University of Glasgow

ANALYSIS OF PHOTOTROPIN MEMBRANE LOCALISATION AND FUNCTION IN *ARABIDOPSIS*

Lisa M Blackwood

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Institute of Molecular, Cell and Systems Biology
School of Life Sciences
College of Medical, Veterinary and Life Sciences
University of Glasgow

September, 2015

Abstract

The ability of plants to respond to environmental cues is crucial for the success of the organism. Light is an important source of energy for the plant as well as providing information for plant growth and development. Plant responses to the electromagnetic spectrum are controlled by a number of different photoreceptors. Phytochromes respond to red and far-red light and are responsible for controlling photomorphogenesis in seedlings (Chen and Chory, 2011). The recently identified UVR8 responds to UV-B light and prevents damage to the plant from these harmful wavelengths (Rizzini et al., 2011). Responses to UVA/blue light are controlled by three different photoreceptors: the cryptochromes that are involved in the circadian clock as well as regulating a number of aspects of photomorphogenesis (Chaves et al., 2011); the Zeitlupe family which control the circadian clock and flowering responses (Demarsy and Fankhauser, 2009) and finally the phototropins which regulate the light-dependent processes that increase photosynthetic efficiency of plants (Christie, 2007, Christie and Murphy, 2013). Within *Arabidopsis* there are two phototropins, phototropin 1 (phot1) and phototropin 2 (phot2), which share approximately 60% sequence identity at the amino acid level (Christie et al., 2002). The phototropins have also been identified in algae, ferns and mosses as well as higher plants where their mode of action appears to be conserved (Onodera et al., 2005). The phototropins function redundantly to control phototropism, chloroplast movement, leaf flattening and positioning, stomatal opening whilst phot1 alone controls the rapid inhibition of hypocotyl growth upon transfer of dark-grown seedlings to light (Christie, 2007).

The phototropins are serine/threonine (Ser/Thr) kinases consisting of two Light, Oxygen and Voltage (LOV) domains in the N terminus and a Ser/Thr kinase at the C terminus (Figure 1). In darkness, the protein associates with the plasma membrane and upon illumination partially internalises to cytosolic strands. Illumination with blue light also results in the the LOV domains forming a covalent linkage with the chromophore, Flavin Mononucleotide (FMN) and the protein undergoes autophosphorylation at a number of serine residues including upstream of the LOV1 domain and between the LOV1 and LOV2 linker region, as

well as within the kinase activation loop (Inoue et al., 2008; Sullivan et al., 2008).

Whilst the physiological functions of the phototropins from *Arabidopsis* are well characterised, the function of the membrane association and subsequent internalisation is unknown. Therefore the aims of this project were to identify the mechanism of phot1 association with the membrane and to determine if there are specific regions of membrane interaction within the kinase domain of phot1, since the C-terminus of phot2 including the kinase domain is known to direct localisation to the plasma membrane (Kong et al., 2007). It was therefore of interest to analyse the kinase domain of various phototropins to assess if there are sequences that may direct protein localisation to the plasma membrane. Chapter 3 describes the Lysine Rich Motif (LRM) that was identified and subsequently mutated to assess localisation when transformed in to the *phot1-5 phot2-1* double mutant as well as subsequent complementation analysis of the physiological responses controlled by phot1. The analysis suggests that phot1 may interact with the membrane through a lipid interaction that is not mediated by the LRM. The consequences of these results are discussed further in the chapter.

Further analysis of the role played by the kinase domain in membrane localisation of phot1 is described in the truncation analysis in Chapter 4. A similar approach to the deletion analysis employed by Kong et al. (2013) was used. A predicted secondary structure was generated to ensure that truncations were performed outside secondary structures that may be important for the protein structure. The insect cell system as well as transient expression in *N. benthamaiana* provided convenient methods to analyse various truncations of the kinase domain of phot1. The method was also used to analyse the membrane association of phot2. The effect of 1-butanol treatment on *Arabidopsis* seedlings was also investigated in relation to phot1 associating with the plasma membrane by interaction with lipids such as phosphatdic acid (PA). The work presented in this chapter suggests that there may be more than one region of phot1 that interacts with the membrane and the complications of this are discussed further.

