

Blue light- and ubiquitin-dependent influence on phototropin 1
abundance and movement at the plasma membrane.

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BLUE LIGHT- AND UBIQUITIN-DEPENDENT INFLUENCE ON PHOTOTROPIN 1
ABUNDANCE AND MOVEMENT WITHIN THE PLASMA MEMBRANE.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF FIGURES	ix
1. CHAPTER 1. PHOTOTROPISM.....	1
a. Light Perception in Plants.....	1
b. Phototropism	2
c. Auxin Transport Proteins	6
d. The Tropic Response to Auxin Transport in the Elongation Zone	10
e. The Blue light Receptors Phototropin 1 and Phototropin 2	13
f. The phot1 Interacting protein NPH3	16
g. Other phot1 Interacting Proteins	20
h. The Role of Blue Light Induced Movement of phot1	22
2. CHAPTER 2. CHARACTERIZATION OF THE MOVMENT OF PHOT1-GFP IN RESPONSE TO BLUE LIGHT.....	28
a. Abstract.....	28
b. Introduction	28
c. Experimental procedures	30
d. Results	34
i. phot1-GFP Accumulates Into Foci at the Plasma Membrane in Response to Blue Light	34
ii. The Blue laser Induces phot1-GFP Movement During Imaging.....	38
iii. phot1-GFP Accumulation Into Foci at the Plasma Membrane Precedes Internalization.....	44

iv. Accumulation of phot1-GFP Into Foci at the Plasma Membrane is Fluence Dependent in Both Lines and is Greater in the <i>phot1-5 nph3-6</i> phot1-GFP Line	48
e. Discussion	58
3. CHAPTER 3. MODULATING PHOT1-GFP ABUNDANCE AND MOVEMENT AT THE PLASMA MEMBRANE.....	61
a. Abstract.....	61
b. Introduction	61
c. Experimental procedures	65
d. Results	66
i. MG132 Induces phot1-GFP Accumulation Into Foci in Darkness	66
ii. Phot1-GFP Accumulation Into Foci in Response to Blue Light Irradiation is Greater in <i>phot1-5</i> phot1-GFP Seedlings Pre- treated With MG132	70
iii. MG132 Increases the Accumulation of phot1-GFP Into Foci at a Moderate to High Fluence Rate and a High Fluence Rate of Blue Light in the <i>phot1-5</i> phot1-GFP Line	75
iv. Red Light Pre-treatment Does Not Prevent Internalization or Accumulation Into Foci at the Plasma Membrane of phot1-GFP in Response to Blue Light Irradiation	81
v. Phot1-GFP Accumulation Into Foci is Reduced in Seedlings Pre- treated With Red Light	84
vi. Low Fluence Rate Blue Light Initiates Little to no phot1-GFP Accumulation Into Foci at the Plasma Membrane or Internalization in the <i>phot1-5</i> phot1-GFP Line	97
e. Discussion	100
4. CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS	104
a. Introduction	104

b. phot1-GFP Accumulates Into foci at the Plasma Membrane Prior to Internalization	106
c. Red Light Pre-treatment Reduces phot1-GFP Accumulation Into Foci at the Plasma Membrane in Response to Blue Light.....	108
d. Monoubiquitination of phot1 May Prevent Accumulation Into Foci in Response to Blue Light Irradiation	109
e. Future Directions.....	112
5. LITERATURE CITED	115
6. ADDENDUM. FURTHER CHARACTERIZATION OF PHOT1-GFP INTERNALIZATION AND ENGINEERING EQUIPMENT TO IMPROVE EFFCIANCY OF STANDARD LAB PROTOCOLS	129
a. Abstract.....	129
b. Introduction	129
c. Experimental procedures	133
d. Results	137
i. TYRA23 Pre-treatment Does Not Prevent phot1-GFP Accumulation Into foci at the Plasma Membrane or Internalization in Response to Blue Light Irradiation	137
ii. Phot1-GFP Accumulation Into Foci at the Plasma Membrane in Response to Blue Light Irradiation is Enhanced in TYR A23 Pre-treated Seedlings.....	142
iii. The ICE-CAPS Fountain Will Allow for High Throughput Growth and Collection of Tissue for Genotyping of <i>Arabidopsis</i> Seedlings.....	145
iv. IR Imaging of Seedlings Allows Accurate Imaging of Seedling Position Before and After Blue Light Irradiation	148
v. Discussion	152
vi. Literature cited	157
7. Vita	160

LIST OF FIGURES

CHAPTER 1 PHOTOTROPISM.....	1
1. Figure 1.1 Auxin redistribution in response to blue light in the zone of cell elongation	5
2. Figure 1.2 Basic phototropin structure and function.....	15
3. Figure 1.3 NPH3 interacts with phot1 and facilitates ubiquitination in response to blue light t.....	19
4. Figure 1.4 Working Model of the events that follow blue light activation of phot1 in the presence and absence of NPH3.....	27
CHAPTER 2 CHARACTERIZATION OF THE MOVEMENT OF PHOT1-GFP IN RESPONSE TO BLUE LIGHT	28
1. Figure 2.1 Images of phot1-gfp fluorescence within the cytosol and at the plasma membrane.....	35
2. Figure 2.2 Accumulation of phot1-GFP into foci and corresponding heat map of above average GFP fluorescence.....	36
3. Figure 2.3 Maximum intensity projections of phot1-GFP fluorescence at the plasma membrane with corresponding heat maps of above average GFP fluorescence.....	37
4. Figure 2.4 Experimental design for using the green laser or blue laser for sample setup.....	39
5. Figure 2.5 The blue laser induces phot1-GFP aggregation in the phot1-5 phot1-GFP line.....	41
6. Figure 2.6 phot1-gfp accumulation into foci occurs in darkness in the phot1-5 nph3-6 phot1-gfp line and the blue laser enhances accumulation	42
7. Figure 2.7 The blue laser initiates phot1-GFP accumulation into foci in the phot1-5 phot1-GFP line and increases foci accumulation in the phot1-5 nph3-6 phot1-GFP line	43
8. Figure 2.8 phot1-GFP accumulates into foci at the plasma membrane prior to becoming internalized	46
9. Figure 2.9 phot1-GFP foci form more quickly in the phot1-5 nph3-6 phot1-GFP line than in the phot1-5 phot1-GFP line	47

10. Figure 2.10 phot1-GFP reorganization at the membrane occurs within minutes of blue light irradiation and increases with fluence	50
11. Figure 2.11 phot1-GFP accumulation into foci is fluence dependent at a moderate fluence rate.....	51
12. Figure 2.12 phot1-GFP accumulation into foci is fluence dependent at a moderate to high fluence rate	53
13. Figure 2.13 phot1-GFP accumulation into foci is fluence dependent at a high fluence rate.....	55
14. Figure 2.14 phot1-GFP accumulation into foci increases with fluence and is enhanced in the phot1-5 nph3-6 phot1-GFP	57
CHAPTER 3 MODULATING PHOT1-GFP ABUNDANCE AND MOVEMENT AT THEPLASMA MEMBRANE	61
1. Figure 3.1 phot1-GFP accumulates into foci in darkness in response to MG132 treatment.....	68
2. Figure 3.2 phot1-GFP accumulates into foci in darkness in phot1-5 phot1-GFP seedlings pre-treated with MG132.....	69
3. Figure 3.3 phot1-GFP accumulation into foci is elevated in phot1-5 phot1-GFP seedlings pre-treated with MG132.....	71
4. Figure 3.4 MG132 pre-treatment of phot1-5 phot1-GFP line increases accumulation of phot1-GFP into foci to a level similar to the phot1-5 nph3-6 phot1-GFP line.....	73
5. Figure 3.5 MG132 pre-treatment elevates phot1-GFP accumulation into foci at the plasma membrane in the phot1-5 phot1-GFP line	76
6. Figure 3.6 MG132 pre-treatment of phot1-5 phot1-GFP seedlings elevates levels of phot1-GFP accumulation into foci similar to levels of phot1-5 nph3-6 phot1-GFP seedlings in response to a moderate to high rate of blue light irradiation.....	77
7. Figure 3.7 MG132 pre-treatment of phot1-5 phot1-GFP seedlings elevates levels of phot1-GFP accumulation into foci similar to levels of phot1-5 nph3-6 phot1-GFP seedlings in response to a high rate of blue light irradiation	79
8. Figure 3.8 phot1-GFP accumulates into foci at the plasma membrane and is internalized in response to blue light irradiation after red light pre-treatment.....	82

9. Figure 3.9 Red light pre-treatment appears to reduce phot1-GFP accumulation into foci in darkness in the phot1-5 nph3-6 phot1-GFP line.....	85
10. Figure 3.10 Red light pre-treatment reduces phot1-GFP accumulation into foci at the plasma membrane in darkness in phot1-5 nph3-6 phot1-GFP seedlings	86
11. Figure 3.11 Red light pre-treatment reduces phot1-GFP accumulation into foci at the plasma membrane in response to blue light irradiation in the phot1-5 phot1-GFP line.....	87
12. Figure 3.12 Red light pre-treatment reduces phot1-GFP accumulation into foci at the plasma membrane in response to blue light irradiation in the phot1-5 nph3-6 phot1-GFP line	88
13. Figure 3.13 Red light pre-treatment reduces phot1-GFP accumulation into foci in response to blue light irradiation at a moderate to high fluence rate in the phot1-5 phot1-GFP line.....	89
14. Figure 3.14 Red light pre-treatment reduces phot1-GFP accumulation into foci in response to blue light irradiation at a moderate to high fluence rate in the phot1-5 nph3-6 phot1-GFP line.....	91
15. Figure 3.15 Red light pre-treatment reduces phot1-GFP accumulation into foci in response to blue light irradiation at a high fluence rate in the phot1-5 phot1-GFP line	93
16. Figure 3.16 Red light pre-treatment reduces phot1-GFP accumulation into foci in response to blue light irradiation at a high fluence rate in the phot1-5 nph3-6 phot1-GFP line	95
17. Figure 3.17 Low fluence rate blue light initiates little to no phot1-GFP aggregation at the plasma membrane in phot1-5 phot1-GFP seedlings	98
18. Figure 3.18 Low fluence rate blue light initiates phot1-GFP accumulation into foci at the plasma membrane in phot1-5 nph3-6 phot1-GFP seedlings	99
CHAPTER 4 CONCLUSIONS AND FUTURE DIRECTIONS	104
1. Figure 4.1 Working Model of the events that follow blue light activation of phot1 in the presence and absence of NPH3	111
ADDENDUM FURTHER CHARACTERIZATION OF PHOT1-GFP INTERNALIZATION AND ENGINEERING EQUIPMENT TO IMPROVE EFFCIANCY OF STANDARD LAB PROTOCOLS.....	129

1. Figure 4.1 phot1-GFP accumulates into foci at the plasma membrane and is internalized in response to blue light irradiation after TYR treatment.....	139
2. Figure 4.2 Phot1-GFP aggregates at the plasma membrane and is internalized in darkness in response to TYR A23 treatment.....	140
3. Figure 4.3 Accumulation of phot1-GFP into aggregates enhanced in TYRA 23 treated seedlings relative to mock treated seedlings	141
4. Figure 4.4 TYR A23 pre-treatment enhances accumulation of phot1-GFP accumulation into foci at the plasma membrane in response to blue light.....	143
5. Figure 4.5 TYR A23 pre-treatment prolongs phot1-GFP accumulation into foci at the plasma membrane in response to blue light.....	144
6. Figure 4.6 Assembled ICE-CAPS fountain	146
7. Figure 4.7 Growth 96-well plate and capture 96-well plate.....	147
8. Figure 4.8 IR imaging camera with IR 88nm bandpass filter installed	149
9. Figure 4.9 IR Imaging apparatus allows imaging of Arabidopsis seedlings before, after and during blue light irradiation	150
10. Figure 4.10 IR Image of Arabidopsis seedlings before and after blue light irradiation	151

CHAPTER 1

PHOTOTROPISM

Light Perception in Plants

Plants lack mobility and therefore have had to develop clever techniques for responding to changes in their environment in order to survive. Two very important aspects of survival are the ability to find or produce food and to acquire water. By utilizing light cues from the sun, plants are able to position themselves to optimize food production from solar energy and find water that collects in cooler parts of the soil away from direct sunlight via a response called phototropism (Galen et al. 2004, 2007; Christie 2007; Inoue et al. 2008; Pedmale et al. 2011). Plants are also able to use the sun to avoid shade, promoting competition for resources and resistance to some bacterial pathogens (Roberts and Paul 2006; Keuskamp et al. 2010). Plants are able to perceive light via photosensory proteins and interpret the cues through a complex network of signaling molecules that allow the plant to most appropriately respond to those cues (Chen and Fankhauser 2004; Franklin et al. 2005; Sullivan and Deng 2003).

In the model organism *Arabidopsis*, photoreceptors have been described that span the spectrum of visible light and influence an array of growth and developmental responses (Chen and Fankhauser 2004; Franklin et al. 2005; Sullivan and Deng 2003). Phytochromes are responsible for red and far-red perception and are primarily involved in germination, de-etiolation, circadian rhythm, flowering time and the shade avoidance response (Casal et al. 1998; Li et al. 2011).

Cryptochrome receptors perceive blue light and wavelengths within the UV-A spectrum and regulate the transition from skotomorphogenesis to photomorphogenesis (Yu et al. 2010). The UV RESISTANCE LOCUS 8 (UVR8) receptor perceives light in the UV-B spectrum and is involved in protection and repair in response to high energy UV light. UVR8 is also believed to be involved in resistance to pathogen growth and herbivore attacks (Jenkins 2009; Ulm and Jenkins 2015). The phototropins, made up of phototropin 1 (phot1) and phototropin 2 (phot2), are UV-A/blue light receptors and are responsible for phototropism (positive and negative depending on the organ of the plant), chloroplast relocation, stomatal opening and leaf positioning (Huala et al. 1997; Jarillo et al. 2001; Oghishi et al. 2004; Christie 2007; Holland et al. 2009). Lastly neochrome, a chimeric photoreceptor comprised of elements of both the phytochrome and phototropin receptors, has been characterized in some algae and ferns (Christie 2007; Jaedicke et al. 2012). Together these light receptors work to guide growth and development. However, the remainder of this chapter will focus on the phototropic response and how it is mediated by the phototropins, specifically phot1.

Phototropism

The bending of a plant in response to light is called phototropism and can be positive or negative depending on if toward or away from the light source (Christie 2007; Holland et al. 2009; Pedmale et al. 2011). Phototropism exhibits a complex fluence-response relationship to light. First positive phototropism results from pulsed blue light and is dependent on fluence, the radiant energy received by a

surface per unit squared. First positive phototropism shows a typical bell-shaped fluence response curve and the magnitude of the response is dependent on the total fluence of light perceived (Zimmerman and Briggs 1963; Janoudi and Poff 1990). Second positive phototropism occurs as a result of prolonged exposure and the magnitude of the response depends on the duration of irradiation (Iino 1990; Janoudi and Poff 1990; Whippo and Hangarter 2006).

Darwin was so intrigued by the ability of a plant to bend in response to its environment that he wrote a book on the subject titled “The Power of movement in plants”. In this work he describes a mysterious “substance” that is transduced throughout the plant in response to light (Darwin 1880). Over 40 years after Darwin’s publication, the “substance” Darwin described was identified by Frits Went and Nicolai Cholodny as the plant hormone indole-3-acetic acid (IAA) or auxin (Cholodny 1928; Went and Thimann 1937). Together these groups illustrated what Darwin had already proposed, that light perception occurred at the apex of the shoot of a plant and the signal was transduced downward initiating a bending response.

Auxin is a plant hormone involved in many aspects of plant growth and development (Benjamins and Scheres 2008; Kharshiing et al. 2010; Zhao 2010; Reemer and Murphy 2014). In dark grown seedlings, auxin movement is fountain-like in that it is primarily synthesized in the apical tip of the stem and is then transported basally and laterally throughout the plant (Goldsmith 1977; Bartel 1997; Kramer and Bennet 2006; Friml 2003; Ganguly and Cho 2013). In phototropism, exposure to directional blue light promotes auxin redistribution

laterally away from tropic stimulus in the shoot via auxin transport proteins (Figure 1.1) (Went and Thimann 1937; Stowe-Evans et al. 1998; Esmon et al. 2006; Liscum et al. 2014). The change in auxin distribution influences transcriptional regulation that results in differential cell growth leading to the phototropic bending response in the elongation zone of the shoot (Esmon et al. 2006; Liscum et al. 2014). Although much is known about the bulk flow of auxin response to light the details of auxin redistribution and differential elongation are still under investigation.

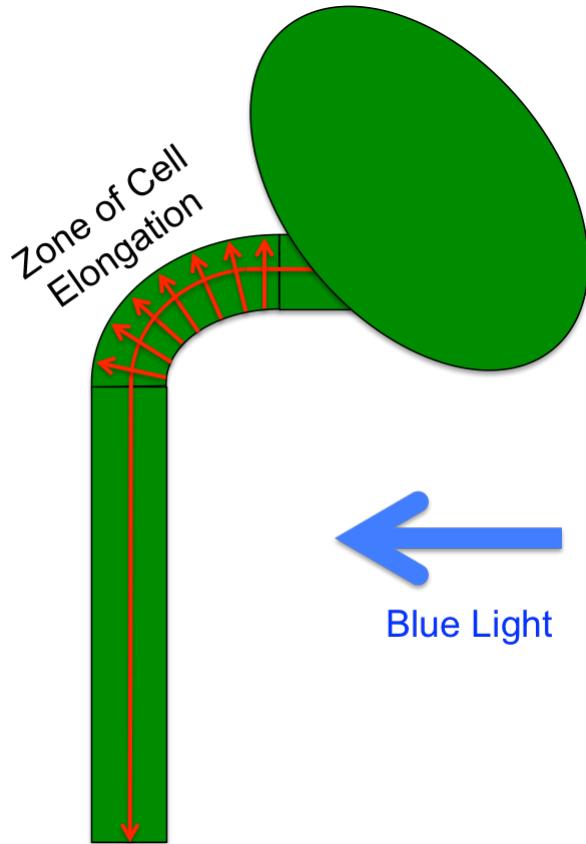


Figure 1.1 Auxin redistribution in response to blue light in the zone of cell elongation.

Illustration of a hypocotyl exhibiting the phototropic response due to directional blue light irradiation. Auxin synthesized in the apical portion of the plant is transported basally toward the roots. Irradiation with directional blue light initiates a change in the pattern of auxin transport that results in the establishment of a concentration gradient within the elongation zone with the highest concentration occurring on the shaded side and the lowest on the lit side. The concentration gradient initiates increased cell elongation of the shaded side relative to the lit side leading to bending toward the light source.

Auxin Transport Proteins

Although the steps between light perception by phototropins and auxin redistribution are still unclear, it is known that there are four types of auxin transporters involved in the transport of auxin: AUXIN1/LIKE-AUX1 (AUX/LAX) permeases, ATP binding cassette B (ABC-B) efflux proteins, the PIN efflux carriers, and the PIN-LIKES (PILS) efflux carriers (Friml 2003; Swarup and Péret 2012; Cho and Cho 2012; Feraru et al. 2012). Auxin is a weak lipophilic acid in its undissociated form (IAAH) and is able to move freely from the acidic extracellular space through the plasma membrane into the cell. Once in the cell, auxin becomes deprotonated (IAA^-) in the neutral cytosolic environment and can no longer move freely into or out of the cell unless facilitated by auxin transporters (Goldsmith 1977; Estelle 1998; Kramer and Bennet 2006). The location of auxin transporters contributes to the natural flow of auxin throughout the plant. The coordinated activity of auxin transport proteins and their relocation in response to tropic stimuli mediate the directional flow of auxin (Petrášek and Friml 2009).

Bennet et al. (1996) first identified the putative auxin influx carrier, AUXIN RESISTANT1 (AUX1). However, it wasn't until almost a decade later that the first report detailing the functional characteristics of AUX1 was published (Yang et al. 2006). AUX1 is a member of the AUX/LAX family in *Arabidopsis* that encode proteins with similar amino acid structure to amino acid transporters (Bennet 1996; Swarup and Péret 2012). The AUX/LAX encoded proteins function as IAA^-/H^+ symporters

that move auxin into the cell (Yang et al. 2006; Swarup et al. 2008; Péret et al. 2012). Although previously reported to function primarily in root tissue, Stone et al. (2008) found that AUX1 loss of function mutants exhibited slight defects in hypocotyl phototropism. However, when combined with a null mutation in the transcriptional activator auxin response factor 7 (ARF7), seedlings exhibited pronounced differences in phototropism. Seedlings with null ARF7 mutations are hypophototropic in response to blue light but phototropism can be rescued by pre-treating with red light. Seedlings lacking AUX1 and ARF7 are hypophototropic even with red light pre-treatment (Bennet et al. 2006; Marchant et al. 1999). This suggests that the role of AUX1 in phototropism is conditional and not the primary method of auxin transport in phototropism.

The second group of auxin transport proteins to be described, the ABCB family of proteins, includes auxin efflux protein ABCB19 (Blakeslee et al. 2007; Verrier et al. 2008). Of the 21 members of the ABCB family only three have been shown to be auxin transporters, and only ABCB19 has been shown to be involved in phototropism (Verrier 2008; Peer et al. 2011; Noh et al. 2003; Nagashima et al. 2008). ABCB19 null mutants exhibit enhanced phototropism in response to blue light further supporting the involvement of ABCB19 in the phototropic response (Noh et al. 2003; Nagashima et al. 2008). Although this does not present a clear picture of how ABCB19 is involved in phototropism, an interaction with another auxin transporter may suggest a model. ABCB 19 has been shown to interact with and stabilize the location of a member of the next family of auxin transporters to be discussed, the auxin efflux carrier PIN-FORMED 1 (PIN1),

further influencing the flow of auxin (Noh et al. 2003; Blakeslee et al. 2007; Titiapiwatanakun et al. 2009).

The PIN efflux transporters are the most well studied group of auxin transporters. Eight different PINs have been identified and are named PIN1-8. Each member is located strategically within different regions of the plant (Grunewald and Friml 2010). The PINS are integral transmembrane proteins with two conserved transmembrane helical domains at both termini and a central hydrophilic loop with variable length depending on the PIN type. PIN1, -2, -3, -4 and -7 are known as long PINS and represent the group with the longest hydrophilic loop. PIN5 and PIN8 both have short loops and PIN6 has a loop of medium length (Křeček et al. 2009; Ganguly et al. 2014). The length of the hydrophilic loop is an indicator of the location of the PIN and subsequent function. Long PIN proteins are found at the plasma membrane and are responsible for auxin efflux from the cytosol. Short PINs are located within the endoplasmic reticulum and contribute to intracellular auxin homeostasis indirectly affecting auxin signaling within the nucleus. (Křeček et al. 2009; Ganguly et al. 2014; Mravec et al. 2009; Cazzonelli et al. 2013).

Although other auxin transport proteins can appear to have polar localization within the cell, the long PIN proteins express frequent polar distribution that can be affected by changes in the environment and auxin concentrations (Grunewald and Friml 2010; Christie and Murphy 2013). The localization of PIN proteins is established by constant cycling between the plasma membrane and the early endosome and endosomal compartments of the TGN. It has also been shown that PIN cycling from the plasma membrane is dependent on clathrin-mediated

endocytosis (CME) and is preceded by phosphorylation (Dhonukshe et al. 2007; Lofke et al. 2013). The current model suggests cycling occurs between the early endosome and the plasma membrane via the action of members of the cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and phospholipid dependent protein kinase C (AGCVIII) kinase subfamily AGC3 and ADP-ribosylation factor GTPase guanine-nucleotide exchange factor (ARF-GEF) proteins (Dhonukshe 2011; Willige et al. 2013; Liscum et al 2014). Tropic stimuli can disrupt the pattern of PIN cycling leading to changes in PIN localization influencing auxin distribution (Grunewald and Friml 2010; Christie and Murphy 2013).

In regard to phototropism, light has been shown to alter the location of PIN transporters within the cell in a phot1 dependent manner. Ding et al. (2011) showed that in darkness PIN3 has an apolar distribution regulated by the AGC3 kinase PINOID (PID). Upon exposure to blue light, PID transcription is reduced and PIN3 is polarized to the inner side of the plasma membrane by the ARF-GEF GNOM-dependent trafficking. Ding et al. (2011) further showed that mutants lacking PID and two other AGC3 kinases, WAG1 and WAG2, exhibited a reduction in PIN3 relocalization in response to blue light and reduced hypocotyl bending. In addition, seedlings carrying a loss of function mutation in GNOM also have reduced hypocotyl bending in response to blue light (DING et al. 2011). This suggests a complex system in which phot1 is the initial step in a cascade of events that leads to PIN relocation within the cell. Understanding the process from phot1 activation to PIN relocalization presents an exciting avenue in the path from light perception to phototropic bending.

The last and most recently discovered group of auxin transporters to be discussed are the PILS. Although PINs were the first to be characterized the PILs family has representatives in single celled algae where PINs have not been found, suggesting that PILs represent an evolutionary older family of auxin transporters (Feraru et al. 2012; Viaene et al. 2013). PILS, like the shorter hydrophilic looped PINs, regulate intracellular accumulation of auxin at the ER but are also capable of transporting auxin across the plasma membrane (Barbez et al. 2012). To date, 7 members of the PILs family have been described in *Arabidopsis* and do not appear to reflect the region specific expression of PIN proteins (Barbez et al 2012). The discovery of the PILs family is an exciting addition to the ever-growing puzzle of auxin regulation. Although PILs research is still in the fledgling stage, it is a promising next step in elucidating the process of auxin transport in response to blue light in the phototropic response.

The Tropic Response to Auxin Transport in the Elongation Zone

Biosynthesis of auxin can occur in most tissues of a plant but the concentration gradient produced in response to directional light in the phototropic response is believed to be a result of auxin transport (Went and Thimann 1937; Goldsmith 1977; Peer et al. 2011; Christie and Murphy 2013; Hohm et al. 2013). As auxin is transported toward the opposite the side of illumination, a series of events occur in response to auxin. Increased auxin levels initiates cell elongation in the zone of elongation on the shaded side of the plant where auxin levels are elevated relative to the lit side resulting in bending toward the light source (Went and

Thimann, 1937; Esmon et al. 2006; Peer et al. 2011; Christie and Murphy 2013; Hohm et al. 2013).

Previously, it was speculated that the plasma membrane and ER protein AUXIN BINDING PROTEIN (ABP1) was an auxin receptor involved in plant phototropism (Jones 1994; Woo et al. 2002; Effendi 2011). However, Gao et al. (2015) demonstrated using *abp1* null mutant *Arabidopsis* plants that ABP1 is not a key component in auxin signaling. However, the nuclear F-box protein TRANSPORT INHIBITOR RESISTANT1/AUXIN BINDING F-BOX (TIR1/AFB), containing an f-box domain that works in complex with SCF to mark proteins for degradation in the proteasome, has been shown to influence transcriptional responses to auxin (Esmon et al. 2006; Tan et al. 2007; Calderon-Villalobos et al. 2010; Peer et al. 2011; Effendi et al. 2011) TIR/AFB proteins facilitate the binding and degradation of repressor proteins that prevent transcription of auxin response elements (AuxRE), regions of DNA that are expressed or repressed in response to auxin.

AuxREs are bound by ARFs that contain an activator or repressor domain (Chapman and Estelle 2009). In low auxin concentrations, the AUX/IAA repressor MASSAGU2 (MSG2/IAA19) binds the ARF responsible for transcriptional activation involved in the phototropic response, NONPHOTOTROPIC HYPOCOTYL4/ARF7 (NPH4/ARF7) (Stowe-Evans et al. 1998; Harper et al. 2000; Tatematsu et al. 2004). Upon blue light perception, the SCFT^{TIR1/AFB-3} E3 ubiquitin ligase complex binds free auxin. Auxin acts as "molecular glue" that adheres the MSG2/IAA19 repressor to the SCFT^{TIR1/AFB-3} complex (Dharmasiri et al. 2005; Guilfoyle 2007; Tan et al. 2007; Calderon-Villalobos et al. 2010). The complex ubiquitinates bound MGS2/IAA19

marking it for degradation in the proteasome (Gray et al. 2001). Removal of the repressor MSG2/IAA19 facilitates the transcription of TROPIC STIMULUS-INDUCED (TSI) genes. (Ulmasov et al. 1999; Harper et al. 2000; Esmon et al. 2006).

Esmon et al. (2006) reported ARF7 initiates transcription of a number of TSI genes, eight of which are involved in the phototropic response. Two of the genes expressed in response to ARF7 activation are members of the α -Expansin family, EXP1 and EXP8, which encode expansin proteins that contribute to cell wall extensibility (Sampedro and Cosgrove 2005; Esmon et al. 2006). This contributes to the differential cell elongation that allows the phototropic response to occur. In addition to α -Expansin, ARF7 initiates transcription of two Glycine max (GH3) genes that encode IAA-amino synthases that catalyze the conjugation of amino acids to free auxin (Staswick et al. 2005; Esmon et al. 2006). Unlike the α -Expansin proteins, GH3.6 and GH3.6 do not accumulate at the forefront of hypocotyl cell elongation but as maximal levels of cell elongation are reached. This suggests that unlike α -Expansins, GH3.5 and GH3.6 do not contribute to initiating cell elongation but may assist in regulating auxin signaling (Esmon et al. 2006). The remaining TSIs await further characterization in the hypocotyl cell elongation response in phototropism.

Although a great deal is known about the auxin response and initiation of differential elongation in phototropism, little is known about the events that directly follow light perception. Upon blue light perception, phot1 is known to initiate the signal that leads to the phototropic response. However, a clear path of signal transduction has yet to be described. The remainder of this chapter will focus on the phototropins, specifically phot1, and the events that follow activation by blue light.

The Blue Light Receptors Phototropin 1 and Phototropin 2

In *Arabidopsis*, phot1 and phot2 are blue light receptors responsible for the phototropic response (Huala et al. 1997; Jarillo et al. 2001; Sakai et al. 2001). Under low light conditions, phot1 is the primary receptor and mediates the positive phototropic response of the stem, negative phototropic response of roots, chloroplast accumulation, stomatal opening and leaf flattening. Under moderate to high light conditions, both photos work redundantly to influence the afore mentioned responses (Liscum and Briggs 1995; Huala et al. 1997; Jarillo et al. 2001; Kinoshita et al. 2001; Sakai et al. 2001; Takemiya et al. 2005; Han et al. 2013). Under high light conditions, phot2 activation initiates chloroplast movement in a way that allows the chloroplasts to avoid high light levels that might cause damage to the organelles (Jarillo et al. 2001; Ishiguro et al. 2001; Kagawa et al. 2001; Sakai et al. 2001; Han et al. 2013). Together, the phototropins facilitate a robust set of responses that allow the plant to most appropriately react to light quality and quantity.

The phototropins are plasma membrane associated and contain two N-terminal Light, Oxygen Voltage (LOV) domains, termed LOV1 and LOV2, and a C-terminal serine/threonine kinase domain (PKD) connected by an α -helical region ($\text{J}\alpha$ -helix) (Figure 1.2) (Gallagher et al. 1988; Huala et al 1997; Harper et al. 2003; Christie 2007). Each LOV domain is associated with a flavin mononucleotide (FMN) that, upon light activation, forms a covalent cysteinal adduct within the LOV domain (Christie et al. 1998; Christie et al. 1999; Salomon et al. 2000; Crosson et al. 2001; Christie et al. 2002). Cysteinal adduct formation in the LOV2 domain disrupts its interaction with the $\text{J}\alpha$ -helix releasing inhibition of the kinase domain (Figure 2.1)

(Christie et al. 2002; Harper et al. 2003, 2004). Although the photos contain two photosensory LOV domains it is currently understood that the LOV 1 domain is involved in protein dimer/multimerization and LOV 2 is the primary light sensory domain that leads to activation of the PKD (Salomon et al. 2004; Nakasako et al. 2008; Nakasone et al. 2013; Christie et al. 2002; Harper et al. 2003, 2004; Jones et al. 2007; Tokutomi et al. 2008).

The PKD of phot1 is a member of the AGC-VIII protein kinase superfamily (Rademacher and Offringa 2012). Experiments in insect cells expressing phot1 show that phot1 is an autophosphorylating protein (Christie et al. 1998). Although both cis- and trans- interactions in phot1 autophosphorylation are implicated, evidence of trans-autophosphorylation has been reported (Kaiserli et al. 2009). Furthermore, mutational studies of *Arabidopsis* phot1 phosphorylation sites SER-849 and SER-851 to alanine results in a loss of phototropism indicating phot1 phosphorylation is necessary for phototropic signaling (Inoue et al. 2008). In addition to phosphorylation, ubiquitination of phot1 is another necessary step in the phototropic response that is dependent on the interacting protein NONPHOTOTROPIC HYPOCOTYL3 (NPH3) (Pedmale and Liscum 2007; Roberts et al. 2011).

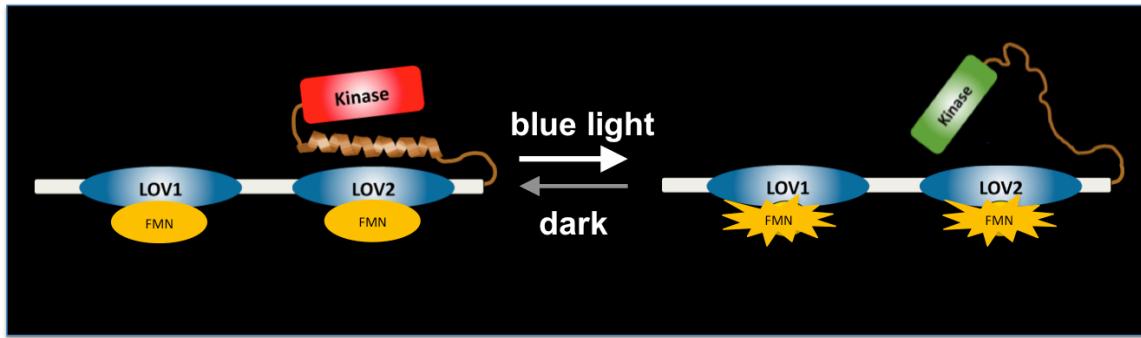


Figure 1.2 Basic phototropin structure and function.

The phototropin proteins consist of two photosensory light oxygen voltage (LOV1 and LOV2) domains at their N-terminus followed by an alpha helical region called the J α helix and a C-terminal protein kinase domain (PKD). In darkness both LOV domains are non-covalently associated with a flavin mononucleotide (FMN) prosthetic group and the activity of the PKD is repressed by the LOV2 domain. Upon absorption of a photon of blue light by the FMNs a covalent cysteinyl adduct is formed between the FMN and a conserved cysteine within each LOV domain. The formation of the cysteinyl adduct within the LOV2 domain results in a conformational change in the protein and unfolding of the J α helix and removal of steric inhibition of the PKD by LOV2 activating the kinase properties of the receptor.

