Agrobacterium tumefaciens* Transformation into *Arabidopsis thaliana*

lrb1-1 lrb2-1 plants were transformed according to the Simplified Arabidopsis Transformation Protocol (Clough & Bent). Briefly, flowering *A. thaliana* plants around 2 months old were cut 4-6 days prior to dipping the plants to allow new inflorescences to rejuvenate. *A.*

tumefaciens containing the LRB-R2D pMDC99 construct was grown in 3 mL LB broth with 0.1 mg/mL Kanamycin for 48 hours. The culture was transferred to 500 mL LB broth with 0.1% Kanamycin and grown until OD600 reached 0.8. The 500 ml culture was centrifuged at 5,000 x g for 10 minutes and the supernatant was discarded. The pellet was resuspended in fresh dipping solution (10 mM $MgCl_2$, 5% sucrose, 0.005% Silwet). The flowers were then dipped in this solution for 2-3 seconds with gentle agitation. A lid was placed over the dipped plants to maintain humidity for 24 hours and plants were stored in the growth chamber and bottom watered. Plants were dipped twice within one week of each other for optimum transformation.

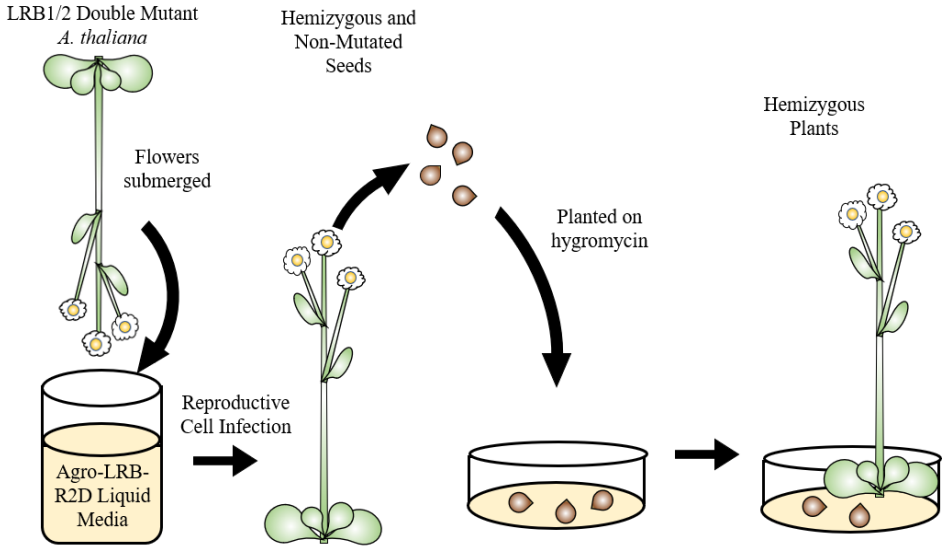

Figure 8: Simplified Arabidopsis Transformation Protocol Mechanism

Transformation of LRB-R2D from *A. tumefaciens* into *lrb1-1 lrb2-1 A. thaliana* plants occurred using the Simplified Arabidopsis Transformation Protocol (Clough & Bent). This consisted of preparing a liquid media containing the *A. tumefaciens* with LRB-R2D and using this media to coat the influoresences of *A. thaliana*. This will initiate the uptake of *A. tumefaciens* into *A. thaliana* via reproductive cell infection. The seeds produced from the infected *A. thaliana* plants will be either successfully hemizygous for LRB-R2D or will be non-mutated. Transformed seeds contain hygromycin resistance.

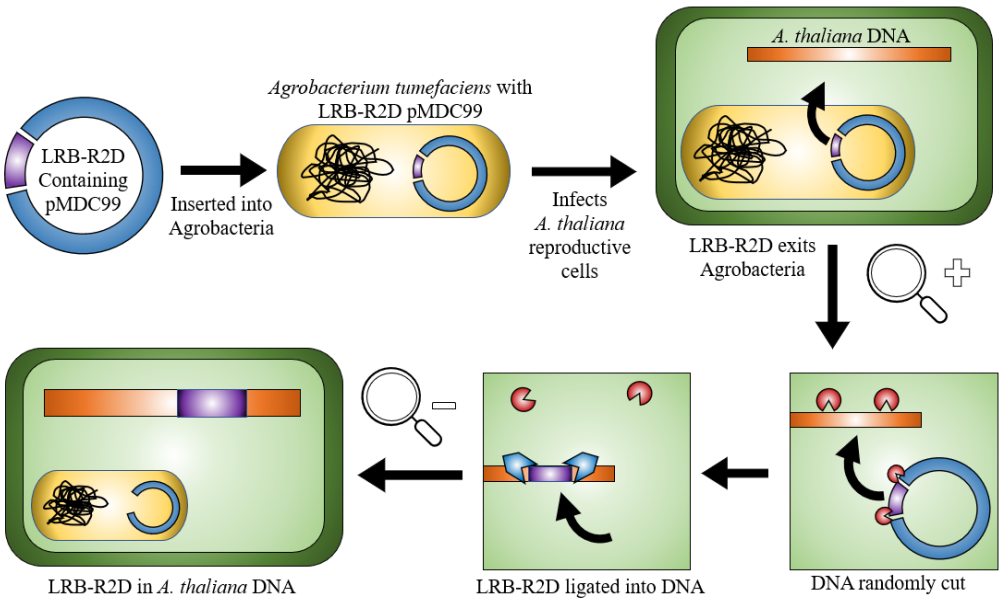

Figure 9: Uptake of *A. tumefaciens* into *A. thaliana* for LRB-R2D Transformation

Pieces of the TI plasmid, pMDC99, are transferred from *A. tumefaciens* to the host plant, *A. thaliana*. These pieces are integrated into the host genome randomly. This allows for the successful transformation of LRB-R2D into *A. thaliana*.

General Seed Sterilization and Plant Growth Conditions

A. thaliana seeds were surface sterilized in a laminar flow hood to prevent contamination. This occurred by washing ~100µl of seeds using 200 µL of 70% ethanol and 400 µL of sterile distilled water. The microfuge tube was inverted to mix. The tube was centrifuged using a tabletop centrifuge for 15 seconds and the supernatant was discarded. The seeds were washed with 70% ethanol and sterile distilled water a total of three times. The seeds were then washed with 200 µL of 50% bleach and the tube was gently inverted to mix. The seeds were incubated at room temperature for 5 minutes with inversion of the tube after every minute. After incubation, 400 µL of sterile distilled water was added to the seeds and the tube was centrifuged using a tabletop centrifuge for 15 seconds. The supernatant was discarded. The seeds were washed with sterile distilled water a total of four times to ensure removal of bleach.

Seeds were plated under a laminar flow hood immediately following surface sterilization. The seeds were plated on Murashige and Skoog plates containing 20% sucrose (MS+Suc). The plate(s) were sealed using porous medical tape and were placed in a growth chamber set at 21℃ with continuous white light for 1-2 weeks.

Seedlings were transplanted into soil after they had shown cotyledon growth and a second pair of true leaves emerging. They were then placed in the growth chamber with distilled water added to fill roughly 1" of the flat, and a humidity dome was placed over the flat. After 3-5 days, the humidity dome was removed. When plants were adapting to soil for 1-2 weeks, the water level in the flat was monitored and maintained often. When plants were mature, they were able to dry out for 2-3 days between waterings.

Selection of Homozygous Dominant LRB-R2D Plants

Homozygous dominant LRB-R2D plants were desired as these plants would have the strongest expression of the LRB-R2D transgene. Transgenic *A. thaliana* plants were identified by growth on hygromycin. Briefly, seeds from Agrobacterium dipped plants (T1 seeds) were sown on MS+Suc+Hygromycin$_{50}$ plates. Plants showing resistance to hygromycin (Hemizygous T1 plants) were then transferred to soil and allowed to self-fertilize. Seeds were collected from individual lines. Progeny from T1 plants (T2 seeds) were then selected for hygromycin resistance and planted into soil. These T2 plants were either hemizygous, or homozygous for the transgene. T2 plants were allowed to self-fertilize. Finally, around 40 seeds from individual T2 plants (T3 seeds) were assessed for hygromycin resistance and separately exposed to red light. Lines were selected that showed 100% hygromycin resistance in the population, and also had significantly longer hypocotyl growth than the control *lrb1/2* plants. Homozygous dominant lines were used to carry out the remainder of the project.

Genotyping Transgenic Plants: The homozygous dominant lines were genotyped via PCR to confirm the presence of the transgene and to ensure that their genetic background contained the *lrb1-1 lrb2-1* mutations. To confirm the presence of the transgene, DNA from each suspected line was amplified using SDM1/2 Genotyping 2 primers with the following conditions: initial denaturation at 95℃ for 30 seconds, then 25 cycles of 95℃ for 30 seconds, 53℃ for 30 seconds, and 72℃ for 1 minute, and final extension at 72℃ for 10 minutes. These PCR products were run on a 1% agarose gel electrophoresis. The presence of the transgene was confirmed by a band present at 350 bp. To confirm these lines contained the *lrb1-1 and lrb2-1* mutations in the genomic DNA, DNA from each transgene line was amplified using *lrb1-1* and *lrb2-1* forward, reverse and Lba1 primers (see Supplemental Table 1). For the *lrb1-1* mutation: initial denaturation at 95℃ for 5 minutes, then 35 cycles at 95℃ for 30 seconds, 53.5℃ for 30 seconds, 72℃ for 1 minute 30 seconds, and final extension at 72℃ for 10 minutes. For the *lrb2-1* mutation: initial denaturation at 95℃ for 5 minutes, then 35 cycles of 95℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 1 minute, and final extension at 72℃ for 10 minutes. The PCR products were run on a 1% gel electrophoresis to confirm the genetic background contained the *lrb1-1 lrb2-1* mutations..