In order to investigate the function of internalised phot1 after irradiation, the protein was constitutively targeted to the membrane using a farnesyl tag. A farnesylation sequence targets proteins to the membrane via a lipid modification (Sorek, Bloch and Yalovsky, 2009). The farnesyl-tagged phot1-GFP was transformed into the *phot1-5 phot2-1* double mutant and the physiological responses controlled by the phototropin were assessed. The results of targeting phot1-GFP to the membrane are shown in Chapter 6. Constitutive targeting of phot1-GFP to the plasma membrane showed that the soluble protein visualised after blue light illumination is, perhaps surprisingly, dispensable for the physiological responses tested. There may however be a role to play in the fine-tuning of the *Arabidopsis* response to illumination. Together these studies provide new perceptions in the possible mechanism of membrane attachment of phot1. Finally, investigation of the phototropin from the algae *Ostreococcus tauri* reveal that, while it can mediate some phot-regulated responses when expressed in the *phot1-5 phot2-1* double mutant the protein is not functional in phototropism providing a unique phototropin that could be used to investigate the downstream control of this response. These findings highlight key differences between the mode of action of plant and algal phototropins that have so far gone unrecognised.

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Publications

Christie J.M., Blackwood L., Petersen J. & Sullivan S. (2014) Plant Flavoprotein Photoreceptors. *Plant and Cell Physiology*, 56(3) pp. 401-413

Part of the work presented in chapter 5 of this thesis has been published as:

Preuten T., Blackwood L., Christie J.M. & Fankhauser C. (2015) Lipid anchoring of *Arabidopsis* phototropin 1 to assess the functional significance of receptor internalization: should I stay or should I go? *New Phytologist*, 1, pp.1-13

Acknowledgements

Firstly, I would like to thank my supervisor Prof. John M. Christie for giving me the opportunity to carry out my PhD and for his help and support over the past four years and especially during the last six months. Also thank you to the BBSRC for funding the project. The many helpful discussions during lab meetings with members of Prof. Gareth I. Jenkins lab helped my project enormously.

I would also like to thank Dr. Katherine Baxter and Peggy Ennis for their help and encouragement at the start of my PhD and continuing throughout. A special thank you to Peggy for her help with the insect cells and answering my many questions about a variety of techniques. The support of Dr. Catherine Cloix throughout my PhD has been invaluable, she has taught me a great deal and made the experience the enjoyable one it has been.

There have been many people in the Brian lab over the last four years who contributed to help make the PhD even more fun. Thanks to Cat, Katherine, Monika, Kirsty, George, Chris, Kirsten, Bobby, Eros, Ashutosh and countless others who have come and gone from the lab. I have definitely made life long friends. I am also grateful to everyone in the Bower building for their assistance on any matter.

To all my friends I have had to ignore whilst writing up my thesis, thank you for all the words of encouragement it means a lot to me. Last, but by no means least, a massive thank you to my mum. I definitely could not have completed this without her support and encouragement. She is certainly one in a million and I cannot thank her enough.

Abbreviations

ABCB	ATP-binding cassette B
ADP	adenosine diphosphate
AP	alkaline phosphatase
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
AUX	AUXIN
BFA	brefeldin A
bHLH	basic helix loop helix
bp	base pair
BR	brassinosteroid
BR1	brassinosteroid insensitive 1
BKI1	BRI1 kinase inhibitor 1
BLUS1	blue light signalling 1
BOG	n-octyl-ß-D-glucoside
BSA	bovine serum albumin
BTB/POZ	broad complex, tram track, bric a brac/pox virus and zinc finger
bZIP	basic leucine zipper
CaMV-35S	Cauliflower mosaic virus 35S promoter
CBP	calmodulin binding peptide
CC	coiled coil
CCT	Cryptochrome C-terminal
CDF	cycling DOF factor
cDNA	complimentary DNA
CHAPS	[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNT	Cryptochrome N-Terminal
CO	Constans
Col	Columbia
COP	constitutively photomorphogenic
CPD	cyclobutane pyrimadine dimer
CDF	CYCLING DOF FACTOR
CPT1	COLEOPTILE PHOTOTROPISM1
cry	Cryptochrome
CV	column volume