The phot1 Interacting protein NPH3

The NPH3 protein includes an N-terminal Broad-complex, Tramtrack and Bric-a-Brac (BTB) domain, a central NPH3 domain, and a C-terminal coiled-coil domain (Motchoulski and Liscum 1999; Pedmale et al. 2011). NPH3 is one of 33 members of the NPH3/RPT2-like family of proteins in *Arabidopsis* characterized by its NPH3 domain (Motchoulski and Liscum 1999; Inada et al. 2004; Pedmale and Liscum 2007; Sakai and Haga 2012). Motchoulski and Liscum (1999) reported that NPH3 is a plasma membrane-associated protein that interacts with the N-terminal LOV containing portion of phot1 through its C-terminal coiled-coil region. The ability of NPH3 to contribute to phototropic signaling is dependent on its phosphorylation state. Pedmale and Liscum (2007) showed that NPH3 is converted from a phosphorylated (NPH3^{DS}) to a dephosphorylated (NPH3^{LS}) state in response to blue light in a phot1-dependent manner. In addition, Roberts et al. (2011) hypothesized phototropic signaling is dependent on dephosphorylation of NPH3 as it facilitates the binding of NPH3 to a Cullin-RING-ubiquitin ligase complex that targets phot1 for ubiquitination.

Ubiquitination, the covalent addition of an ubiquitin molecule, of a protein regulates the stability, function and location of the protein (Finley and Chau 1991). Ubiquitination occurs by the action of three enzymes: an activating enzyme, E1; a conjugating enzyme, E2; and a ligating enzyme, E3. The E1 enzyme activates ubiquitin in an ATP dependent mechanism and transfers the activated ubiquitin to the active site of an E2 enzyme. The E2 enzyme works together with the E3 enzyme to transfer the ubiquitin to a lysine of a target protein (Hershko and Ciechanover

1998). Roberts et al. (2011) reported that NPH3 interacts with Cullin3, the core component of a cullin-RING-based E3 ubiquitin-protein ligase complex (CRL3^{NPH3}), acting as a substrate adaptor facilitating phot1 ubiquitination in response to blue light (Figure 1.3). Furthermore Roberts et al. (2011) also showed the type of ubiquitination is dependent on the intensity of blue light and can lead to receptor signaling or degradation in the proteasome.

Ubiquitination of receptors can influence several intracellular processes including protein trafficking and proteasomal degradation (Miranda and Sorkin 2007; Chen and Sun 2009). The type of ubiquitination can dictate a protein's ultimate fate. Polyubiquitination, multiple ubiquitin molecules in a chain attached to a target lysine within a substrate, can result in targeting the substrate protein for degradation by the proteasome. In contrast, monoubiquitination, single ubiquitin molecules attached to a single or multiple lysine residues, can result in protein internalization and trafficking to intercellular targets (Miranda and Sorkin 2007; Komander 2009). Roberts et al. (2011) showed that under high light conditions, similar to what a plant would experience under direct sunlight, phot1 is polyubiquitinated marking the protein for degradation in the proteasome. Therefore, polyubiquitination and subsequent degradation of phot1 may be a means of receptor desensitization in response to high light conditions. Under low light conditions phot1 is monoubiquitinated presumably representing the active signaling form (Figure 1.3) (Roberts et al. 2011). How monoubiquitination influences phot1 signaling is still unknown. However, ubiquitin moieties have been

known to facilitate receptor binding to signaling molecules (Miranda and Sorkin 2007). Perhaps ubiquitination allows for interaction with substrate proteins.

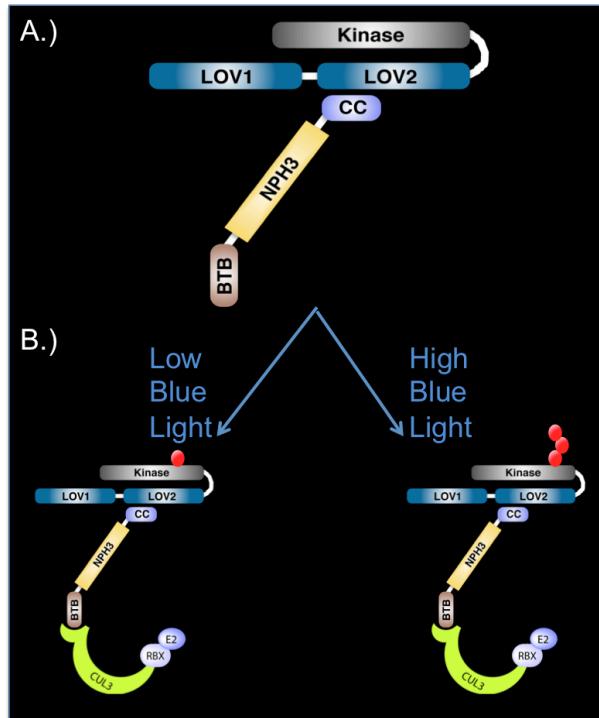


Figure 1.3 NPH3 interacts with phot1 and facilitates ubiquitination in response to blue light.

(A) NPH3 consists of an N-terminal Broad-complex, Tramtrack and Bric-a-Brac (BTB) domain, a central NPH3 domain, and a C-terminal coiled-coil (CC) domain. NPH3 interacts with the LOV2 domain of phot1 via its CC domain. (B) NPH3 acts as a substrate adaptor for a cullin-RING-based E3 ubiquitin-protein ligase complex (CRL3^{NPH3}) that catalyzes the addition of ubiquitin molecules (red balls) to a target lysine in the PKD of phot1. This occurs via an interaction between its BTB domain and cullin3, the core of the ubiquitin-protein ligase complex. Activation of phot1 by low blue light leads to monoubiquitination of the receptor by the ubiquitin-protein ligase complex whereas activation by high blue light initiates polyubiquitination of the receptor.

Other phot1 interacting proteins involved in phototropism

Although path from light perception by phot1 to lateral auxin redistribution is not completely clear phot1 interacting proteins and substrates have been identified (Liscum et al. 2014). The PHYTOCHROME KINASE SUBSTRATE (PKS) proteins PKS1, PKS2 and PKS4 have been shown to interact with phot1 at the plasma membrane and are also involved in phototropic signaling (Lariguet et al. 2006; de Carbonnel et al. 2010; Demarsy et al 2012). Additionally, PKS4 is phosphorylated by phot1 in response to activation by blue light (Demarsy et al. 2012). Kami et al. (2014) reported that PKS4 null mutants exhibited reduced phototropism under low light conditions. Further analysis showed that PKS1 and PKS4 influence phototropism at low fluence blue light ($0.1 \mu\text{mol m}^{-2} \text{ sec}^{-2}$) and PKS1 and PKS2 influence phototropism at high fluence light ($10 \mu\text{mol m}^{-2} \text{ sec}^{-2}$). This suggests that PKS1 plays a role in both phot1 and phot2 mediated phototropic responses and PKS 4 and PKS2 are involved in phot1 or phot2 mediated responses respectively.

The phot1 interacting protein ROOT PHOTOTROPISM2 (RPT2) makes up the second half of the family name in the NPH3/RPT2-like family of proteins (Motchoulski and Liscum 1999; Inada et al. 2004; Pedmale and Liscum 2007; Sakai and Haga 2012). RPT2 was first described in a mutant screen for plants deficient in root phototropism and has since been shown to be involved in hypocotyl phototropism under high light conditions (Okada and Shimura 1992; Sakai et al. 2000). RPT2 shares similar structure to NPH3 possessing an N-terminal BTB domain, a central NPH3 domain and a C-terminal coiled-coil domain (Inada et al.

2004). Although it is still unclear the exact role that RPT2 plays in the phototropic response, yeast-two-hybrid assays show that RPT2 interacts with the N-terminal LOV domain portion of phot1 through its N-terminal domain and not the C-terminal coiled-coiled domain as seen in NPH3 (Motchoulski and Liscum 1999; Inada et al. 2004). Furthermore, Inada et al. (2004) reported that RPT2 interacts with phot1 and NPH3 at the plasma membrane. This has led to speculation that RPT2 may be involved in phot1 ubiquitination under high light conditions in a CRL3^{NPH3/RPT2} complex (Hohm et al. 2013; Liscum et al. 2014).

The auxin efflux protein ABCB19 was shown by Christie et al. (2011) to be phosphorylated by phot1 in response to blue light. In HeLa cell systems, phosphorylation by phot1 inhibits auxin efflux activity suggesting an interaction in response to blue light that regulates auxin transport (Christie et al. 2011). Given that ABCB19 has also been implicated in stabilizing PIN1 at the plasma membrane it is possible that phot1 phosphorylation of ABCB19 could have an impact on the interaction between ABCB19 and PIN1 influencing the flow of auxin in the phototropic response (Noh et al. 2003; Blakeslee et al. 2007; Titiapiwatanakun et al. 2009).

It is still unclear how phot1 activation leads to auxin redistribution and subsequent phototropic bending. The dynamic nature of cell signaling and the robust array of phot1 interacting proteins open many avenues of research to be explored in the coming years. In addition to interacting with other proteins phot1 exhibits another behavior that has puzzled researchers for many years, internalization in response to blue light.

The Role of Blue Light Induced Movement of phot1

Upon sensing blue light, phot1 undergoes a conformational change and autophosphorylates rendering the receptor active (Christie et al. 1998; Kaiserli et al. 2009; Inoue et al. 2008). Microscopic evidence using a GFP tagged phot1 construct (phot1-GFP) in the phot1 null *phot1-5* background shows that phot1-GFP signal moves from the membrane to yet a to be identified intracellular compartment or structure in response to blue light (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Sullivan et al. 2010; Preuten et al. 2015).

Autophosphorylation is not only necessary for phototropic signaling but is a prerequisite for receptor internalization. Kaiserli et al. (2009) reported confocal imaging of phot1-GFP carrying the D806N mutation, a mutation that inactivates the kinase, shows phot1-GFP retention at the membrane after blue light irradiation. In addition, images of phot1-GFP carrying a constitutively active kinase mutation, I608E, show internalization in the absence of blue light (Kaiserli et al. 2009). Although phot1 activation appears to be necessary for movement, the purpose and target of phot1 internalization is still unclear.

As described earlier, Roberts et al. (2011) showed that in addition to phosphorylation phot1 is also ubiquitinated in response to blue light. Previously, it was reported using cell fractionation studies that blue light causes movement of phot1 and phot1-GFP from the microsomal to the soluble fraction (Sakamoto and Briggs 2002; Knieb 2004; Roberts 2012). Roberts (2012) showed that phot1 does not appear in the soluble fraction in fractionations performed on seedlings lacking NPH3. This suggests ubiquitination is necessary for internalization of phot1.

However, confocal imaging shows phot1-GFP being internalized in response to blue light in seedlings lacking NPH3 (Roberts 2012). Perhaps phosphorylation is sufficient to induce receptor internalization but ubiquitination is necessary to target internalized phot1 to the correct endosomal compartment. Whether phot1 internalization is functionally relevant in signaling is still unclear.

It has been known for many years that pre-treating seedlings with a red light enhances the phototropic response (Curry 1957; Briggs 1963; Zimmerman and Briggs 1967). Han et al. (2008) reported that pre-treating *Arabidopsis* seedlings with red light prior to blue light also prevents phot1-GFP internalization in a phyA dependent mechanism. Although phyA has been shown to interact with phot1 when co-expressed in onion epidermal cells, no interaction has been reported in *Arabidopsis* (Jaedicke et al. 2012). The role of phyA in enhancing phototropism and retaining phot1 at the membrane is still under investigation. However, it appears that phot1 signaling can occur without internalization suggesting that internalization in response to blue light may serve a purpose other than to facilitate signaling.

Another recent study by Preuten et al. (2015) further supported that phot1 signaling may occur from the membrane. The group constructed transgenic *Arabidopsis* lines that expressed GFP labeled myristoylated or farnesylated phot1. Myristylation and farnesylation have been shown to attach otherwise soluble proteins to the plasma membrane (Thompson and Okuyama 2000). The lipid anchored phot1-GFP constructs rescued phototropism and other phot1 mediated responses but were not internalized in response to blue light. Furthermore, Han et

al. (2008) speculated that phot1-GFP retention at the plasma membrane in response to red-light pre-treatment contributes to enhanced phototropism. In contrast, Preuten et al. (2015) did not report enhanced phototropism in constructs retained at the membrane. Currently, the evidence in regard to retention at the plasma membrane enhancing signaling is inconclusive. Although the role of phot1 internalization in response to blue light in phototropic signaling is still unclear, there is evidence that suggests receptor movement may be functionally important.

The role of phot1 movement in light dependent responses cannot be completely ruled out at this time. Kong et al (2013) reported that both phot1 and phot2 associate with the chloroplast outer membrane in response to blue light. As mentioned previously, phot2 mediates the chloroplast avoidance response to high intensity blue light (Jarillo et al. 2001; Kagawa et al. 2001; Sakai et al. 2001; Kong and Wada 2016). Kong et al. (2013) showed that phot2 mediated chloroplast avoidance requires association with the plasma membrane, Golgi membrane and chloroplast outer membrane. In addition, phot1 was able to initiate the chloroplast avoidance response to high intensity blue light in the phot2 null mutant, presumably due to association with the chloroplast outer membrane (Luesse et al. 2010; Kong et al. 2013). Although not directly related to phototropic bending, this still suggests a role for the movement of phot1 in response to blue light. Further characterization of phot1 movement may be necessary to understand the biological relevance as internalization may not be the only form of phot1 movement in response to blue light.

Recently, Liscum (2016) reported findings from our lab that indicate that in addition to internalization, punctate accumulation of phot1-GFP appears at the plasma membrane in response to blue light. The accumulation of phot1-GFP into foci builds into mosaic patterns at the plasma membrane in response to continuous blue light irradiation and returns to a random distribution when seedlings are returned to darkness. The function of the accumulation that forms in response to blue light is currently unclear but could represent putative signaling centers or perhaps a method of receptor desensitization. The effects of post-translational modification on receptor movement may aid in our understanding of the functional role of phot1 movement in response to blue light.

According to Roberts (2012) the post-translational modification ubiquitination may influence the movement of phot1 in response to blue light. Roberts (2011) showed that when NPH3 is present and phot1-GFP is polyubiquitinated in response to high blue light that some phot1-GFP can be found in the soluble fraction after blue light irradiation. However, when NPH3 is not present phot1-GFP is not found in the soluble fraction in response to blue light irradiation. Contrary to the biochemistry, Roberts (2012) showed using confocal microscopy that phot1-GFP is still internalized in seedlings lacking NPH3. Given that phot1 moves from the microsomal to the soluble fraction in seedlings possessing NPH3 but not in lines lacking NPH3 is interesting. This suggests that the movement of ubiquitinated phot1 is different than the non-ubiquitinated form. Further characterization of phot1-GFP movement in lines possessing and lacking NPH3 may aid in our understanding of the role of phot1 internalization in response to blue light

and assist in furthering our understanding of the signaling events that follow phot1 activation.

Given that phot1 appears to be internalized in response to blue light in the ubiquitinated form and non-ubiquitinated form, it is unclear the role that internalization might play in signaling. According to the reports by Roberts et al. (2011) and Roberts (2012) the model in Figure 1.4 describes our current hypotheses of the role of phot1 internalization. Luckily genetic tools exist that can aid in determining the difference in phot1 movement between the ubiquitinated and non-ubiquitinated forms and allow us to test this model. The purpose of this dissertation is to test the hypotheses presented in our current working model using the GFP tagged phot1 construct in the phot1 null *phot1-5* background and the phot1 and NPH3 null *phot1-5 nph3-6* background. Using confocal microscopy the internalization and movement at the plasma membrane of phot1-GFP in response to blue light will be fully characterized in the ubiquitinated and non-ubiquitinated form. The goal will be to determine if by utilizing the differences in signaling and movement between the ubiquitinated and non-ubiquitinated form we can determine the functional role of phot1 movement in response to blue light.

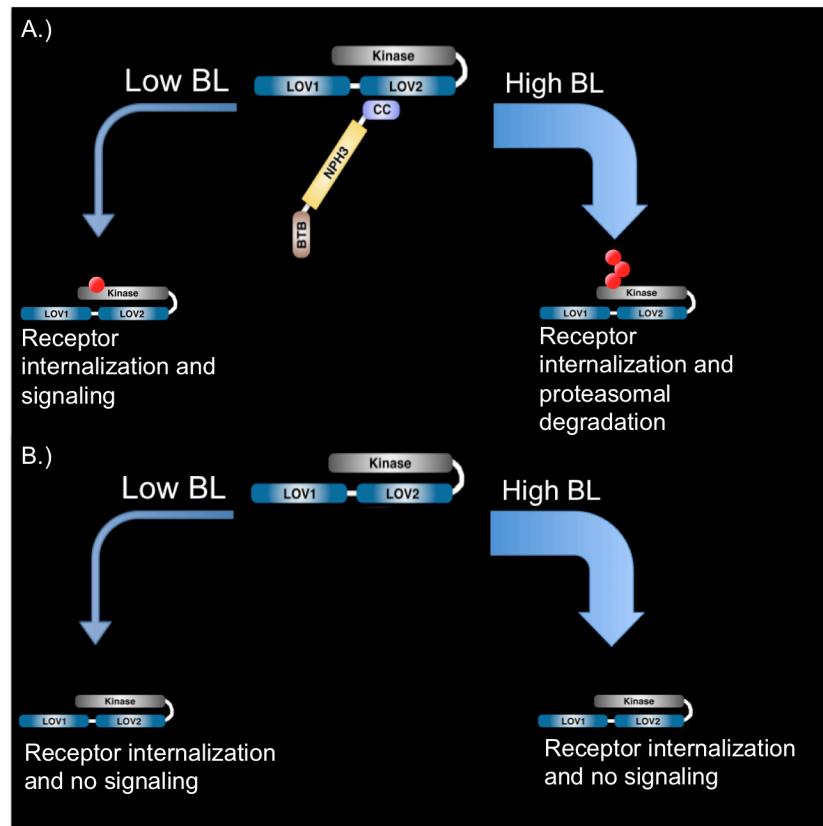


Figure 1.4 Working Model of the events that follow blue light

activation of phot1 in the presence and absence of NPH3.

This working model represents what we hypothesize to occur after blue light activation of phot1 in seedlings where NPH3 is either present or absent. (A) When NPH3 is present phot1 is ubiquitinated after activation by blue light. Under low light conditions phot1 is monoubiquitinated leading to receptor internalization and signaling. Under high light conditions phot1 is polyubiquitinated leading to receptor internalization and degradation. (B) When NPH3 is absent phot1 is not ubiquitinated in response to blue light irradiation. Although phot1 still appears to be internalized in the non-ubiquitinated form signaling that leads to phototropism does not occur.

CHAPTER 2

CHARACTERIZATION OF THE MOVEMENT OF PHOT1-GFP IN RESPONSE TO BLUE LIGHT

Abstract

Phototropism, the bending of a plant in response to light, is mediated by the blue light photoreceptor phototropin 1. Previous reports indicate that in response to blue light, phot1-GFP is internalized to a yet to be identified intracellular target. Here we propose a technique for imaging phot1-GFP to reduce light contamination and show that prior to internalization phot1-GFP accumulates into foci at the plasma membrane in response to moderate and high blue light. Internalization appears to originate from the foci that form and target adjacent regions of the plasma membrane as opposed to intracellular targets. Quantification of phot1-GFP foci suggests that the accumulation is fluence dependent and diminishes over time when seedlings are returned to darkness. Mutants lacking NPH3, necessary for the ubiquitination of phot1 and receptor signaling, show phot1-GFP accumulation into foci in darkness and enhanced phot1-GFP accumulation into foci in response to blue light. This suggests a role of NPH3 in the cycling and maintenance of phot1 levels in darkness and in response to blue light irradiation.

Introduction

The blue light photoreceptor phototropin 1 (phot1) is the primary receptor that mediates phototropism under low light condition (Liscum and Briggs 1995;

Sakai et al. 2001). In etiolated seedlings, phot1 is associated with the internal face of the plasma membrane (Gallagher et al. 1988; Sakamoto and Briggs 2002; Christie 2007). Several labs have reported that upon blue light activation GFP tagged phot1 (phot1-GFP) becomes internalized to a currently unidentified intracellular target (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Preuten et al. 2015). This is supported by reports that show in darkness phot1 is found exclusively in the membrane fraction but upon blue light irradiation appears in the soluble fraction (Sakamoto and Briggs 2002; Knieb 2004; Roberts 2012). Kaiserli et al. (2009) reported that, in addition to being necessary for signaling in phototropism, receptor autophosphorylation is necessary for phot1-GFP internalization. Likewise ubiquitination of phot1 dependent on the substrate adaptor protein NONPHOTOTROPIC HYPOCOTYL 3 (NPH3) is necessary for signaling and may influence the movement of phot1 as well (Motchoulski and Liscum 1999; Roberts et al. 2011; Roberts 2012). This suggests post-translational modification is involved in both phot1 signaling in the phototropic response and influencing receptor movement in response to blue light.

Roberts et al. (2011) showed that the phot1 interacting protein NPH3 acts as a substrate adaptor for a cullin-RING-based E3 ubiquitin-protein ligase complex (CRL3^{NPH3}) responsible for the ubiquitination of phot1 in response to blue light. Later Roberts (2012) reported that phot1 does not appear in the soluble fraction in response to blue light in seedlings lacking functional NPH3. However, confocal images show phot1-GFP is still internalized in response to blue light (Roberts 2012). This suggests that phot1 phosphorylation is sufficient to induce receptor

internalization but not movement into the soluble fraction. This is an interesting phenomenon given that ubiquitination can target receptor proteins for internalization in other model systems (Miranda and Sorkin 2007; Haglund and Dikic 2012).

The type of ubiquitination can influence a receptors ability to interact with other proteins and destination within the cell. Monoubiquitination can facilitate interaction with signaling components or mark a protein for endocytosis and endosomal sorting. Additionally polyubiquitination of a receptor can lead to degradation in the proteasome (Miranda and Sorkin 207). Roberts et al. (2011) reported that phot1 is polyubiquitinated in response to high fluence blue light leads to degradation in the proteasome. Furthermore, phot1 is monubiquitinated under low light conditions and may represent the active signaling form of the receptor (Roberts et al. 2011). In this study our goal is to further characterize the movement of phot1 under moderate and high blue light conditions in *Arabidopsis* seedlings with and without NPH3. This will allow further characterization of the movement of phot1-GFP in the ubiquitinated and nonubiquitinated from in response to blue light and perhaps contribute to our understanding of the functional relevance of phot1-GFP internalization.

Experimental procedures

Plant Materials and Growth Conditions

Phot1-5 seedlings of *Arabidopsis thaliana* (Columbia, *gl-* background) that were transformed with a Phot1-GFP construct by Sakamoto and Briggs (2002) and

phot1-5 nph3-6 phot1-GFP plants acquired Christian Fankauiser's group were used in all experiments. The Phot1-GFP expression has been shown to compliment the mutant phenotype for blue light induced phototropism (Sakamoto and Briggs 2002). The *phot1-5 nph3-6* phot1-GFP line was created by crossing the *phot1-5* phot1-GFP line with a line lacking functional NPH3, *nph3-6*. The result is the phototropic null *phot1-5 nph3-6* phot1-GFP line lacking functional phot1 and NPH3 and carrying GFP tagged phot1.

Seeds were sterilized in 30% bleach solution for 15 minutes and rinsed 3 times in sterile water. The seeds were then allowed to dry on filter paper. Once dry, the seeds were transferred individually to half-strength Murishige and Skoog medium agar plates with the tip of sterilized hemostats (approximately 60 seedlings per plate). Plates were then placed at 4° C in a dark box for 48 hours. Plates were then subjected to 1 hour fluorescent lighting under red Plexiglas to induce germination. Seedlings were grown in darkness for 72 hours prior to imaging on confocal microscope. All handling of seedlings outside of darkness was performed under dim green light conditions to prevent unnecessary photoreceptor activation.

Light treatment of seedlings

Prior to imaging on the confocal microscope, seedlings were either mock treated or irradiated with unilateral blue light at a total fluence of 100 umol m⁻², 1,000 umol m⁻², 2,000 umol m⁻² or 10,000 umol m⁻². Total fluence was achieved at a fluence rate of 5 umol m⁻² sec⁻¹ (moderate fluence rate), 10 umol m⁻² sec⁻¹ (moderate to high fluence rate) or 20 umol m⁻² sec⁻¹ (high fluence rate). Seedlings were blue light treated by fluorescent lighting through one layer of blue acrylic (Rohm and

Haas No. 2424, 3.18Mmm thick; Cope Plastics). Seedlings were then placed in darkness for 5, 15, 25, 35 or 45 minutes prior to mounting on slides in distilled water and imaged on confocal microscope.

Confocal microscopy

Confocal images were obtained on an Olympus IX-71 Inverted Microscope using a 60X/1.3NA oil immersion lens. Microscope was equipped with Yokagawa Spinning Disc component (CSUX 1-A1N-E). GFP was excited with either Spectra Physics 515 nm laser at 30% power ($10 \text{ umol m}^{-2} \text{ sec}^{-1}$ fluence rate) or Spectra Physics 488 nm laser at 15% power ($10 \text{ umol m}^{-2} \text{ sec}^{-1}$ fluence rate). Fluorescence was imaged using Andor iXon Ultra EMCCD camera (DU-8979-CSO-#BV-500) between 500 and 550nm. The 515 nm laser (green laser) was used for sample setup and the 488 nm laser (blue laser) was used for imaging except when noted in the results section. All images and z-stack parameters were manipulated using Andor IQ3 software version 3.3. Fluorescence of phot1-GFP was imaged at the outer periclinal and anticlinal plasma membrane of cortical cells within the elongation zone of etiolated seedlings as described previously (Sakamoto and Briggs, 2002; Wan et al. 2008). Great care was taken to measure the same cell in each seedling. This was done by moving from the apical hook into the elongation zone and taking images of the first cell after the transition from the apical hook to the elongation zone that exhibited the elongated characteristic of a cell in the elongation zone and not the cuboidal characteristic of a cell in the apical hook.

Fluorescence measurements

All measurements were taken using Fiji Is Just ImageJ (FIJI) software version 2.0.0-rc-49/1.51f. Prior to measuring phot1-GFP fluorescence, maximum intensity projections of Z-stack images were generated using the Z-Project option in FIJI. Average fluorescence intensity at the plasma membrane was established by measuring fluorescence across the plasma membrane of phot1-GFP that exhibited a homogenous distribution. The “polygon section freehand tool” was then used to draw regions of interest around the plasma membrane of cells in the Z-project images. Phot1-GFP foci were then measured in Z-project images using the “threshold” function in FIJI to determine the percentage of the plasma membrane that exhibited above average fluorescence, representing accumulation into foci, within the drawn regions of interest.

Graphical representation and statistics

Graphs were constructed using DeltaGraph 7 version 7.1.0 build 2, from Red Rock Software, Inc., Salt Lake City Utah www.redrocksw.com. Each point on graphs represents six measurements. Each measurement was taken from an individual cell within a seedling. Each experiment was performed on six total seedlings yielding one measurement per seedling. Each experiment consisted of two seedlings and was repeated in three biological replicates. Statistical analysis was performed using SPSS Statistics Software Version 24.0.0.0, from IBM Corporation, Armond Ney York www.IBM.com.

Results

phot1-GFP Accumulates Into Foci at the Plasma Membrane in Response to Blue Light

Prior to collecting image data, we noticed that GFP signal accumulates into foci at the plasma membrane in response to blue light. Movement of phot1-GFP at the plasma membrane has briefly described by Wan et al. (2008) but has not been fully characterized as previous imaging of phot1-GFP has focused on the cytosol (Sakamoto and Briggs 2002; Han et al. 2008; Kaiserli et al. 2009; Preuten et al. 2015). Figure 2.1 illustrates the difference between images of phot1-GFP fluorescence taken within the cytosol and at the plasma membrane. The foci that formed at the plasma membrane were more predictable than the internalized strands of GFP signal and offered a more consistent method of measuring phot1-GFP movement in response to blue light.

Measurements of phot1-GFP foci that accumulate at the plasma membrane were taken using FIJI software. Image stacks generated using Andor IQ3 Software were used to generate maximum intensity projections in FIJI. The freehand tool was used to draw a region of interest and the threshold function of FIJI was used to generate a heat map of areas of the plasma membrane that exhibited above average GFP fluorescence (Figure 2.2). Figure 2.3 shows a series of maximum intensity projections with corresponding heat maps to illustrate varying degrees of phot1-GFP accumulation into foci at the plasma membrane.

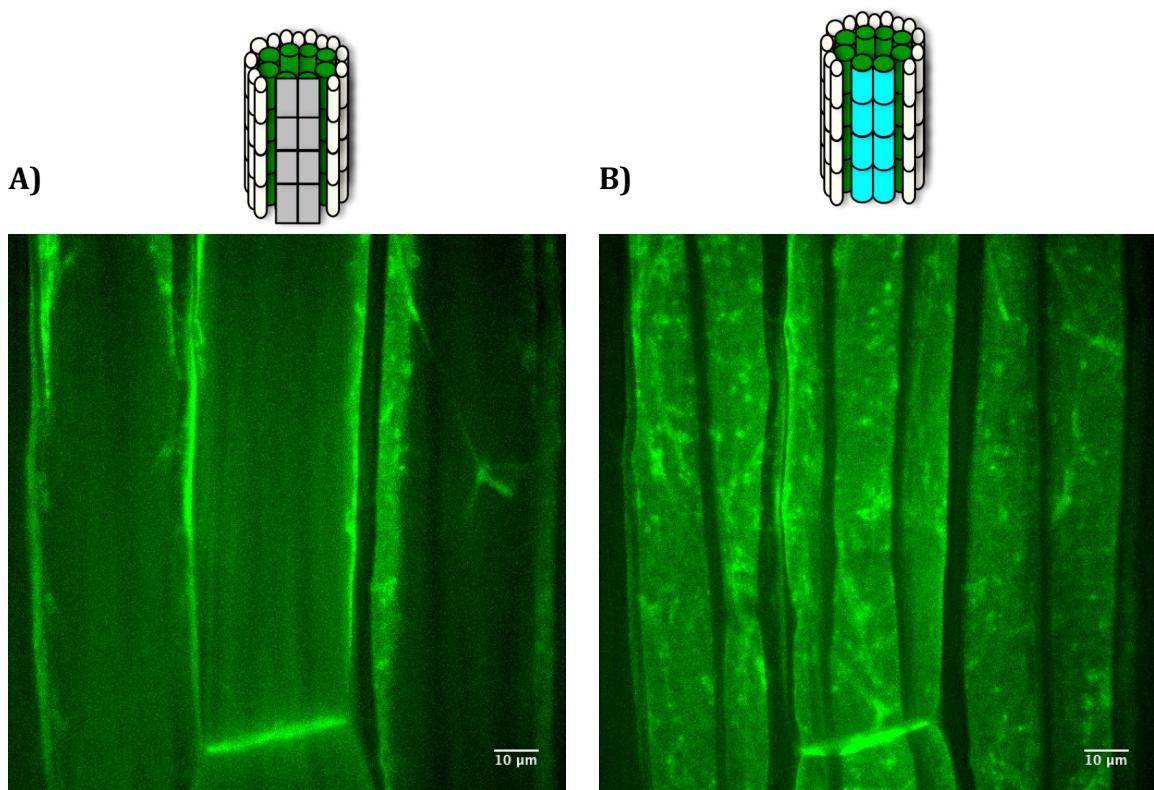


Figure 2.1 Images of phot1-GFP fluorescence within the cytosol and at the plasma membrane.

Fluorescence of phot1-GFP was imaged in the cytoplasm (approximately 20 μm within the cell) (A) and at the plasma membrane (B). The illustrations above each column represent the focal plane of the cells that were imaged.

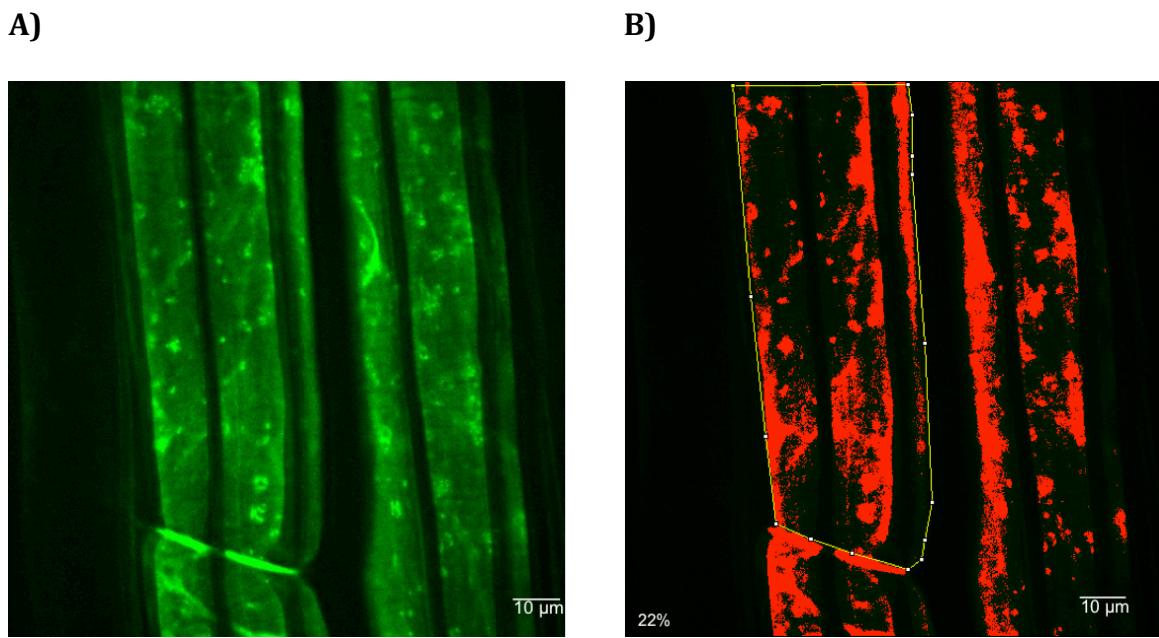


Figure 2.2 Accumulation of phot1-GFP into foci and corresponding heat map of above average GFP fluorescence.

Maximum intensity projection of phot1-GFP fluorescence at plasma membrane of seedling irradiated with $10,000 \mu\text{mol m}^{-2}$ at a moderate to high fluence rate and returned to darkness for 15 minutes (A) and corresponding heat map of above average GFP fluorescence (B). FIJI was used to generate a maximum intensity projection of image stack taken at the plasma membrane. A corresponding heat map was generated in FIJI using the threshold function to determine regions of plasma membrane expressing above average GFP fluorescence. A region of interest was drawn around the plasma membrane using the freehand tool. Percentage indicates the percentage of the plasma membrane calculated by FIJI expressing above average GFP fluorescence.