LRB-R2D Protein Expression Analysis

In order to measure LRB-R2D protein expression, immunoblotting was performed. Seeds from each line, including GFP-LRB1 as the positive control and Col-0 and *lrb1/2* as negative controls, were sterilized and plated on MS+sucrose plates containing a cellophane overlay to allow for easy tissue collection. The cellophane sheets were first incubated in 5mM EDTA for 30 minutes on a shaker to remove any metal impurities and then washed with diH_2O 4 more times to

remove the residual EDTA, then sterilized in the autoclave. The sterilized seeds were plated on the cellophane and were exposed to red light for 4 days.

Tissue was collected after 4 days of growth in red light conditions. It was flash frozen and then ground up in MOPS protein extraction buffer (100mM MOPS pH 7.6, 50mM fresh sodium metabisulfite, 2% SDS, 20% glycerol, 4mM EDTA, 0.068% water, 0.01mM bromophenol blue, and 10mM beta-mercaptoethanol) and then boiled to further break up cellular components and further denature the proteins. The ground tissue samples were centrifuged at max speed for 5 minutes and the supernatant was loaded onto a protein gel for analysis.

Protein samples were run on a 10% resolving polyacrylamide gel (40% H_2O, 0.1M acrylamide, 0.5M Tris pH 8.8, 1mM SDS, 0.5mM fresh ammonium persulfate (APS), and 4mM tetramethylethylenediamine (TEMED)), and a 4% polyacrylamide stacking gel (64.5% H_2O, 36.4mM acrylamide, 0.15M Tris pH 6.8, 0.91mM SDS, 0.45mM APS, and 3.60mM TEMED). The tissue samples were run at 180V at room temperature along with a protein ladder (Chameleon 700 Pre-stained, LI-COR). The proteins were then transferred to a PVDF membrane overnight in TG buffer at 30V in 4℃. Following the transfer, the membrane was stained with ponceau stain to confirm the transfer was successful. The membrane was blocked with 1:1 Odyssey blockchain buffer (LI-COR) and PBS. Primary antibody consisted of 1:1 PBS:Odyssey blocking buffer, 0.1% Tween. Both anti-Green Fluorescent Protein (GFP) Ab290 (Abcam) and PBA1 antibodies (Vierstra Lab, Madison Wisconsin) were used at a 1:2000 dilution. After incubation with primary antibodies, the membrane was washed with 1X PBST 4 times. The secondary antibody consisted of 1:1 PBS:Odyssey blocking buffer, 0.1% Tween, and 0.02% SDS) IRDye 800CW goat anti-rabbit and was used at a 1:2000 dilution. After incubation with secondary antibodies, the membrane was washed with 1X PBST 4 times, and one final wash of

1X PBS. The immunoblotted membrane was then viewed under Oddessey FC to examine protein expression via fluorescence at 700 or 800 nm.

Measuring GFP Localization into the Nucleus

The localization of GFP into the nucleus was measured using confocal microscopy. Seeds of the GFP-LRB1 (positive control), the *lrb1/2* mutant (negative control), LRB-R2D 1, and LRB-R2D 2 were plated on a MS+Suc plate and were placed in the white light growth chamber for 24 hours to initiate germination. After, the plate was wrapped three times in aluminum foil and grown in the dark an additional 5 days.

Following the 5 days of growth in the dark, the seedlings were viewed under a Nikon A1 confocal microscope. Seedlings were placed on a slide and covered by a cover slip with water added to prevent the seedlings from drying out. The slide was placed on the stage of the microscope and the roots/root tips were analyzed under 20X magnification. The light was excited with a 488 nm laser and a pinhole size of 1.9 was set for the objective. Images of each line were taken both under bright field and when excited with a 488 nm laser. GFP-LRB1 was used as a control as this was previously determined to be nuclear localized.

Red Light Experiment

To determine how the transgenic LRB-R2D plants respond to red light, 100 seeds from each homozygous dominant line, Col-0 and the *lrb1-1 lrb2-1* were sterilized as previously described. Sterile seeds were stored in a dark, 4°C environment for a span of three days in 200 µL water. After three days, 50 seeds per line were plated on MS+Suc and placed in white light for 24 hours before being placed in continuous red light (20 uMol/m/sec) (RL) or darkness (DK) for 4 days. After 4 days of growth, the seedlings were photographed using the UCam Plus

application under a Leica MS5 stereomicroscope and the hypocotyls were measured using ImageJ software.

Two-Tailed T-Test for Significance

A two-tailed T-test was performed to determine the significance between hypocotyl measurements of Col-0 and both LRB-R2D lines. The Statistical Package for the Social Sciences (SPSS) was used to conduct the T-tests. The data from the T-tests was used to create two separate graphs on Microsoft Excel to show the mean differences amongst the LRB-R2D lines and Col-0.

CHAPTER 4: RESULTS

The overall purpose of this experiment was to determine the function of Region 2 for LRB1 in *A. thaliana.* This was completed by removing the majority of the flanking regions surrounding Region 2 outside of the nuclear localization sequence to create the LRB-R2D construct and assessing the function of this protein in *A. thaliana.*

Arabidopsis Thaliana

A. thaliana is a part of the *Brassicaceae* family, which also includes cultivated species such as cabbage, mustard, radish, and other crops. Aside from its relationship to common cultivated species, *A. thaliana* has a relatively quick generation time of about six weeks and can successfully grow under laboratory conditions requiring limited amounts of light. This plant is also inexpensive to grow and produces many seeds allowing for a large sample size to easily be produced. *A. thaliana* also has a small genome with significantly less introns and duplication events than several other plant types, which provides a simple model to work with that can be translated into more complicated plant genomes (Saeidfirozeh et al., 2018). Genes responsible for light sensing and responses have also been shown to be highly conserved amongst other agriculturally important plants such as soybeans, corn, rice, etc., which resulted in deciding to use *A. thaliana* as a model plant for these experiments.

Site Directed Mutagenesis and Cloning of LRB-R2D

Site directed mutagenesis was done to remove the Region 2 portions that were flanking the NLS to produce the desired LRB-R2D construct. It was not desired to disturb the 5 core amino acids that make up the NLS (RKRRR, See Fig. 6), as this was thought to lead to inadequate nuclear localization. It was predicted that these core amino acids were key to the LRB1 function to degrade PhyB in the nucleus. Two amino acids were left intact on either side

of the core NLS (R_{71}HRKRRRED$_{79}$) to buffer the NLS from the deletions. To determine how much flanking sequence to delete, the alignment of the LRBs with RBX1 (Fig. 7) was used as a guide since the similarities between these two proteins might play a role in the importance of Region 2's function. 10 amino acids upstream of the NLS (E_{61}GCTSIADWA$_{70}$) and 16 amino acids downstream of the NLS (N_{80}KKDNGVAISDIVACA$_{96}$) were deleted.

Two sequential site directed mutagenesis reactions were performed on the pENTR plasmid containing a full length copy of the LRB1 cDNA. The first site directed mutagenesis was completed to remove the portion of Region 2 that was *upstream* of the NLS, thus creating pENTR SDM1. The second site directed mutagenesis was performed to remove the remaining portion of Region 2 downstream of the NLS and produce pENTR SDM1/2 (Fig. 10).

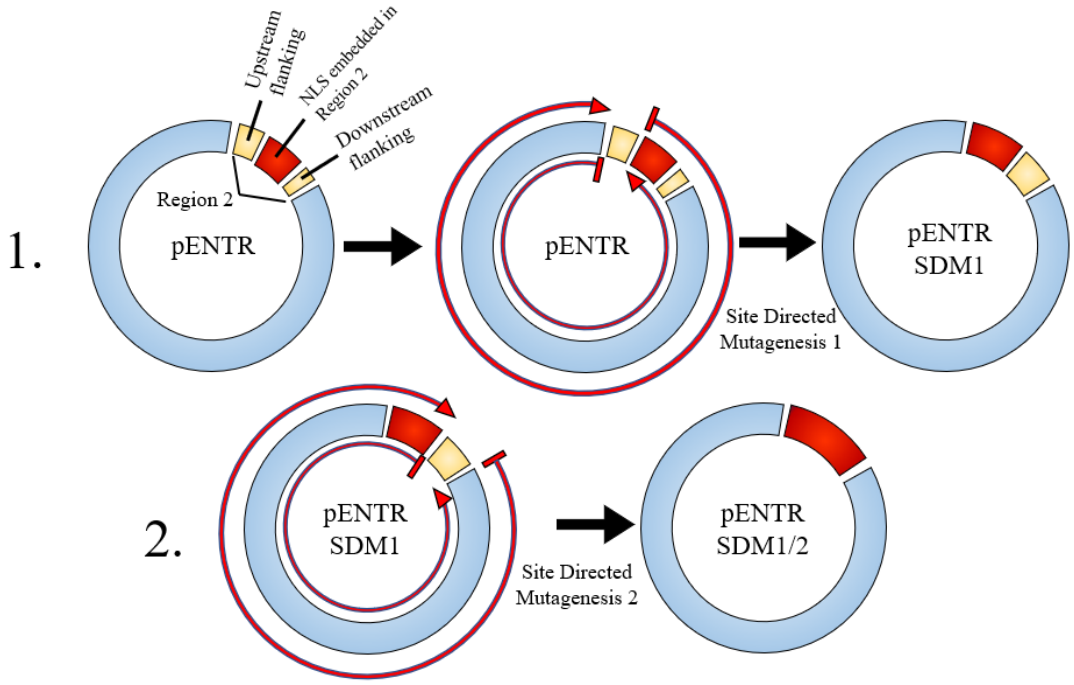

Figure 10: Site Directed Mutagenesis Schema

Site directed mutagenesis was used to successfully remove the portions of Region 2 flanking the NLS within the LRB1 gene. In the first site directed mutagenesis, a mutant of pENTR SDM1 was produced containing the NLS and the remaining Region 2. In the second site directed mutagenesis, the remaining portion of Region 2 was removed producing pENTR SDM1/2.