DASH	Drosophila Arabidopsis Synechocystis Homo
DM	n-decyl-ß-D-Maltopyranoside
DDM	n-dodecyl-ß-D-Maltoside
DHN1	dehydrin 1
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	1,4-Dithiothreitol
EGTA	ethylene glycol tetra-acetic acid
EMS	ethyl methanesulfonate
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide
FKF1	flavin-binding, kelch repeat, F-box 1
FMN	Flavin Mononucleotide
Fos-9	Fos choline 9
FT	Flowering locus T
GFP	Green Fluorescent Protein
GP-64	glycoprotein 64
GI	GIGANTEA
GPCR	G protein-coupled receptors
GRK	G protein-coupled receptor kinases
GST	glutathione-S-transferase
GUS	ß-glucuronidase
HRP	horse radish peroxidase
HY5	long hypocotyl 5
HYH	long hypocotyl5-like
IAA	indole-3-acetic acid
IPTG	isopropyl ß-D-galactopyranoside
kDa	kilo Daltons
LAX	LIKE AUX1
LB	Luria broth medium
LEI	leaf expansion index
LKP2	LOV, Kelch protein 2
LOV	Light, Oxygen or Voltage

LRM	lysine rich motif
mRNA	messanger RNA
MS	Murashige and Skoog salts
MTHF	methenyltetrahydrofolate
NCBI	National Centre for Biotechnology Information
neo	neochrome
NES	nuclear export signal
NLS	nuclear localisation signal
nm	nanometres
NPH	non-phototropic hypocotyl
NPL	non-phototropic hypocotyl-like
NRL	NPH3/RPT2-Like
OD	optical density
PA	phosphatidic acid
PAR	photosynthetically active radiation
PAS	Per, ARNT, Sim
PC	Phosphatidylcholine
PCR	polymerase chain reaction
Pfr	far-red light absorbing form of phytochrome
pH	-log10 (hydrogen ion concentration)
phot	Phototropin
PHR	photolyase-related
phy	phytochrome
PID	pinoid
PIF	phytochrome interacting factor
PIN	pin-formed
PIP_2	phosphatidyl-inositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKS	phytochrome kinase substrate
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethylsulphonyl fluoride
Pr	red light absorbing form of phytochrome
rbcL	Rubisco large subunit

RPT2	root phototropism 2
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SAXS	small angle X-ray scattering
SCF	Skp Cullin F-box
SDS	sodium dodecyl sulphate
Sf9	*Spodoptera frugiperda*
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SYMREM1	SYMBIOTIC REMORIN 1
TAE	Tris-acetate EDTA
TBS-T	Tris buffered saline triton-X
TBS-TT	Tris buffered saline triton-X Tween
T-DNA	transfer DNA
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TOC	Timing of CAB expression
Tris	Tris(hydroxymethyl)aminomethane
UGPase	UDP-glucose pyrophosphorylase
UV	ultra violet
UVR8	ultra violet resistance 8
v/v	volume/volume
w/v	weight/volume
WT	wild-type
ZTL	Zeitlupe

Chapter 1: Introduction to photoreception and signalling in plants

1.1 The requirement of light for plant signalling and growth

Plants are sessile organisms that must respond to a variety of environmental signals. One of the most important is light, which directs a number of responses that are important for plant growth and development. Germination of seedlings in soil occurs in darkness and is directed by gravity to ensure that seedlings emerge from the soil to become auxotrophic (Chen, Chory and Fankhauser, 2004). The change in gene expression when plants sense light is known as photomorphogenesis and encompasses a wide variety of physiological responses. Several photoreceptors have evolved to respond to the different wavelengths of the light spectrum. Phytochromes respond to red (600-700 nm) and far-red (700-750 nm) light and are responsible for controlling photomorphogenesis in seedlings (Chen and Chory, 2011) whilst the more recently identified UV Resistance Locus 8 (UVR8) responds to UV-B wavelengths (280-315 nm) and prevents damage to the plant from these harmful rays (Rizzini et al., 2011, Jenkins, 2014). Plant responses to UV-A/blue light are controlled by three different photoreceptors: the cryptochromes which are involved in a wide number growth and developmental roles (Chaves et al., 2011); the Zeitlupe family which control circadian responses and the flowering pathway (Demarsy and Fankhauser, 2009) and finally the phototropins which regulate the light-dependent processes that increase photosynthetic efficiency of plants (Christie 2007; Briggs 2014; Christie et al. 2014). These include phototropism, chloroplast movement, leaf flattening and positioning, stomatal opening and rapid inhibition of hypocotyl growth upon transfer of dark-grown seedlings to light (Demarsy and Fankhauser, 2009). Phototropins, as well as cryptochromes and phytochromes, have been identified in algae, ferns and mosses as well as higher plants where their mode of action appears to be conserved (Montgomery & Lagarias 2002; Onodera et al. 2005; Losi & Gärtner 2012). The known photoreceptors and their function will be described below.