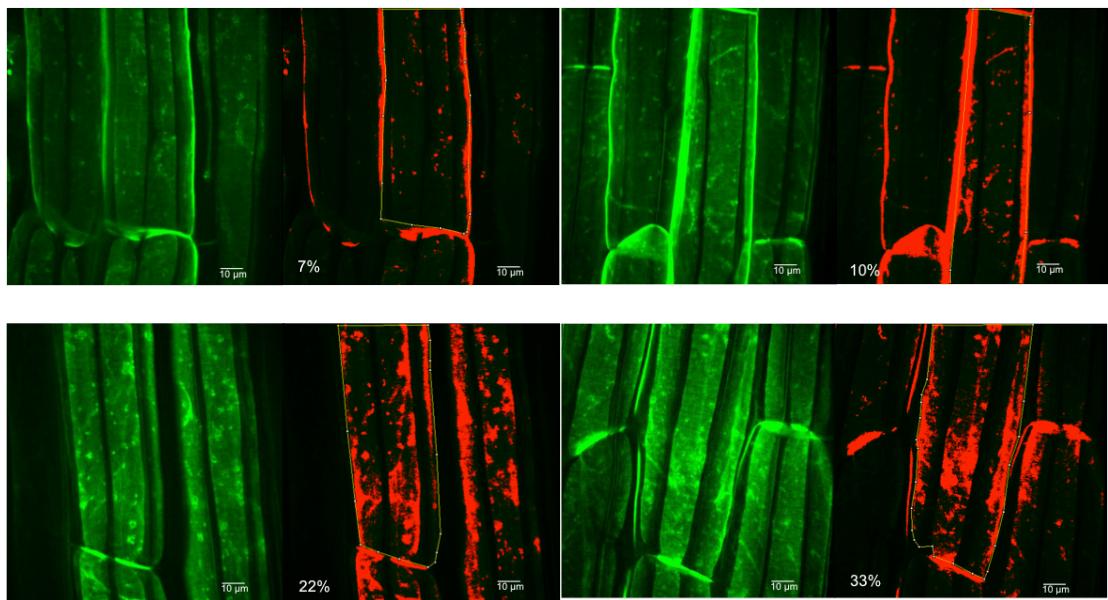


Figure 2.3 Maximum intensity projections of phot1-GFP fluorescence at the plasma membrane with corresponding heat maps of above average GFP fluorescence.

Maximum intensity projection of phot1-GFP fluorescence at plasma membrane of seedling irradiated with varying amounts of blue light and corresponding heat map of above average GFP fluorescence. FIJI was used to generate a maximum intensity projection of image stack taken at the plasma membrane. A corresponding heat map was generated in FIJI using the threshold function to determine regions of plasma membrane expressing above average GFP fluorescence. A region of interest was drawn around the plasma membrane using the freehand tool. Percentage indicates the percentage of the plasma membrane calculated by FIJI expressing above average GFP fluorescence.

The Blue Laser Induces phot1-GFP Movement During Imaging

Given that the excitation wavelength of the blue laser, 488 nm, generally used for GFP imaging falls within the activation spectrum of phot1, 300-500 nm, the blue laser could be introducing light contamination prior to imaging (Tsien 1998; Kasahara et al. 2002; Kaiserli 2009). Therefore, we speculate phot1-GFP movement can be initiated by the blue laser. In order to test this hypothesis, we devised an alternate technique that utilizes the green laser to aid in phot1-GFP imaging. The green wavelength of the green laser falls outside of the activation spectra of phot1 but still excites the GFP fluorophore (Tsien 1998; Kaiserli et al. 2009).

Generally, transmitted white light and the blue laser are used to position a sample for imaging introducing light contamination (Preuten et al. 2015). Here we imaged phot1-GFP using two different methods, both of which do not require transmitted white light. Prior to imaging, the green laser or the blue laser was used for sample setup. After sample setup, the blue laser was used to acquire images in both methods (Figure 2.4). The green laser was sufficient to visualize GFP signal for sample setup but imaging with the green laser yielded low quality images (data not shown).

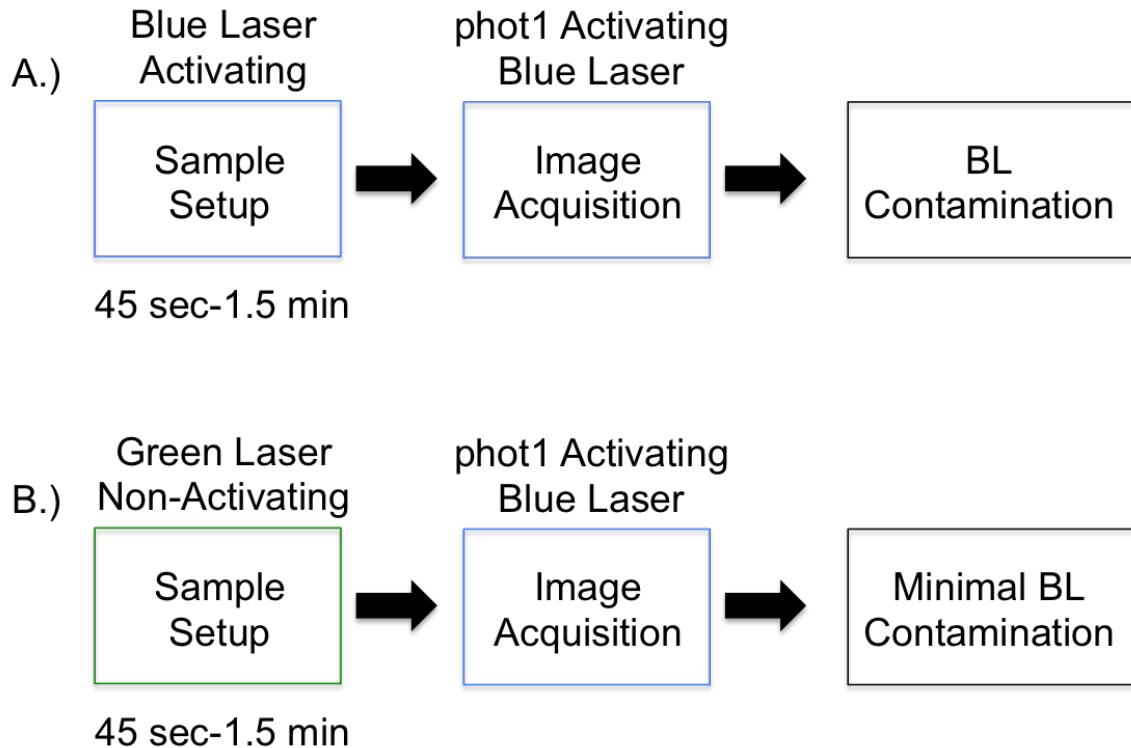


Figure 2.4 Experimental design for using the green laser or blue laser for sample setup.

The diagram illustrates the protocol for image setup and image acquisition. (A) The protocol traditionally used for imaging phot1-GFP involves using the blue laser for sample setup and image acquisition. This method may introduce blue light contamination via the blue laser during sample setup. (B) The modified protocol employs the green laser for sample setup and blue laser for image acquisition. Using the green laser for sample setup instead of the blue laser limits blue light contamination during imaging.

Our data shows using the blue laser for sample setup induced phot1-GFP movement (Figure 2.5). Therefore, we used a hybrid technique in which the green laser was used for sample setup and the blue laser was used only for imaging. As shown in figure 2.5, the hybrid technique induced little to no phot1-GFP movement. Interestingly, phot1-GFP foci were present in the *phot1-5 nph3-6* phot1-GFP line using both techniques (Figure 2.6). However, accumulation into foci was greater when the blue laser was used for sample setup.

In order to determine the amount of phot1-GFP movement at the plasma membrane, we used FIJI image processing software to quantify phot1-GFP accumulation into foci. As indicated by Figure 2.7, when the blue laser was used for sample setup phot1-GFP accumulation into foci occurred in the *phot1-5* phot1-GFP line and was enhanced in the *phot1-5 nph3-6* phot1-GFP. However, when the green laser was used for sample setup phot1-GFP accumulation into foci did not occur in the *phot1-5* phot1-GFP line and was reduced in the *phot1-5 nph3-6* phot1-GFP line.

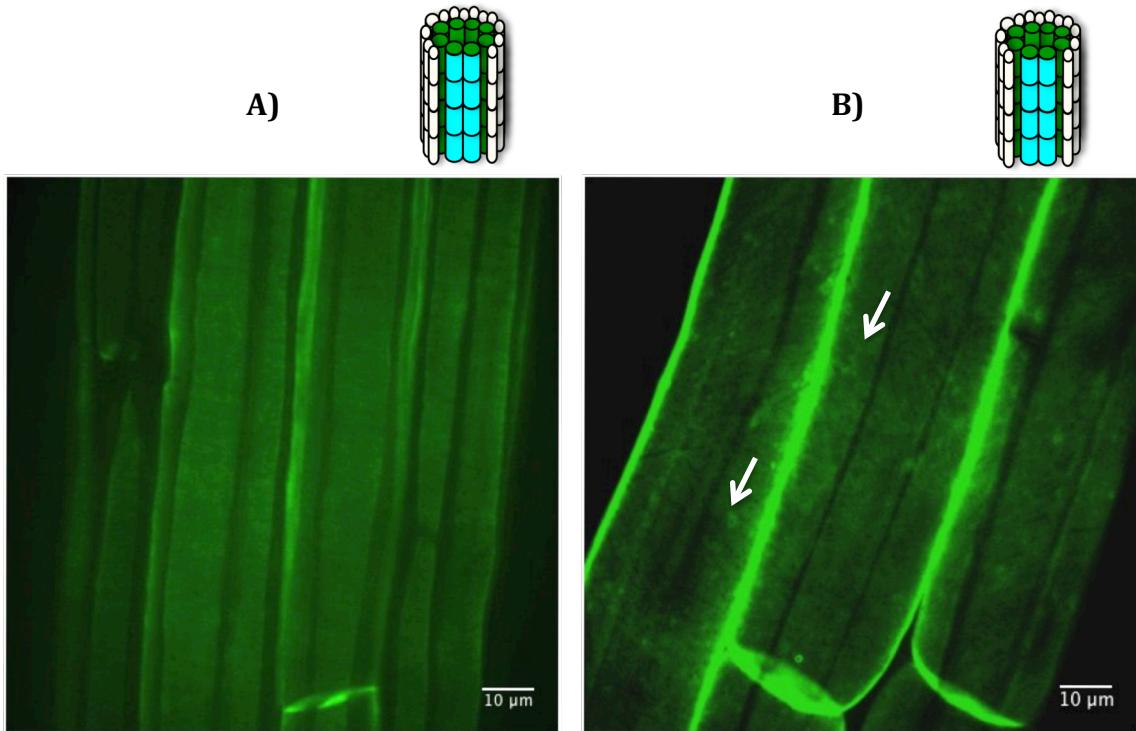


Figure 2.5 The blue laser induces phot1-GFP aggregation in the *phot1-5* phot1-GFP line.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP *Arabidopsis* seedlings taken from darkness. Using the green laser for sample setup shows little to no phot1-GFP accumulation into foci at the plasma membrane (A). However, using the blue laser for sample setup does show phot1-GFP accumulation into foci at the plasma membrane (B). Foci depicted by white arrows.

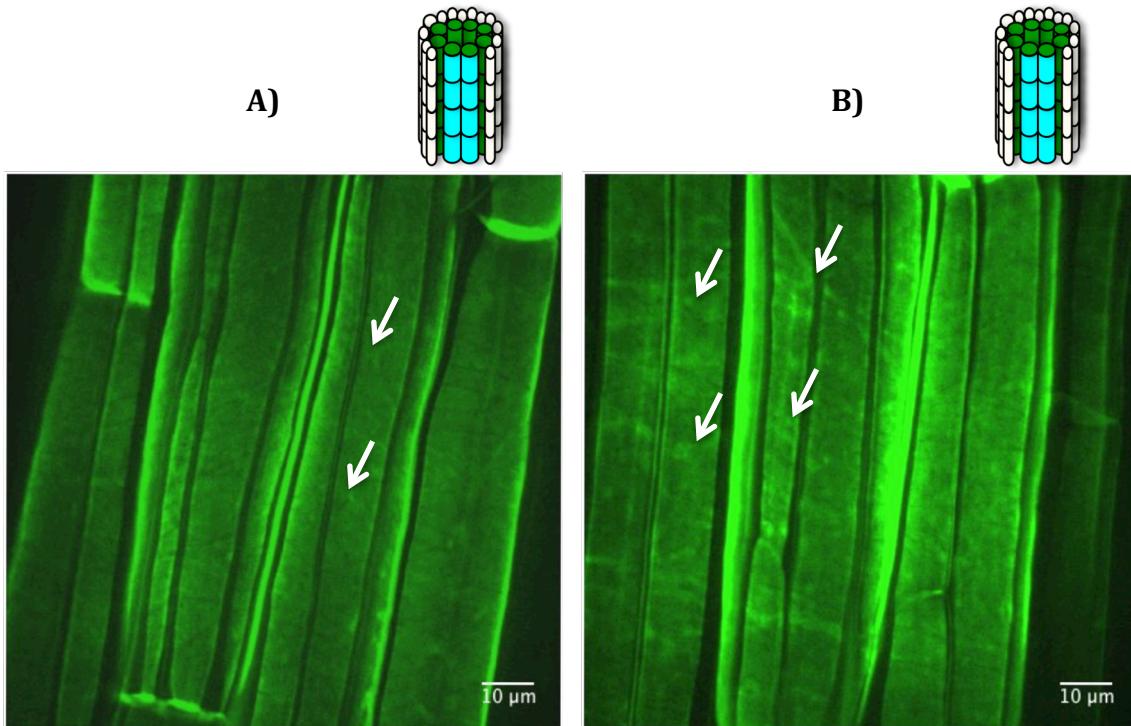


Figure 2.6 phot1-GFP accumulation into foci occurs in darkness in the *phot1-5 npn3-6 phot1-GFP* line and the blue laser enhances accumulation.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 npn3-6 phot1-GFP* *Arabidopsis* seedlings taken from darkness. Using the green laser for sample setup shows phot1-GFP accumulation of phot1-GFP into foci (A). Additionally, using the blue laser for sample setup shows an increase in accumulation of phot1-GFP into foci (B). Foci depicted by white arrows.

Green Vs. Blue Laser for Sample Setup

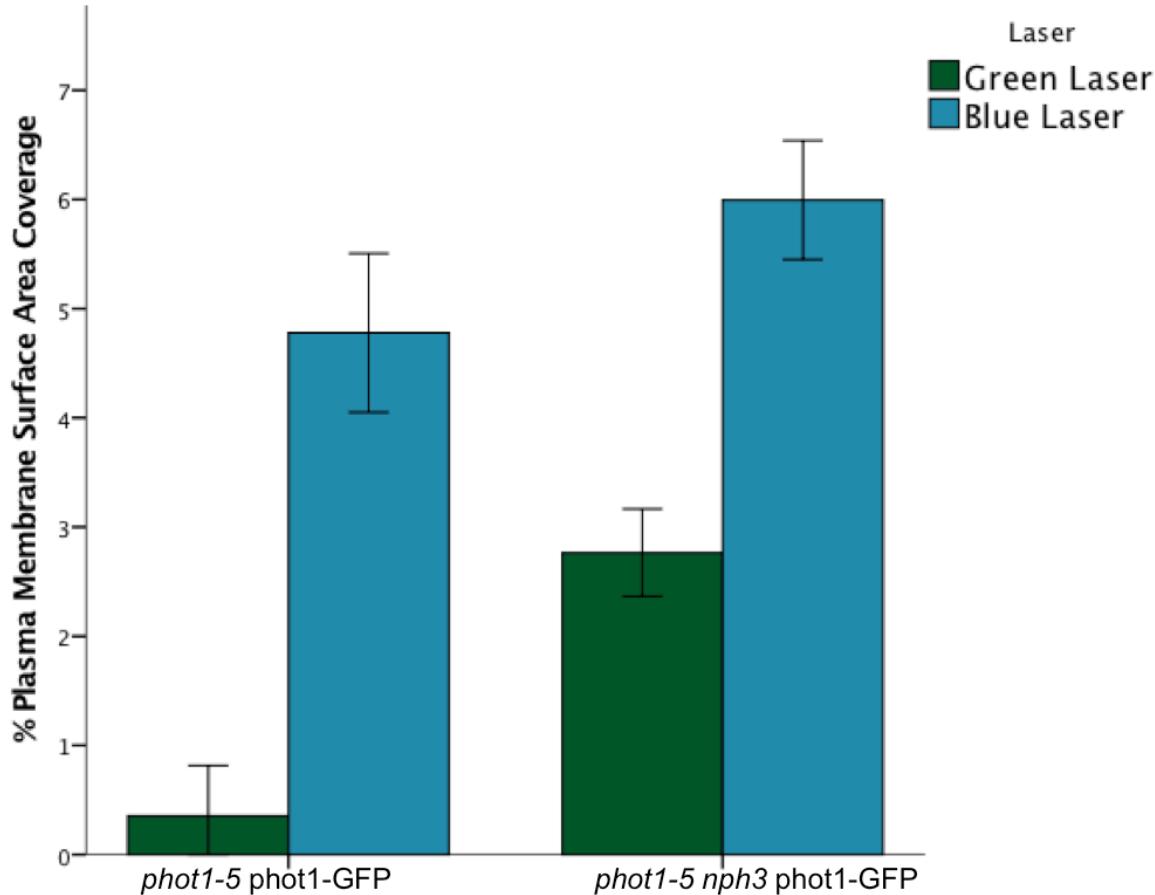


Figure 2.7 The blue laser initiates phot1-GFP accumulation into foci in the phot1-5 phot1-GFP line and increases foci accumulation in the phot1-5 nph3-6 phot1-GFP line.

phot1-GFP fluorescence was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP *Arabidopsis* seedlings taken from darkness. Image setup was performed using the green laser (green bars) or the blue laser (blue bars). Error bars represent 95% confidence interval of the mean.

Phot1-GFP Accumulation Into Foci at the Plasma Membrane Precedes Internalization

To date, phot1-GFP movement in response to blue light has been described mostly as internalization (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Preuten et al. 2015). We examined phot1-GFP movement in response to several minutes of exposure to the blue laser and were able to visualize phot1-GFP internalization as described before (Figure 2.8). However, prior to internalization we show phot1-GFP accumulates into foci at the plasma membrane. After prolonged exposure to the blue laser, the foci pool into long strand like structures parallel to the plasma membrane. The structures appear to flow laterally along the length of the cell until they reach the apex apical and basal borders. At the border between adjacent cells, the flow changes direction and begins to move toward the opposite apex of the cell. The movement appears to be phot1-GFP cycling within the cell.

Internalization of phot1-GFP is less frequent than the movement at the membrane and generally occurs near apical borders of the cell. Internalization occurs in long strands that emerge from one area of the plasma membrane and merge with an adjacent region of the plasma membrane (Figure 2.8). Intracellular movement did occur away from apical borders but was less frequent and did not appear to interact with internal structures. Rather, internalized phot1-GFP signal would continue through the cell until it reached an adjacent side of the plasma membrane. Unfortunately, due to the three-dimensional shape of the cells and rapid

movement of the phot1-GFP signal, this was difficult to capture in a single image or z-stack.

The movement of phot1-GFP was almost identical in the *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP lines. The only difference we observed was that phot1-GFP foci appeared more quickly and in greater numbers in the *phot1-5 nph3-6* phot1-GFP line (Figure 2.9). This suggests that although NPH3 dependent ubiquitination of phot1 is a necessary step in the signaling cascade that leads to the phototropic response, it does not appear to be necessary for phot1-GFP aggregation or internalization in response to blue light.

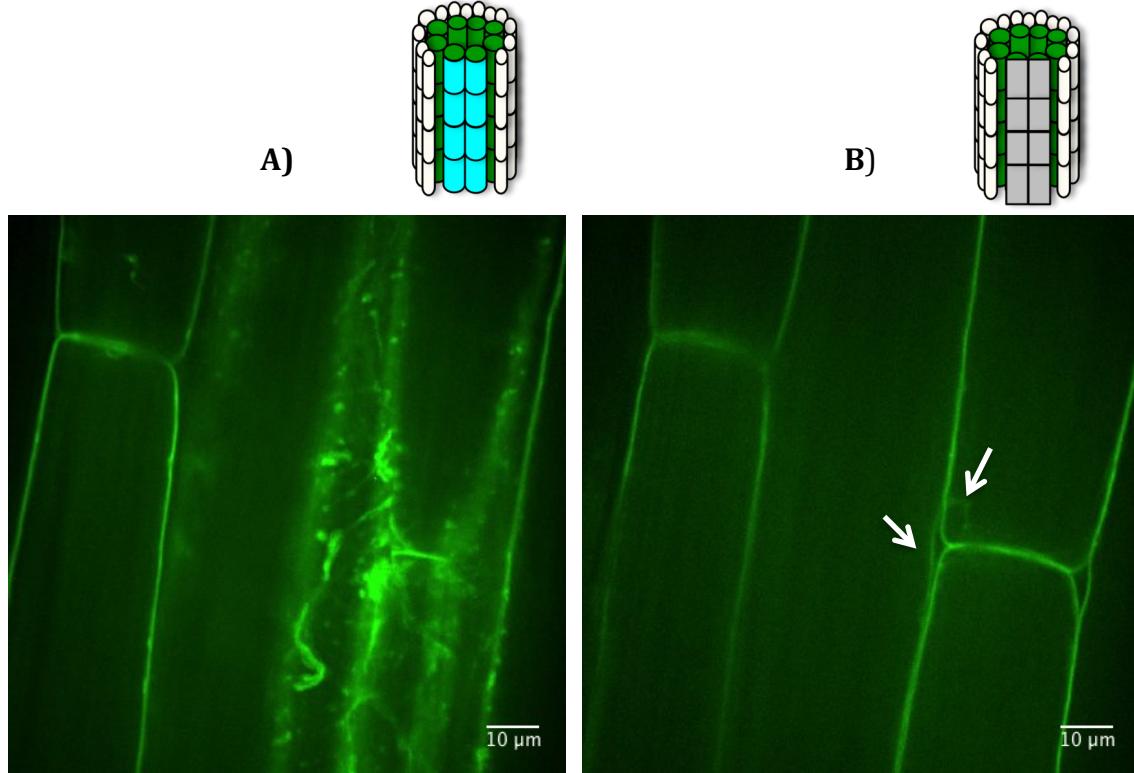


Figure 2.8 phot1-GFP accumulates into foci at the plasma membrane prior to becoming internalized.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP *Arabidopsis* seedlings. Seedlings were imaged after several minutes of exposure to the blue laser. Images were taken at the plasma membrane adjacent to the epidermal layer (A) and approximately 20 μm within the cortical cells (B). White arrows indicate internalized phot1-GFP merging with adjacent region of the plasma membrane.

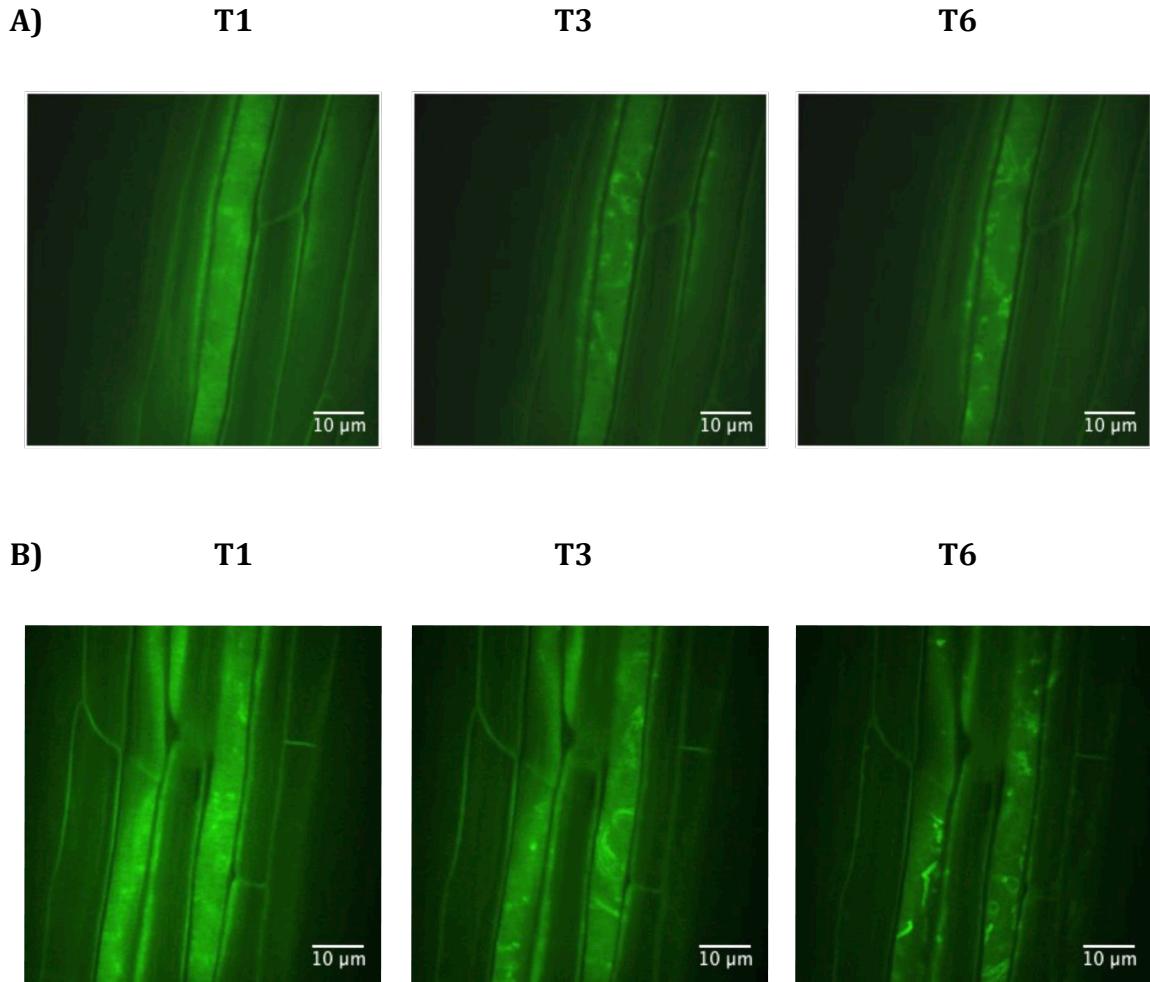


Figure 2.9 *phot1*-GFP foci form more quickly in the *phot1-5 nph3-6* *phot1*-GFP line than in the *phot1-5* *phot1*-GFP line.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* *phot1*-GFP (A) and *phot1-5 nph3-6* *phot1*-GFP (B) *Arabidopsis* seedlings. Each series represents an image taken after one minute, three minutes and six minutes of exposure to blue laser respectively. Accumulation of *phot1*-GFP into foci occurs more quickly in the *phot1-5 nph3-6* *phot1*-GFP line.

Accumulation of phot1-GFP Into Foci at the Plasma Membrane is Fluence Dependent in Both Lines and is Greater in the *phot1-5 nph3-6* phot1-GFP Line Relative to *phot1-5* phot1-GFP Line

Wan et al. (2008) reported that the time of onset of internalization of phot1-GFP is dependent on the total fluence of irradiation by blue light. Their data indicated that after exposure to 100 $\mu\text{mol m}^{-2}$ fluence of blue light no phot1-GFP internalization was detectable after up to 100 minutes post irradiation. Here we show that in *phot1-5* phot1-GFP seedlings reorganization of phot1-GFP at the plasma membrane occurs within five minutes at 100 $\mu\text{mol m}^{-2}$ fluence blue light. In accordance with Wan et al. (2008), the degree to which the reorganization occurs is fluence dependent (Figure 2.10).

In order to support our claims that phot1-GFP reorganization and accumulation into foci is dependent on fluence, accumulation into foci was measured using FIJI software. The results were then graphed using DeltaGraph. Seedlings were subjected to 100 $\mu\text{mol m}^{-2}$, 1,000 $\mu\text{mol m}^{-2}$, 2,000 $\mu\text{mol m}^{-2}$ or 10,000 $\mu\text{mol m}^{-2}$ fluence of blue light at a moderate fluence rate of 5 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$. Seedlings were returned to darkness for 5, 15 or 25 minutes post blue light irradiation prior to imaging to observe the accumulation of phot1-GFP into foci over time in response to controlled amounts of blue light irradiation. The graphs show that phot1-GFP accumulation into foci appears to be fluence dependent and that accumulation is enhanced in the *phot1-5 nph3-6* phot1-GFP line. Accumulation into foci of phot1-GFP peaked at around 15 minutes post irradiation in *phot1-5* phot1-GFP lines and began to subside. However, in *phot1-5 nph3-6* phot1-GFP lines,

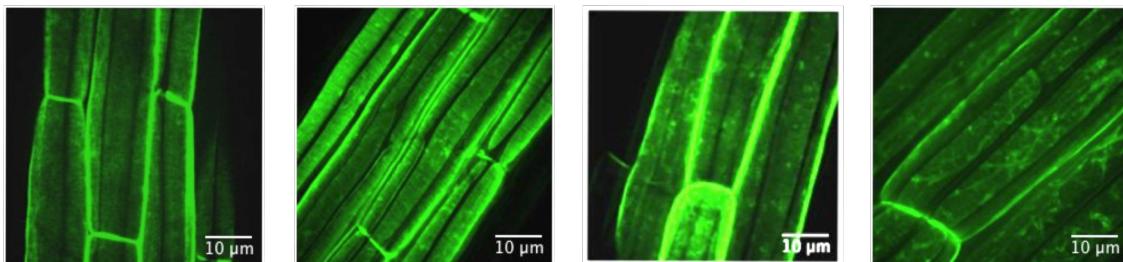
accumulation continued to increase until 25 minutes post irradiation before subsiding (Figure 2.11).

Next we investigated whether the pattern of *phot1*-GFP aggregation was similar under higher fluence rates of blue light. This was done as described above with a moderate to high fluence rate of $10 \text{ umol m}^{-2} \text{ sec}^{-1}$ (Figure 2.12), and a high fluence rate of $20 \text{ umol m}^{-2} \text{ sec}^{-1}$ (Figure 2.13). Additional time points of 35 and 45 minutes in darkness post blue light irradiation were measured. Again, accumulation peaked at 15 minutes post blue light irradiation in the *phot1-5* *phot1*-GFP line and at between 25 minutes in the *phot1-5 nph3-6* *phot1*-GFP line. Accumulation into foci was again higher in the *phot1-5 nph3-6* *phot1*-GFP line. The images of *phot1*-GFP fluorescence show an increase in *phot1*-GFP aggregation with increasing total fluence and foci are visibly higher in the *phot1-5* *phot1*-GFP line (Figure 2.14).

A)

100 umol m⁻² **1,000 umol m⁻²** **2,000 umol m⁻²** **10,000 umol m⁻²**

2



B)

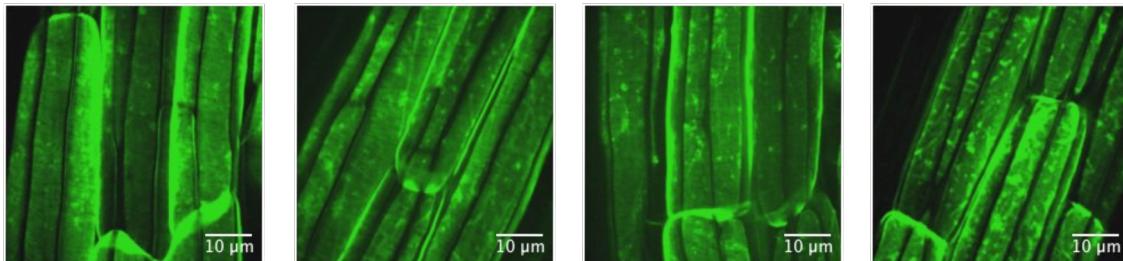


Figure 2.10 phot1-GFP reorganization at the membrane occurs within minutes of blue light irradiation and increases with fluence.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP (A) and *phot1-5 nph3-6* phot1-GFP (B) *Arabidopsis* seedlings. Each series represents images taken after five minutes in darkness post blue light irradiation at a fluence rate of 5 $\text{umol m}^{-2} \text{ sec}^{-1}$ to yield a total fluence of 100 umol m^{-2} , 1,000 umol m^{-2} , 2,000 umol m^{-2} or 10,000 umol m^{-2} respectively. Images taken at plasma membrane adjacent to epidermal cell layer.

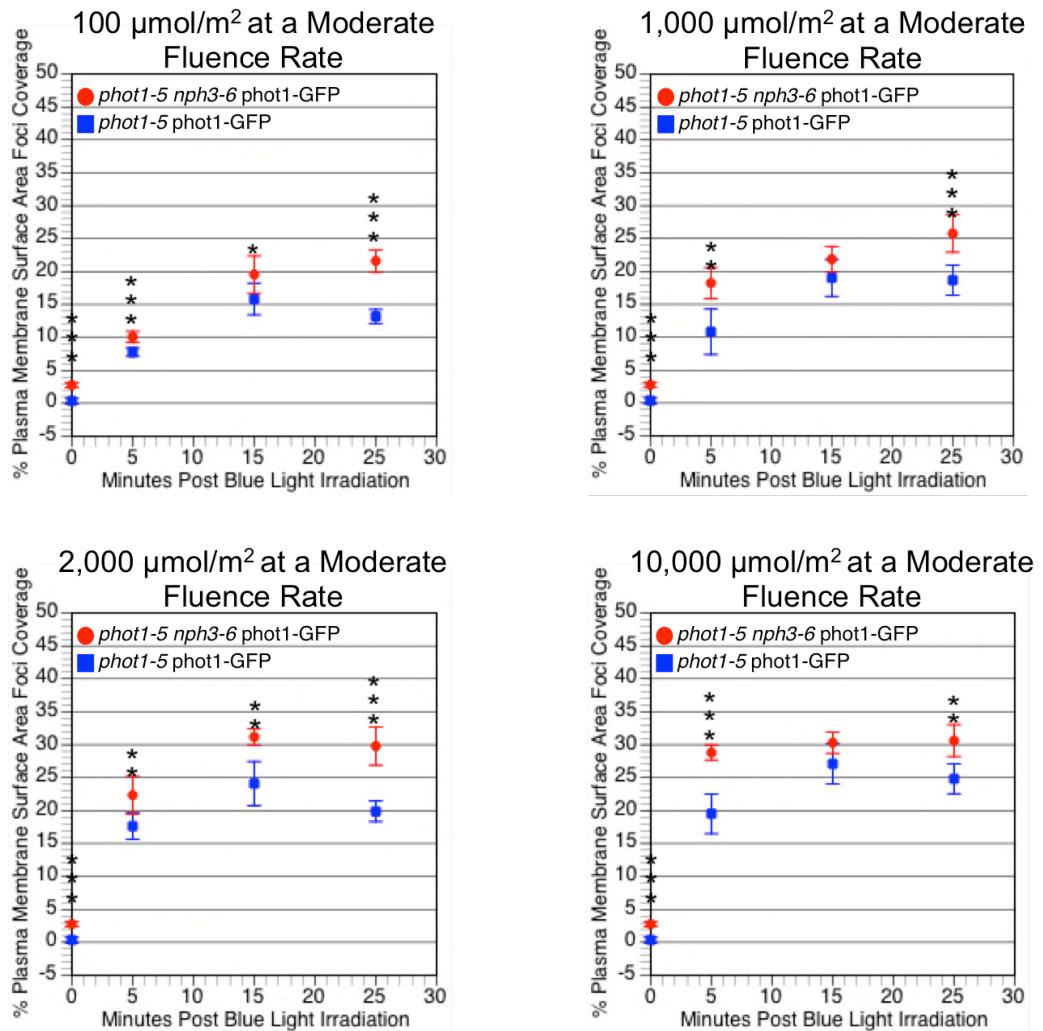


Figure 2.11 phot1-GFP accumulation into foci is fluence dependent at a moderate fluence rate.

phot1-GFP aggregation at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 phot1-GFP* (blue squares) and *phot1-5 nph3-6 phot1-GFP* (red circles) *Arabidopsis* seedlings. Seedlings were treated with the indicated fluence of blue light at a moderate fluence rate of $5 \text{ umol m}^{-2} \text{ sec}^{-1}$ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation into foci

at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) and genotype ($p \leq 0.001$) for all total fluences. Significant differences between genotypes were determined using a 2-tailed t-test for equality of means *post-hoc* test (p , * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001).