Successful site directed mutagenesis was confirmed after each reaction, using two mechanisms: colony PCR and sequencing. Colony PCR was performed on the bacteria transformed with the pENTR LRB1-SDM plasmids (SDM1 and SDM1/2). This allowed for detection of the 5' end of the LRB1 gene where Region 2 resides. The gel electrophoresis resulting from the LRB1-SDM1 PCR showed successful banding around 1000 bp, which was the predicted size of the amplified fragment, indicating the bacteria were successfully transformed with the pENTER SDM1 reaction (Fig. 11). With this gel, it was not able to be confirmed that there was a deletion, since the deletion was rather small (34 bp) compared to the overall size of the full length PCR product. However, the deletion of the correct nucleotides was analyzed by sequencing. This confirmed that pENTR SDM1 was successfully mutagenized in colony 2H (Fig. 12, and supplemental Figure 2).

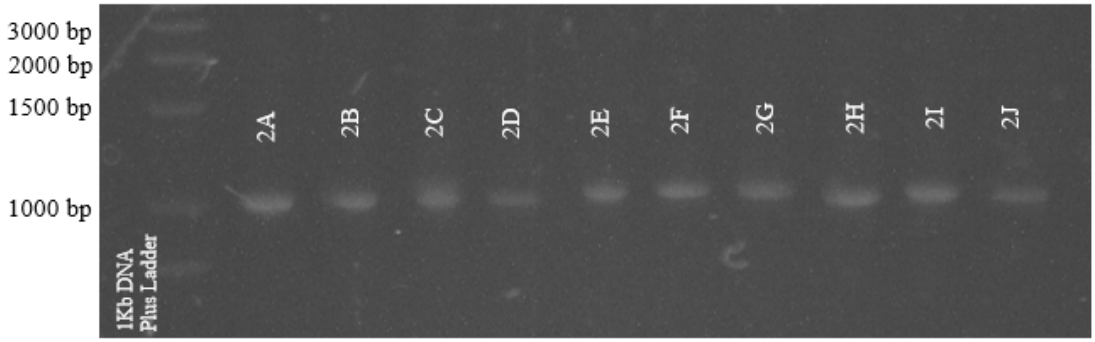

Figure 11: Gel Electrophoresis from Colony PCR of pENTR LRB/cds-SDM1

Gel electrophoresis was performed following the colony PCR to identify the colonies that were transformed with pENTR SDM1. The PCR products were predicted to be around 1000 bp. All colonies had a band at 1000 bp. Colony 2H was chosen for the second site directed mutagenesis reaction.

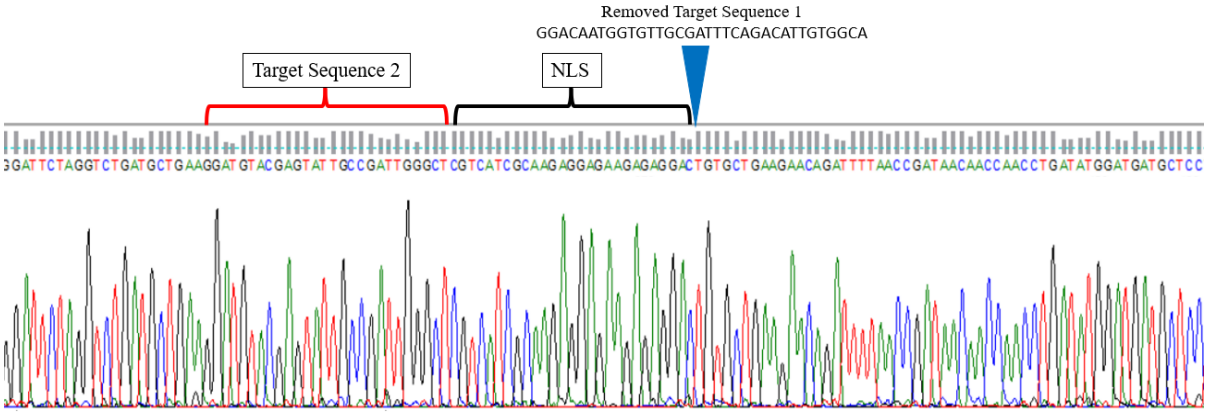

Figure 12: Sequencing Report to Confirm pENTR LRB/cds-SDM1

The successful removal of one portion of Region 2 flanking the NLS downstream was confirmed by sequencing. In the sequencing report analyzed on FinchTV (shown here), the DNA sequence corresponding to the NLS is labeled above. The flanking sequence downstream of Region 2 was shown to be deleted without changing the reading frame of the protein.

Colony 2H was chosen to perform site directed mutagenesis 2 as it was found (through sequencing) to have the SDM1 mutation (Fig. 12). Following the second mutagenesis, the presence of pENTR SDM1/2 was determined by colony PCR and sequencing. The gel electrophoresis resulting from the colony PCR showed successful banding around 1000 bp, which was the predicted size of the amplified fragment. This indicates that the bacteria were successfully transformed with the pENTR SDM1/2 reaction (Fig. 13). As this gel was unable to confirm that the *deletion* was successful, sequencing was performed on colony 2HC (Fig. 14, and supplemental Figure 2). This confirmed that both the upstream and downstream flanking sequences were now deleted. The entirety of the LRB gene was also analyzed after sequencing and it was confirmed that there were no other mutations present in the LRB1 gene aside from the deleted SDM1/2 regions.

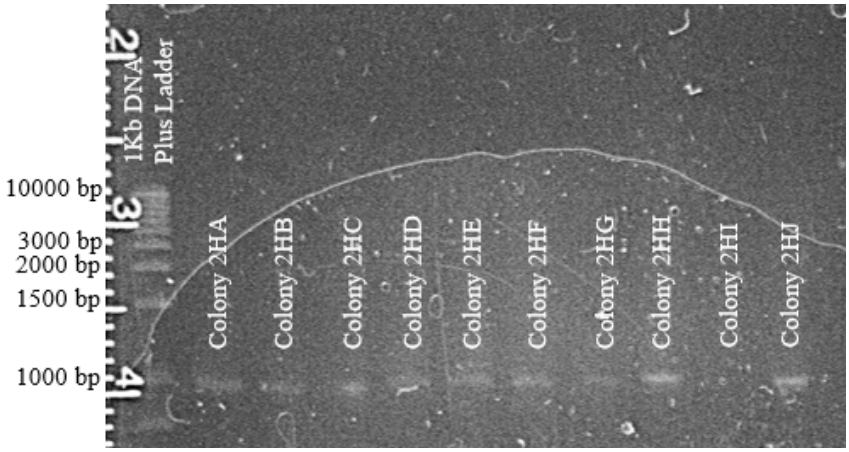

Figure 13: Gel Electrophoresis from Colony PCR of pENTR LRB/cds-SDM1/2

Gel electrophoresis was performed following the colony PCR to identify the colonies that were successfully transformed with pENTR SDM1/2. The PCR products were predicted to be slightly less than 1000 bp. All colonies had a band just below 1000 bp. Colony 2HC was chosen for sequencing.

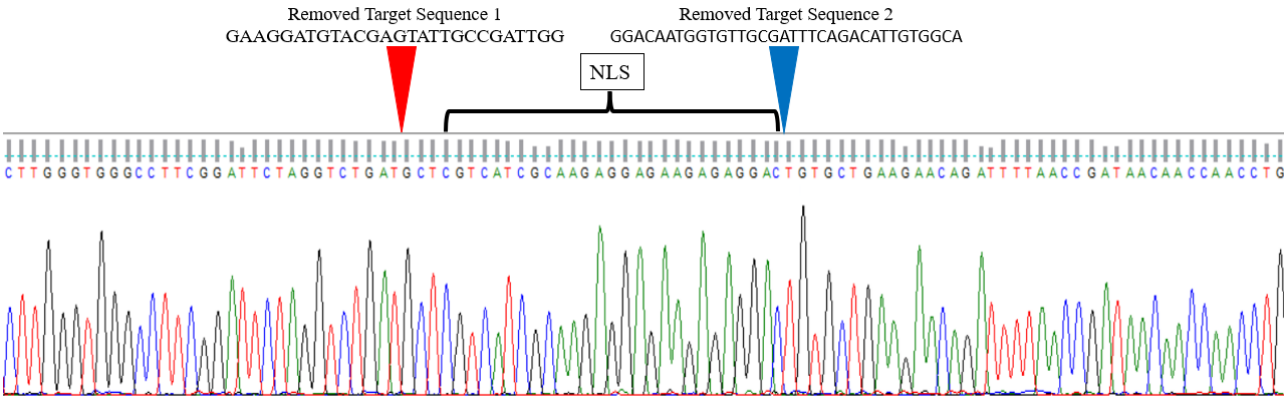

Figure 14: Sequencing Report to Confirm LRB/cds-SDM1/2

The successful removal of the remaining portion of Region 2 flanking the NLS was confirmed by sequencing. In the sequencing report analyzed on FinchTV software V1.4.0 (Geospiza Inc., Seattle, WA) shown here, the DNA sequence corresponding to the NLS is labeled above. The entire flanking sequences upstream and downstream of Region 2 were confirmed to be deleted without changing the reading frame of the protein..

After confirmation that the flanking regions surrounding the NLS were deleted in the LRB1 gene (now called LRB-R2D), an LR reaction was performed in order to insert the

LRB-R2D gene into a modified pMDC99 vector, the destination vector (Fig. 15). This plasmid has been previously modified (Christians et al, 2012) with N-terminal GFP in frame with the insert, and this is expressed by the highly active *Arabidopsis thaliana Ubiquitin 10* promoter (*pUBC10*). Sequencing was performed in the pMDC99 vector to ensure the construct was inserted in frame with the GFP gene.