1.2 Phytochromes and red light signalling

Phytochromes (phys) are the red and far-red photoreceptors that are involved in photomorphogenesis, a set of development responses that seedlings undergo when first exposed to light. Since the identification of phytochrome in the late 1950s (Butler et al., 1959), phytochrome light sensing domains have been identified in bacteria and fungi coupled to other signalling domains (Montgomery and Lagarias, 2002). *Arabidopsis* contains five phytochromes, *PHYA*, *PHYB*, *PHYC*, *PHYD* and *PHYE* (Clack, Mathews and Sharrock, 1994). The phys contain an N-terminal photosensory domain combined with a C-terminal dimerization/localisation domain (Nagatani, 2010). Three of the phys, PHYA, PHYB and PHYD exist as homodimers (Sharrock and Clack, 2004) whilst PHYC and PHYE are found only as heterodimers with PHYB and PHYD (Clack et al., 2009). The phytochromes bind the linear tetrapyrrole bilin chromophore via a covalent thioether linkage (Rockwell, Su and Lagarias, 2006, Bae and Choi, 2008). The bilin chromophore enables phytochromes to photoconvert between the inactive red-absorbing (Pr) and active far-red-absorbing (Pfr) forms (Rockwell, Su and Lagarias, 2006). This interplay between Pr and Pfr light conveys information about the photosynthetic energy available to ensure that the plant receives enough light for growth (Rockwell, Su and Lagarias, 2006).

In the darkness all five phytochromes are found in the cytoplasm and upon illumination with light they translocate into the nucleus within minutes (Kircher et al., 2002), with phyA and phyB localising to photobodies that contain a multitude of transcription factors and E3 ligases that are involved in the photomorphogenic response of seedlings (Chen, 2008). This accumulation in the nucleus triggers phosphorylation of the Phytochrome Interacting Factors (PIFs) and their subsequent rapid degradation by the ubiquitin proteasome (Al-Sady et al., 2006). PIFs are transcription factors that negatively regulate the photomorphogenic response (Lucas and Prat, 2014) by binding to the G-box motif of light regulated genes (Chen and Chory, 2011). One of the main physiological responses of these light regulated genes that are controlled by the phytochromes is shade avoidance. This provides information to the plants about the quality of the light and also any neighbouring plants. Shade signals increase the abundance of PIF4 and PIF5 resulting in the synthesis and redirection of the

hormone auxin as well as increased expression of the other hormones gibberellins and brassinosteroid for plant growth (Casal, 2012). It has also been proposed that phytochromes act as kinases to phosphorylate a number of downstream targets including Aux/IAA auxin transporters and Phytochrome Kinase Substrate PKS1 (Fankhauser et al. 1999; Chen et al. 2000) as well as interaction with the blue light photoreceptor cryptochromes (Ahmad et al. 1998; Más et al. 2000). This allows integration of the photoreceptor signalling pathways.

1.3 UVR8 and UV-B light

UV-B irradiation has the potential to damage DNA and cells as well as the ability to modulate photomorphogenesis at low fluence rates (Brosche & Strid 2003; Ulm & Nagy 2005; Frohnmeyer & Staiger 2003). In response to damaging levels of UV-B light plants produce flavonoid compounds that protect the plant by accumulating in the epidermal layer and preventing transmittance to the cells below (Winkel-Shirley, 2002, Jenkins, 2014). Conversely non-damaging levels of UV-B causes a suppression of hypocotyl extension and root growth as well as cotyledon opening (Jenkins, 2014). The photoreceptor involved in these processes remained unknown for a number of years until 2002 when the *uvr8-1* mutant was identified in a screen for mutants sensitive to UV-B and was found to be deficient in flavonoid biosynthesis (Kliebenstein et al., 2002). The gene was subsequently found to encode a seven-ß-propeller protein that regulates expression of over 70 genes in *Arabidopsis* leaf tissue (Brown et al., 2005) and several hundred genes in *Arabidopsis* seedlings (Favory et al., 2009).