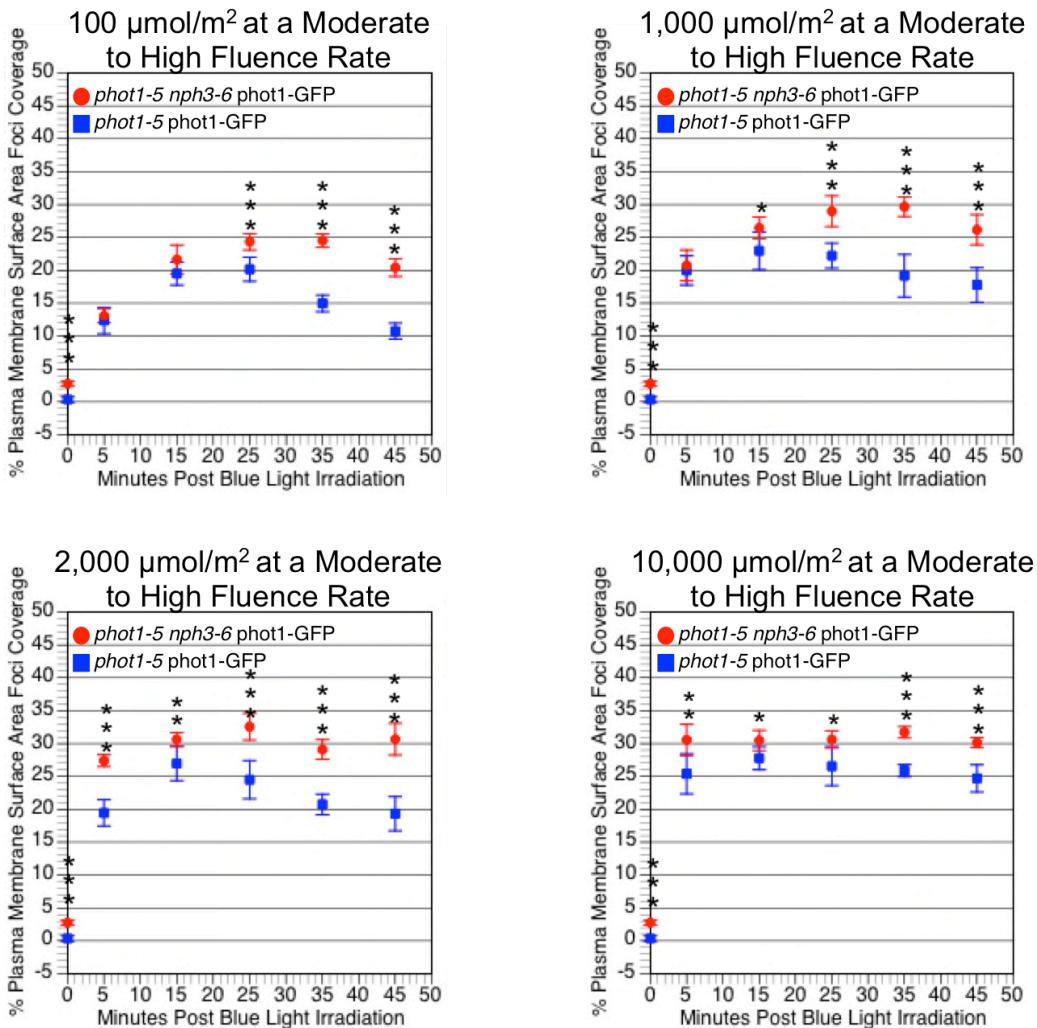


Figure 2.12 phot1-GFP accumulation into foci is fluence dependent at a moderate to high fluence rate.

phot1-GFP aggregation at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 phot1-GFP* (blue squares) and *phot1-5 nph3-6 phot1-GFP* (red circles) *Arabidopsis* seedlings. Seedlings were treated with the indicated fluence of blue light at a moderate to high fluence rate of 10 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation

into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) and genotype ($p \leq 0.001$) for all total fluences. Significant differences between genotypes were determined using a 2-tailed t-test for equality of means *post-hoc* test (p , * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.005).

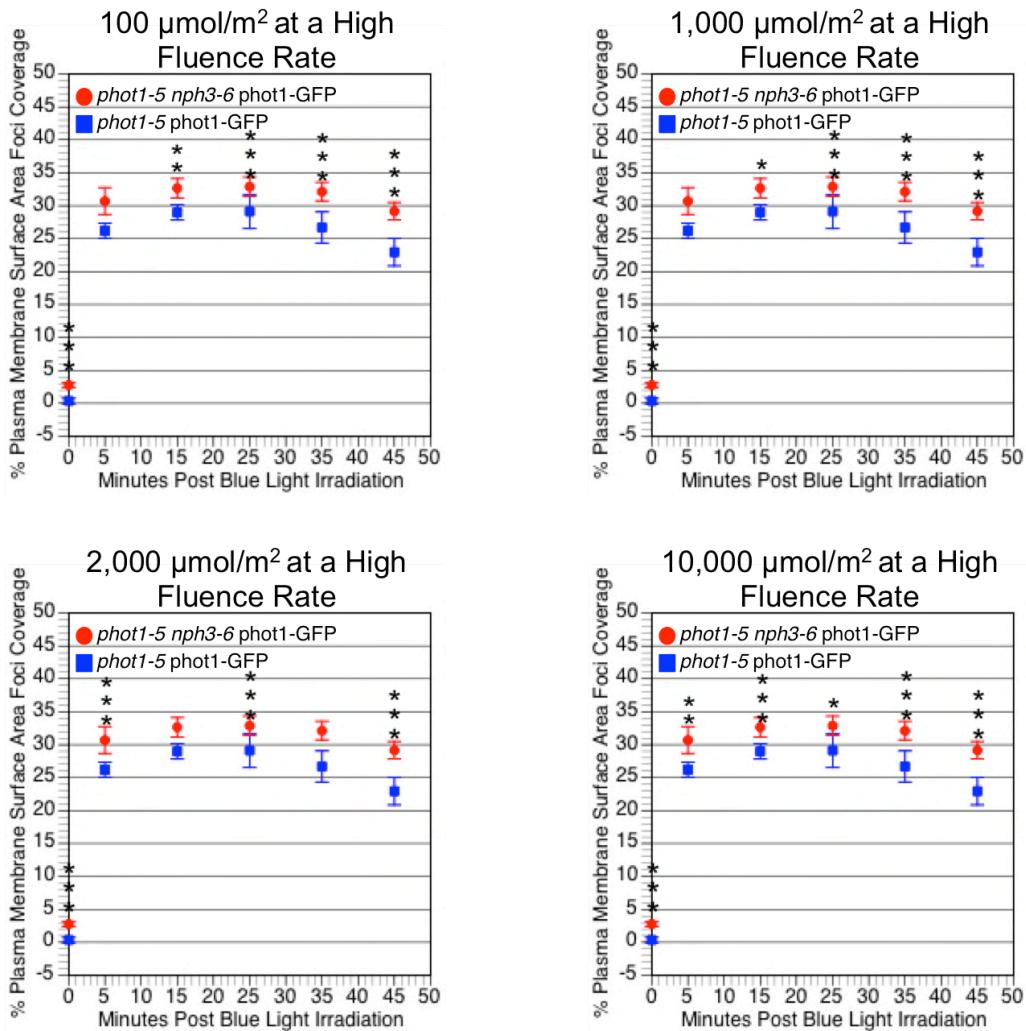


Figure 2.13 phot1-GFP accumulation into foci is fluence dependent at a high fluence rate.

phot1-GFP aggregation at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 phot1-GFP* (blue squares) and *phot1-5 nph3-6 phot1-GFP* (red circles) *Arabidopsis* seedlings. Seedlings were treated with the indicated fluence of blue light at a high fluence rate of 20 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation into foci at

the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) and genotype ($p \leq 0.001$) for all total fluences. Significant differences between genotypes were determined using a 2-tailed t-test for equality of means *post-hoc* test (p , * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001).

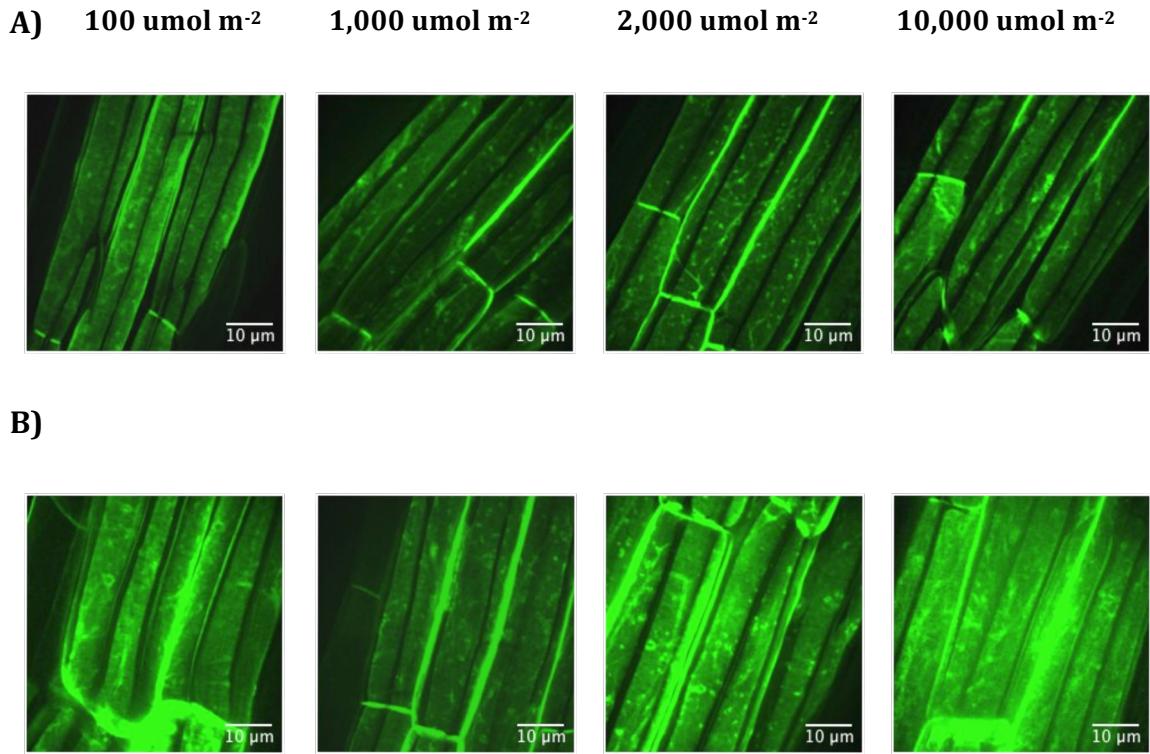


Figure 2.14 phot1-GFP accumulation into foci increases with fluence and is enhanced in the *phot1-5 nph3-6* phot1-GFP.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP (A) and *phot1-5 nph3-6* phot1-GFP (B) *Arabidopsis* seedlings. Each series represents images taken after 15 minutes in darkness post blue light irradiation at a fluence rate of 10 $\text{umol m}^{-2} \text{ sec}^{-1}$ to yield a total fluence of 100 umol m^{-2} , 1,000 umol m^{-2} , 2,000 umol m^{-2} or 10,000 umol m^{-2} respectively. Images taken at plasma membrane adjacent to epidermal cell layer.

Discussion

Previous reports on phot1-GFP movement in response to blue light irradiation have focused on internalized signal (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Preuten et al. 2015). Although Wan et al. (2008) alludes to phot1-GFP reorganization at the plasma membrane in response to blue light the movement at the plasma membrane has yet to be characterized. Here we report that phot1-GFP accumulates into foci at the plasma membrane in response to blue light and the formation of foci precedes internalization (figure 2.8). Our data further shows that the internalization of phot1-GFP in response to blue light does not target an internal structure as previously suggested (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009). Instead, internalized phot1-GFP signal appears to target adjacent regions of the plasma membrane. This suggests that phot1-GFP internalization is a cycling event and rather than being involved in signaling may represent a method of receptor desensitization and recycling.

Identifying phot1-GFP accumulation into foci at the plasma membrane also allowed repeatable measurements of phot1-GFP reorganization in response to blue light. Internalization of phot1-GFP in response to blue light varies greatly and is difficult to image consistently. However, the foci that accumulate at the plasma membrane in response to blue light are predictable and imaging can be performed consistently making foci an ideal target for measurements. Our initial measurements of phot1-GFP accumulation into foci at the plasma membrane supported a caveat introduced by Preuten et al. (2015) in imaging phot1-GFP

movement, that the blue laser used to excite the GFP fluorophore induces phot1-GFP activation and reorganization (figure 2.7).

Traditionally, sample setup for confocal microscopy involves transmitted white light to position the sample over the objective and excitation of phot1-GFP for further positioning and imaging with the blue laser (Preuten et al. 2015). Given that phot1 is a blue light photoreceptor sample setup for confocal microscopy introduces light contamination during imaging. Our method introduced here excluding transmitted light and utilizing the green laser for sample setup allows for accurate imaging of phot1-GFP movement without introducing light contamination. Figure 2.7 shows that using the green laser for sample setup eliminates phot1-GFP accumulation into foci in darkness for the *pho1-5* phot1-GFP line. However, although using the green laser reduces the amount of foci that appear in the *phot1-5 nph3-6* phot1-GFP line some accumulation still occurs. This suggests a difference in the movement or abundance of phot1-GFP in the NPH3 deficient line.

Further analysis of the amount of phot1-GFP accumulation into foci in response to blue light supports previous work by Wan et al. (2008) that suggests phot1-GFP movement is fluence dependent (Figures 2.11, 2.12 and 2.13). The data also supports that phot1-GFP accumulation into foci is consistently higher in the *phot1-5 nph3-6* phot1-GFP line. This suggests that NPH3 may be involved in the ability of phot1-GFP to accumulate into foci in response to blue light or that NPH3 influences the abundance of phot1-GFP. Roberts et al. (2011) showed that NPH3 acts as a substrate adaptor for an E3 ubiquitination complex that ubiquitinates phot1 in response to blue light irradiation. Additionally, high light initiates

polyubiquitination of phot1 which leads to degradation in the proteasome (Roberts et al. 2012). This suggests that the increased accumulation phot1-GFP foci that appear in darkness and in response to blue light may be a result of elevated levels of phot1-GFP present in the NPH3 null line. This hypothesis will be tested in the next group of experiments using the proteasome inhibitor MG132 to determine if preventing phot1-GFP degradation in the *phot1-5* phot1-GFP line increases the amount of foci that appear in response to blue light irradiation.

Chapter 3

MODULATING PHOT1-GFP ABUNDANCE AND MOVEMENT AT THE PLASMA MEMBRANE

Abstract

Previously we showed that shortly after activation by blue light phot1-GFP accumulates into foci at the plasma membrane. The function of these foci is currently unknown but could represent active signaling centers or a method of receptor desensitization. The accumulation of phot1-GFP into foci appears to be greater in *Arabidopsis* seedlings that lack the NPH3 protein (*phot1-5 nph3-6 phot1-GFP*) than in seedlings possessing NPH3 (*phot1-5 phot1-GFP*). Here we show using the proteasomal inhibitor MG132 that the elevated level of phot1-GFP foci that appear at the plasma membrane in the line lacking NPH3 is due to receptor abundance. Additionally, pre-treating seedlings with red light, known to enhance phototropism, and treatment with low fluence blue light, 0.1 umol m^{-2} , reduces the amount of foci that form in response to blue light. This suggests that the accumulation of phot1-GFP into foci in response to blue light is a means of receptor desensitization and that preventing foci from forming may prolong signaling.

Introduction

The blue light photoreceptor phototropin 1 (phot1) localizes to the plasma membrane in etiolated *Arabidopsis* seedlings (Gallagher et al. 1998; Sakamoto and Briggs 2002; Christie 2007). Upon activation by blue light, GFP tagged phot1 (phot1-

GFP) has been reported to become internalized to a yet to be determined intracellular target (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Roberts 2012). This is supported by work done by Sakamoto and Briggs (2002) showing that a small portion of phot1 appears in the soluble fraction following blue light irradiation. Kaiserli et al. (2009) showed that autophosphorylation of phot1 precedes receptor internalization, which is necessary for receptor signaling as well (Inoue et al. 2008). Ubiquitination of phot1 is also necessary for receptor signaling and is dependent on the plasma membrane localized protein NONPHOTOTROPIC HYPOCOTYL3 (NPH3) (Motchoulski and Liscum 1999; Pedmale and Liscum 2007; Roberts et al. 2011).

Ubiquitination of a receptor can influence its function and location within a cell (Miranda and Sorkin 2007; Haglund and Dikic 2012). NPH3 has been shown to interact with phot1 at the plasma membrane and act as a substrate adaptor for a cullin-RING-based E3 ubiquitin-protein ligase complex (CRL3^{NPH3}) responsible for ubiquitinating phot1 in response to blue light (Motchoulski and Liscum 1999; Roberts et al. 2011). Roberts et al. (2011) showed that phot1 is monoubiquitinated under low light conditions and polyubiquitinated under high light conditions. The group hypothesized that monoubiquitinated phot1 represented the active signaling form where polyubiquitination marked the receptor for degradation in the proteasome (Roberts et al. 2011). Although ubiquitination has been shown to influence receptor internalization in other models, the role of ubiquitination in phot1 movement in response to blue light has yet to be determined (Miranda and Sorkin 2007; Chen and Sun 2009, Haglund and Dikic 2012).

Previous reports using crude microsomal fractionation indicate that in darkness phot1 is found exclusively in the membrane fraction but upon blue light irradiation appears in the soluble fraction (Sakamoto and Briggs 2002; Krieb 2004; Roberts 2012). Roberts (2012) reported that phot1 does not move into the soluble fraction in response to blue light in *Arabidopsis* lines lacking NPH3. However, confocal imaging shows that phot1-GFP is still internalized in response to blue light in seedlings lacking NPH3 (Roberts 2012). This is supported by our data presented in Chapter 2 where phot1-GFP accumulates into foci at the plasma membrane and is internalized in *Arabidopsis* lines possessing and lacking NPH3. We further showed that phot1-GFP accumulation into foci was enhanced in the lines lacking NPH3. Due to the inability of NPH3 deficient lines to undergo NPH3 dependent degradation of phot1 in the proteasome we postulated that the enhanced phot1-GFP accumulation into foci could be due to an abundance of phot1-GFP. One of the goals of this study is to determine if inhibiting proteasomal degradation of phot1-GFP will increase the amount of foci measured at the plasma membrane in response to blue light irradiation. The remainder of the study will focus on the function of the foci that form in response to blue light.

The role of phot1 movement in signaling has been a topic of discussion for many years (Liscum 2016). In fact, it has been suggested that retention of phot1 at the plasma membrane may enhance phototropism (Han et al. 2008). Han et al. (2008) reported that pre-treating *Arabidopsis* seedling with red light prevents phot1-GFP internalization, a technique that has also been shown enhance the phototropic response (Curry 1957; Zimmerman and Briggs 1967; Han et al. 2008;

Sullivan et al. 2016). Han et al. (2008) hypothesized that phot1 retention at the membrane may contribute to the enhanced phototropic response. However, recent research suggests otherwise.

Preuten et al. (2015) constructed lipid-anchored phot1-GFP constructs that they reported were not internalized in response to blue light irradiation. The lipid anchored phot1-GFP constructs complemented the *phot1-5* mutant phenotype and were degraded over time in response to prolonged blue light irradiation (Preuten et al 2015). This agrees with earlier reports by Han et al. (2008) that phot1-GFP retention at the membrane does not prevent phototropism, however, tethering phot1 to the membrane did not enhance the phototropic response (Preuten et al. 2015). Neither Preuten et al. (2015) nor Han et al. (2008) mention if their methods of preventing internalization of phot1-GFP also prevents aggregation at the plasma membrane. Although we do not currently posses lipid anchored phot1-GFP constructs, we can test the affect that red light pre-treatment has on phot1-GFP aggregation in response to blue light to further Asses the function of the foci that form at the plasma membrane in response to blue light.

Lastly, to date the experiments performed to visualize phot1-GFP movement in response to light have focused on high fluence rates of light ($20\text{-}25 \text{ umol m}^{-2} \text{ sec}^{-1}$) when the phototropic response is not necessary for a plant to collect solar energy (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaisereli et al. 2009; Roberts 2012; Preuten et al. 2015). Here we investigate the effects of low fluence rate light ($0.1 \text{ umol m}^{-2} \text{ sec}^{-1}$), when the phototropic response would be necessary for optimal light collection, on phot1-GFP movement. Our goal is to construct a

model for phot1-GFP movement based on the data presented in this dissertation to describe the possible function of phot1 movement in response to blue light.

Experimental procedures

Plant materials and growth conditions

Plant materials and growth conditions were performed as described in Chapter 2. Red light pre-treated seedlings were either mock irradiated or treated with 10 seconds of 5 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$, to equal a total of 50 $\mu\text{mol m}^{-2}$ fluence, as described previously (Han et al. 2008). MG132 pre-treated seedlings were subjected to 2 hours of MG132 (Adipogen AG-CP3-0011-M005) treatment prior to blue light irradiation. A 50 μmol MG132 working solution was made by diluting 10 μl of 50 mmol MG132 dissolved in DMSO (Sigma D4540) in 10 ml of half-strength Murashige and Skoog liquid medium and pouring onto plates housing seedlings. For DMSO treated control seedlings, 10 μl of DMSO was placed into 10 ml of half-strength Murashige and Skoog liquid medium and poured onto plates housing seedlings. Plates were then placed on shaker (Lablive Orbit Shaker) at 60 RPM in darkness.

Light treatment of seedlings

Prior to imaging on the confocal microscope, seedlings were either mock treated or irradiated with unilateral blue light at a total fluence of 100 $\mu\text{mol m}^{-2}$, 1,000 $\mu\text{mol m}^{-2}$, 2,000 $\mu\text{mol m}^{-2}$ or 10,000 $\mu\text{mol m}^{-2}$. Total fluence was achieved at a fluence rate of 0.1 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$ (low fluence rate light), 10 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$ (moderate to high fluence rate light) or 20 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$ (high fluence rate light).

Seedlings were light treated by fluorescent lighting through one layer of blue acrylic (Rohm and Haas No. 2424, 3.18Mmm thick; Cope Plastics) or red acrylic (Rohm and Haas No. 43196, 3.18Mmm thick; Cope Plastics). Seedlings were then placed in darkness for 5, 15, 25, 35 or 45 minutes prior to mounting on slides in distilled water and imaged on confocal microscope.

Confocal microscopy

Confocal microscopy was performed as described in Chapter 2, however, the green laser (515 nm) was used for positioning and the blue laser (488 nm) was used for image acquisition in every experiment.

Fluorescence measurements, graphical representation and statistics

Measurements of phot1-GFP accumulation into foci, graphical representation and statistics were performed as described in Chapter 2.

Results

MG132 Induces phot1-GFP Accumulation Into Foci in Darkness

The tripeptide aldehyde N-Benzoyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal (MG132) is a potent inhibitor of the proteasome and is an effective agent to prevent protein degradation (Tsubuki et al. 1996). Pre-treatment of etiolated *Arabidopsis* seedlings with MG132 was performed to demonstrate if accumulation of phot1-GFP into foci in the *pho1-5* phot1-GFP line would be increased as a result of preventing phot1-GFP degradation in the proteasome. Previously, we showed that phot1-GFP is homogeneously distributed at the plasma membrane in darkness in the *phot1-5* phot1-GFP (Figure 2.5). In contrast, phot1-GFP in the *phot1-5 nph3-6* phot1-GFP line

accumulates into foci occurs in darkness (Figure 2.6). We postulated that NPH3 may influence receptor abundance and seedlings lacking NPH3 therefore exhibit an increase in *phot1*-GFP accumulation into foci.

Our results show that in *phot1-5* *phot1*-GFP lines pre-treated with MG132, *phot1*-GFP accumulation appears to occur in darkness (Figure 3.1). In fact, pre-treating *phot1-5* *phot1*-GFP lines with MG132 brought the amount of *phot1*-GFP accumulation in darkness to a level similar to that of the *phot1-5* *nph3-6* *phot1*-GFP line (Figure 3.2). The DMSO vehicle control had no apparent effect on *phot1*-GFP accumulation into foci of the *phot1-5* *phot1*-GFP line (Figure 3.1 and 3.2). This suggests that NPH3 may contribute to the maintenance of *phot1* levels in darkness and that increased accumulation may be due to an abundance of *phot1*-GFP in seedlings lacking NPH3.

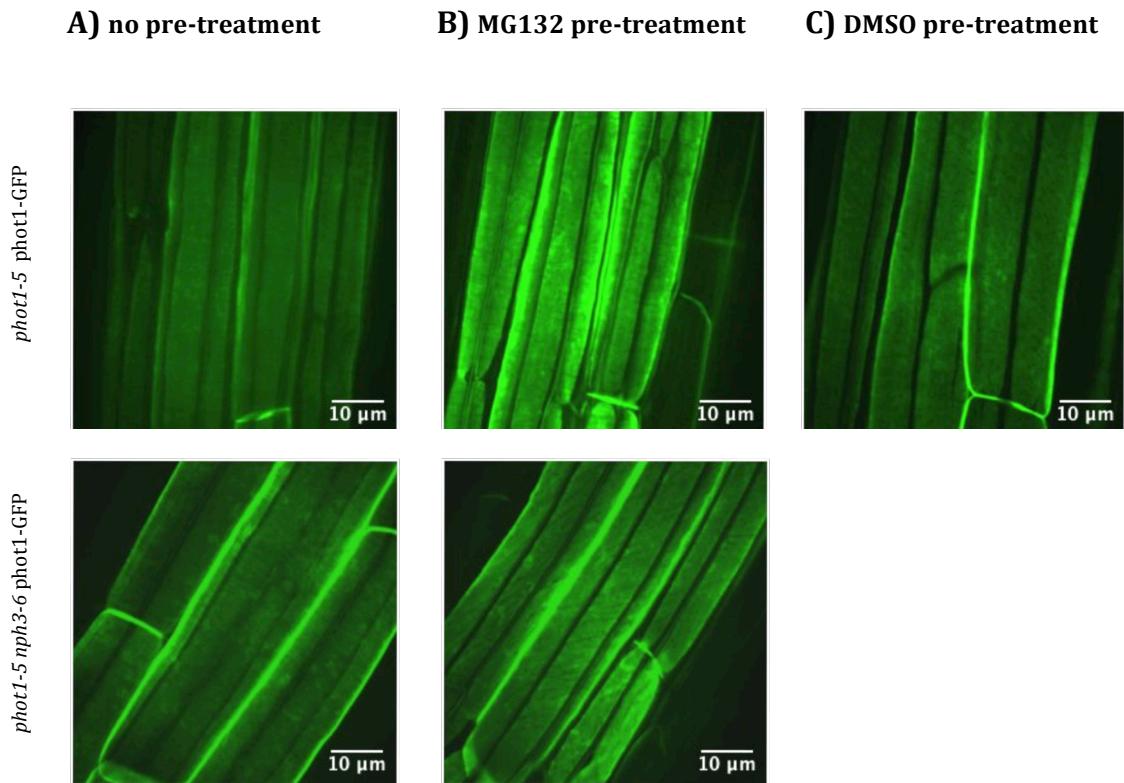


Figure 3.1 phot1-GFP accumulates into foci in darkness in response to MG132 treatment.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP *Arabidopsis* seedlings taken from darkness. Seedlings were mock treated (A), pre-treated with the proteasome inhibitor MG132 2 hours prior to imaging (B) or pre-treated with the vehicle control DMSO 2 hours prior to imaging (C). Images represent maximum intensity projections of the plasma membrane of cortical cells adjacent to the epidermal layer.

phot1-GFP Accumulation Into Foci in Darkness

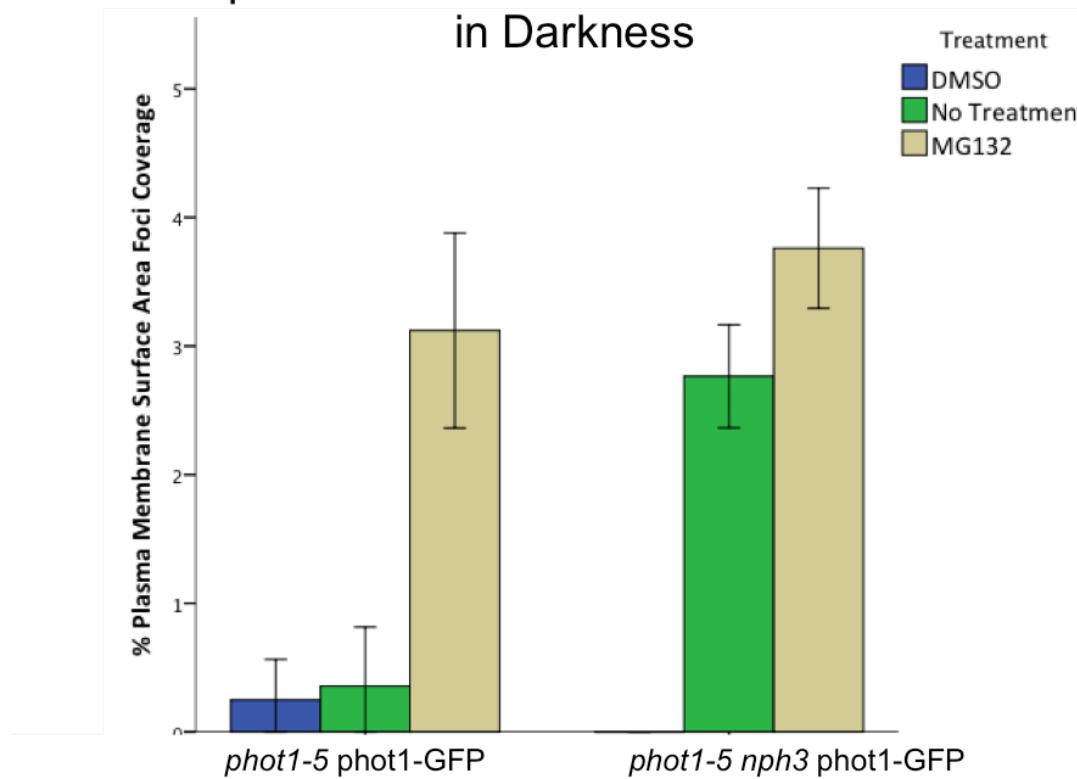


Figure 3.2 *phot1-GFP* accumulates into foci in darkness in *phot1-5* *phot1-GFP* seedlings pre-treated with MG132.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* *phot1-GFP* and *phot1-5 nph3-6* *phot1-GFP* *Arabidopsis* seedlings taken from darkness. Seedlings were treated with the vehicle control DMSO 2 hours prior to imaging (blue bar), mock treated (green bars) or pre-treated with 50 mmol MG132 two hours prior to imaging (yellow bars). Each bar represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent 95% confidence interval of the mean.

phot1-GFP Accumulation Into Foci in Response to Blue Light Irradiation is Greater in *phot1-5* phot1-GFP Seedlings Pre-treated with MG132

Previously, we showed that phot1-GFP accumulation in response to blue light irradiation at the plasma membrane was greater in the *phot1-5 nph3-6* phot1-GFP line than the *phot1-5* phot1-GFP line (Figures 2.11, 2.12 and 2.13). Here we show that pre-treatment with MG132 of the *phot1-5* phot1-GFP line appears to increase the accumulation of phot1-GFP in response to blue light irradiation but pre-treating with the vehicle control DMSO does not (Figure 3.3). Pre-treating the *phot1-5 nph3-6* phot1-GFP line with MG132 had little to no effect on phot1-GFP accumulation at the membrane in darkness or in response to blue light irradiation. According to quantitative data, pre-treating the *phot1-5* phot1-GFP line with MG132 enhances phot1-GFP accumulation at the membrane similar to the level of the *phot1-5 nph3-6* phot1-GFP line mock treated and pre-treated with MG132 (Figure 3.4). Taken together, this supports that enhanced accumulation of phot1-GFP into foci in response to blue light irradiation could be due NPH3 dependent maintenance of the abundance of phot1-GFP.