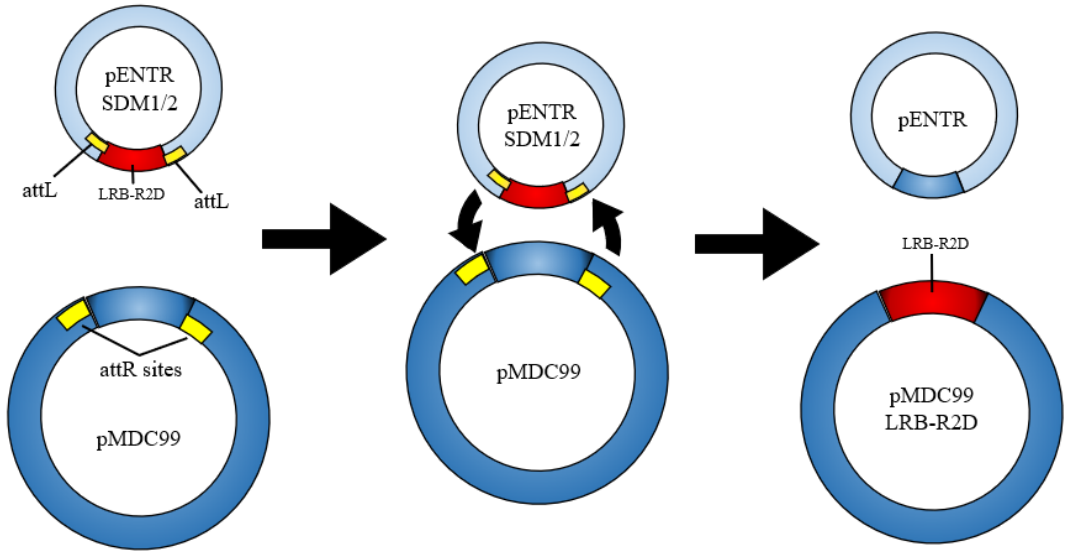

Figure 15: LR Reaction Producing pMDC99 containing LRB-R2D (formerly SDM1/2) from pENTR

The LR reaction was performed to recombine the desired LRB1-SDM1/2 gene (LRB-R2D) out of the pENTR plasmid and insert it into the pMDC99 plasmid. The LR reaction results in the recombination between an entry clone containing a gene of interest flanked by attL sites and a destination vector containing attR sites to generate an expression clone. In this case, pENTR was used as the entry clone containing the LRB1-SDM1/2 gene of interest and pMDC99 was used as the destination vector. The final expression clone created from this LR reaction was pMDC99 containing LRB-R2D.

LRB-R2D pMDC99 plasmid was transformed into *Agrobacterium tumefaciens,* and colonies were selected on Kanamycin. Attempts to perform a colony PCR on the *Agrobacterium* to confirm the correct construct present in the isolated colonies were unsuccessful. Purification of the DNA from the Agrobacteria was attempted via miniprep, but that also failed.

Transformation of LRB-R2D into *A. thaliana* occurred without this confirmation and would later be confirmed via antibiotic selection and genetic testing in the plants themselves.

Selection of Transformed *A. thaliana* Containing LRB-R2D

In order to assess the effects of LRB-R2D mutation on red light responses, homozygous dominant lines bearing the R2D mutation in the *lrb1-1 lrb2-1* genetic background were generated. To do this, hygromycin resistance was used as a marker for transformation, as the transgenic DNA contained the hygromycin resistance gene (Fig. 16). First, seeds collected from the dipped *lrb1-1 lrb2-1* plants (T0) were grown on hygromycin selection media (MS + Suc + $Hygromycin_{50}$) to select for transformed seeds. The transformed seedlings (hygromycin resistant) were considered T1 and were allowed to self pollinate. This produced a population of seeds that were 1:2:1 wild type: hemizygous: homozygous dominant.

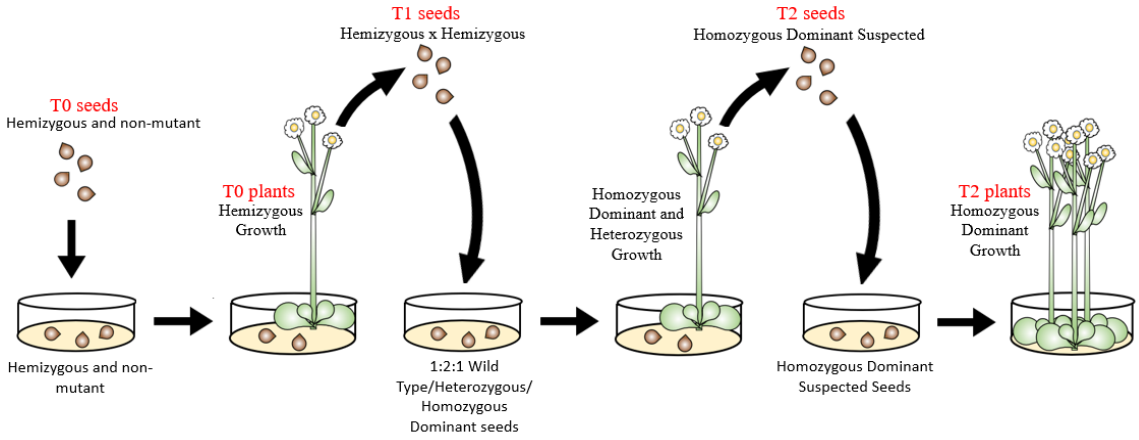

Figure 16: Selection of Homozygous Dominant LRB-R2D *A. thaliana*

The selection of homozygous dominant LRB-R2D *A. thaliana* occurred by selection through a total of four generations. The T0 generation was composed of the *lrb1-1 lrb2-1* plants, the T1 generation was composed of hemizygous plants, and the T2 generation was composed of homozygous dominant and heterozygous plants. The homozygous dominant T2 plants were used for further analysis.

When these seeds germinated, wild type plants (non-transformed plants) withered and died on the media whereas homozygous dominant and hemizygous plants grew (Fig. 17). Seeds from these plants (T2) were collected and grown again on the same selection media. If the T2 plant was hemizygous, then seeds would be 1:2:1 wild type: hemizygous: homozygous dominant. If the T2 plant was homozygous dominant, then 100% homozygous plants would be produced in the next generation. These ratios were detected by selection on hygromycin. The seeds from the homozygous dominant suspected plants were collected and grown once more on selection media to ensure that ~100% of seedlings were hygromycin resistant. These plants were used for various experiments to analyze the function of LRB Region 2.

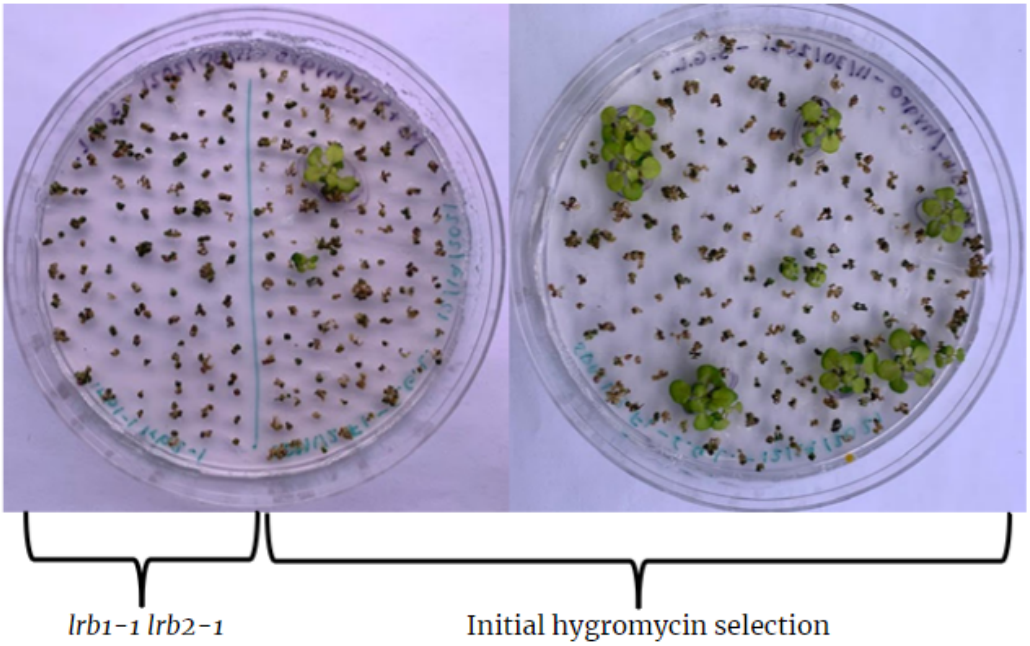

Figure 17: Identification of Dominant LRB-R2D Expressed *A. thaliana*

The identification of hygromycin resistant plants occurred by growing seeds on hygromycin selection plates for two weeks and analyzing the phenotypic differences between the seedlings. Homozygous recessive seedlings were identified as germinating but very little growth when exposed to hygromycin. Hemizygous dominant seedlings were identified as germinating and experiencing significant growth (leaf greening) and first true leaf development compared to the homozygous recessive seedlings.

Five lines from five different transgene insertion events were assessed and homozygous dominant lines were generated from them: T2-3, T2-4, T2-5, T2-6, and T2-8. To confirm the presence of the transgene, genotyped plants were amplified using primers targeting the 5' end of the LRB1 gene (SDM1/2 Genotyping 2 primers). The amplified sequences were visualized with gel electrophoresis (Fig. 18).