The UVR8 structure was recently solved (Christie et al., 2012, Di Wu et al., 2012) and revealed UVR8 to be the first photoreceptor that did not have an external chromophore but instead perceived light via conserved tryptophan residues. The protein exists as both a monomer and a dimer (Rizzini et al., 2011) with the interface between the two monomers grouped so they form a salt bridge of electrostatic interactions on the opposing monomer. This dimer to monomer switch upon UV-B perception is thought to allow UVR8 to translocate from the cytoplasm to the nucleus upon irradiation (Kaiserli and Jenkins, 2007). GFP-UVR8 is found to remain in the nucleus 24 hours after dark acclimation suggesting that

there may be a homeostasis in plants growing in the natural environment (Jenkins, 2014). In the nucleus UVR8 also interacts with the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), a major regulator of plant photomorphogenesis in *Arabidopsis* (Favory et al., 2009). UVR8 and COP1 are also able to interact in yeast and do so in a UV-B dependent manner (Rizzini et al., 2011). This interaction with COP1 therefore allows UVR8 to act upon downstream transcription factors affecting photomorphogenesis of seedlings (Jenkins, 2014).

1.4 Blue light photoreceptors

Plant responses to blue wavelengths of light (390nm-500nm) are wide ranging and are mediated by three different groups of photoreceptors: the cryptochromes (Chaves et al., 2011), phototropins (Christie, 2007) and the Zeitlupe family (ztl, fkf1 and lkp2) (Suetsugu and Wada, 2013). The model plant *Arabidopsis* has been employed to understand the molecular basis of plant blue-light photoreceptors and how this correlates with the function. Previous to the use of *Arabidopsis* and genetics, the action spectra for several blue-light responses were investigated. The action spectra for phototropism is similar to the absorption spectrum of a flavoprotein with maximal activity between 400 and 500 nm with a major peak at 450 nm and shoulders at 430 and 470 nm (Briggs and Iino, 1983). This property is shared with plant blue-light receptors as each binds the molecule flavin as a light absorbing chromophore (Conrad, Manahan and Crane, 2014). Nomenclature for photoreceptors was first presented for *Arabidopsis* phytochromes and phototropins (Quail et al., 1994, Briggs et al., 2001) and will be followed in this text. The holoprotein with its chromophore is lower case (cry, phot, ztl) and in upper case when the protein without the chromophore is described (CRY, PHOT, ZTL). Uppercase italics are used for genes encoding the photoreceptor apoproteins (*CRY, PHOT, ZTL*) whilst lower case italics are used for mutants (*cry, phot, ztl*).

1.4.1 Cryptochromes

Cryptochromes (cry) are one set of photoreceptors that are responsible for plant responses to blue and UV-A light. Cryptochromes were first identified through

the isolation of the hy4 gene. The *hy4* mutant was insensitive to inhibition of hypocotyl elongation specifically under blue light. Plants grown under blue light were seen to have long hypocotyls whereas this insensitivity was not seen when plants were grown under red light as the seedlings grew similar to wild type (Ahmad and Cashmore, 1993). The protein encoded by the hy4 gene was found to be similar to photolyases, a special group of flavoproteins that mediate redox reactions in response to the absorption of blue light to repair DNA damage (Sancar, 1994). Subsequently, the HY4 protein was found to bind a chromophore, flavin adenine dinucleotide (FAD) and was renamed cryptochrome 1 (CRY1) (Lin et al., 1995). Since then cryptochromes have been identified in prokaryotes, archaea and many eukaryotes (Chaves et al., 2006, 2011, Hendrischk et al., 2009) and have a function in the animal circadian clock (Sancar, 2004). To date there have been three cryptochromes identified in *Arabidopsis*, cry1, cry2 and cry3 where they are involved in a number of processes including hypocotyl growth inhibition; photomorphogenic development; floral initiation; regulation of primary root elongation and entrainment of the circadian clock (Li and Yang, 2007). Overexpression of *CRY1* in *Arabidopsis* results in the seedlings being hypersensitive to blue light with unusually short hypocotyls when grown in the light whilst there is no difference when seedlings are grown in the dark (Lin, Ahmad and Cashmore, 1996). This is in contrast to the double mutant *cry1 cry2*, which does not show a phototropic response (Lascève et al., 1999) whilst also being highly drought tolerant as the stomata show a reduced blue light response compared to wild type (Lascève et al., 1999). The single *cry2* mutants show reduced blue light dependent hypocotyl inhibition as well as cotyledon opening, with flowering occurring later than wild type (Guo et al., 1998). Cry3 is a cry-DASH (Drosophila-Arabidopsis-Synechocystis-Human) protein that in *Arabidopsis* localises to the chloroplast and mitochondria (Kleine, Lockhart and Batschauer, 2003) and is reported to repair UV-induced lesions in single-stranded DNA (Selby and Sancar, 2006).