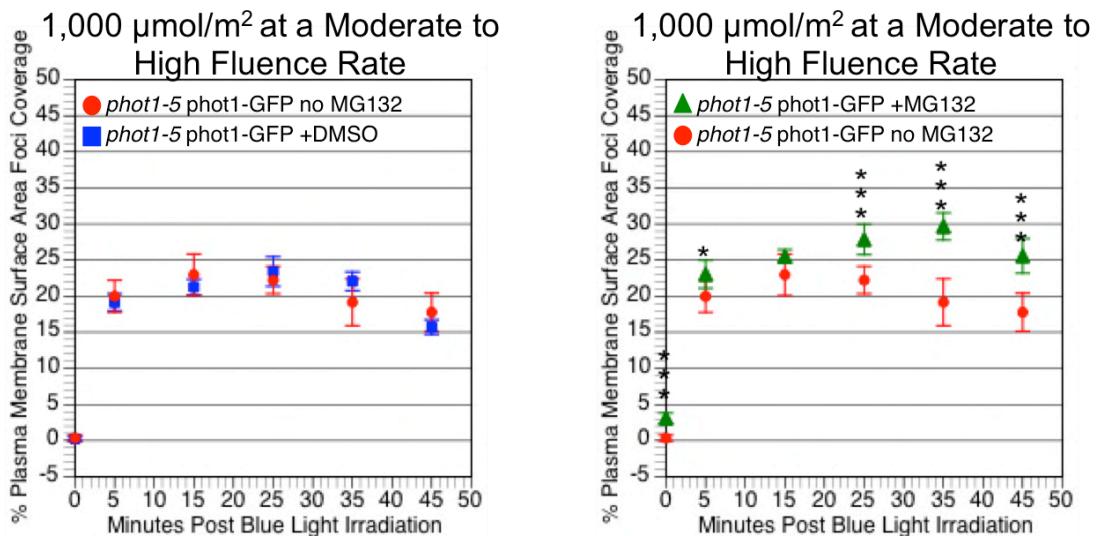


Figure 3.3 phot1-GFP accumulation into foci is elevated in phot1-5 phot1-GFP seedlings pre-treated with MG132.

phot1-GFP accumulation into foci at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 phot1-GFP* *Arabidopsis* seedlings. The graph on the left shows seedlings mock treated (red circles) or pre-treated with the vehicle control DMSO (blue squares) prior to blue light irradiation. The graph on the right shows seedlings mock treated (red circles) or pre-treated with MG132 (green triangles) prior to blue light irradiation. Seedlings were irradiated with a total fluence of 1,000 $\mu\text{mol m}^{-2}$ blue light at a rate of 10 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq$

0.001) for mock treated, DMSO pre-treated and MG132 pre-treated seedlings and between mock treated and MG132 pre-treated groups ($p \leq 0.001$). Two-way repeated ANOVA did not reveal a significant effect between mock treated and DMSO pre-treated groups. Significant differences between treatments were determined using a 2-tailed t-test for equality of means *post-hoc* test ($p, * \leq 0.05, *** \leq 0.05$).

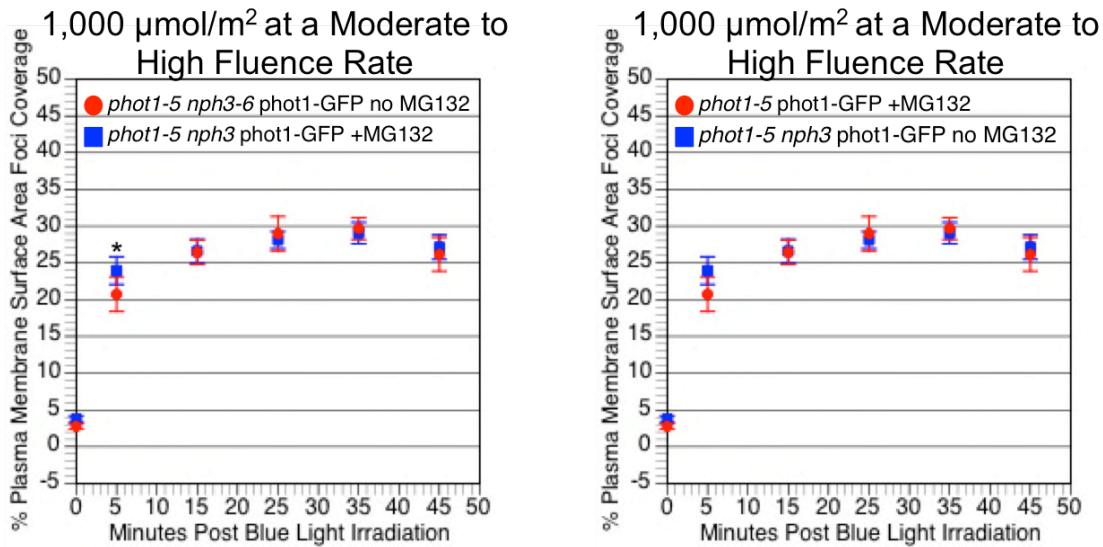


Figure 3.4 MG132 pre-treatment of *phot1-5 phot1-GFP* line increases accumulation of *phot1-GFP* into foci to a level similar to the *phot1-5 nph3-6 phot1-GFP* line.

phot1-GFP accumulation into foci at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 phot1-GFP* and *phot1-5 nph3-6 phot1-GFP* *Arabidopsis* seedlings. Seedlings were irradiated with 1,000 umol m⁻² at a fluence rate of 10 umol m⁻² sec⁻¹ and returned to darkness for the varying amounts of time. The graph on the left shows *phot1-5 nph3-6 phot1-GFP* seedlings mock treated (red circles) and pre-treated with MG132 (blue squares). The graph on the right shows *phot1-5 nph3-6 phot1-GFP* seedlings mock treated (blue squares) and *phot1-5 phot1-GFP* seedlings pre-treated with MG132 (red circles). Each data point represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time

($p \leq 0.001$) for mock treated and MG132 pre-treated seedlings. Two-way repeated ANOVA did not reveal a significant effect between mock treated and MG132 treated *phot1-5 nph3-6* phot1-GFP seedlings or between mock treated *phot1-5 nph3-6* phot1-GFP and MG132 pre-treated *phot1-5* phot1-GFP seedlings. Significant differences between treatments were determined using a 2-tailed t-test for equality of means *post-hoc* test ($p^* \leq 0.05$).

MG132 Increases the Accumulation of phot1-GFP Into Foci at a Moderate to High Fluence Rate and a High Fluence Rate of Blue Light in the *phot1-5 phot1-GFP* Line

Previously, we reported that phot1-GFP accumulation into foci at the plasma membrane in response to blue light irradiation is fluence dependent at both moderate to high and high fluence rates (Figures 2.12 and 2.13). The accumulation in the *phot1-5 nph3-6* phot1-GFP line was also consistently higher relative to the *phot1-5* phot1-GFP line. Figure 3.5 shows that after 15 minutes of darkness post blue light irradiation, pre-treating the *phot1-5* phot1-GFP line with MG132 elevates phot1-GFP accumulation into foci. This appears consistent over all total fluence treatments at both a moderate to high fluence rate (Figure 3.6) and a high fluence rate (Figure 3.7). In addition, the pattern of phot1-GFP accumulation into foci at the plasma membrane over time in the *phot1-5* phot1-GFP line of the MG132 pre-treated seedlings changed to match that of the *phot1-5 nph3-6* phot1-GFP line. In Chapter 2 we showed that in the *phot1-5* phot1-GFP line phot1-GFP accumulation into foci at the plasma membrane increased until about 15 minutes in darkness after blue light irradiation before subsiding. In the *phot1-5 nph3-6* phot1-GFP line accumulation continued to increase until 25-35 minutes in darkness post blue light irradiation before subsiding. Here we see that phot1-GFP accumulation into foci in the *phot1-5* phot1-GFP line pre-treated with MG132 continues until 25-35 minutes in darkness after blue light irradiation before subsiding similar to the untreated *phot1-5 nph3-6* phot1-GFP line.

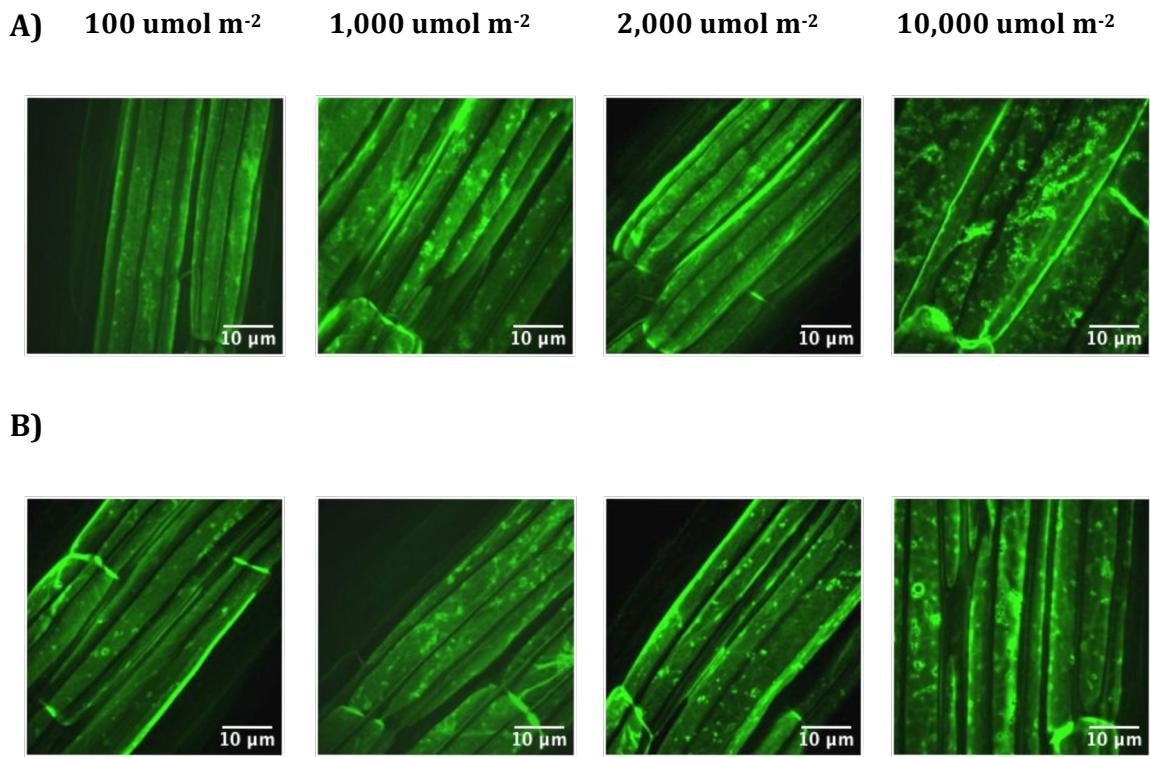


Figure 3.5 MG132 pre-treatment elevates phot1-GFP accumulation into foci at the plasma membrane in the *phot1-5* phot1-GFP line.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP *Arabidopsis* seedlings mock treated (A) and pre-treated with MG132 (B) two hours prior to blue light irradiation. Images were taken at the plasma membrane of cortical cells after 15 minutes of darkness post irradiation at fluence rate of 10 umol m⁻² sec⁻¹ to yield a total fluence of 100 umol m⁻², 1,000 umol m⁻², 2,000 umol m⁻² or 10,000 umol m⁻² respectively.

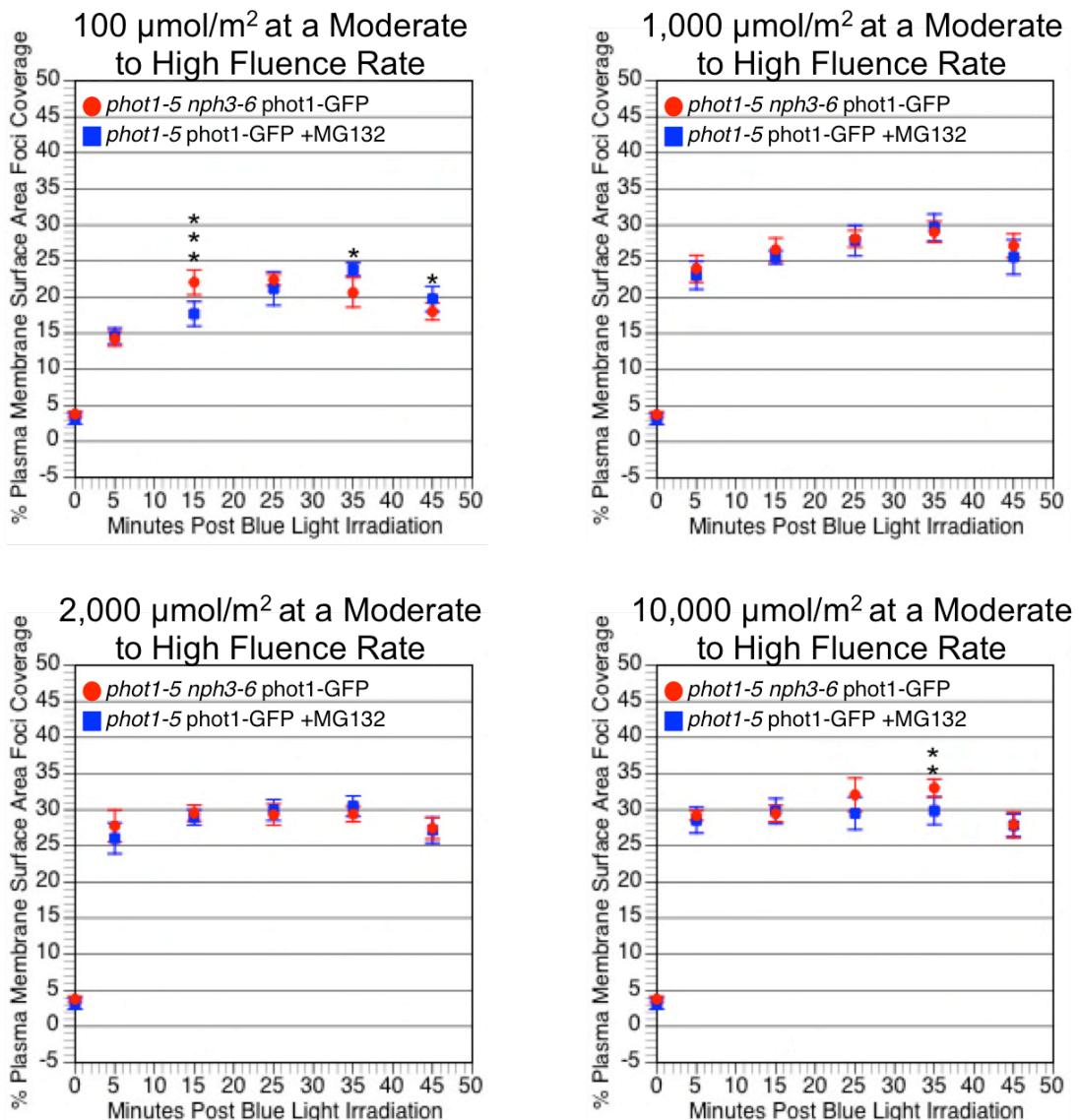


Figure 3.6 MG132 pre-treatment of *phot1-5 phot1-GFP* seedlings

elevates levels of *phot1-GFP* accumulation into foci similar to

levels of *phot1-5 nph3-6 phot1-GFP* seedlings in response to a

moderate to high rate of blue light irradiation.

phot1-GFP accumulation into foci at the plasma membrane was measured in cortical

cells of the hypocotyl elongation zone in 3-day-old etiolated *Arabidopsis* seedlings in

the *phot1-5* phot1-GFP line pre-treated with MG132 (blue squares) and the *phot1-5* *nph3-6* phot1-GFP line mock treated (red circles) prior to blue light irradiation.

Seedlings were treated with the indicated fluence of blue light at a rate of 10 umol m⁻² sec⁻¹ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) for mock treated and MG132 pre-treated seedlings. Two-way repeated ANOVA did not reveal a significant effect between mock treated and MG132 treated seedlings. Significant differences between treatments were determined using a 2-tailed t-test for equality of means *post-hoc* test (p , * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.005).

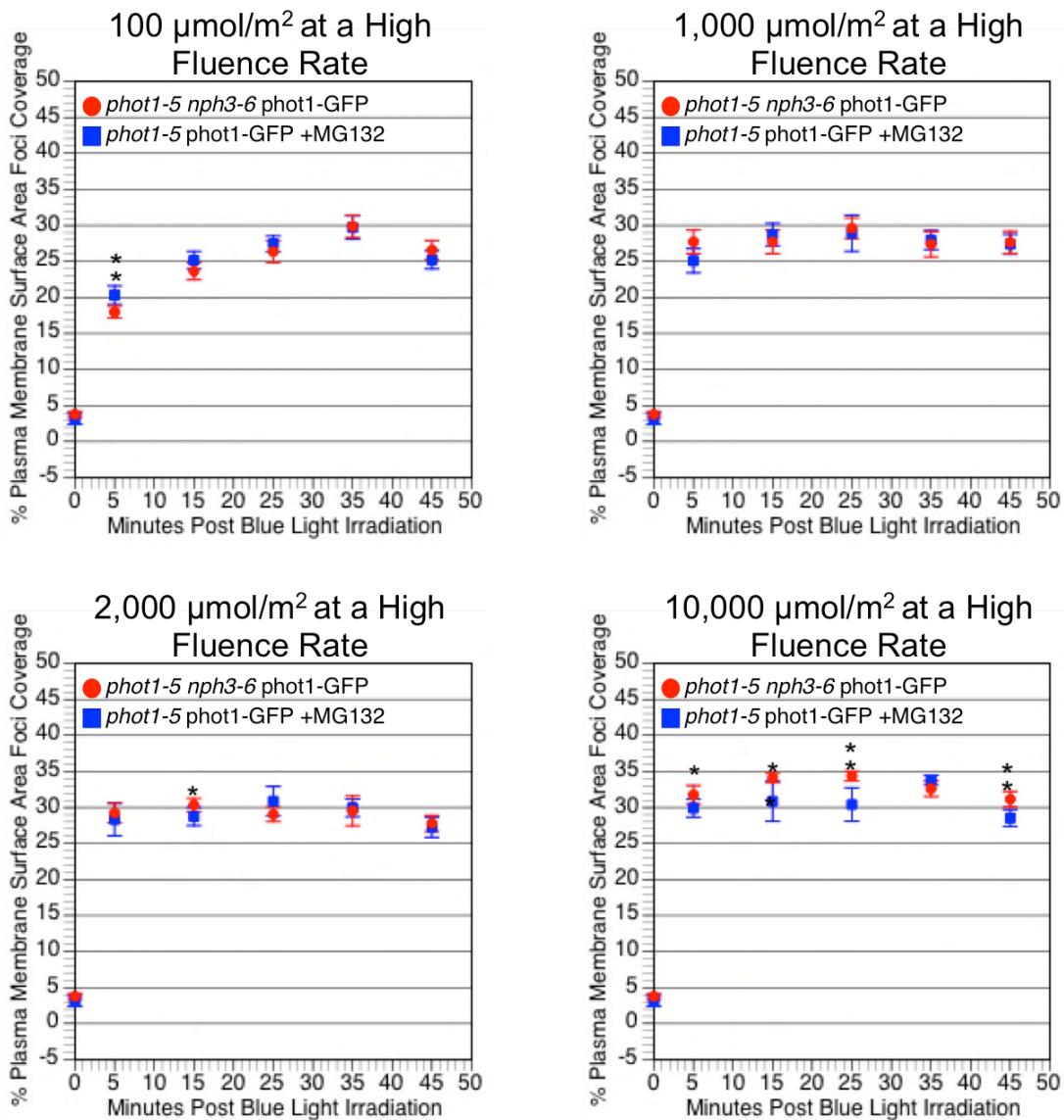


Figure 3.7 MG132 pre-treatment of *phot1-5 phot1-GFP* seedlings elevates levels of *phot1-GFP* accumulation into foci similar to levels of *phot1-5 nph3-6 phot1-GFP* seedlings in response to a high rate of blue light irradiation.

phot1-GFP accumulation into foci at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *Arabidopsis* seedlings in

the *phot1-5* phot1-GFP line pre-treated with MG132 (blue squares) and the *phot1-5* *nph3-6* phot1-GFP line mock treated (red circles) prior to blue light irradiation.

Seedlings were treated with the indicated fluence of blue light at a rate of 20 umol m⁻² sec⁻¹ and returned to darkness for varying amounts of time prior to imaging.

Each data point represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) for mock treated and MG132 pre-treated seedlings. Two-way repeated ANOVA did not reveal a significant effect between mock treated and MG132 treated seedlings. Significant differences between treatments were determined using a 2-tailed t-test for equality of means *post-hoc* test (p , * ≤ 0.05 , ** ≤ 0.01).

Red Light Pre-treatment Does Not Prevent Internalization or Accumulation

Into Foci at the Plasma Membrane of phot1-GFP in Response to Blue Light

Irradiation

Han et al. (2008) reported that pre-treating *phot1-5* phot1-GFP *Arabidopsis* seedlings with 50 umol m⁻² red light prevents phot1-GFP internalization in response to blue light irradiation. Figure 3.8 shows pre-treating with red light does not prevent phot1-GFP internalization nor does it prevent accumulating into foci at the plasma membrane in response to blue light. Phot1-GFP in both the *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP lines begins to accumulate into foci at the plasma membrane and becomes internalized after three minutes of exposure to the blue laser. However, phot1-GFP accumulation into foci appears to be diminished in red light pre-treated seedlings.

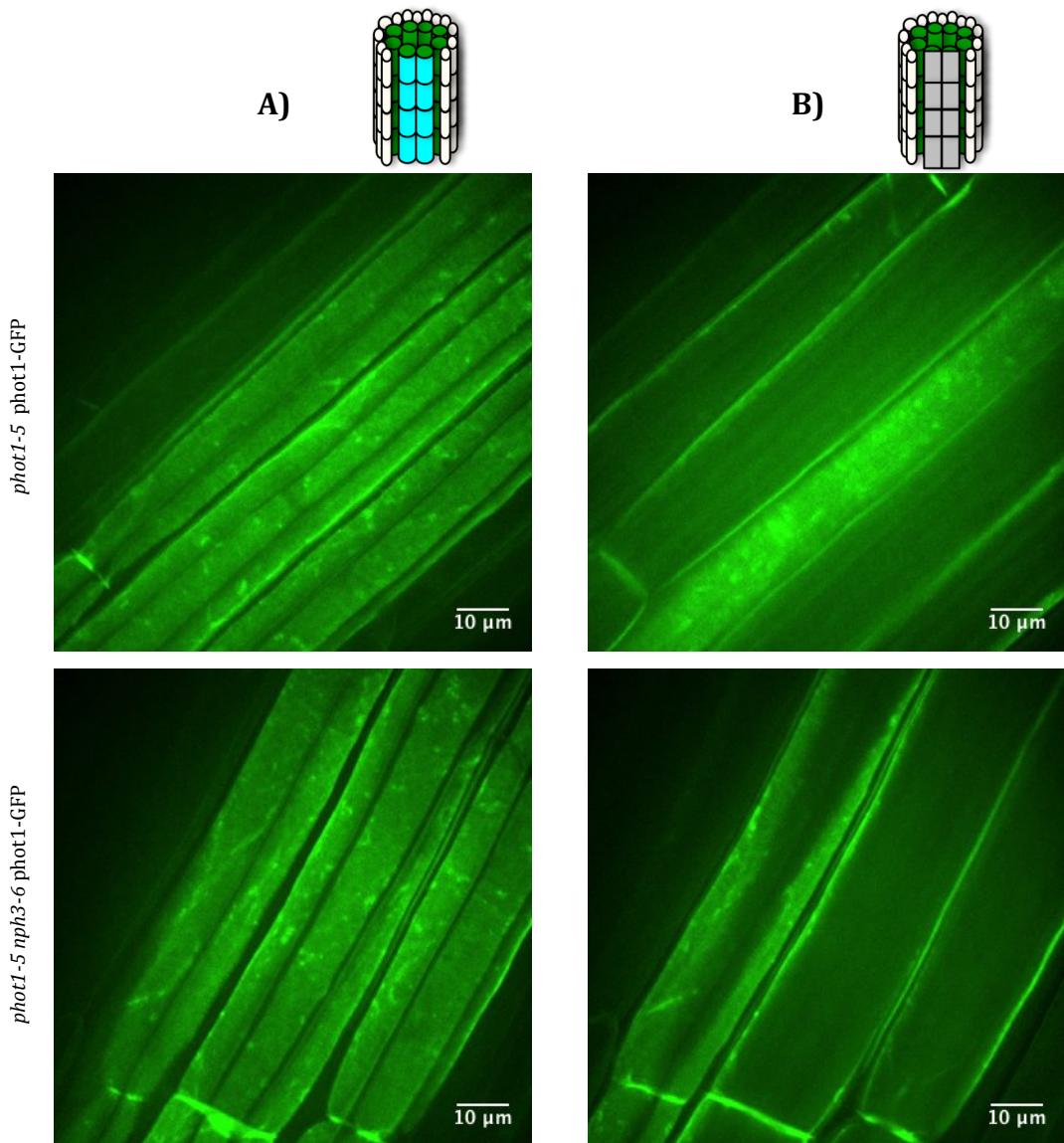


Figure 3.8 phot1-GFP accumulates into foci at the plasma membrane and is internalized in response to blue light irradiation after red light pre-treatment.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP *Arabidopsis* seedlings pre-treated with 50 $\mu\text{mol m}^{-2}$ red light. Images taken at the

plasma membrane (A) and approximately 20 um into cortical cell layer (B) after three minutes of exposure to the blue laser.

Phot1-GFP Accumulation Into Foci is Reduced in Seedlings Pre-treated With Red Light

Although accumulation of phot1-GFP at the plasma membrane in response to blue light still occurs in seedlings pre-treated with red light, the extent of accumulation is reduced. In Chapter 2 we reported that phot1-GFP accumulates into foci in darkness in the *phot1-5 nph3-6* phot1-GFP line (Figure 2.7). Images taken of seedlings from darkness indicate phot1-GFP aggregation is diminished in the *phot1-5 nph3-6* phot1-GFP line (Figure 3.9). Quantitative data shows pretreating with red light reduces the amount of phot1-GFP aggregation in darkness in the *phot1-5 nph3-6* phot1-GFP line (Figure 3.10).

Phot1-GFP accumulation into foci at the plasma membrane is reduced in response to blue light irradiation in both *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP seedlings pre-treated with red light. Figures 3.11 and 3.12 indicate that even after 15 minutes post blue light irradiation in darkness, when foci are at their highest in *phot1-5* phot1-GFP and still climbing in *phot1-5 nph3-6* phot1-GFP seedlings not pre-treated with red light, phot1-GFP foci at the plasma membrane are reduced. Measurements taken of phot1-aggregation at the plasma membrane of seedlings pre-treated with red light two hours prior to irradiation at a moderate to high fluence rate (Figures 3.13 and 3.14) and a high fluence rate (Figures 3.15 and 3.16) show reduction of phot1-GFP foci at the plasma membrane.

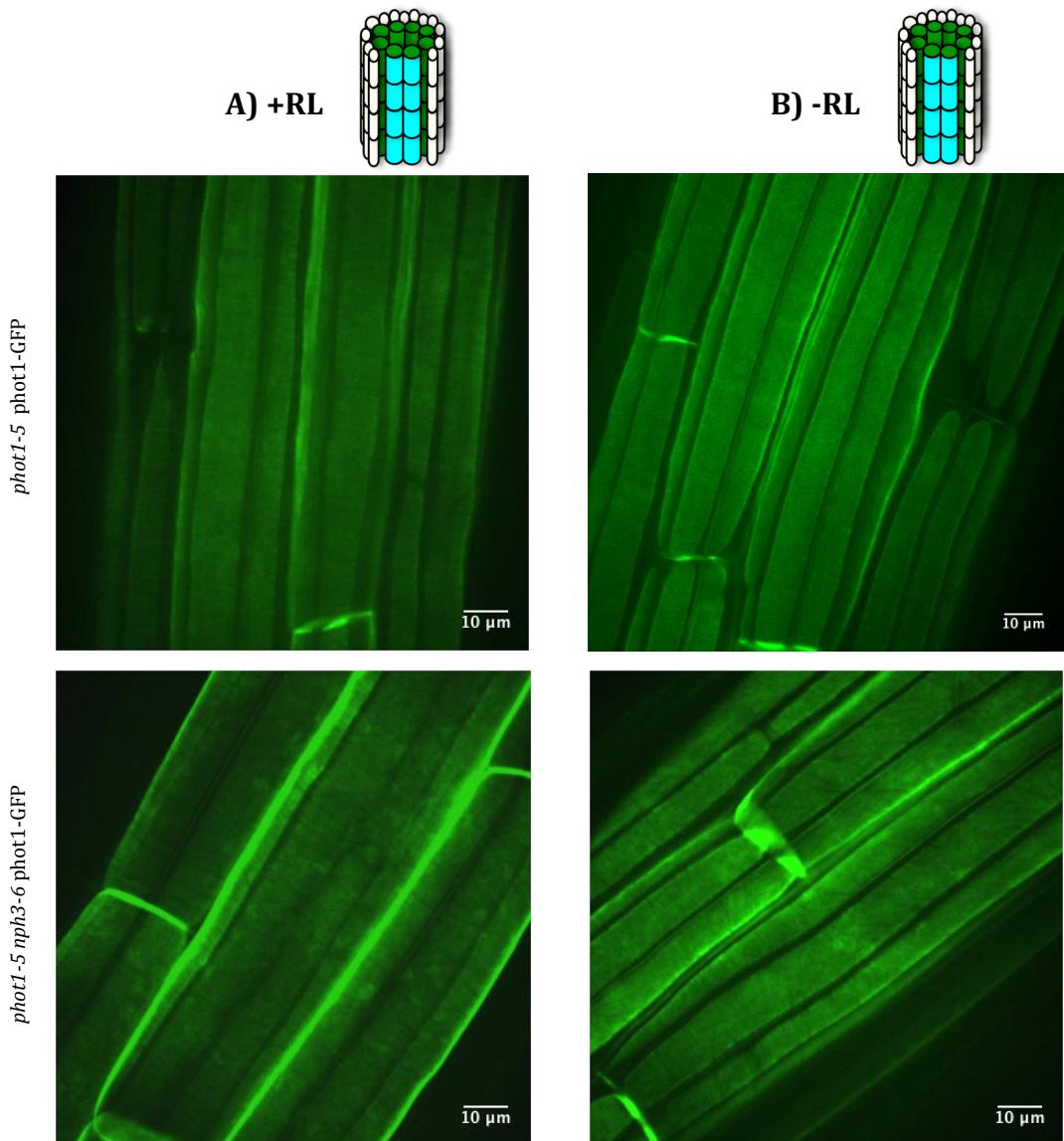


Figure 3.9 Red light pre-treatment appears to reduce *phot1*-GFP accumulation into foci in darkness in the *phot1-5 nph3-6 phot1*-GFP line.

phot1-GFP fluorescence was imaged at the plasma membrane in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 phot1*-GFP and *phot1-5 nph3-6 phot1*-GFP *Arabidopsis* seedlings taken from darkness. Seedlings were either mock treated (A) or pre-treated with 50 $\mu\text{mol m}^{-2}$ red light (B) two hours prior to imaging.

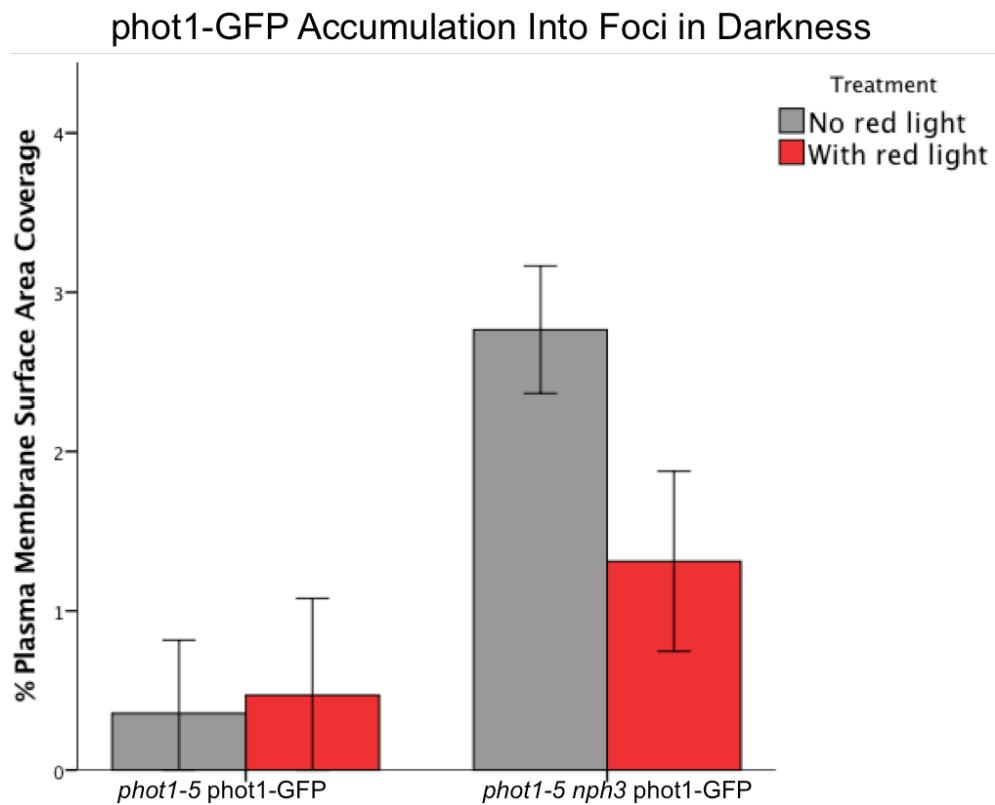


Figure 3.10 Red light pre-treatment reduces phot1-GFP accumulation into foci at the plasma membrane in darkness in *phot1-5 nph3-6 phot1-GFP* seedlings.

phot1-GFP fluorescence was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 phot1-GFP* and *phot1-5 nph3-6 phot1-GFP* *Arabidopsis* seedlings taken from darkness. Seedlings were either mock treated (grey bars) or pre-treated with 50 $\mu\text{mol m}^{-2}$ red light (red bars) prior to imaging. Each bar represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent 95% confidence interval of the mean.