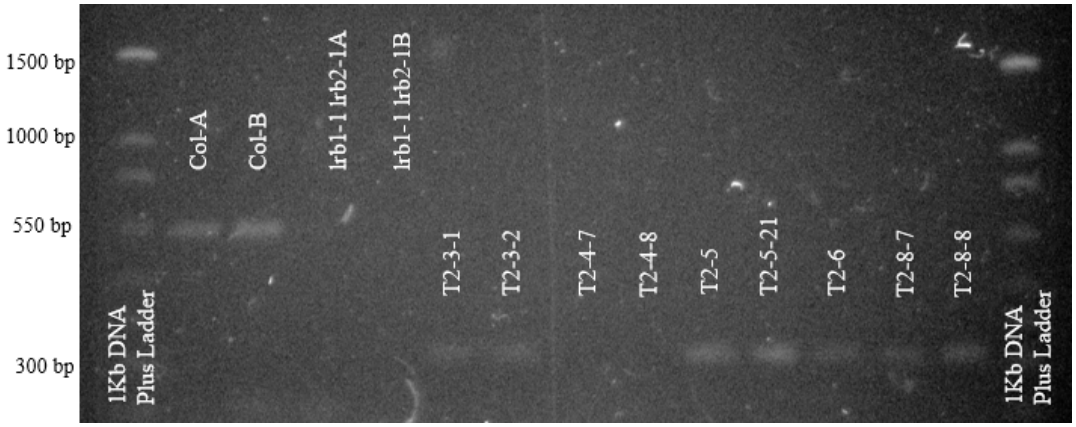

Figure 18: Transgene Presence in Homozygous Dominant Suspected Lines

Gel electrophoresis was performed following the PCR to identify the transgene in the chosen lines that were suspected to be homozygous dominant for LRB-R2D. The positive controls were Col-A and Col-B whereas the negative controls were *lrb1-1 lrb2-1* A and *lrb1-1 lrb2-1* B. Two samples from most lines were analyzed for transgene presence. Col-A and B showed banding around 550 bp as expected. There was no banding present in *lrb1-1 lrb2-1* A or B as expected. In all experimental line samples except line T2-4, there was banding around 350 bp indicating the presence of LRB-R2D.

The primers recognized the 5' end of the LRB1-cds. As expected, there were no bands present in *lrb1-1 lrb2-1* A and B reactions. Genomic DNA was used for amplification and the large *lrb1-1 lrb2-1* T-DNA insertion mutation lies between the primers used for PCR, and thus under the PCR conditions used, a product was not expected to be produced. In the transgenic plants, this sequence is expected to be only 350 bp long. However, Col-0 is larger (550 bp) as there is an intron between the primer pair. The lines T2-3, T2-5, T2-6, and T2-8 were confirmed

to have had successful transformation of LRB-R2D, whereas one of the lines thought to be transgenic initially, did not seem to show the expected transgenic LRB1-R2D DNA. T2-3 (referred to as LRB-R2D Line 1) and T2-6 (referred to as LRB-R2D Line 2) were used for the remainder of the study.

It was important that the transgenic plants contain the genetic background of *lrb1-1 lrb2-1*. These plants have no functional LRB protein in them, so the LRB-R2D transgene could be inserted and the functional complementation capabilities could be analyzed. To confirm the transgenic plants contained the *lrb1-1/lrb2-1* genetic background, they were genotyped via PCR. Four PCR reactions were carried out using different primers to target the wild type and mutations of the gene and a gel electrophoresis analysis was performed (Fig. 19).

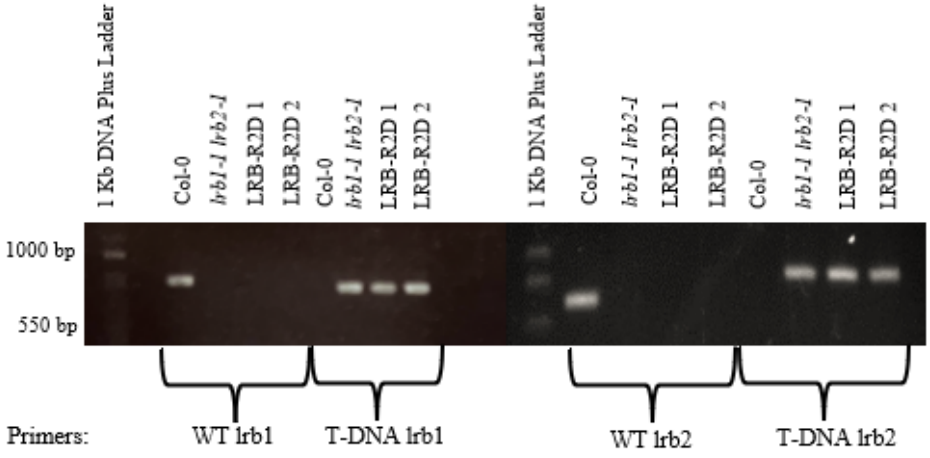

Figure 19: Confirmation of *lrb1-1 lrb2-1* Mutant Background

The presence of *lrb1-1 lrb2-1* mutant background in the LRB-R2D lines was confirmed using the lrb1 and lrb2 primers (WT) with the T-DNA primer (T-DNA). This allowed for the detection of the T-DNA insertions of *lrb1-1* and *lrb2-1* in the LRB-R2D lines. The wild type LRB1 and LRB2 genes were detected using the WT lrb1 and WT lrb2 primer pairs. These were detected only in Col-0.

It was found that the wild type primers produced a product only in the wild type plants but not in the mutants, while the primers used to detect the mutation produced a band that was

found in both the *lrb1-1 lrb2-1* plants, as well as the transgenic plants. This confirmed that the mutant plants contained were homozygous for both the *lrb1-1* and *lrb2-1* mutations.

Analysis of Homozygous LRB-R2D Transformants

Protein Expression Analysis: Expression of LRB-R2D protein was determined by immunoblot analysis. Due to the nature of the constructed pMDC99 plasmid, GFP is covalently bound to LRB1-R2D (GFP-LRB1-R2D). GFP antibodies were used to determine the expression of LRB-R2D as LRB1 antibodies were not available (Fig. 20). Protein from four day old LRB-R2D 1 and LRB-R2D 2 plants (exposed to red light conditions) were separated on a 10% polyacrylamide gel and then transferred onto a PVDF membrane. The expression of GFP-LRB1-R2D and PBA1, a subunit of the proteasome, was determined. PBA1 expression was used as a loading control.

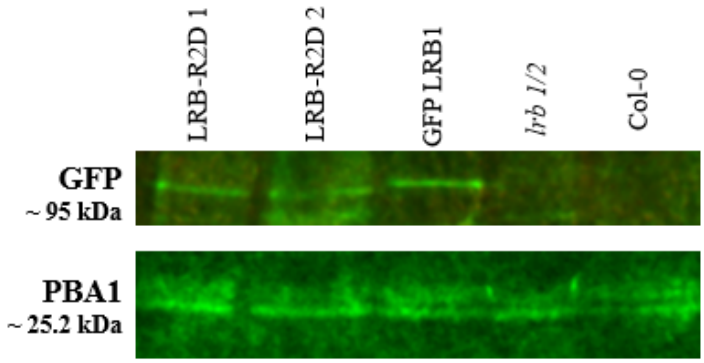

Figure 20: Protein Expression of GFP-LRB1-R2D via Immunoblot
The expression of GFP-LRB1-R2D was analyzed using immunoblotting techniques. Total Protein extracts from four-day-old seedlings grown in red light was assessed. LRB-R2D was measured using anti-GFP primary antibody followed by GAR800 secondary antibody. PBA1 was used as a loading control and was measured using anti-PBA1 primary antibody followed by GAR800 secondary antibody.

When using GFP antibodies, bands are present for GFP-LRB1 as well as in both lines of the GFP-LRB1-R2D present at approximately 95 kDa (30 kDa for GFP + 65 kDa for LRB1). In addition, the expression of both transgenic lines of GFP-LRB1-R2D were comparable to that of

GFP-LRB1. This transgenic line (GFP-LRB1) was constructed previously, and was reported to express GFP attached to the Full-Length LRB1 protein (Christians et al, 2012). GFP-LRB1 is driven by the same promoter as the GFP-LRB1-R2D. The full length GFP-LRB1 protein was shown to fully complement the *lrb1-1 lrb2-1* mutant (Christians et al, 2012). While the mutated GFP-LRB1-R2D protein *was expected* to be expressed at roughly the same level as the GFP LRB1 protein, this experiment confirms this. In addition, the GFP-LRB1-R2D proteins run slightly lower than the GFP-LRB1 (full length LRB1 protein) because of the deletions introduced into the construct. No bands were detected in Col-0 and *lrb1-1 lrb2-1* as expected. Regarding PBA1, the loading control shows relatively equal loading of each sample.

Nuclear Localization of the GFP-LRB1-R2D Protein: Flanking regions close to the five amino acids (RKRRE) determined by Salanoubut et al. as the NLS were deleted, but the NLS itself remained intact. To determine if the NLS was still functional in the GFP-LRB1-R2D protein, the localization of the protein was investigated. The localization of GFP-LRB-R2D into the nucleus was measured via fluorescence of GFP using confocal microscopy. Seeds from GFP-LRB1, *lrb1-1 lrb2-1*, LRB-R2D 1, and LRB-R2D 2 were grown in dark conditions for 4 days prior to GFP localization analysis. The roots from each line were analyzed under the confocal microscope. Photos of both brightfield and GFP fluorescence were taken (Fig. 21).