1.4.2 Cryptochrome structure and function

Cryptochromes are closely related structurally to photolyases, ancient flavoproteins involved in light dependent DNA repair (Sancar, 1994). Cry proteins have two conserved domains, an N-terminal PHR domain (Photolyase

Homologous Region) that binds the FAD chromophore, and a CCT domain (cryptochrome C-terminus), which appears to be unstructured but is important for the function and regulation of crys (Yu et al., 2010). Purification of the PHR domain from cry1 bound to the chromophore FAD (CRY1-FAD) and structural analysis showed that the domain is composed of two subdomains, an N-terminal α/ß domain and a C-terminal α-helical subdomain where the FAD chromophore binds (Brautigam et al., 2004). FAD binds the CRY1-PHR in an unusual U-shaped confirmation, which buries the chromophore within the protein. Brautigam et al. (2004) also discovered that the PHR domain bound ATP whereby the adenine moiety penetrates into the FAD cavity, whereas the phosphates are located near to the surface of the protein. In contrast to the photolyase family, which have a positively charged protein surface, the CRY1-PHR has a mainly negatively charged suraface that may explain the differences in enzymatic function and why cry1 does not repair UV-damaged DNA. It has also been proposed that cry1 and cry2 bind the cofactor pterin derivative 5,10-methenyltetrahydrofolate (MTHF) however the binding site for this is usually lost when the proteins are purified (Christie et al., 2014). Pterin is proposed to act as an accessory cofactor that transports light energy from the UV to the flavin. The crystal structure of cry3 from *Arabidopsis* showed that it bound to MTHF along a dimer interface and shows that MTHF transfers energy excitation to FAD (Klar et al., 2007).

The CCT domain of cry is a distinct sequence that is unrelated to other protein domains and is not present in photolyase (Cashmore et al., 1999, Yang et al., 2000). A crystal structure has yet to be solved for cry1 and cry2 since it is mostly unstructured. Unstructured proteins are hypothesised to be important for protein-protein interaction since the unordered nature means the conformation of the protein can change and they are therefore able to interact with a large number of different interacting proteins (Dyson and Wright, 2005). It has been proposed that the CCT domain and the PHR domain interact and the phosphorylation of the cry protein upon blue light exposure may cause the repulsion of the mainly negatively charged PHR domain from the phosphorylated CCT domain and initiate the photomorphogenic responses (Yu et al., 2007).

The exact mechanism of photoexcitation of the cryptochromes remains unclear, however the photoexcited cryptochromes do undergo structural rearrangements,

protein modifications and protein interactions upon illumination (Yu et al., 2010). Using recombinant baculovirus expressed cry1 from *Arabidopsis,* Giovani et al. (2003) showed that in the darkness, which is the ground state of FAD, cry1 binds oxidised FAD that absorbs blue light (λmax 450nm). Upon blue light absorption the chromophore is semi-reduced (semiquinone) to FADH•, which is generated by the photoreduction and subsequent protonation of the FAD chromophore, a reaction that occurs within microseconds (Langenbacher et al., 2009). In the absence of light FADH• is evidently unstable and can revert back to FAD ground state within minutes (Chaves et al., 2011). The photoreduction of FAD also occurs in photolyases and is proposed to occur on a triad of tryptophan residues (Aubert et al., 2000). Sequence alignment and comparison of structure between photolyases and cryptochromes suggests that W324, W377 and W400 are the tryptophan residues in the triad of *Arabidopsis* cry1 (Giovani et al., 2003). Mutation of W324 and W400 to phenylalanine resulted in impairment of the electron transfer. The mutations expressed in plants resulted in a lack of blue light dependent inhibition of hypocotyl elongation as well as anthocyanin accumulation (Zeugner et al., 2005). The cry1 protein in these seedlings did undergo autophosphorylation albeit at a reduced level compared to wild type (Zeugner et al., 2005). This suggests that the photochemistry of the cryptochromes is closely related to its function.