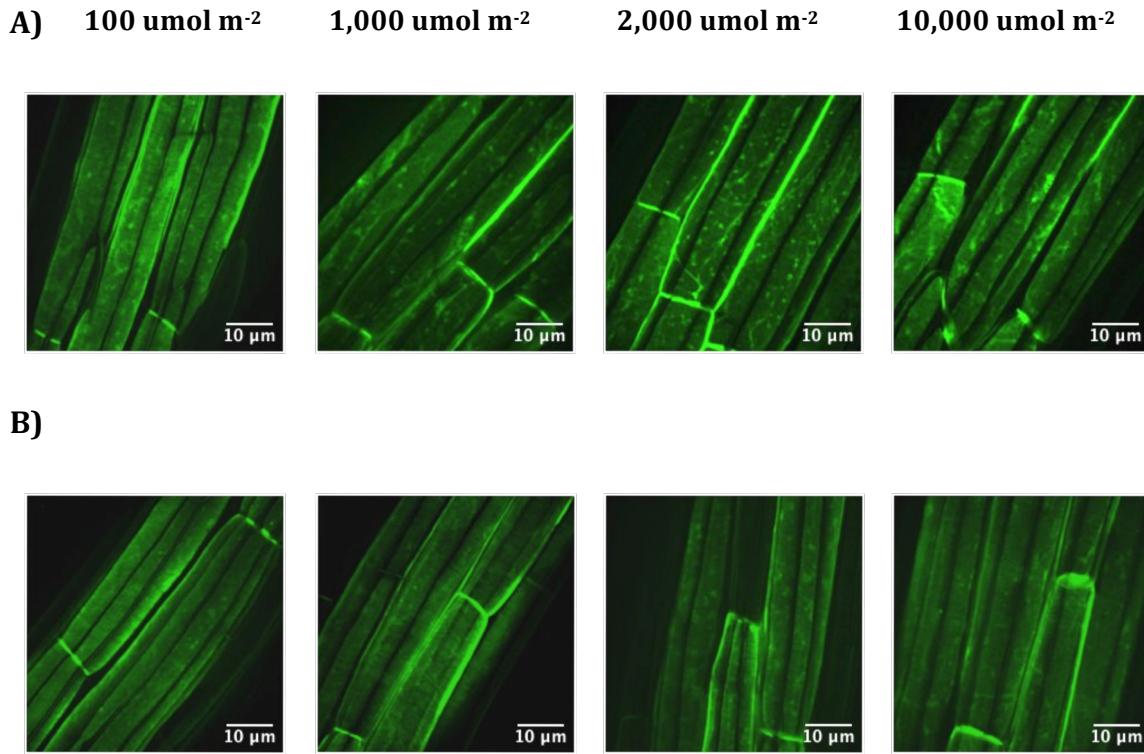


Figure 3.11 Red light pre-treatment reduces phot1-GFP accumulation into foci at the plasma membrane in response to blue light irradiation in the *phot1-5* phot1-GFP line.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP *Arabidopsis* seedlings mock treated (A) and pre-treated with 50 umol m^{-2} red light (B) two hours prior to blue light irradiation. Images were taken at the plasma membrane of cortical cells after 15 minutes of darkness post irradiation at fluence rate of $10 \text{ umol m}^{-2} \text{ sec}^{-1}$ to yield a total fluence of 100 umol m^{-2} , $1,000 \text{ umol m}^{-2}$, $2,000 \text{ umol m}^{-2}$ or $10,000 \text{ umol m}^{-2}$ respectively.

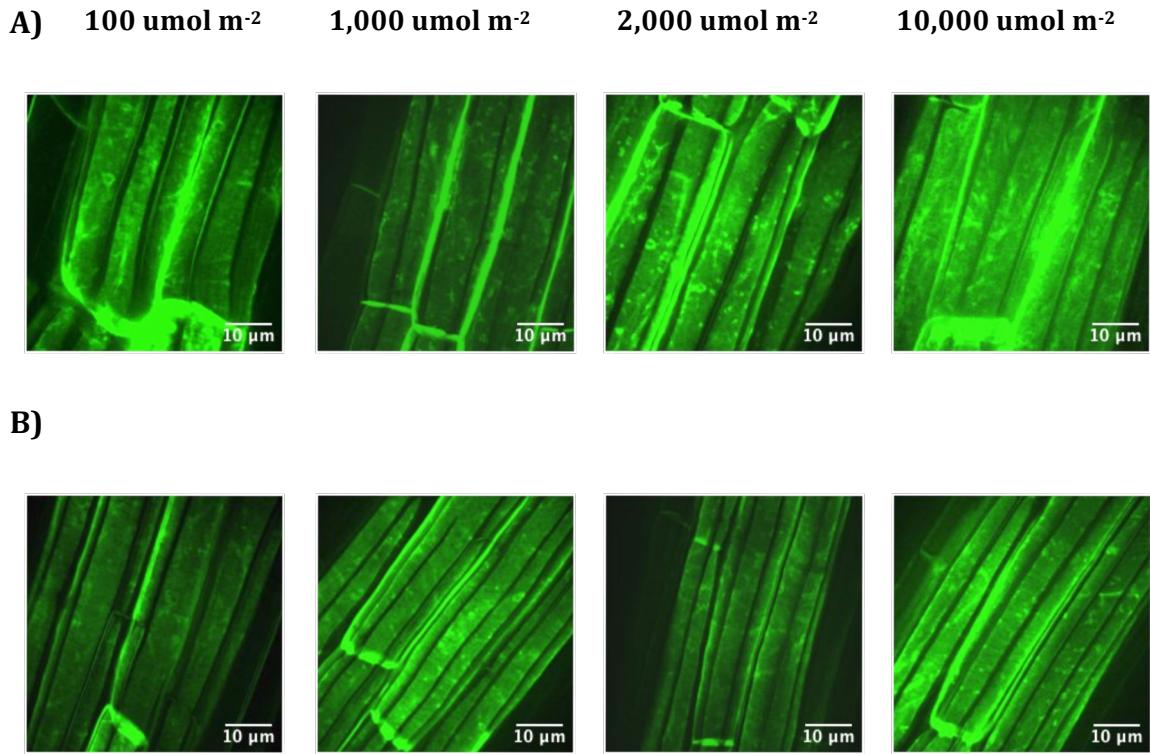


Figure 3.12 Red light pre-treatment reduces phot1-GFP accumulation into foci at the plasma membrane in response to blue light irradiation in the *phot1-5 nph3-6* phot1-GFP line.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 nph3-6* phot1-GFP *Arabidopsis* seedlings mock treated (A) and pre-treated with 50 umol m^{-2} red light (B) two hours prior to blue light irradiation. Images were taken at the plasma membrane of cortical cells after 15 minutes of darkness post irradiation at fluence rate of $10 \text{ umol m}^{-2} \text{ sec}^{-1}$ to yield a total fluence of 100 umol m^{-2} , $1,000 \text{ umol m}^{-2}$, $2,000 \text{ umol m}^{-2}$ or $10,000 \text{ umol m}^{-2}$ respectively.

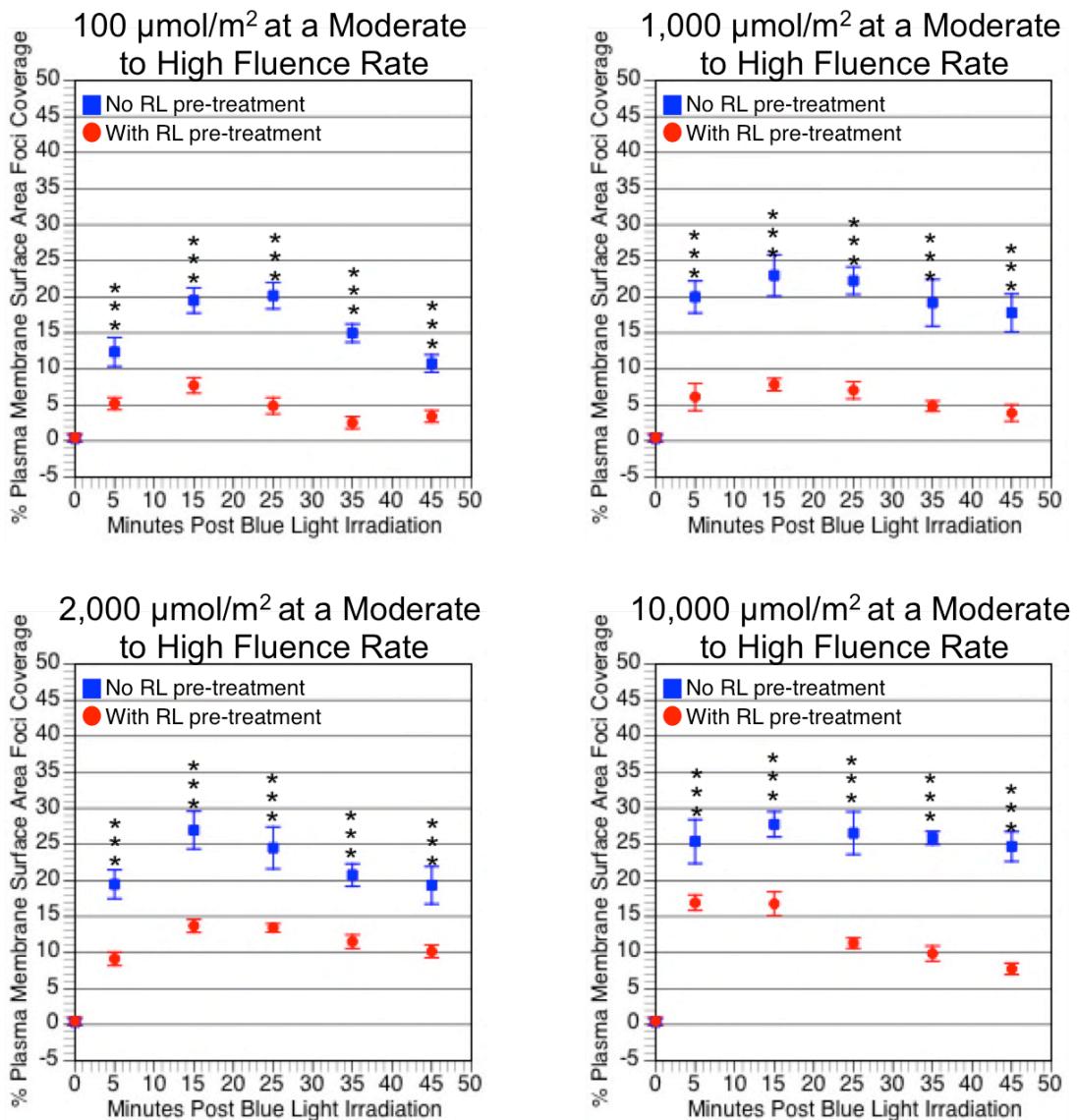


Figure 3.13 Red light pre-treatment reduces *phot1-GFP* accumulation into foci in response to blue light irradiation at a moderate to high fluence rate in the *phot1-5 phot1-GFP* line.

phot1-GFP accumulation into foci at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 phot1-GFP*

Arabidopsis seedlings mock treated (blue squares) and pre-treated with 50 $\mu\text{mol m}^{-2}$ (red circles) two hours prior to blue light irradiation. Seedlings were treated with the indicated fluence of blue light at a rate of 10 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) and treatment ($p \leq 0.001$) for all total fluences. Significant differences between genotypes were determined using a 2-tailed t-test for equality of means *post-hoc* test ($p, * \leq 0.05, ** \leq 0.01, *** \leq 0.001$).

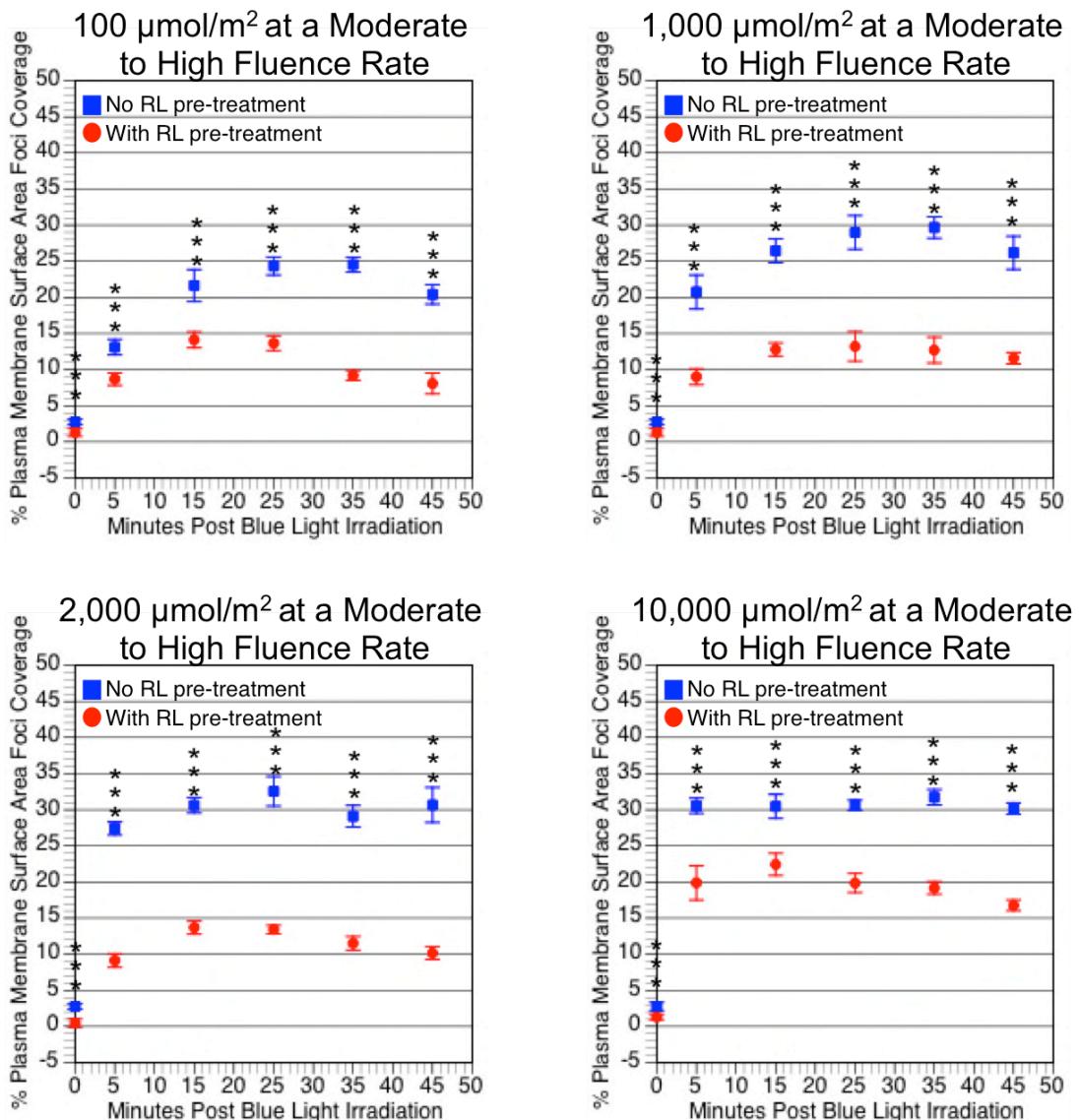


Figure 3.14 Red light pre-treatment reduces *phot1-GFP* accumulation into foci in response to blue light irradiation at a moderate to high fluence rate in the *phot1-5 nph3-6 phot1-GFP* line.

phot1-GFP accumulation into foci at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 nph3-6 phot1-*

GFP *Arabidopsis* seedlings mock treated (blue squares) and pre-treated with 50 umol m⁻² (red circles) two hours prior to blue light irradiation. Seedlings were treated with the indicated fluence of blue light at a rate of 10 umol m⁻² sec⁻¹ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) and treatment ($p \leq 0.001$) for all total fluences. Significant differences between genotypes were determined using a 2-tailed t-test for equality of means *post-hoc* test ($p, * \leq 0.05, ** \leq 0.01, *** \leq 0.05$).

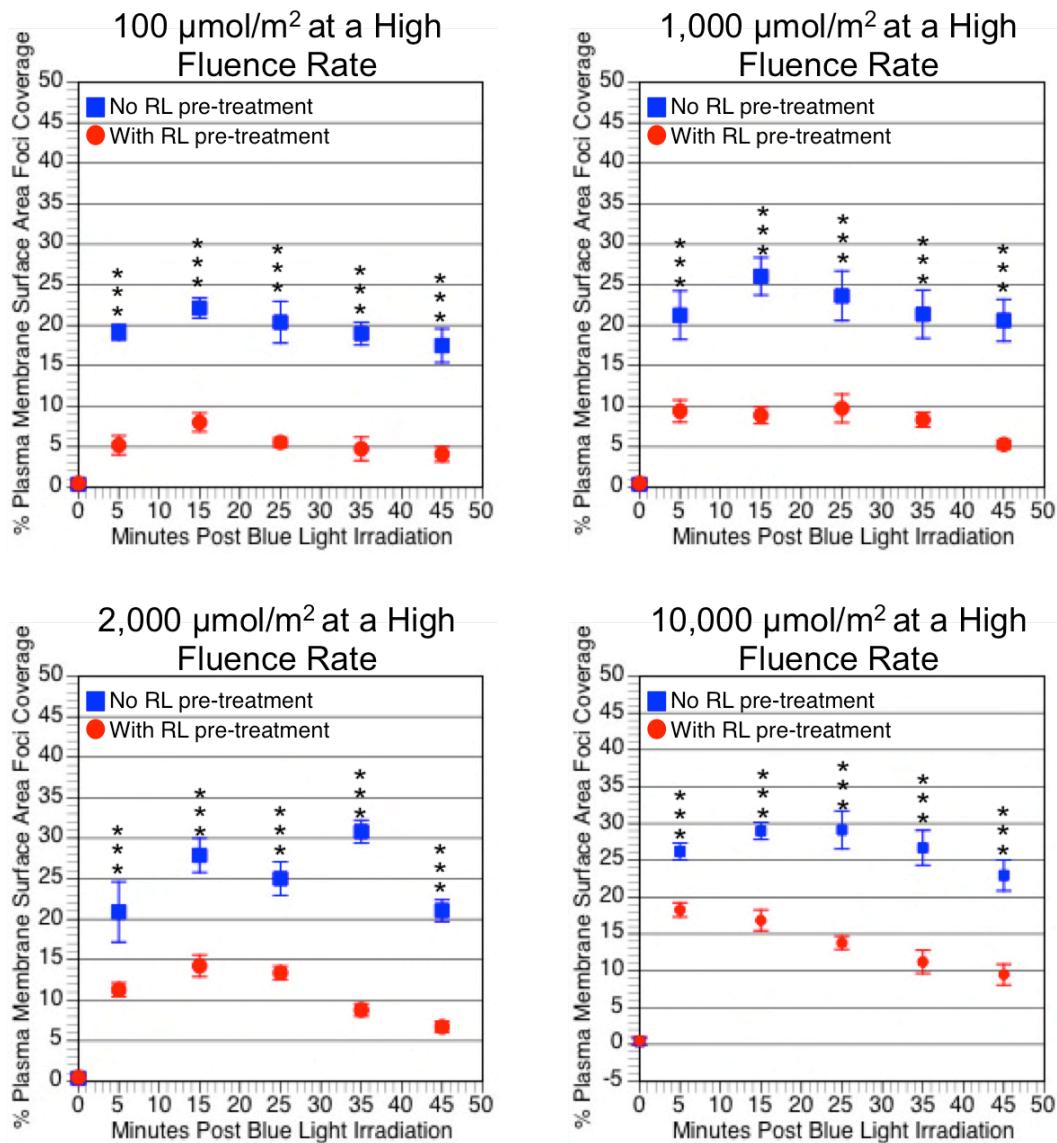


Figure 3.15 Red light pre-treatment reduces *phot1*-GFP accumulation into foci in response to blue light irradiation at a high fluence rate in the *phot1-5* *phot1*-GFP line.

phot1-GFP accumulation into foci at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP *Arabidopsis* seedlings mock treated (blue squares) and pre-treated with 50 $\mu\text{mol m}^{-2}$

(red circles) two hours prior to blue light irradiation. Seedlings were treated with the indicated fluence of blue light at a rate of $20 \text{ umol m}^{-2} \text{ sec}^{-1}$ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) and treatment ($p \leq 0.001$) for all total fluences. Significant differences between genotypes were determined using a 2-tailed t-test for equality of means *post-hoc* test ($p, * \leq 0.05, ** \leq 0.01, *** \leq 0.005$).

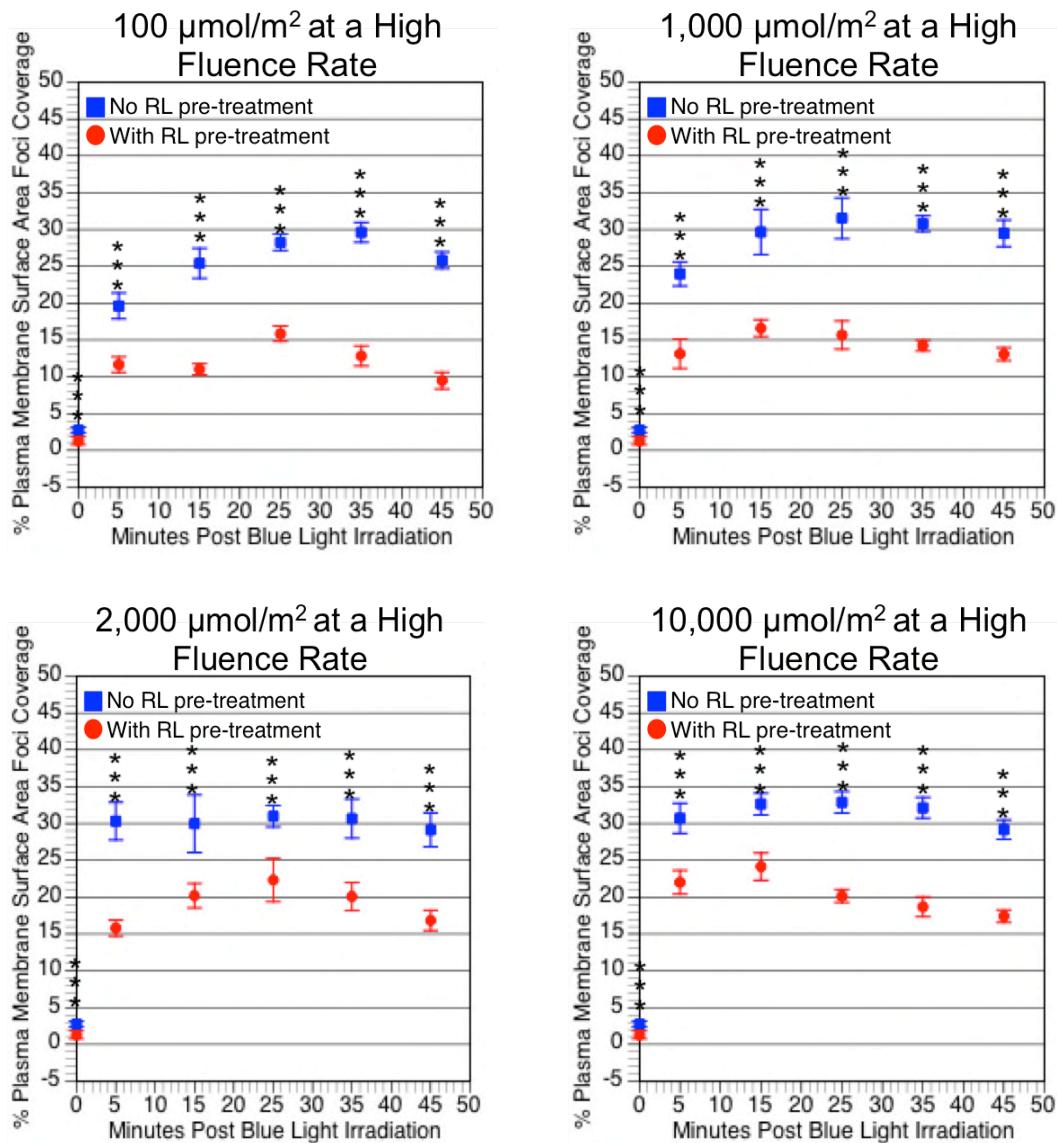


Figure 3.16 Red light pre-treatment reduces phot1-GFP accumulation into foci in response to blue light irradiation at a high fluence rate in the *phot1-5 nph3-6* phot1-GFP line.

phot1-GFP accumulation into foci at the plasma membrane was measured in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 nph3-6* phot1-GFP *Arabidopsis* seedlings mock treated (blue squares) and pre-treated with 50

umol m⁻² (red circles) two hours prior to blue light irradiation. Seedlings were treated with the indicated fluence of blue light at a rate of 20 umol m⁻² sec⁻¹ and returned to darkness for varying amounts of time prior to imaging. Each data point represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean. Two-way repeated measures ANOVA revealed a significant effect of time ($p \leq 0.001$) and treatment ($p \leq 0.001$) for all total fluences. Significant differences between genotypes were determined using a 2-tailed t-test for equality of means *post-hoc* test ($p, * \leq 0.05, ** \leq 0.01, *** \leq 0.05$).

**Low Fluence Rate Blue Light Initiates Little to No phot1-GFP Accumulation
Into Foci at the Plasma Membrane or Internalization in the *phot1-5* phot1-GFP
Line**

Previous reports on phot1-GFP movement have primarily focused on reorganization in response to high fluence rate blue light ($20\text{-}25 \text{ umol m}^{-2} \text{ sec}^{-1}$) (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Preuten et al. 2015). To date, images of phot1-GFP reorganization in response to blue light at a low fluence rate, when phot1 acts as the primary receptor in phototropism, have not been reported (Liscum and Briggs 1995; Sakai et al. 2001). Therefore, we imaged *Arabidopsis* seedlings from both the *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP lines after treatment with low fluence rate blue light ($0.1 \text{ umol m}^{-2} \text{ sec}^{-1}$). Seedlings were treated with a total of 100 umol m^{-2} or $1,000 \text{ umol m}^{-2}$ and returned to darkness prior to imaging. The results show little to no reorganization at the membrane in response to low fluence rate blue light in the *phot1-5* phot1-GFP line but accumulation into foci at the plasma membrane is evident in the *phot1-5 nph3-6* phot1-GFP line (Figures 2.11 and 2.12). In addition, internalized phot1-GFP signal was not detected in either line.

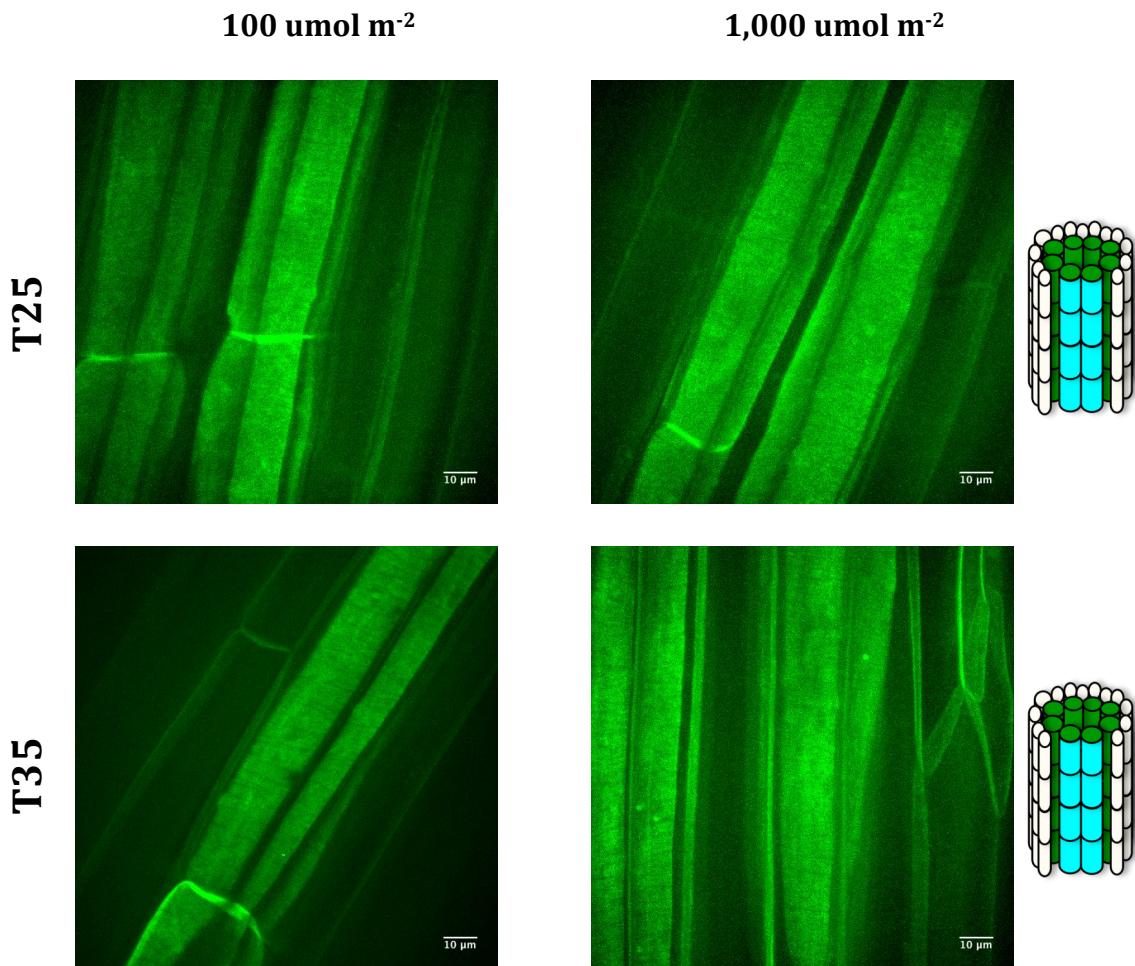


Figure 3.17 Low fluence rate blue light initiates little to no phot1-GFP accumulation into foci at the plasma membrane in *phot1-5* phot1-GFP seedlings.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5* phot1-GFP *Arabidopsis* seedlings. Seedlings were subjected to blue light irradiation at a fluence rate of 0.1 $\text{umol m}^{-2} \text{ sec}^{-1}$ to yield a total fluence of 100 umol m^{-2} (A) or 1,000 umol m^{-2} (B). Images were images taken after 25 or 35 minutes in darkness post blue light irradiation at plasma membrane adjacent to epidermal cell layer.

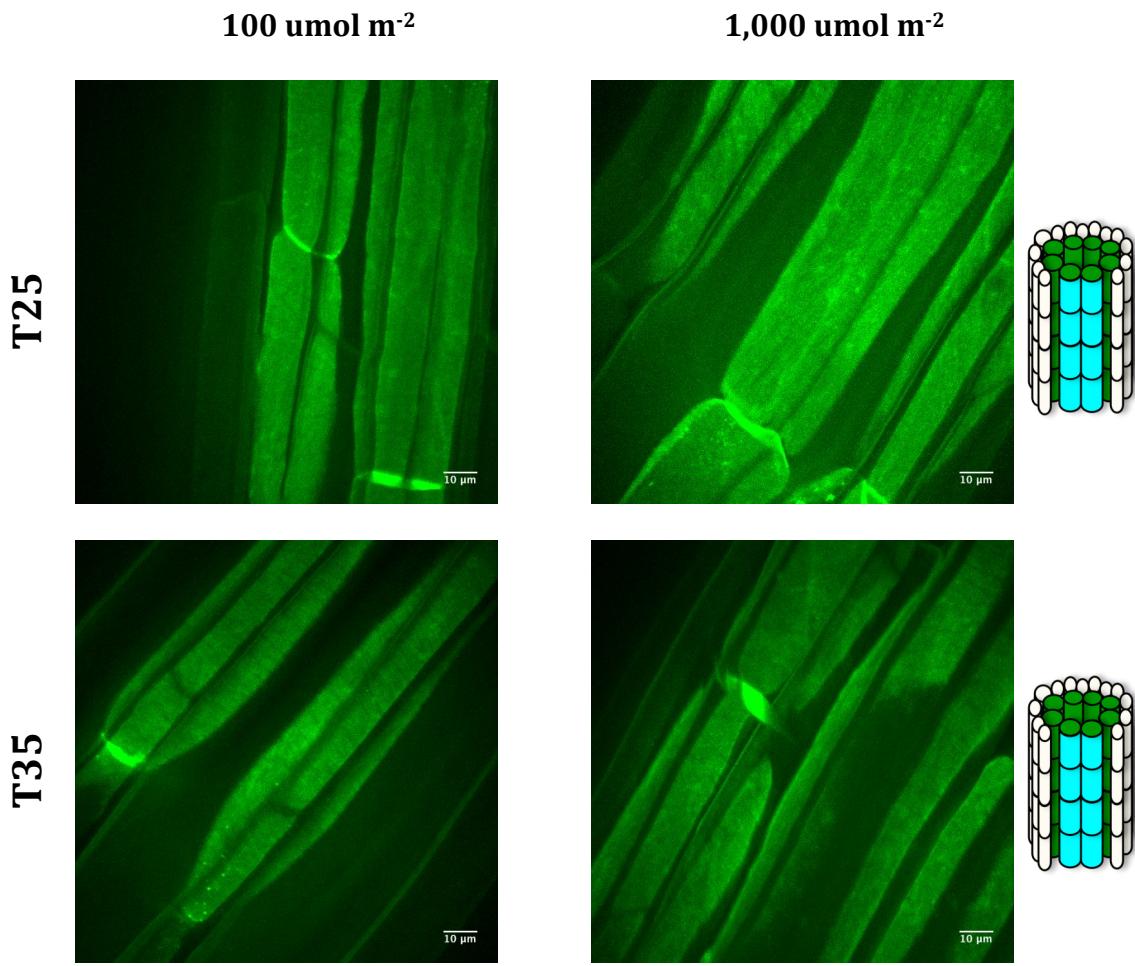


Figure 3.18 Low fluence rate blue light initiates phot1-GFP accumulation into foci at the plasma membrane in *phot1-5 nph3-6* phot1-GFP seedlings.

phot1-GFP fluorescence was imaged in cortical cells of the hypocotyl elongation zone in 3-day-old etiolated *phot1-5 nph3-6* phot1-GFP *Arabidopsis* seedlings. Seedlings were subjected to blue light irradiation at a fluence rate of $0.1 \text{ umol m}^{-2} \text{ sec}^{-1}$ to yield a total fluence of 100 umol m^{-2} (A) or $1,000 \text{ umol m}^{-2}$ (B). Images were taken after 25 or 35 minutes in darkness post blue light irradiation at the plasma membrane adjacent to epidermal cell layer.

Discussion

In Chapter 2 we reported that the accumulation of phot1-GFP in darkness and in response to blue light irradiation is higher in the *phot1-5 nph3-6 phot1-GFP* line relative to the *phot1-5 phot1-GFP* line. Roberts et al. (2011) showed that under high light conditions phot1 is polyubiquitinated leading to receptor degradation in the proteasome. Because the Iphot1-5 nph3-6 *phot1-GFP* line lacks the NPH3 protein, a substrate adaptor for an E3 ubiquitin ligase responsible for ubiquitinating phot1 in response to blue light irradiation, we hypothesized the increased accumulation into foci could be due to protein abundance (Roberts et al. 2012). Therefore, we pre-treated both lines with the proteasomal inhibitor MG132 to determine if preventing the proteasomal degradation of phot1-GFP would influence accumulation into foci at the plasma membrane. Our data shows that not only does MG132 pre-treatment not have an effect on the accumulation of phot1-GFP in the *phot1-5 nph3-6 phot1-GFP* line but it also elevates the accumulation in the *phot1-5 phot1-GFP* line to the level of the *phot1-5 nph3-6 phot1-GFP* line (Figure 3.4). This is consistent in darkness and throughout all fluences at all fluence rates tested (Figures 3.2, 3.6 and 3.7). Taken together this data supports that NPH3 influences the level of phot1-GFP within the cell and that the greater accumulation of phot1-GFP at the plasma membrane in the line lacking NPH3 was due to elevated levels of phot1-GFP relative to the line possessing NPH3. However, the function of phot1-GFP accumulation into foci is still unclear.