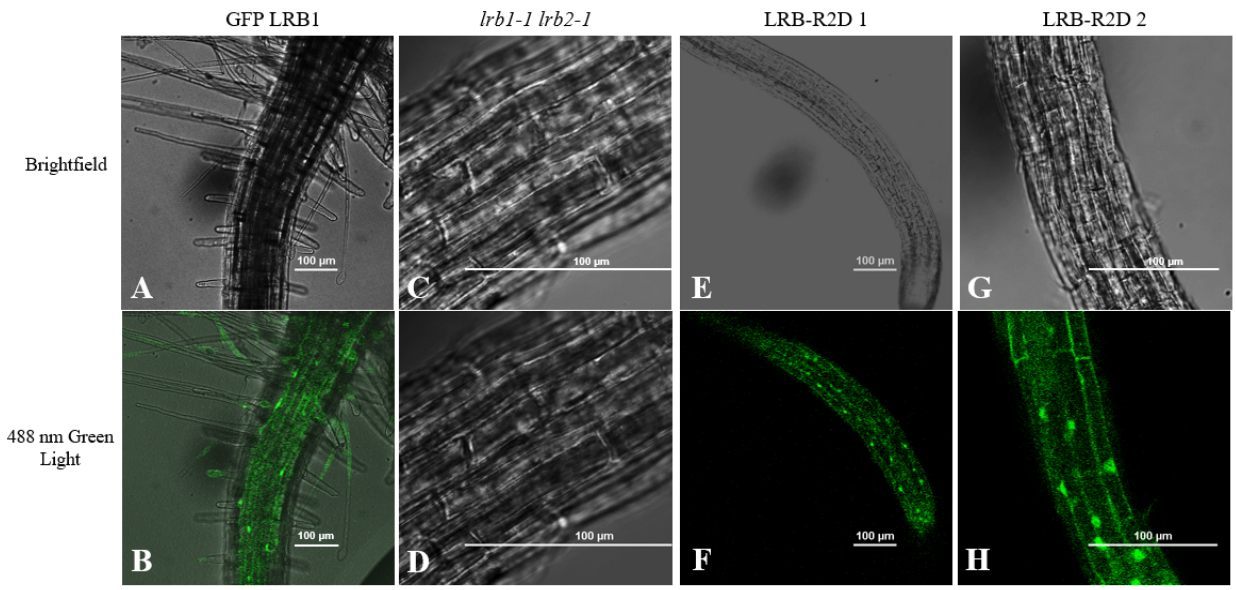

Figure 21: Nuclear Localization of GFP-LRB1-R2D

Localization of GFP-LRB1-R2D was determined by viewing four-day-old seedling roots using a confocal microscope under brightfield and 488 nm laser light. Photos from GFP-LRB1, *lrb1-1 lrb2-1*, LRB-R2D 1, and LRB-R2D 2 were captured at 20X magnification.

As expected, the *lrb1-1 lrb 2-1* roots displayed no fluorescence, which was expected as it does not contain any GFP protein. GFP-LRB1 was found to be localized in discrete structures, which were previously determined to be the nucleus (Christians et. al., 2012). LRB-R2D is present in the same pattern as the GFP-LRB1. Discrete structures of green fluorescence scattered throughout the root tissue are present within the cells. From this, it can be assumed that the LRB-R2D protein is localizing into the nucleus in a similar fashion to GFP-LRB1. This suggests that the NLS is functional, even after deleting the conserved regions directly surrounding it. Overall, the NLS in GFP-LRB1-R2D is still functional which allows it to go into the nucleus where the LRB1 protein is thought to function.

Red Light Analysis: To determine if LRB-R2D can functionally complement the *lrb1-1 lrb2-1* mutant while missing much of the conserved sequence of region 2, *A. thaliana* seeds that were

homozygous for LRB-R2D were grown in either red light (20 uMol/m/sec) or dark conditions. After four days of growth, hypocotyls were measured for both red light and dark exposed seedlings (Fig. 22A-B). Hypocotyl lengths of each seedling were measured using ImageJ and the data was compared amongst the lines (Fig. 22C-D).

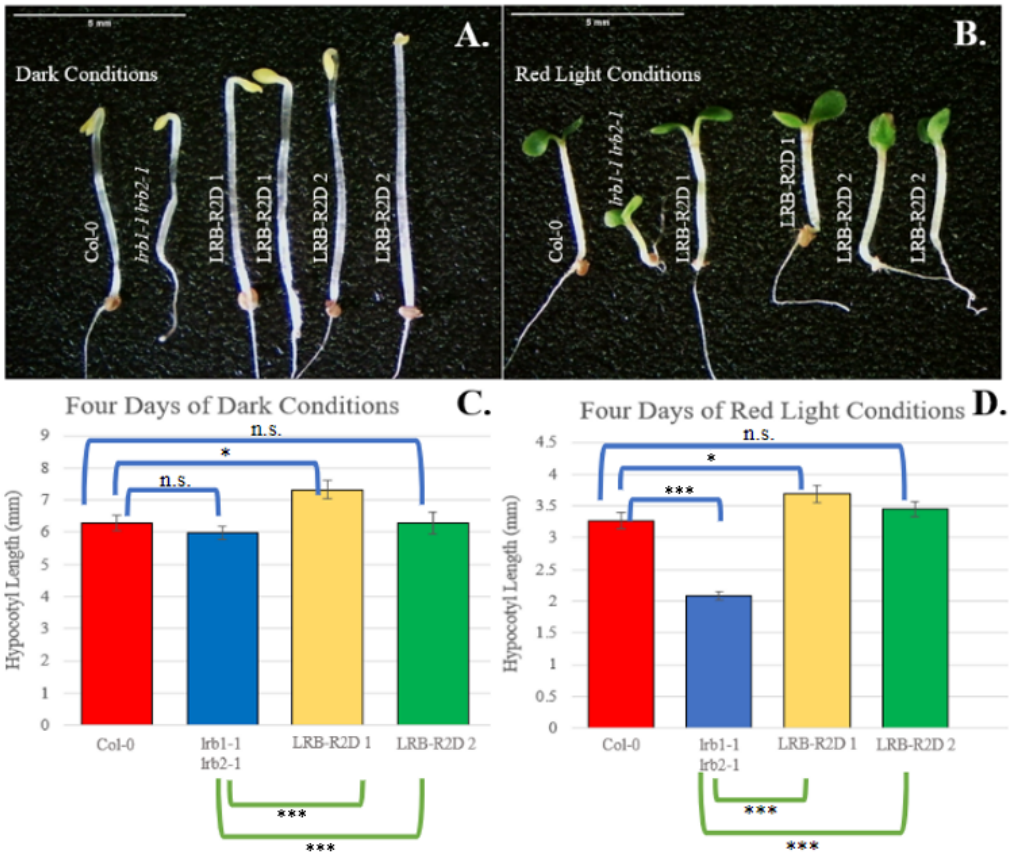

Figure 22: LRB-R2D Hypocotyl Growth and Measurements

Differences in hypocotyl lengths of each line were viewed under a Leica MS5 stereomicroscope and captured using the UCam Plus application. **A.** Seedlings grown for 4 days in dark conditions. **B.** Seedlings grown for 4 days in red light conditions. Hypocotyl lengths were measured and compared amongst all lines. Col-0 was used as the wild type control **C.** Hypocotyl lengths of lines after being exposed to dark conditions for 4 days. **D.** Hypocotyl lengths of lines after being exposed to red light conditions for 4 days. Each bar represents the average height. Error bars represent standard deviation. N=30. A two tailed t-test was performed between Col-0 vs. LRB-R2D 1 and 2 (blue brackets) and between *lrb1-1 lrb2-1* vs. LRB-R2D 1 and 2 (green brackets). No significance is indicated as "n.s.", significance at a p-value of <0.05 is indicated with one asterisk, significance at a p-value of <0.01 is indicated with two asterisks, and significance at a p-value of <0.001 is indicated with three asterisks.

In dark conditions, the hypocotyls all grew quite long, at around 5mm-6 mm tall. All hypocotyls in LRB-R2D lines were shown to be of similar length or longer than the wild type Col-0. After 4 days in red light conditions, all hypocotyls were overall shorter than their dark grown counterparts (between 2 and 4 mm) due to light sensitivity. The *lrb-1 lrb2-1* plants were considerably shorter than the other lines since this mutant has been shown to be hypersensitive to red light (Christians et al, 2012). Interestingly, both LRB-R2D lines had hypocotyls that were longer than the *lrb1-1 lrb2-1* line and were similar in height to the Col-0 plants. This indicated that LRB-R2D 1 and LRB-R2D 2 may have experienced a reversion to wild type in red light conditions after 4 days, which would suggest that the LRB-R2D protein is functional in red light responses. For further analysis, a two tailed T-test was performed on LRB-R2D 1 and LRB-R2D 2 in comparison to Col-0 and *lrb1-1 lrb2-1*. LRB-R2D 1 was similar in height but statistically different when compared to Col-0, whereas LRB-R2D 1 was not similar in height but was statistically different when compared to the *lrb1-1 lrb2-1* double mutant ($p=0.02759$ and 0.00001 respectively). LRB-R2D 2 was not significantly different from Col-0 but was compared to the *lrb1-1 lrb2-1* double mutant ($p=0.3029$ and 0.00001 respectively).

These results convey three important findings. Firstly, based on immunoblot analysis, protein expression of GFP-LRB1-R2D is high. To ensure that the NLS was still functional after removing flanking amino acids surrounding it, confocal microscopy determined that GFP-LRB1-R2D contained a functional NLS. Most importantly, plants containing LRB-R2D were grown and compared to the *lrb1-1 lrb2-1* mutant and it was shown that LRB-R2D can functionally complement the *lrb1-1 lrb2-1* plants.

CHAPTER 5: CONCLUSION

It was hypothesized that Region 2 was important for LRB functioning in red light signaling. To test this, a mutated LRB1 protein lacking portions of Region 2 flanking the NLS was created and transformed into *A. thaliana*. This allowed for the transgenic plants to be exposed to red light conditions and observed for any differences. If these regions are important for LRB function in red light, the plants would not be able to complement the *lrb1-1 lrb2-1* mutant. If Region 2 flanking the NLS is not important for LRB function in red light, the plants would revert back to a Col-0 WT phenotype, indicating the LRB-R2D is a fully functional LRB. From these experiments, there were several important findings. First, the expression of LRB-R2D was investigated using immunoblot techniques. It was determined that several of the experimental lines were able to successfully express LRB-R2D. The expression of the LRB-R2D lines were comparable to the expression of the positive control (Full-Length LRB1). In order to ensure the NLS was still functional after the flanking sequences composed of Region 2 were removed, the localization of LRB-R2D was assessed using the GFP tag that was attached. After viewing the roots from each plant line under a confocal microscope, it was evident that both of the LRB-R2D lines were able to locate into the nuclei. This indicated that the removal of the regions flanking the NLS did not disrupt the function of the NLS. After determining successful LRB-R2D expression and nuclear localization, red light experiments were conducted. After growing seedlings in red light conditions for four days and comparing hypocotyl lengths, LRB-R2D had longer hypocotyls than *lrb1-1 lrb2-1* and similar length hypocotyls to Col-0. Through T-test evaluation, it was determined that both the LRB-R2D lines were significantly different from *lrb1-1 lrb2-1*. This evaluation also determined that LRB-R2D 1 and 2 were both functional and found to be more similar to wild type than the *lrb1-1 lrb2-1* mutant. Interestingly,

the LRB-R2D 2 plants were found to be taller than the wild type which indicates that this reversion worked even better than LRB-R2D 1. This is thought to be influenced by the insertion location of LRB-R2D 2 into the *A. thaliana* genome.