1.4.3 Cryptochrome signalling

Around 5-25% of genes in the *Arabidopsis* genome change expression levels in response to blue light most of which is regulated by cry1 and cry2 (Yu et al., 2010). However the expression of many of these genes are also regulated by other signalling pathways, such as auxin, brassionosteroids and other phytohormones, which indicates the intricate nature of the signalling networks. *CRY1* and *CRY2* genes are expressed ubiquitously in all cell types and organs examined (Ahmad and Cashmore, 1993) however the cellular level of cry2 is negatively regulated by light, whereby the protein is rapidly degraded in as little as 15 minutes upon exposure to 5 μmol m^{-2} s^{-1} blue light (Lin et al., 1998). In contrast cry1 protein levels are not affected by light and the protein can be found in both dark and light conditions. Fusion of the cry1 protein to GFP and a nuclear localisation signal (NLS) or nuclear export signal (NES) resulted in

determination that nuclear, and not cytoplasmic, localised cry1 could rescue both the hypocotyl phenotype and the cotyledon expansion phenotype of the *cry1* mutant (Wu and Spalding, 2007). The function of cry2 in the detiolation response is probably limited to lower fluence of blue light (<10 μmol m^{-2} s^{-1}) due to the faster turnover of cry2 under higher fluence rates of light (Lin et al., 1998, Yu et al., 2010). cry2-GFP has been shown to associate with condensed chromosomes in mitotic cells whilst accumulating in the nucleoplasm of non-dividing cells indicating that it can control the expression of a variety of genes for the photomorphogenic response (Cutler et al., 2000, Yu et al., 2009).

The signalling of cryptochromes in photomorphogenesis is mediated by the negative regulation of COP1 by direct interaction with CRY1 through the CCT domain (Wang et al., 2001). The C-terminal domain of cry1 or cry2 fused to the enzymatic reporter ß-glucuronidase (GUS) showed a Constitutively Photomorphogenic (COP) phenotype similar to *cop1* and *cop9* mutants indicating that cryptochromes interact with COP proteins to affect downstream signalling (Yang et al., 2000). CRY1 mediates the blue light suppression of COP1 degradation of the transcription factors LONG HYPOCOTYL5 (HY5) and LONG HYPOCOTYL5-LIKE (HYH), which regulate genes involved in the de-etiolation response (Smirnova, Stepanenko and Shumny, 2012). Cryptochromes are also involved in flowering initiation, a response that is controlled by cry2. Under blue light, cry2 is involved in accumulation of the protein CONSTANS (CO), a positive regulator of flowering in long days through the activation of the florigen gene FT (Valverde et al., 2004). In darkness COP1 targets CO for degradation by ubiquitination thereby preventing flowering. However upon exposure to blue light cry2 interacts with COP1 lifting the repression on CO.

An important mechanism in the function of the cryptochromes is dimerization of the protein, a step that is required for light activation of the C-terminus by changing the electrostatic interaction (Yu et al., 2007). Both CRY1 and CRY2 exist as homodimers, which is important for function (Sang et al., 2005a, Yu et al., 2007). Blue light also causes the protein to become phosphorylated in a fluence dependent manner. cry1 expressed and purified from insect cells can also be autophosphorylated (Yu et al., 2010). Mutations that prevent dimerization of cry1 are not phosphorylated in a light dependent manner and

seedlings with this mutation show little inhibition of hypocotyl elongation therefore suggesting that dimerization and subsequent phosphorylation is required for function (Sang et al., 2005b). Phosphorylation also appears to trigger ubiquitination of the cry2 protein and targets it for degradation.

1.4.4 The Zeitlupe family

Another family of blue light photoreceptors is the Zeitlupe family comprising the Zeitlupe (ztl), Flavin-binding kelch repeat F-box 1 (fkf1) and LOV kelch protein 2 (lkp2) (Ito, Song and Imaizumi, 2012, Suetsugu and Wada, 2013). These proteins play a role in the circadian clock and control photoperiodic flowering of the plant (Ito, Song and Imaizumi, 2012). Recent studies have also indicated that the proteins function as E3 ubiquitin ligases and target proteins for degradation in a light dependent manner (Kim et al., 2007, Sawa et al., 2007). Structurally, the proteins contain a Light, Oxygen or Voltage (LOV) domain, an F box domain and a Kelch repeat domain (Ito, Song and Imaizumi, 2012). The recombinant LOV domain of the proteins binds the flavin cofactor Flavin Mononucleotide (FMN) and undergoes a self-contained photocycle. The dark recovery of this photocycle is much slower than that found for the *Arabidopsis* phototropins and takes the order of days to return to the ground state (Zikihara et al., 2006), however there may be other factors involved *in vivo* to change the slow recovery measured in the recombinant proteins. This slow dark recovery measured is thought to be the result of an additional nine amino acid insertion in the proteins from the ztl family that is not present in the phototropins, which will be discussed later. This insertion causes the formation of a loop structure that is only found in slow dark recovery LOV domains (Zikihara et al., 2006). The other domains of the proteins are involved in other functions. The F-boxes of the ztl family are associated with the Skp Cullin F-box (SCF)-type E3 ubiquitin ligases, which target proteins for degradation through the ubiquitin-proteosome (Ito, Song and Imaizumi, 2012) whilst the kelch repeats mediate protein:protein interactions and heterodimerisation between lkp2 and ztl/fkf1 (Takase et al., 2011).