In order to determine a possible function for the phot1-GFP foci that form in response to blue light we quantified the accumulation into foci in seedlings pre-

related with red light prior to blue light and in seedlings irradiated with low fluence blue light. Pre-treating seedlings with red light prior to blue light has been reported to enhance the phototropic response (Curry 1957; Zimmerman and Briggs 1967; Sullivan et al. 2016). Han et al. (2008) reported that in addition to enhancing phototropism red light pre-treatment also promoted *phot1*-GFP retention at the plasma membrane in response to blue light irradiation. The group also suggested that the retention of *phot1*-GFP at the plasma membrane might be responsible for the enhanced phototropic response. Therefore, we wished to determine if red light pre-treatment prevents accumulation into foci at the plasma membrane or if it only prevented internalization.

Our data shows that although internalization and accumulation of *phot1*-GFP still occur in response to blue light after red light pre-treatment, both appear to be reduced (Figures 3.8 and 3.9). This is supported by quantification of foci that accumulate at the plasma membrane in both the *phot1-5* *phot1*-GFP and the *phot1-5 nph3-6* *phot1*-FP line pre-treated with red light prior to blue light irradiation (Figures 3.13, 3.14, 3.15 and 3.16). Additionally, red light pre-treatment also reduces the amount of *phot1*-GFP foci that appear in darkness in the *phot1-5 nph3-6* *phot1*-GFP (Figure 3.10). This not only shows that red light pre-treatment influences the accumulation of *phot1*-GFP into foci at the plasma membrane but also suggests that accumulation into foci represents a method of receptor desensitization.

Our final experiment focused on the effects of low fluence rate blue light irradiation on the accumulation of *phot1*-GFP into foci at the plasma membrane.

Previous reports on phot1-GFP movement have focused on high fluence rate blue light (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Preuten et al. 2015). Roberts et al. (2011) reported that under high fluence rate blue light conditions phot1 is polyubiquitinated marking it for degradation in the proteasome. Given that under high fluence rate light conditions phototropism would not be a necessary response for a plant the foci that appear in response to high light may not be involved in receptor signaling (Huala et al. 1997; Jarillo et al. 2001). Roberts et al. (2011) also reported that under low fluence rate blue light phot1 is monoubiquitinated presumably representing the active signaling form. Because low fluence rate blue light represents a condition in which a plant would benefit most from the phototropic response it makes sense to visualize phot1-GFP behavior under these conditions (Huala et al. 1997; Jarillo et al. 2001).

Our data shows that in response to low fluence blue light little to no phot1-GFP accumulation into foci occurs at the plasma membrane in the *phot1-5* phot1-GFP line (Figure 3.17). However, in the *phot1-5 nph3-6* pht1-GFP line some accumulation into foci at the plasma membrane in response to blue light does occur (Figure 3.1). The *phot1-5* phot1-GFP line possesses the NPH3 protein that acts as a substrate adaptor allowing the monoubiquitination of phot1-GFP in response to low fluence rate blue light. The *phot1-5 nph3-6* phot1-GFP line does not possess NPH3 and therefore phot1-GFP is not monoubiquitinated in response to low fluence rate blue light. This suggests that the monoubiquitination of phot1-GFP in the *phot1-5* phot1-GFP line retains phot1-GFP in a random distribution at the plasma membrane preventing it from accumulating into foci. Taken together with the red light pre-

treatment data this further supports that phot1 signals when randomly distributed within the plasma membrane and accumulation into foci represents a method of receptor desensitization.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Introduction

Plants lack motility and have therefore developed adaptations to procure water and nutrients in order to survive. One such adaptation is the phototropic response in which the plant bends to position the aerial organs toward a light source to optimize collection of solar energy and its roots away from direct sunlight to acquire moisture that collects in cooler regions of the soil (Galen et al. 2004, 2007; Christie 2007; Inoue 2008; Pedmale et al. 2011).

The phototropic response is mediated by two blue light receptors, phototropin 1 (phot1) and phototropin 2 (phot2), in the model organism *Arabidopsis* (Christie 2007; Holland et al. 2009; Pedmale et al. 2011). The phototropins work redundantly under moderate to high light conditions with phot1 acting as the primary blue light receptor under low light conditions when strategic placement of photosynthetic organs is most crucial (Huala et al. 1997; Jarillo et al. 2001; Liscum and Briggs 1995; Sakai et al. 2001). The steps that lead from blue light perception to phototropic bending are still under investigation however; it is known that the response begins with phototropin activation (Liscum et al. 2014).

Upon activation by blue light phot1 undergoes a conformational change that leads to autophosphorylation of the receptor, an event that is necessary for the phototropic response to occur (Christie et al. 1998; Kaiserli et al. 2009; Inoue et al. 2008). In addition to autophosphorylation, the post-translational modification

ubiquitination is also necessary for phototropic signaling. Ubiquitination of phot1 in response to blue light depends on the interacting protein NPH3 (Motchoulski and Liscum 1999; Pedmale et al. 2011). NPH3 also interacts with Cullin3, the core component of a cullin-RING-based E3 ubiquitin-protein ligase complex (CRL3^{NPH3}), responsible for ubiquitinating phot1 in response to blue light (Roberts et al. 2012). Roberts et al. (2011) further showed that under high light conditions phot1 is polyubiquitinated marking it for degradation in the proteasome. Under low light conditions phot1 is monoubiquitinated, presumably representing the active signaling form (Roberts et al. 2011). In addition to signaling, post-translational modification also influences the behavior of activated phot1.

Shortly after activation by blue light, phot1-GFP becomes internalized from the plasma membrane to what has been reported as “a yet to be identified internal target” (Sakamoto and Briggs 2002). Kaiserli et al. (2009) showed using transiently expressed phot1-GFP in tobacco leaves that autophosphorylation is necessary for internalization in response to blue light. Kinase dead phot1-GFP was not phosphorylated nor was it internalized in response to blue light. Likewise the constitutively active kinase mutant of phot1-GFP was internalized in darkness (Kaiserli et al 2009). In addition to phosphorylation, ubiquitination appears to influence receptor movement as well. Sakamoto and Briggs (2002) reported that in darkness phot1 is found exclusively in the membrane fraction but upon activation by blue light a small amount of phot1 moves to the soluble fraction. However, Roberts (2012) showed that phot1 in the *nph3-6* background, seedlings lacking NPH3 which are not ubiquitinated in response to blue light, remains in the

microsomal fraction even after prolonged blue light exposure. However, the functional relevance of the internalization of phot1 in response to blue light is still unknown.

Since the creation of the phot1-GFP construct, almost a decade and a half ago, researchers have puzzled over the possible function of phot1-GFP internalization (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Sullivan et al. 2010; Preuten et al. 2015). Liscum (2016) has even gone so far as to question if there is any functional relevance at all. The goal of this dissertation project was to further characterize the movement of phot1-GFP in response to blue light and to determine if any functional relevance behind that movement exists. The following sections will discuss our findings and present a new working model in regard to phot1 movement and the function of said movement in response to blue light irradiation.

phot1-GFP Accumulates Into Foci at the Plasma Membrane Prior to Internalization

Historically, phot1-GFP movement in response to blue light has been described primarily as internalization (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Sullivan et al. 2010; Preuten et al. 2015). Our data shows that prior to internalization phot1-GFP accumulates into foci at the plasma membrane. Internalization arises from the foci that form at the plasma membrane and rather than targeting an internal structure target areas of adjacent plasma membrane (Figure 2.8). Given that internalized phot1-GFP appears to be

cycling from one area of the plasma membrane to another this mode of transport most likely represents a method of receptor desensitization or recycling.

Internalization was also inconsistent and relatively sporadic compared to the foci that formed at the plasma membrane. Therefore, we focused our attention on the accumulation of phot1-GFP into foci at the plasma membrane.

The *phot1-5 nph3-6* phot1-GFP line, lacking NPH3 and subsequent receptor ubiquitination, exhibited greater phot1-GFP accumulation into foci than the *phot1-5* phot1-GFP line. This was consistent across all measurements taken regardless of total fluence or fluence rate (Figures 2.11, 2.12 and 2.13). Given that phot1 is polyubiquitinated under high light conditions we hypothesized that the elevated levels of foci formation may be due to phot1-GFP abundance in the line lacking NPH3. Our follow up experiments using the proteasomal inhibitor NPH3 confirmed our suspicions. Pre-treating the *phot1-5 nph3-6* phot1-GFP line with MG132 had little to no effect on the number of foci that formed at the plasma membrane in response to blue light (Figure 3.4). Conversely, pre-treating the *phot1-5* phot1-GFP line with MG132 increased the number of foci that formed at the plasma membrane in response to blue light to that of the *phot1-5 nph3-6* phot1-GFP line (Figures 3.6 and 3.7). This supports the data presented by Roberts et al. (2011) that the abundance of phot1 is mediated by NPH3 dependent polyubiquitination of phot1 in response to high blue light.

Red Light Pre-treatment Reduces phot1-GFP Accumulation Into Foci at the Plasma Membrane in Response to Blue Light

In order to determine the function of phot1-GFP accumulation into foci at the plasma membrane we visualized and quantified the change in foci that appeared when seedlings were pre-treated with red light. Red light pre-treatment has been shown to enhance the phototropic response to blue light (Curry 1957; Zimmerman and Briggs 1967; Sullivan et al. 2016). Han et al. (2008) reported that in addition to enhancing the phototropic response red light pre-treatment also inhibits phot1-GFP internalization in response to blue light. The group speculated that the retention of phot1-GFP at the plasma membrane might be the cause of the enhanced phototropic response (Han et al. 2009). Therefore we investigated the effect that red light pre-treatment has on phot1-GFP accumulation into foci at the plasma membrane.

Our results show that although red light pre-treatment does not prevent accumulation into foci at the plasma membrane, it does inhibit accumulation (Figures 3.8 and 3.9). This is consistent for genotypes, *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP, across all fluences regardless and fluence rates (Figures 3.14, 3.15, 3.16 and 3.17). This suggests that since red light pre-treatment enhances phototropism and also reduces the number of foci that form at the plasma membrane in response to blue light that the accumulation of phot1-GFP into foci may represent a method of receptor desensitization. This is an interesting finding as the function of phot1-GFP movement in response to blue light has been disputed for many years (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Sullivan et al. 2010; Preuten et al. 2015; Liscum 2016). Given that

internalized phot1-GFP arises from the foci that form at the plasma membrane and meet up with foci at an adjacent region of the plasma membrane the foci and the internalized strands of phot1-GFP most likely represent desensitized receptor as well.

Monoubiquitination of phot1 May Prevent Accumulation Into Foci in Response to Blue Light Irradiation

Historically the movement of phot1-GFP in response to blue light has focused on the response to high fluence rate blue light (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009; Sullivan et al. 2010; Preuten et al. 2015). Roberts et al. (2011) reported that high fluence rate blue light initiates polyubiquitination of phot1 and subsequent degradation of phot1 in the proteasome. Roberts et al. (2011) also reported that under low fluence rate blue light phot1 is monoubiquitinated, which they presumed is the active signaling form. Therefore, imaging phot1-GFP movement under high light conditions and looking for functional relevance of movement does not seem appropriate. To overcome this conundrum we visualized the movement of phot1-GFP in response to low fluence rate blue rate, when a majority of activated phot1 is in the monoubiquitinated form.

Our data shows that the movement of phot1-GFP is virtually nonexistent when phot1-GFP is monoubiquitinated in the *phot1-5* phot1-GFP line. After prolonged exposure to low fluence rate blue light little to no phot1-GFP accumulation into foci at the plasma membrane can be detected in the *phot1-5* pot1-GFP line (Figure 3.17). However, in the *phot1-5 nph3-6* phot1-GFP line phot1-GFP

accumulation into foci at the plasma membrane does occur in response to low fluence rate blue light (Figure 3.18). This suggests that monoubiquitination may prevent phot1-GFP from accumulating into foci preventing receptor desensitization. Given that the *phot1-5 nph3-6* phot1-GFP line still exhibits phot1-GFP accumulation into foci, receptor phosphorylation is sufficient to induce accumulation. This would further suggest that monoubiquitination might act as rheostat that works against the influence of autophosphorylation driving the activated receptor into desensitizing foci.

This model seems reasonable when applied to a plant's environment. If a plant were situated in a high light environment it would not be necessary for the plant to expend the resources to induce the phototropic response. Therefore it would desensitize the photoreceptor that initiates the response and mark the receptor for degradation. Under low light conditions, when the phototropic response would be most beneficial desensitizing and degrading the receptor would be counterproductive. Taken together, the above information is presented in our new working model of phot1 ubiquitination and signaling (Figure 4.1).

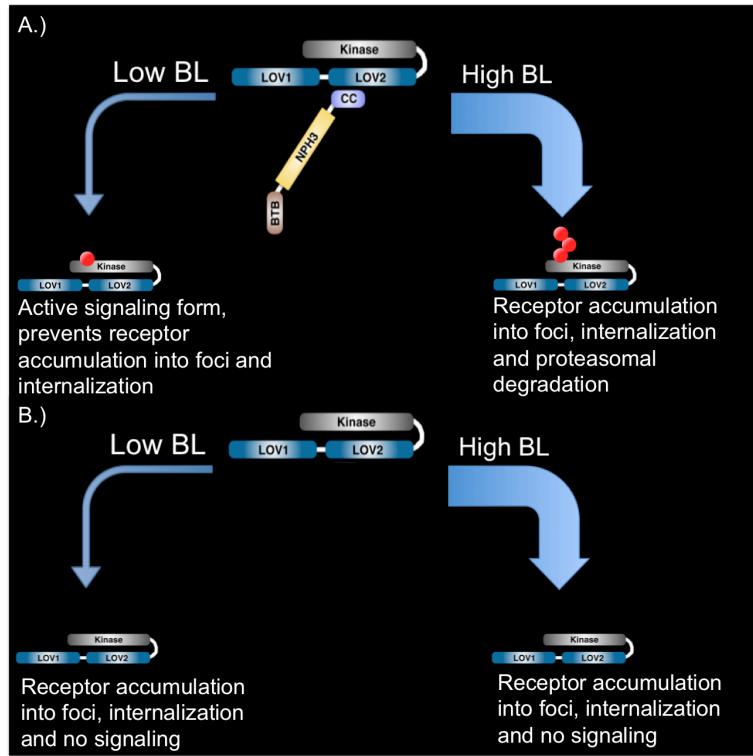


Figure 4.1 Working Model of the events that follow blue light

activation of phot1 in the presence and absence of NPH3.

This modified working model represents what we hypothesize to occur after blue light activation of phot1 in seedlings where NPH3 is either present or absent. (A) When NPH3 is present phot1 is ubiquitinated after activation by blue light. Under low light conditions phot1 is monoubiquitinated representing the active signaling form as well as prevents receptor accumulation into foci and internalization. Under high light conditions phot1 is polyubiquitinated leading to receptor accumulation into foci, internalization and degradation. (B) When NPH3 is absent phot1 is not ubiquitinated in response to blue light irradiation but accumulates into foci and is internalized.

Future Directions

Although the functional relevance of phot1 movement in response to blue light is becoming clearer, there is still work to be done. Several obstacles arise from studying light sensitive proteins, especially those activated with the same spectrum of light used to excite an attached fluorophore. We presented a technique for imaging phot1-GFP that minimizes the amount of light contamination while imaging. However, during imaging the seedling is exposed to several seconds of high fluence rate blue light and is therefore not useful for follow up experiments.

Generating a phot1 fluorescent construct that possesses a fluorophore with an excitation spectrum outside the activation spectrum of phot1 would be a very useful tool. This would allow for imaging phot1 movement live and may elucidate temporal movements that are not as visible in single images. To meet this end our lab is currently working on fluorescently labeled mCHY phot1 construct. The goal will be to confirm the data presented in this dissertation and to further explore the behavior of phot1 in response to blue light. In addition to this construct we are also working on fluorescent-labeled phot1 mutants as well.

Kaiserli et al. (2009) reported that phot1-GFP transiently expressed in tobacco cells becomes internalized in response to blue light as it does in planta. Their work also showed that kinase dead phot1-GFP is not internalized in response to blue light and that a mutation that renders the phot1-GFP kinase constitutively active results in receptor internalization in darkness. This suggests that phosphorylation of phot1-GFP is sufficient and necessary for receptor internalization. It will be interesting to see if similar results are obtained when the

kinase dead or constitutively active phot1-GFP protein are expressed in planta. This would support the hypothesis that phosphorylation of phot1 triggers internalization of the receptor. Introducing the constitutively active form of phot1-GFP into *Arabidopsis* will also allow us to test the type of ubiquitination of phot1-GFP that occurs in response to a constitutively active kinase. If phot1-GFP is polyubiquitinated in darkness that would suggest that the degree of phosphorylation influences the type of ubiquitination phot1-GFP undergoes ultimately affecting signaling by and internalization of the receptor.

Lastly, given our new knowledge of the accumulation of phot1-GFP into foci at the plasma membrane that precedes receptor internalization it would be prudent to repeat the experiments performed by Preuten et al. (2015) using lipid-anchored phot1-GFP. Preuten et al. (2015) reported that lipid-anchored phot1-GFP is not internalized in response to blue light, is degraded at the membrane in response to prolonged high fluence rate blue light and complements phot1 signaling but does not result in an enhanced phototropic response. The group suggested that the retention of phot1-GFP at the plasma membrane reported by Han et al. (2008) did not influence enhanced phototropic curvature given that the preventing phot1-GFP internalization via lipid anchors did not result in enhanced phototropism. However, Preuten et al. (2015) made no mention of the accumulation of phot1-GFP into foci at the plasma membrane suggesting that accumulation may still occur in the lipid-anchored constructs. Determining that lipid-anchored phot1-GFP still accumulates into foci at the plasma membrane in response to blue light would further support our claim that the foci represent a method of receptor desensitization and that

preventing the accumulation of phot1-GFP into foci may prolong phototropic signaling and lead to enhanced phototropism. If this turns out to be true, it may offer a technique for generating plants with an enhanced phototropic response.

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ADDENDUM

FURTHER CHARACTERIZATION OF PHOT1-GFP INTERNALIZATION AND ENGINEERING EQUIPMENT TO IMPROVE EFFCIANCY OF STANDARD LAB

PROTOCOLS

Abstract

The blue light photoreceptor phototropin 1 (phot1) has been shown to be internalized from the plasma membrane in response to blue light irradiation to a yet to be identified target. The vesicle coat clathrin has been implicated in phot1 internalization. Clathrin co-immunoprecipitates with phot1 in pull down experiments and pre-treatment with the phosphotyrosine analog tyrphostin A23 (TYR A23) has been reported to prevent phot1-GFP movement in response to blue light. Here we show that pre-treatment with TYR A23 does not prevent phot1-GFP internalization and accumulation into foci at the plasma membrane in response to blue light. Additionally, we describe the construction and utilization of the Ice-Caps fountain for streamlining growth and collection of tissue from *Arabidopsis* seedlings for genotyping and an IR imaging system for real-time phototropism measurements in darkness.

Introduction

Traditional models of plasma membrane receptor internalization involve the vesicle coat protein clathrin. Upon receptor activation a series of post-translational modifications occur initiating the binding of adaptor proteins that allow formation of the clathrin cage around the budding vesicle. This leads to vesicle scission from

the plasma membrane and subsequent internalization and trafficking between endosomal compartments (Valencia et al. 2016). Although clathrin independent mechanisms for internalization can occur, clathrin-mediated endocytosis (CME) is the best characterized and most widely used method in plants and animals (Otto and Nichols 2011).

In CME, receptor internalization begins with the activation of a plasma membrane localized receptor. Generally, once activated the receptor undergoes post-translational modifications that can include phosphorylation and ubiquitination (Valencia et al. 2016). Ubiquitin and phosphorylation motifs are recognized by adaptor and accessory proteins and can act as sorting signals that dictate the ultimate target of the budding vesicle (Mittal and McMahon 2008). Once bound, adaptor and accessory proteins initiate formation of the clathrin triskelia made up of multiple subunits consisting of 3 heavy chain and 3 light chain clathrin molecules to create a 3-dimensional clathrin cage that resembles the pattern of a soccer ball (Pearse 1976). There are many known adaptor and accessory proteins that vary between plants and animals but the AP-2 adaptor protein has been shown to be necessary for CME in animals and involved in CME in plants. The adaptor protein AP-2 initiates formation of the clathrin cage around budding vesicles by binding both cargo proteins and clathrin (Honing et al. 2005). Plants have retained an ancestral adaptor protein called TPLATE that, similar to AP-2 in animals, is necessary for survival in plants (Gadeyne, 2016). Once the clathrin cage has formed the large GTPase dynamin assembles into a multimeric helical structure around the point of contact between the budding vesicle and plasma membrane and, through its

mechanical action powered by GTP hydrolysis, synches abscising the forming vesicle from the plasma membrane (Chappie and Dyda 2013). Once vesicle abscission has occurred the clathrin coat disassembles and the vesicle is free to fuse with endosomal compartments (Valencia et al. 2016).

Phototropin 1, the primary plant photoreceptor responsible for the phototropic response under low light conditions, is a membrane associated blue light receptor that initiates a cell signal cascade resulting in phototropic bending toward a light source (Gallagher et al. 1988; Huala et al 1997; Harper et al. 2003; Christie 2007). In response to blue light, phot1-GFP constructs have been shown to be internalized to a yet to be discovered internal target (Sakamoto and Briggs 2002; Han et al. 2008; Wan et al. 2008; Kaiserli et al. 2009). In addition, clathrin co-immunoprecipitates with phot1 suggesting a role for CME in phot1 internalization (Kaiserli et al. 2009; Roberts et al. 2011).

The phosphotyrosine analog tyrphostin A23 (TYRA23) is a pharmacological agent that is used as an inhibitor of AP-2 binding preventing clathrin cage formation in both plants and animals (Crump et al. 1998; Banbury et al. 2003). Kaiserli et al. (2009) reported that treating *Arabidopsis* seedlings with the CME inhibitor TYR A23 prevents phot1-GFP internalization in response to blue light. In Chapter 2 we characterized phot1-GFP accumulation into foci at the membrane prior to internalization we sought to further study the effects of tyrphostin A23 on phot1-GFP movement in response to blue light.

In addition to characterizing the movement of phot1-GFP we have also been working to improve the efficiency of experiments performed within the lab by

engineering new equipment to reduce the amount of time necessary for essential lab protocols. This was done by constructing an Ice-Caps fountain to streamline genotyping and by constructing an infrared imaging system to capture images of seedlings using wavelengths of light that fall outside of the spectrum absorbed by photoreceptors within plants.

Developing the necessary lines of plants can require transformation or crossing of plants with the desired genes of interest. This in turn requires genotyping to ensure that the correct genes are passed on to the line of plants in an experiment. In *Arabidopsis*, traditional genotyping methods require collecting tissue from individual plants once the plants have reached a level of maturity that allows for safe collection of leaf tissue. If genotyping hundreds or thousands of plants this can introduce a bottleneck into the pipeline of proceeding with an experiment.

Krysan (2004) Introduced an idea called ICE-CAPS, a method for growing seedlings in 96-well plates filled with growth media set upon a second 96-well plate within a reservoir of circulating fresh water. The wells of the top 96-well plate do not have a floor allowing roots to protrude into the wells of the bottom 96-well plate. The wells of the bottom 96-well plate fill with fresh water from the reservoir and once the roots have extended into the water the top and bottom 96-well plates are removed from the reservoir together and the water within the bottom plate is frozen allowing the root tissue to be firmly embedded within the ice before the top plate housing the seedlings is removed. This results in fresh root tissue within each well of the bottom plate ready for DNA purification and subsequent genotyping and seedlings

that can be stored for up to 2 weeks at 4°C housed within the top plate (Krysan, 2004; Clark and Krysan 2007; Clark et al. 2011).

Due to the array of photoreceptors possessed by plants and the overlap of there subsequent signaling pathways, imaging seedlings grown in darkness can prove challenging without introducing light contamination (Liscum et al. 2014). The traditional method of phototropism analysis is to capture images of a large number of seedlings before irradiation and a large number of different seedlings after irradiation (Firn, 1994). This method, although effective, is time consuming, as it requires a large number of seedlings be hand measure. In addition, temporal information can be overlooked due to the limitations of single end point measurements. Real time imaging of phototropic bending using IR imaging can allow for time point measurements and direct comparison of the amount of curvature prior to and after blue light irradiation of the same seedling. This greatly reduces the necessary number of seedlings to be measured saving time and space in the growth chamber or greenhouse (Miller et al. 2007; Wang et al. 2009).

Experimental procedures

Plant Materials and Growth Conditions

Plant materials and growth conditions were performed as described previously in Chapter 2. A working solution of 30 µM Tyrphostin A23 (TYRA23) (RG-50810, Sigma-Aldrich) was made by adding 5 µl of 25 mM stock solution TYRA23 to 5 ml half-strength Murishige and Skoog liquid medium. The resulting

5ml solution was added directly to agar plates housing seedlings two hours prior to blue light irradiation of seedlings.

Light treatment of Seedlings

Light treatment of seedlings was performed as described in Chapter 2.

Confocal microscopy

Confocal microscopy was performed as described in chapter 2. Seedlings treated with TYRA23 were placed on microscope slide in 80 µl of half-strength Murashige and Skoog liquid medium containing 30 µM TYRA23 for imaging.

Fluorescence Measurements

Fluorescent measurements performed as described in Chapter 2.

Graphical representation

Graphs were constructed and statistical data was generated using SigmaPlot version 13.0, from Systat Software, Inc., San Jose California USA, www.systatsoftware.com. Each point on graphs represents six individual measurements. Each measurement was taken from an individual cell within a seedling.

Construction and protocol of Ice-Caps fountain

Clear Plexiglas sheets were purchased from the local business Koonse Glass (Columbia, MO). Plexiglas was cut and bonded together using acetone to construct one large and one slightly smaller reservoirs. Shelving brackets and long steel bolts were purchased from Home Depot (Columbia, MO) and metal components that come in contact with liquid were coated in Performix Plastic Dip purchased from Home Depot (Columbia, MO). Metal components were constructed into an

adjustable structure that is housed within larger bottom reservoir and supports smaller reservoir above. An 80 GPH Submersible Fountain Pump, model M60HD, was purchased from Home depot (Columbia, MO) and placed in the bottom reservoir. Vacuum tubing was used to route water from pump up to top reservoir and to route water from top reservoir back down to bottom reservoir.

The 96-well plates lacking well floors (SigmaAldrich cat No. 278012) were filled with half-strength Murashige and Skoog medium by first applying parafilm to the bottoms of the plates with a sealing roller tool. Sterile seed was transferred individually to each of the 96 wells of the top plate. The top 96-well plates were then placed on bottom 96-well plates (SigmaAldrich cat. No 278129) before placing in the top reservoir of the Ice-Caps fountain. After 10-14 days of growth small wooden skewers were carefully placed between top and bottom 96-well plates and transferred to liquid nitrogen bath. Once the liquid in the bottom plate was frozen the top plate was carefully removed from the bottom plate. The bottom plate housing the root tissue was then ready for DNA purification and genotyping while the top plate housing the seedlings could be covered in foil and stored for up to 2 weeks at 4°C to minimize growth or can be placed back under white light and allowed to grow.

Construction and protocol of IR imaging system

The Cognex Insight Color Starter Toolset was purchased from Power Motion Sales, item no. COG IS7010-01, that included the AF 16mm camera, a 24V DC power supply, communication cables, camera mounting plate and Cognex Insight EasyBuilder Software Version 4.7. In addition an Edmund 8.5 mm Techspec 2/3"

fixed focal length lens, item no. COG LEC-58000, a 25.5 mm OPPT bandpass filter for IR wavelength of 880 nm, item no. COG IMIF-BP880-25.5, and a 6"X6" 880nm IR strobe backlight, item no. AI BL0606-880-I3-M12, were also purchased from Power Motion Sales. The imaging apparatus was constructed so that the camera faces backlit patri plates housing seedlings at a distance of approximately 12". The camera and IR backlight are controlled through the Cognex InSight easyBuilder software so that the IR backlight is only lit while acquiring images. In order to prevent light contamination from computer components the PC running the camera and software is housed within a light tight cabinet and operated remotely via GotomyPC software version 5.0.

Arabidopsis seed was sterilized as described previously in Chapter 2 and placed on vertical plates containing half-strength Murishige and Skoog medium using the tip of sterilized hemostats. Plates were cold and red light treated and placed in darkness as described previously. After 72 hour in darkness plates were placed in front of IR back light and imaged in darkness before irradiation for 4 hours at 0.1 umol m⁻² sec⁻¹ fluence rate. At the end of the 4 hour irradiation plates were placed in front of IR backlight and imaged again.

Results

TYR A23 Pre-treatment Does Not Prevent phot1-GFP Accumulation Into Foci at the Plasma Membrane or Internalization in Response to Blue Light Irradiation

Phot1-GFP has been reported to become internalized from the plasma membrane in response to blue light irradiation (Sakamoto and Briggs 2002; Han et al. 2008; Kaiserli et al. 2009; Preuten et al. 2015). Previous reports show that phot1 interacts with clathrin heavy chain at the plasma membrane suggesting phot1-GFP movement is clathrin mediated (Kaiserli et al., 2009; Roberts et al., 2011). Confocal images published by Kaisereli et al. (2009) indicate that phot1-GFP internalization in response to blue light irradiation in *Arabidopsis* seedlings is inhibited by the phosphotyrosine analog TYR A23 further supporting the involvement of clathrin. Conversely, here we show TYR A23 treatment of *Arabidopsis* does not prevent internalization or accumulation into foci of phot1-GFP at the plasma membrane in response to blue light irradiation.

After several minutes of irradiation of TYR A23 pre-treated seedlings with the 488 nm laser, phot1-GFP begins to form foci at the plasma membrane and becomes internalized as described previously. It appears as though the amount of aggregation and internalization is greater in TYR A23 treated seedlings in both the *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP lines and that the pattern appears slightly different to that of mock treated seedlings (Figure 4.1). In addition to forming foci and becoming internalized in response to blue light irradiation, phot1-GFP also appears to accumulate into foci and become internalized in darkness (Figure 4.2). This was unexpected given the nature of TYR A23 treatment inhibiting

CME. Quantification of foci forming at the plasma membrane in darkness compared to mock treated seedlings shows a large increase in the amount of phot1-GFP aggregation in darkness (Figure 4.3).

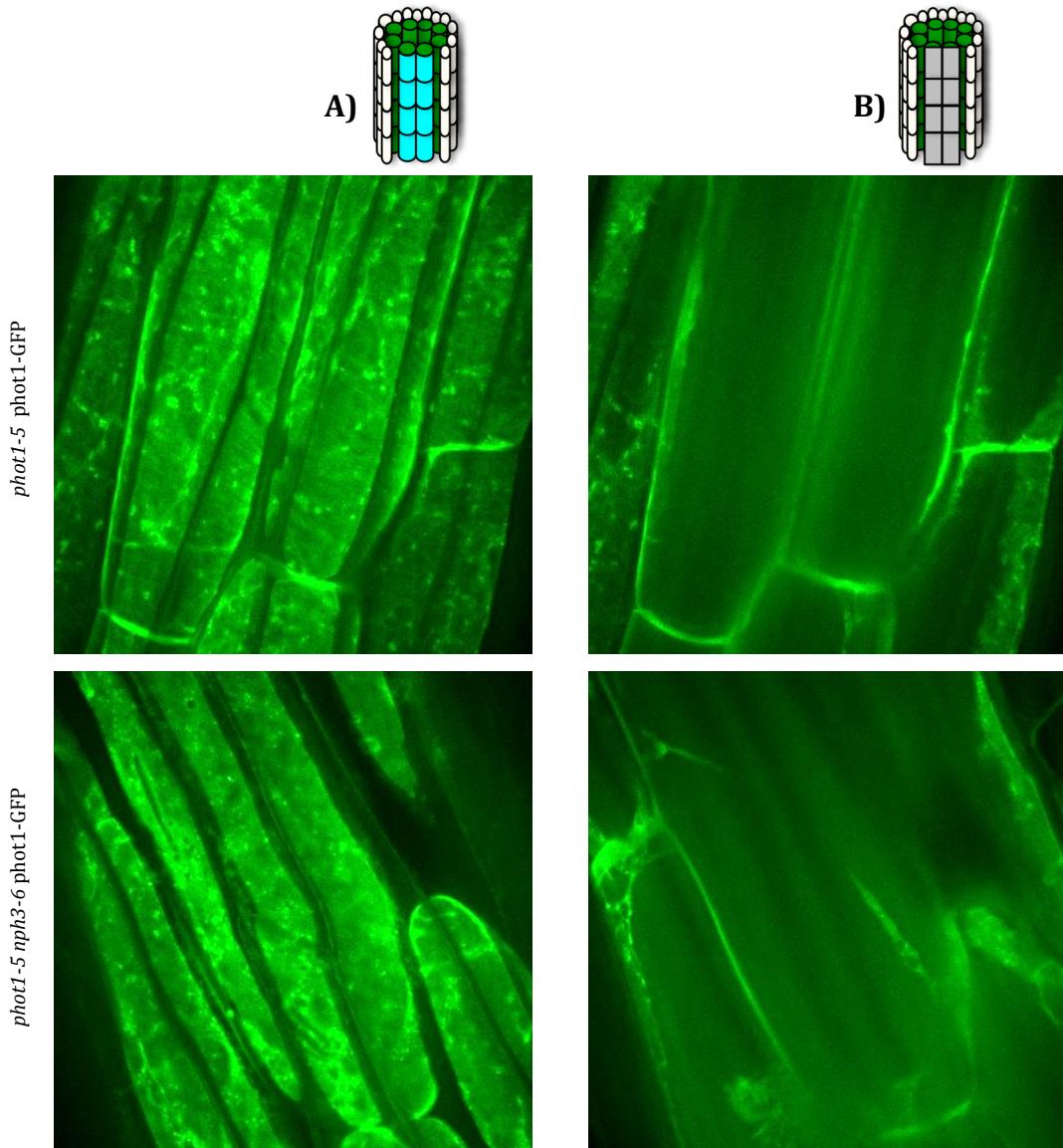


Figure 4.1 phot1-GFP accumulates into foci at the plasma membrane and is internalized in response to blue light irradiation after TYR treatment.

Phot1-GFP fluorescence was imaged in 3-day-old etiolated *phot1-5 phot1-GFP* and *phot1-5 nph3-6 phot1-GFP* seedlings pre-treated with TYR A23 two hours prior to imaging. Images taken after several minutes of exposure to the 488 nm laser at the plasma membrane (A) or approximately 20 μm into cortical cell layer (B).