It was hypothesized that the lack of region 2 flanking the NLS would impact the ability to degrade phytochromes when exposed to red light conditions. Although phyB degradation was not directly studied, these plants were shown to respond normally to red light, therefore it can be assumed that PhyB is degraded by LRB-R2D. The experiments showed that the hypothesis was not supported and that the flanking regions around the NLS do not seem to function in red light perception. One line (LRB-R2D 1) did have significantly longer hypocotyls in both red light and dark conditions compared to Col-0. Perhaps the LRB1-R2D transgene is inducing increased cell elongation in these plants. However, since the other transgenic line (LRB-R2D 2) did not show this phenotype, perhaps it is because of positional differences of the transgene itself in the *A. thaliana* genome. Although the flanking regions surrounding the NLS do not seem to be important for red light signaling, there is still much to understand about Region 2. For instance, there is no direct evidence that the LRBs are required to go into the nucleus to interact with the Phys. In this study, it was assumed that the NLS is important for red light responses as it allows for the localization of LRB into the nucleus. It was also assumed that the LRBs interact with phytochrome *only* in the nucleus, and it is in this compartment where the phytochromes are ubiquitinated. However, these assumptions have never been tested empirically, and might be worth further investigation.

It is known that the flanking regions and the NLS region show strong conservation between different plant species and therefore, may have an important function. Interestingly, this region shows some similarity to a portion of RBX1 protein. It is unknown if this aspect of

Region 2 plays a role in the function of the LRB protein. Perhaps it can somehow regulate the E3 ligase complex in some way. However, there may be other unfamiliar functions of the LRBs. During the generation of homozygous transgenic plants, it was observed that these plants wilted and some even perished when exposed to dry soil conditions that typically would not cause harm to WT plants based on previous observations (Unpublished, Christians lab). A formal experiment to test the phenotype of hypersensitivity to drought was never performed, although this may allude to an unknown function of Region 2 and the LRB protein. In the future, LRB-R2D plants can be tested against drought conditions, as well as other conditions, to further determine if Region 2 has a function related to this.

This study focused mainly on determining the function of Region 2 flanking the NLS. However, Regions 1 and 3 are also present on the LRB protein and little is known about these regions. They may play an important role in conjunction with Region 2. When deleted, Region 1 does not rescue the *lrb1-1 lrb2-1* mutant which suggests that it is important for red light signaling (Christians lab). Previous research in the Christians lab found that Region 1 is very similar in sequence to a region of Cul1, a protein similar to Cul3 but forms different types of E3 ligases (Christians lab). It is thought that having two regions, Region 1 and 2, that are similar to other E3 ligase components is a clue to the overall function of the N-terminal region of the LRBs. Region 3 has not been investigated, but it is highly conserved and located between Region 2 and the BTB region. This region is smaller, but still may play an important role in red light signaling. The same experiments that have been performed in this study could be applied to Region 3 to determine its function. Further understanding of Regions 1 and 3 may contribute to the overall understanding of the LRB protein as well as Region 2.

Unique interacting factors that are involved with these regions could be explored and may allow more insight into the overall function of the flanking regions outside of the NLS. Region 2 most likely interacts with nuclear import factors, but more details regarding how the interaction occurs and what exact nuclear import factors it interacts with are unknown. Interacting factors can be identified in all three regions of the LRB protein using mass spectrometry techniques, particularly MALDI (Matrix-Assisted Laser Desorption/Ionization) mass spectrometry. This allows for interacting particles to be identified by comparing these particles to a proteome database (Singhal et al., 2015). By identifying the interacting factors involved in these protein regions, more light can be shed on possible functions these regions may have in regards to the E3 ligase as a whole.

Overall, this study found that Region 2 is not important in red light signaling, although the NLS was not tested directly. This was confirmed by the LRB1-R2D protein functionally complementing the *lrb1-1 lrb2-1* mutant. Although the function of Region 2 flanking the NLS was not determined through this study, there are many questions to further investigate to discover the purpose of this highly conserved portion of the LRB protein and ultimately shed light on the function of Region 2.

APPENDICES

Appendix 1: Primer Sequences Table

Primer Name	Primer Sequence	Pair Name
pMDC99 Forward pMDC99 Reverse	5'-CGCCAAGCTATCAAACAAG-3' 5'-GGAATTATCGAACCACTTTGTAC-3'	pMDC99
AtLRB1 Cterm 1 Forward LRB1 GT 5' Reverse	5'-GGGGACAAGTTTGTACAAAAAAGCAGG-3' 5'GGCCCACCCAAGATCTCGATCCGG 3'	AtLRB1
LRB1 SDM 1.1 Forward LRB1 SDM 1.2 Reverse	5'-AGCATCAGACCTAGAATCC-3' 5'-CGTCATCGCAAGAGGAGA-3'	LRB1 SDM1
LRB1 SDM 2.1 Forward LRB1 SDM 2.2 Reverse	5'-GTCCTCTCTTCTCCTCTT-3' 5'-TGTGCTGAACAGATTTTAAC-3'	LRB1 SDM2
SDM1/2 Genotype Forward SDM1/2 Genotype Reverse	5'-TTCCTCCGAAGGTGATTTTG-3' 5'-CTGAAATCGCAACACCATTG-3'	SDM1/2 Genotype
New SDM1/2 Genotype Forward New SDM1/2 Genotype Reverse	5'-TGACCCTAAGACGGAGATGG-3' 5'-CCCCAGTTTGGCTCACTAGA-3'	New SDM1/2 Genotype
M13 Forward M13 Reverse	5'-GTAAAACGACGGCCAGT-3' 5'-CCAGGAAACAGCTATGACC-3'	M13
lrb1-1 Forward lrb1-1 Reverse	5'-CCGCCCTGTTACCATAACCGT-3' 5'-GCTTTTTGCGGCCAAGATAGG-3'	WT lrb1
lrb2-1 Forward lrb2-1 Reverse	5'-ATGAGAGGTACTACTGAGAAT-3' 5'-CAGTAAGGTACACATGAGAGC-3'	WT lrb2
Lba1	5'-TGGTTCACGTAGTGGGCCATCG-3'	T-DNA

Appendix 2: Site Directed Mutagenesis Sequences

SDM1 Forward Sequence:

NNNNNNTNNGNTCGGGNNNAAATAATGANTTTATTTTGACTGATAGTGACCTGTTCGT
TGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTATAATAAAGTTGAA
CGAGAAACGTAAAATGATATAAATATCAATATATTAAATTAGATTTTGCATAAAAAACA
GACTACATAATACTGTAAAACACAACATATCCAGTCACTATGAATCAACTACTTAGATG
GTATTAGTGACCTGTAGTCGACCGACAGCCTTCCAAATGTTCTTCGGGTGATGCTGCC
AACTTAGTCGACCGACAGCCTTCCAAATGTTCTTCTCAAACGGAATCGTCGTATCCAG
CCTACTCGCTATTGTCCTCAATGCCGTATTAAATCATAAAAAGAAATAAGAAAAAGAG
GTGCGAGCCTCTTTTTTGTGTGACAAAATAAAAACATCTACCTATTCATATACGCTAGT
GTCATAGTCCTGAAAATCATCTGCATCAAGAACAATTTCACAACTCTTATACTTTTCTC
TTACAAGTCGTTCGGCTTCATCTGGATTTTCAGCCTCTATACTTACTAAACGTGATAAA
GTTTCTGTAATTTCTACTGTATCGACCTGCAGACTGGCTGTGTATAAGGGAGCCTGAC
ATTTATATTCCCCAGAACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGGCT
GAGATCAGCCACTTCTTCCCCGATAACGGAGACCGGCACACTGGCCATATCGGTGGT
CATCATGCNCNNNNCTTTCATCCCCGATATGCACCACCGGGTAAAGTTCACGGGAGAC
TTTATCTGACAGCAGACGTGCACTGGCCAGGGGGATCACCATCCGTCGCCCGGGCGT
GTCAATAATATCACTCTGTACATCCACAAACAGACGATAACGGCTCTCTCTTTTTATAG
GTGTAAACCTTAAACTGCATTTTCACCAGCCCCCTTGTTCTCGTCAGCAAAAAGAGCC
NNNCATTTTNNATAAACCNGGGNGANCCTCANNNATCCCTTNCTGATTTTTTNCCGNT

TTTCCANNNNGTTCNGGCACGNAGACNNANGNNNNTCNNTTTNNNNGNNATGNNNN
NGCNTTNNCCNANNNNNNNATNNNNNCATTCANAATANNNNNNNTNNNNNNCCA

SDM1 Reverse Sequence:

NNNNNNNNNNGNNTNGCGGNGCCCTGCAGCTGGATGGCAAATAATGATTTTATTTTG
ACTGATAGTGACCTGTTCGTTGCAACAAATTGATAAGCAATGCTTTCTTATAATGCCAA
CTTTGTACAAGAAAGCTGAACGAGAAACGTAAAATGATATAAATATCAATATATTAAAT
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GAGACGTTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAAATAAGATCACT
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TNTTNNANCGCNNNGNNGAANNNNTGCTGATNNNNCNNNNNGAATTTCNNNNNCAT
CANNNNNNTNNNNTNNNNNNNNNNTCGGNNGNANNNNNNNNNNNNNCCNN

SDM1/2 Forward Sequence:

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CTTCACCATGAGAGGTTCCAATAACACCGATCTATTTGACCCTAAGACGGAGATGGAT
TCCAATTTCTCTCGCCATGGTTCTTCCTCCGAAGGTGATTTTGGTTTCGCTTTCAATGA
CAGTAACTTCTCCGATCGTTTGCTCCGGATCGAGATCTTGGGTGGGCCTTCGGATTCT
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AGCCAAACTGGGGTATTGATTGTTCTACTGTTGTTAGAGTTAAAGAACTGCATATTAGT
TCTCCTATCTTGGCCGCAAAAAGCCCCTTTTTCTACAAGCTGTTTTCTAATGGAATGAG
GGAATCAGAACAAAGACATGTAACCCTTAGAATTAGTGCACAAGAGGAAGGTGCTTT
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NN

SDM1/2 Reverse Sequence:

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AAAGCAGTGGAACGAGCTTTGCTGGTCCATGTTGCAGTGTGCTGAGAGGAAGAAAC
CTTGACCTCCTAAGTGGAAAGCCTGAGAATAGACTCTTCCTGAAGGGAATAGTCCTG
CGCATTCCTCTCGCTTCAAGTCCAGGTACACTACACATTGCGGGCGAGGAAGCTCAA
ACTCCACCACTTTTACGGGTCTGTATTTGTAAGCCCTCTCTATGAAACGGCGGTTCATG
GAGTCCGATCCTTCAGCTGCGAGGATGCGTTGCCTGTGTGGGGCTTCTGCTTTGAAG
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TACCTTTTTTCAGCTTTCGACACGTCATGTATGGGAAGCGGATGTAGAGTGCAAGGCG
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AGACAAAATCATAAACAGCATCCTCAGAAAGCAATTTGGAGATCATCGCTNNATAGT
NTCGNNTNTANNNCAGCCNAATGNNNNNNCNATAANCCTCATCATGAAANTTGGTAA
TATCCCNTNNNANCGNNNGNNNNNNCTGNTTTGCTNGNNATNNGNNNANANNNGCN
CTGNNTTNNANNCNNNNNANNNCTANNNGNNNNNNNNAGNANNNNNNNNNNNNN
NNNNNNNNNTNNANNGNNNNATANTTN

BIBLIOGRAPHY

Ahmad, K. F., Engel, C.K., & Privé, G.G. Crystal structure of the BTB domain from PLZF. Proc. Natl. Acad. Sci. U. S. A. 95, (1998) 12123–12128

Al-Sady, B., Ni, W., Kircher, S., Schäfer, E., & Quail, P.H. (2006). Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. Mol Cell 23: 439–446

Bauer, M., Gingerich, D., & Gingerich, J. (2019, May 01). Creation LRB (Light-Response BTB) /PIF (Phytochrome-Interacting Factor) Mutant Lines in Arabidopsis thaliana. Retrieved from https://minds.wisconsin.edu/handle/1793/79607

Boylan, M.T., & Quail, P.H. (1991). Phytochrome A overexpression inhibits hypocotyl elongation in transgenic Arabidopsis. Proc Natl Acad Sci USA 88: 10806–10810

Casal, J.J. (2007). Phytochromes, Cryptochromes, Phototropin: Photoreceptor Interactions in Plants. Photochemistry and Photobiology, 71(1), 1-11. doi:10.1562/0031-8655(2000)0710001pcppii2.0.co2

Chen, M., Chory, J., & Fankhauser, C. (2004) Light Signal Transduction in Higher Plants. Annu. Rev. Genet. 38, 87–117.

Christians, Matthew J., Gingerich, Derek J., Hua, Z., Lauer, Timothy D., & Vierstra, Richard D. (2012) "The light-response BTB1 and BTB2 proteins assemble nuclear ubiquitin ligases that modify phytochrome B and D signaling in Arabidopsis." Plant Physiology 160.1 : 118-34.

Clough, S., & Bent, A. (n.d.). Simplified Arabidopsis Transformation Protocol. Retrieved from https://plantpath.wisc.edu/simplified-arabidopsis-transformation-protocol/

Columbia University Zuckerman Institute. (2008). Nikon A1R Confocal: Cellular Imaging Guide. Retrieved from https://internal.zi.columbia.edu/sites/default/files/content/Zuckerman%20Institute/Cellular%20Imaging/A1R%20Guide%202017.pdf

CUL3 gene - Genetics Home Reference - NIH. (n.d.). Retrieved from https://ghr.nlm.nih.gov/gene/CUL3

Franklin, K. A., & Quail, P. H. (2009). Phytochrome functions in Arabidopsis development. Journal of Experimental Botany, 61(1), 11-24. doi:10.1093/jxb/erp304

Franklin, K. A., & Whitelam, G. C. (2005). Phytochromes and shade-avoidance responses in plants. Annals of Botany, 96(2), 169–175. https://doi.org/10.1093/aob/mci165

Gao, C., Pallett, M., Croll, T., & Graham, S. (2019). Molecular basis of cullin-3 (Cul3) ubiquitin ligase subversion by vaccinia virus protein A55. Journal of Biological Chemistry, 294(16), 6416-6429. doi:10.1074/jbc.RA118.006561

Gray, W. M., Hellmann, H., Dharmasiri, S., & Estelle, M. (2002). Role of the arabidopsis Ring-h2 Protein Rbx1 in RUB modification and SCF Function. The Plant Cell, 14(9), 2137-2144. doi:10.1105/tpc.003178

Hartmann, T., Pan, Z.-Q. & Sarikas, A. (2011)The cullin protein family. Genome Biol. Online Ed.12, 220.

Hua, Z., & Vierstra, R.D. (2011). The cullin-RING ubiquitin-protein ligases. Annu Rev Plant Biol 62: 299–334

Hwang, H., & Lai, E. (2017). Agrobacterium-Mediated Plant Transformation: Biology and Applications. In 975983646 756833418 M. Yu (Ed.), The Arabidopsis Book. The American Society of Plant Biologists. doi:10.1199/tab.0186

Kutschera, U., & Briggs, W. R. (2013). Seedling development in buckwheat and the discovery of the photomorphogenic shade-avoidance response. Plant Biology, 15(6), 931-940. doi:10.1111/plb.12077

Lazzarin, M., Meisenburg, M., Meijer, D., van Ieperen, W., Marcelis, L. F. M., Kappers, I. F., van der Krol, A. R., van Loon, J. J. A., & Dicke, M. (2021). LEDs make it resilient: Effects on plant growth and defense. Trends in Plant Science, 26(5), 496–508. https://doi.org/10.1016/j.tplants.2020.11.013

Leivar, P., Monte, E., Cohn, M. M. & Quail, P. H. (2012) Phytochrome Signaling in Green Arabidopsis Seedlings: Impact Assessment of a Mutually Negative phyB–PIF Feedback Loop. Mol. Plant 5, 734–749.

Li, J., Li, G., Wang, H., & Wang Deng, X. (2011). Phytochrome signaling mechanisms. The Arabidopsis Book, 9. doi:10.1199/tab.0148

Madeira, F., Pearce, M., Tivey, A. R., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A., & Lopez, R. (2022). Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Research, 50(W1). doi:10.1093/nar/gkac240

Nandi, D., Tahiliani, P., Kumar, A., & Chandu, D. (2006). The ubiquitin-proteasome system. Journal of Biosciences, 31(1), 137-155. doi:10.1007/bf02705243

Orosa, B., He, Q., Mesmar, J., Gilroy, E. M., Mclellan, H., Yang, C., . . . Sadanandom, A. (2017). BTB-BACK Domain Protein POB1 Suppresses Immune Cell Death by Targeting Ubiquitin E3 ligase PUB17 for Degradation. PLOS Genetics, 13(1). doi:10.1371/journal.pgen.1006540

Pham, V., Kathare, P., & Huq, E. (2017). Phytochromes and Phytochrome Interacting Factors. Plant Physiology, 176(2), 1025-1038. doi:10.1104/pp.17.01384

Qu, N., Gan, W., Bi, D., Xia, S., L,i X., & Zhang, Y. (2010). Two BTB proteins function redundantly as negative regulators of defense against pathogens in Arabidopsis. Botany 88: 953–960

Saeidfirozeh, H., Shafiekhani, A., Cifra, M., & Masoudi, A. A. (2018). Endogenous chemiluminescence from germinating *Arabidopsis thaliana* seeds. Scientific Reports, 8(1). doi:10.1038/s41598-018-34485-6

Salanoubat, M., Lemcke, K., Rieger, M., Ansorge, W., Unseld, M., Fartmann, B., Valle, G., & Blöcker, H. (2000). Sequence and analysis of chromosome 3 of the plant *Arabidopsis thaliana*. Nature, 408(6814), 820–823. https://doi.org/10.1038/35048706

Shi, H., Shen, X., Liu, R., Xue, C., Deng, X., & Zhong, S. (2016). The Red Light Receptor Phytochrome B Directly Enhances Substrate-E3 Ligase Interactions to Attenuate Ethylene Responses. Developmental Cell, 39(5), 597-610. doi:10.1016/j.devcel.2016.10.020

Singhal, N., Kumar, M., Kanaujia, P. K., & Virdi, J. S. (2015). MALDI-Tof Mass Spectrometry: An emerging technology for microbial identification and diagnosis. Frontiers in Microbiology, 6. https://doi.org/10.3389/fmicb.2015.00791

Smalle, J., & Vierstra, R.D. (2004). The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol 55: 555–590

Thelen, M. (2018, October 02). Michigan State University Extension: Wind Damage in Your Corn Field. Retrieved from https://www.canr.msu.edu/news/wind_damage_in_your_corn_field

Thelen, M. (2020, May 21). Michigan State University Extension: Evaluating Wind-Damaged Crops. Retrieved from https://www.canr.msu.edu/news/evaluating_wind_damaged_crops

Toettcher, J. E., Gong, D., Lim, W. A., & Weiner, O. D. (2011). Light Control of Plasma Membrane Recruitment Using the Phy–PIF System. Methods in Enzymology Synthetic Biology, Part A, 409-423. doi:10.1016/b978-0-12-385075-1.00017-2

Wiersma, C. & Christians, M., (2015). "Modification of LRB Complex Members in Arabidopsis thaliana" Student Summer Scholars Manuscripts. 157. https://scholarworks.gvsu.edu/sss/157