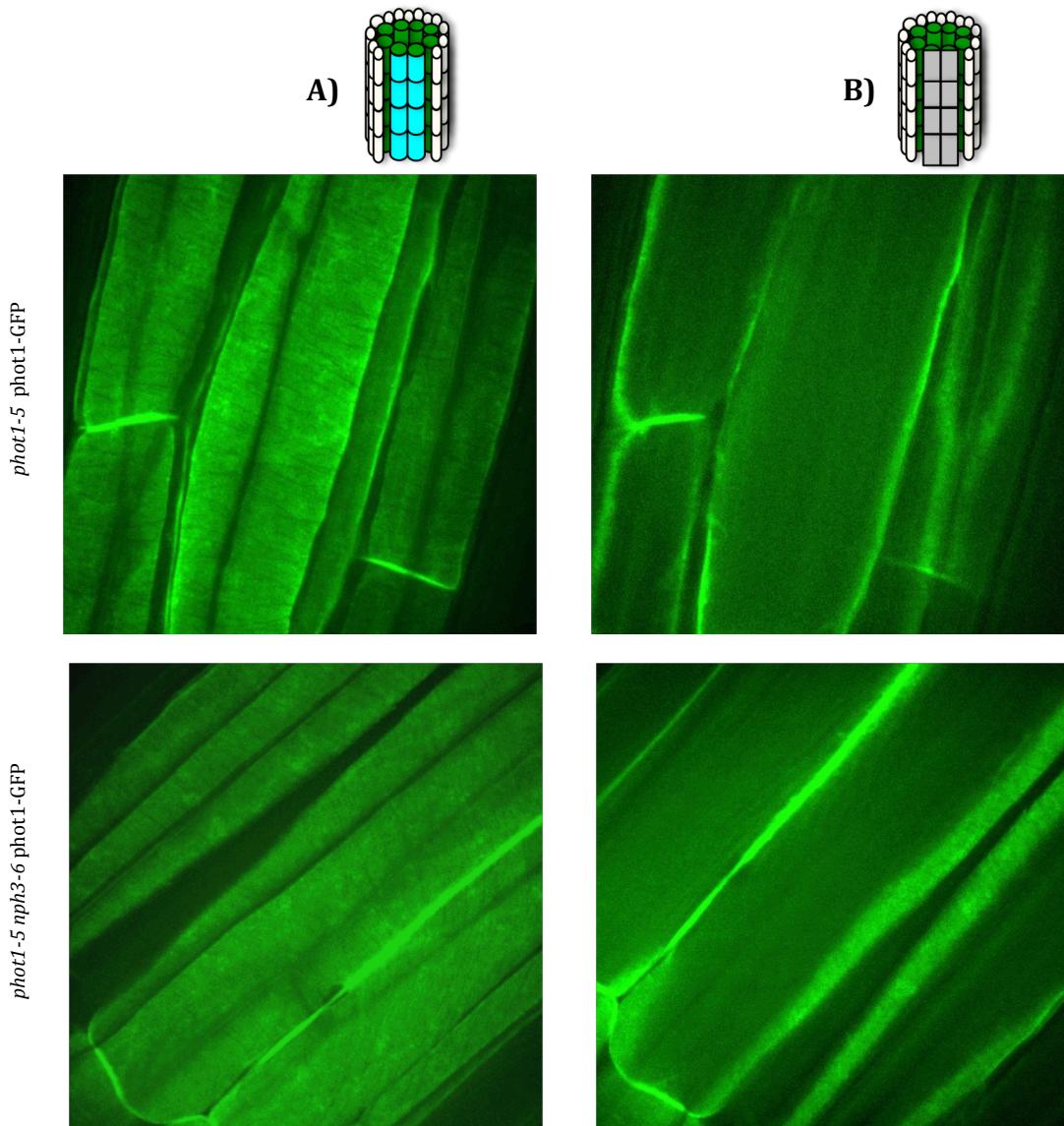


Figure 4.2 Phot1-GFP accumulates at the plasma membrane and is internalized in darkness in response to TYR A23 treatment.

Phot1-GFP fluorescence was imaged in 3-day-old etiolated *phot1-5 phot1-GFP* and *phot1-5 nph3-6 phot1-GFP* seedlings treated with TYR A23 two hours prior to imaging. Images taken from darkness at the plasma membrane (A) or approximately 20 μm into cortical cell layer (B).

Phot-GFP1 aggregation in darkness in samples with TYR A23 or no pre-treatment

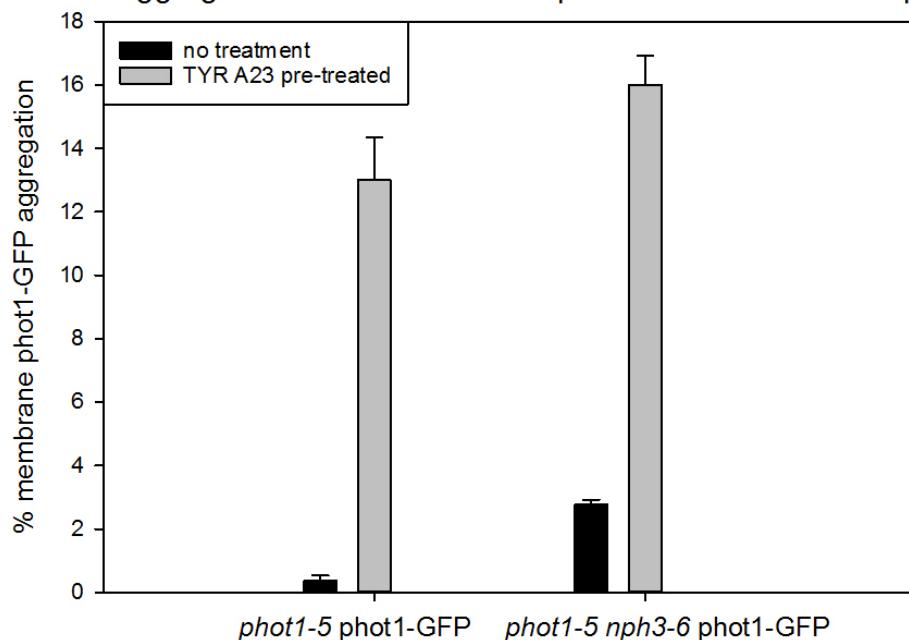


Figure 4.3 Accumulation of phot1-GFP into foci is greater in TYRA 23 treated seedlings relative to mock treated seedlings.

Phot1-GFP fluorescence was imaged in 3-day-old etiolated *phot1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP seedlings mock treated or pre-treated with TYR A23 2 hours prior to imaging. Each bar represents six measurements of accumulation into foci at the plasma membrane of six individual cells. Error bars represent standard error of the mean.

Phot1-GFP Accumulation Into Foci at the Plasma Membrane in Response to Blue Light Irradiation is Enhanced in TYR A23 Pre-treated Seedlings

In order to determine if the amount of phot1-GFP aggregation in response to blue light irradiation was in fact enhanced in TYR A23 pre-treated seedlings, we quantified the amount of aggregation at the plasma membrane. In both the *pho1-5* phot1-GFP and *phot1-5 nph3-6* phot1-GFP lines, phot1-GFP accumulation into foci was greatly enhanced in response to blue light (figure 4.4). Phot1-GFP accumulation into foci at the plasma membrane began prior to blue light irradiation and continued beyond 35 minutes in darkness post blue light irradiation. The enhanced accumulation into foci at the plasma membrane in response to blue light is apparent in images taken at intervals in darkness post blue light irradiation (Figure 4.5)

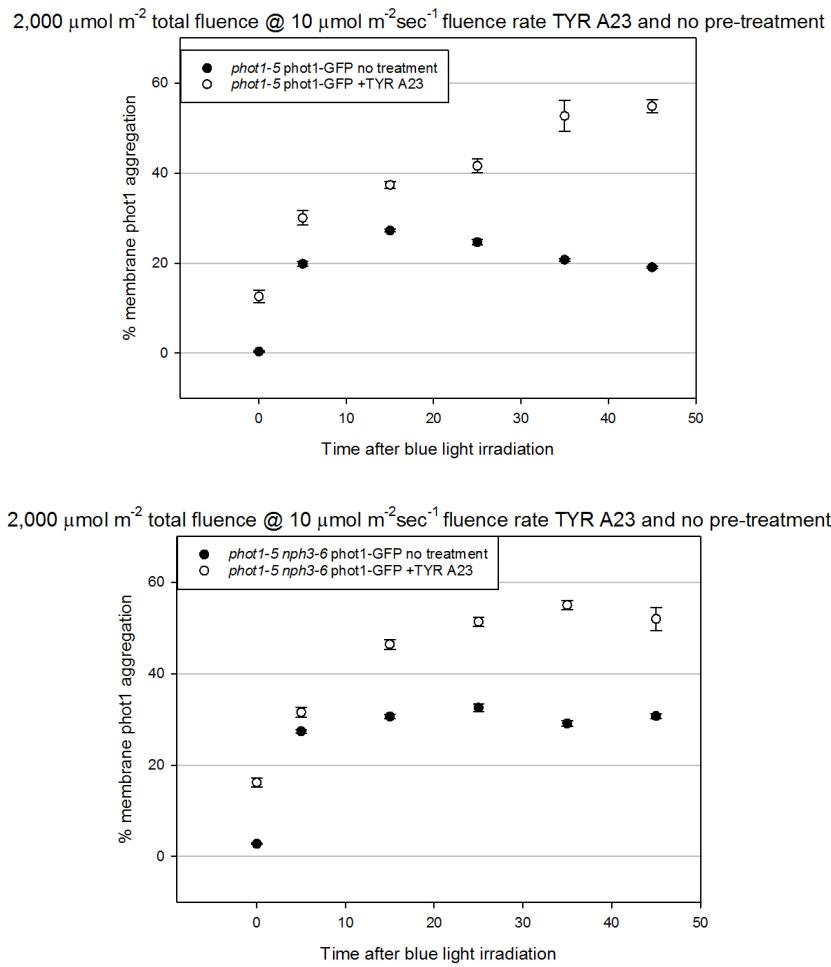


Figure 4.4 TYR A23 pre-treatment enhances accumulation of phot1-GFP into foci at the plasma membrane in response to blue light.

Phot1-GFP fluorescence was imaged in 3-day-old etiolated *Arabidopsis* seedlings in both the *phot1-5 phot1-GFP* (top graph) and *phot1-5 nph3-6 phot1-GFP* (bottom graph) lines. Seedlings were either mock treated (solid circles) or pre-treated with TYR A23 2 hours prior to blue light irradiation (open circles). Seedlings were then placed in darkness for varying amounts of time after irradiation prior to imaging. Error bars represent standard error of the mean.

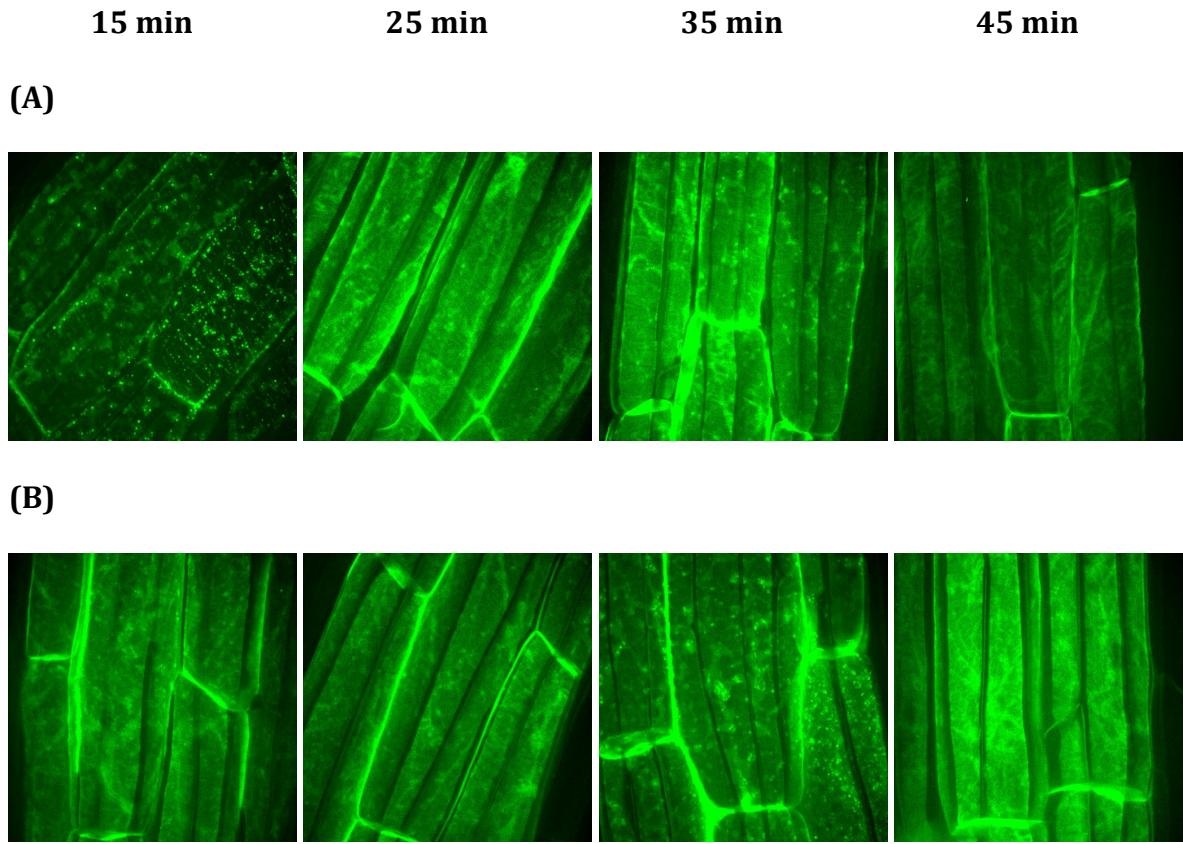


Figure 4.5 TYR A23 pre-treatment prolongs phot1-GFP accumulation into foci at the plasma membrane in response to blue light.

Phot1-GFP fluorescence was imaged in 3-day-old etiolated *Arabidopsis* seedlings pre-treated with TYRA23 two hours prior to imaging from the *phot1-5* *phot1-GFP* (A) and *phot1-5 nph3-6* *phot1-GFP* (B) lines. Seedlings were treated with a total fluence of 2,000 $\mu\text{mol m}^{-2}$ at a fluence rate of 10 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$ and returned to darkness 15, 25, 35 or 45 minutes prior to imaging.

The ICE-CAPS Fountain Allows High Throughput Growth and Collection of Tissue for Genotyping of *Arabidopsis* Seedlings

The ICE-CAPS fountain will allow for growth and collection of *Arabidopsis* tissue within 10-14 days with minimal space and resource requirements. Growing *Arabidopsis* seedlings in 96-well plates reduces the amount of space needed for growth and collection of root tissue can be performed well before leaf tissue is ready to be harvested. The fountain has an area of approximately 6 ft², about the same area as 1 flat of seedlings (Figure 4.6). The Ice-Caps fountain can support the growth of 9 plates allowing for the growth of 864 seedlings simultaneously. A flat of seedlings containing 5 seedlings per pot only allows for the growth of 90 seedlings. The 96-well plates allow for the growth of seedlings in agar within the top plate and seedlings to protrude through into the bottom plate (Figure 4.7). Additionally, collection of root tissue to be analyzed for genotyping can be done immediately for every seedling in a 96-well plate in contrast to collecting individual leaf punches from the traditional method of tissue collection.

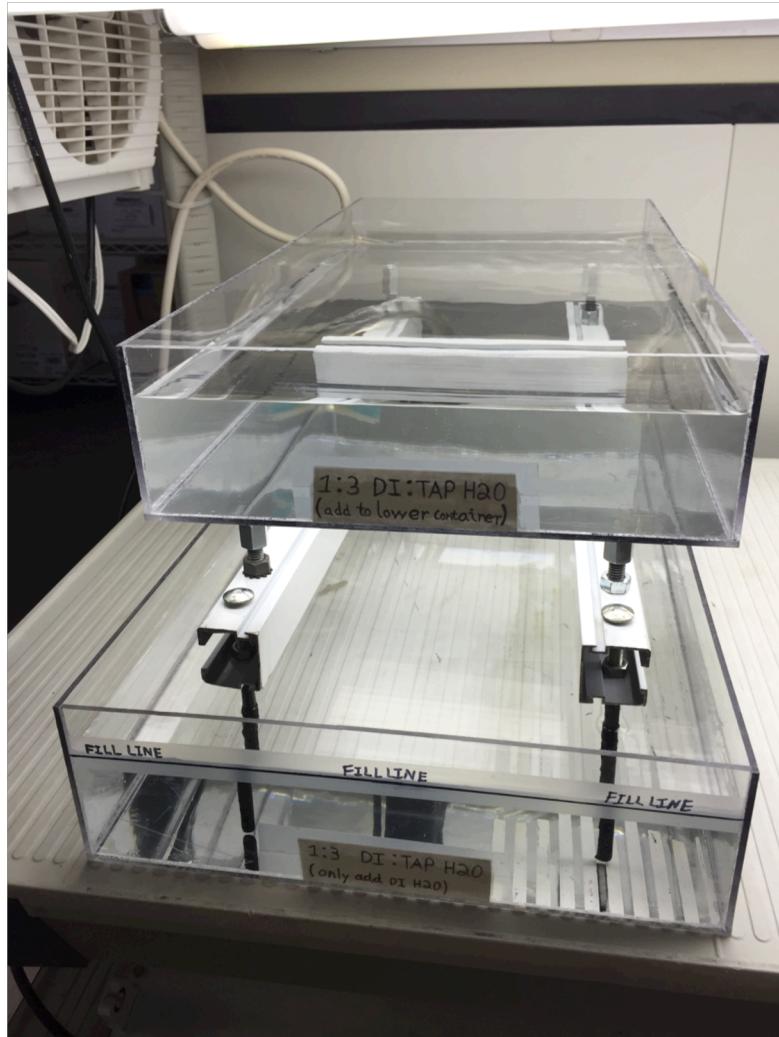


Figure 4.6 Assembled ICE-CAPS fountain.

The Ice-Caps fountain consists of a water reserve reservoir on bottom and growth reservoir on top constructed from clear Plexiglas supported by shelving material. Water is circulated from the bottom reserve reservoir to the top growing reservoir via an 80 GPH Submersible Fountain Pump and flows back down via rubber tubing into the reserve reservoir.

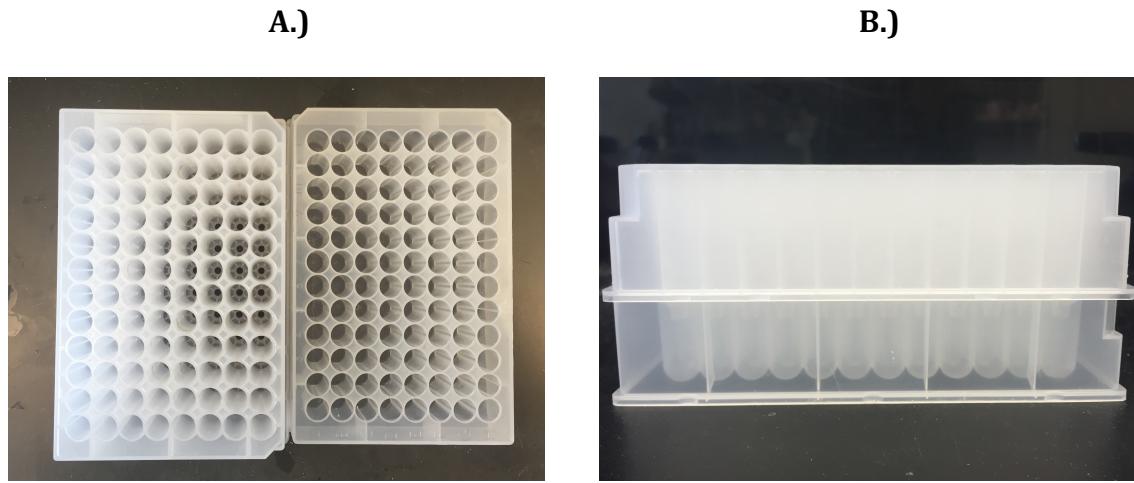


Figure 4.7 Growth 96-well plate and capture 96-well plate.

Images of growth plate and collection plate respectively (A) and growth plate placed on top of capture plate (B). Each well of the growth plate is filled with half-strength Murishige and Skoog agar medium and placed on top of the collection plate. When placed into the ICE-CAPS fountain the capture plate fills with water. Roots that protrude from the growth plate into the corresponding well of the capture plate will be submerged in water allowing for collection when water in capture plate is frozen.

IR Imaging of Seedlings Allows Accurate Imaging of Seedling Position Before and After Blue Light Irradiation

The IR imaging apparatus was constructed to facilitate positional imaging of *Arabidopsis* seedlings grown on vertical plates before, during and after blue light irradiation. The Cognex 16mm camera is equipped with a bandpass filter that restricts wavelengths of light outside of the 880 nm wavelength (Figure 4.8). The 880 nm wavelength strobe light allows for illumination of seedlings with a wavelength of light that is not perceivable by the photoreceptors within the plant (Figure 4.9). The camera does not directly image the seedling but rather the shadow that the seedling casts against the IR backlight. Seedlings can be imaged before and after blue light irradiation allowing for direct comparison between the position of the seedling before and after blue light irradiation (Figure 4.10). Direct comparison facilitates a more accurate means of determining phototropic bending in response to blue light irradiation.



Figure 4.8 IR imaging camera with IR 88nm bandpass filter installed.

Image of AF 16mm camera with 25.5 mm OPPT IR wavelength 880 nm bandpass filter installed. Camera is mounted on custom made wooden block.

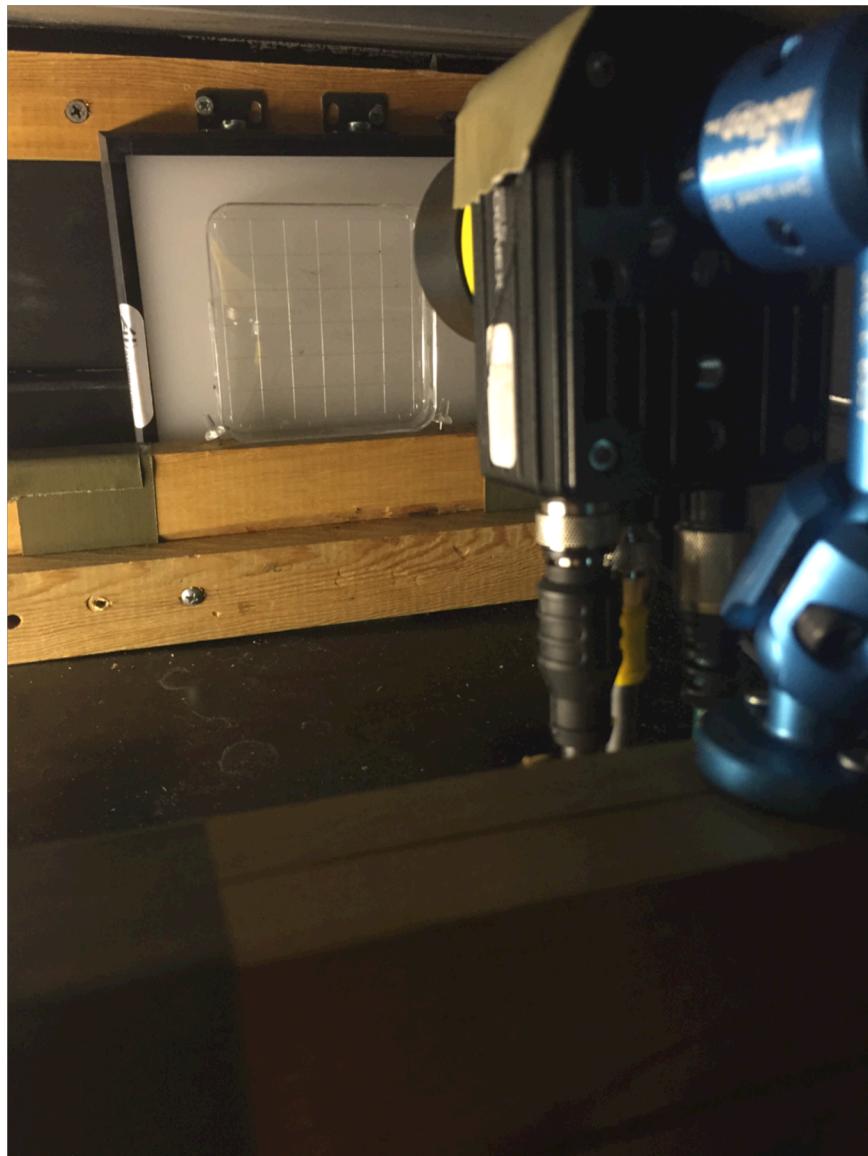


Figure 4.9 IR Imaging apparatus allows imaging of *Arabidopsis* seedlings before, after and during blue light irradiation.

Image of IR imaging setup with IR backlight and IR imaging 16mm camera. Vertical plate is placed in front of IR backlight to acquire image of shadow cast by seedlings growing on plates.

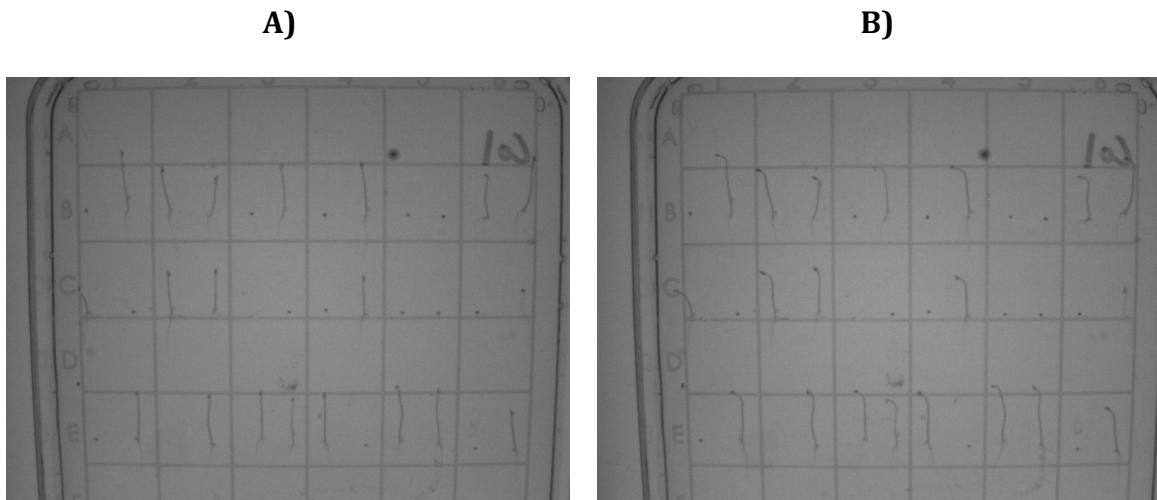


Figure 4.10 IR Image of *Arabidopsis* seedlings before and after blue light irradiation.

3-day old Etiolated *Arabidopsis* seedlings grown on vertical plates were imaged from darkness (A) and after 4 hours blue light irradiation at a fluence rate of 0.1 umol m⁻² sec⁻¹ from the left (B) using the IR imaging system. IR imaging allows for direct comparison of seedlings before and after blue light irradiation without introducing light contamination.

Discussion

Previously Kaiserli et al. (2009) reported that pre-treating *Arabidopsis* seedlings with 30 µM TYR A23 prevents phot1-GFP internalization in response to blue light irradiation. Here we show that pre-treatment with TYRA23 does not prevent the internalization of phot1-GFP in response to blue light irradiation. Phot1-GFP also accumulates into foci at the plasma membrane in response to blue light irradiation when pretreated with TYR A23 (Figure 4.1). Accumulation and internalization of phot1-GFP occurred within minutes of irradiation with the 488 nm laser but the amount of accumulation and internalization appeared to be greater compared to mock treated seedlings. Internalization and accumulation into foci at the plasma membrane also appeared in seedlings imaged directly from darkness (Figure 4.2). Quantification indicates that accumulation into foci at the plasma membrane is increased when compared to mock treated seedlings imaged from darkness (Figure 4.3). This data is interesting considering that previous reports suggest phot1-GFP movement is dependent on CME (Sullivan et al. 2009; Kaiserli et al. 2009; Roberts et al. 2011).

Further quantification shows that phot1-GFP accumulation into foci at the plasma membrane is also enhanced in seedlings irradiated with blue light that have been pre-treated with TYR A23 (Figure 4.4). Not only is the accumulation increased but the pattern appears to be altered as well. In mock treated lines, phot1-GFP accumulation peak at around 15-25 minutes in the *phot1-5* phot1-GFP line and around 25-35 minutes in the *phot1-5 nph3-6* phot1-GFP line after blue light irradiation. After peaking, phot1-GFP accumulation begins to dissipate as phot1-GFP

returns to the homogenous distribution or is degraded. In lines pre-treated with TYR A23, phot1-GFP aggregation builds quickly and reaches a maximum that is 2-3 fold that of its mock treated counterparts in both lines. This suggests that although AP-2 initiated CME nucleation is not necessary for phot1-GFP aggregation it may be involved in the process or phot1-GFP trafficking.

The phosphotyrosine analog pharmacological agent TYR A23 prevents CME by inhibiting binding of the adaptor protein AP-2 to cargo proteins and clathrin thereby preventing clathrin cage formation and subsequent CME (Honing et al., 2005; Crump et al., 1998; Banbury et al., 2003). Perhaps phot1-GFP aggregation and internalization occurs via a clathrin dependent mechanism for endocytosis that proceeds without the aid of AP-2 in initiating the CME nucleation complex. Fan et al. (2013) showed that although *Arabidopsis* lines deficient in AP-2 exhibited some developmental defects they were still viable suggesting that plants are able to overcome the lethality of the loss of AP-2 seen in animal and fungal cells (Mitsunari, 2005). Recently Gadeyne et al. (2014) identified an eight-core component complex in *Arabidopsis* called TPLATE that precludes AP-2 binding and may function as a substitute nucleation complex in plants lacking a functional AP-2. Although this may help explain the ability of phot1-GFP to accumulate into foci and become internalized in response to blue light, it does not explain the fact that phot1-GFP forms foci and is internalized in darkness. Although AP-2 may not be necessary for initiating CME nucleation in phot1-GFP movement, perhaps it is necessary for trafficking of phot1-GFP to and from the membrane in the absence of light. Unfortunately, due to resolving limitations of temporal recruitment of the factors

involved in CME, it is difficult to fully explain the behavior of phot1-GFP in seedlings treated with TYR A23 in regard to CME (Godlee and Kaksonen 2013).

Identifying genotypes and phenotypes of organisms used in experiments are two examples of maintenance work that needs to be done before data generating experiments can be performed. Generating *Arabidopsis* lines that posses a particular phenotype can be cumbersome and can necessitate large amounts of. Likewise once a genotype has been established confirmation of a specific phenotype must be completed before an experiment can be performed. Therefore, efficiency in performing maintenance tasks is essential for obtaining experimental specimens.

In order to promote efficiency in the lab we constructed a variation of the Ice-Caps fountain described previously by Krysan (2004). The Ice-Caps fountain consists of a growth reservoir on top circulated with water from a reserve reservoir on bottom fed by a small fountain pump (Figure 4.6). The Ice-Caps setup allows the growth nearly ten times the seedlings as a traditional flat in the same amount of space. In addition the Ice-Caps fountain utilizes ice to “capture” root tissue that extends from the growth plate down into the capture plate (Figure 4.7). This not only allows for an exponential amount of seedlings that can be grown but also reduces materials costs and streamlines tissue collection for genotyping. After 10-14 days the roots have extended from the growth plate housing the growth media and developing seedling into the capture plate where each well is constantly circulated with fresh water from the fountain. Moving the growth and capture plates to liquid nitrogen allows for rapid freezing and capturing of root tissue in ice which can be subjected to genotyping in the same 96 well plate. The corresponding plant

in the growth plate to the PCR reaction that shows a positive result can then be transferred to soil for propagation. This is ideal for situations where growth chamber space is limited or where punching individual leaves of mature plants for tissue collection can be time consuming and cumbersome. However, once a genotype of interest has been established the expected phenotype must also be confirmed.

Phototropism is profoundly important to our work and any deficiencies or enhancements must be well characterized in a plant line before moving forward with an experiment. Therefore, we developed an IR imaging system to capture images of seedlings before, during and after blue light irradiation. The traditional method of “pick and stick” phototropism assays involves growing seedlings on horizontal plates and “picking” and subsequently “sticking” them to a piece of tape. Although this method has served our lab we throughout the years it is time consuming and requires large numbers of seedlings as before and after images of the same plant cannot be obtained. Here we described constructing an apparatus that allows for imaging of seedlings using IR light that is invisible to plants.

Plants host an array of photoreceptors that span the visible light spectrum (Liscum et al. 2014). Therefore imaging seedlings without contaminating light is difficult. Wavelengths in the IR spectrum are not perceivable by plants and make great wavelengths to capture backlit images with the proper camera and filter set. Miller et al. (2007) previously described a method to image dark grown seedlings using IR wavelengths of light. Here we constructed a similar apparatus consisting of a camera capable of imaging in the IR range with an appropriate filter set for the

camera that restricts all but wavelengths of 880 nm (Figure 4.8) and a light source that emits a wavelength of 880nm. The apparatus is assembled so that the IR light source faces the camera and vertical plates are placed in between the light source and the camera (Figure 4.9). Seedlings are grown vertically on the vertical plate and are backlit by the IR light source causing a shadow to be cast on the lens of the camera indicating the seedlings position. Images can be taken before, after and during blue light irradiations and allow for direct comparison of a seedling before and after irradiation (Figure 4.10). This also allows for resolution of temporal events that can occur during irradiation that would not be present in end point measurements.

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VITA

I was born in a small mining community in rural Montana on January 1st 1984. The town that I spent the first few years of my life no longer exists; it disappeared with the mine many years ago. Neither of my parents received a formal education but knew of its importance and always encouraged me to attend college. Upon graduation I threw all of my belongings into my S-10 pickup truck and moved to Springfield, MO. After working as a mechanic for a year saving money for tuition I began working the night shift at a remanufacturing plant so I could attend classes during the day. Unfortunately, I was laid off from work and could not afford an apartment and tuition so I spent the better part of my freshman year living in my car. My grades were not the best because of my living situation but I worked very hard and a few of my professors took notice. I have been very lucky in that I have met very supportive people throughout my life and academic career that have encouraged me and pushed me to achieve goals I would have otherwise thought impossible.

Upon completing my undergraduate degree at Missouri State University the head of the Biomedical Sciences, Dr. Collette Witkowski, pushed me to continue my education by pursuing a Master's degree. Luckily, I was offered an assistantship teaching position and was able to continue to develop my skills as a scientist. I completed my Master's degree and taught for a year at Missouri State University before applying to the Ph.D. program at Mizzou. I began my Ph.D. work in September

of 2012 and shortly after settled down in Mannie Liscum's lab and began working as a photobiologist. I am now completing my program and am excited to soon become Dr. Scott Askinosie and begin my career as an industry scientist.