The role of the ztl family of proteins appears to be in the control of the circadian rhythm in plants as well as being involved in flowering. Flowering Locus T (FT) is an important regulator of flowering during long and short days (Khan, Ai and Zhang, 2014) and its expression is regulated by the CONSTANS (CO)

protein in *Arabidopsis*. ztl, fkf1 and lkp2 are all involved in the regulation of flowering through the regulation of FT and CO (Imaizumi et al., 2003, Sawa et al., 2007). The transcriptional repressor family CYCLING DOF FACTOR (CDF) targets the CO gene for degradation by direct binding to DOF binding sites in the CO promoter (Imaizumi et al., 2005). The large nuclear protein GIGANTEA (GI) then interacts with the CDF1 on the *CO* promoter, upon blue light absorption in the fkf1 LOV domain fkf1 interacts with GI degrading CDF1 and results in *CO* promoter activation (Ito, Song and Imaizumi, 2012). This interaction occurs in late afternoon resulting in *CO* transcription at the end of the day (Sawa et al., 2007). The other two proteins, ztl and lkp2, are also thought to be involved in regulation of *CO* expression as overexpression of either results in downregulation of *CO* transcription, possibly by sequestering of GI in the cytosol as opposed to the nucleus (Ito, Song and Imaizumi, 2012).

Control of the circadian clock is very important for regulation of developmental processes in plants (Shim and Imaizumi, 2015). There are a number transcriptional regulators involved in control of circadian genes including the transcriptional repressor Timing of CAB expression 1 (TOC1) (Gendron et al., 2012). A number of studies have shown that ztl is involved in the degradation of TOC1, a process that is inhibited by blue light (Más et al., 2003, Kiba et al., 2007, Fujiwara et al., 2008). The binding of GI to ztl prevents its SCF activity resulting in an accumulation of TOC1 (Sawa et al., 2007). This close interaction of the ztl family of blue light photoreceptors with the circadian clock illustrates the complex interplay between light and plant growth and development.

1.5 Phototropins

Blue light induced bending in etiolated coleoptiles was first noted by Darwin more than 100 years ago when he described a mobile signal which moved from the site of perception to the site of bending (Darwin, 1880). Briggs and colleagues noted that a plasma membrane associated protein of around 120kDa became phosphorylated in response to blue light (Gallagher et al., 1988, Reymond et al., 1992, Palmer et al., 1993), but it would be another five years before the photoreceptor for phototropism was identified (Christie et al., 1998). Screens of *Arabidopsis* seedlings (mutated by ethyl methanesulfonate (EMS) and

T-DNA insertional mutagenesis) had earlier identified eight mutant lines impaired in phototropism (Liscum and Briggs, 1995). These were designated *non-phototropic hypocotyl 1 (NPH1), NPH2, NPH3* and *NPH4*. NPH1 was characterised further as the seedlings had lost the light-induced phosphorylation of the 120 kDa plasma membrane protein. Further characterisation of NPH1 in insect cells showed that the protein undergoes light-induced autophosphorylation and that it bound the cofactor flavin mononucleotide (FMN) (Christie et al., 1998). A further search of the *Arabidopsis* genome identified a gene with high sequence similarity to *NPH1,* which was designated *NPL1* (*NPH1*-Like) (Jarillo, Ahmad and Cashmore, 1998). Following the identification of genes homologous to *NPH1* and *NPL1* in other species it was decided to rename the genes using the nomenclature in place for phytochromes. *NPH1* became *PHOT1* whilst *NPL1* became *PHOT2* after the main response which they were identified from (Briggs et al., 2001). From this point, wild-type genes are designated *PHOT1* and *PHOT2*; mutated genes are *phot1* and *phot2*; holoproteins with bound FMN are designated phot1 and phot2, whilst the apoproteins are PHOT1 and PHOT2.

1.5.1 Phototropin structure

Phototropins are Serine/Threonine protein kinases belonging to the conserved AGC kinase family (cAMP dependent kinases; cGMP dependent kinases and diacylglycerol-activated/phospholipid-dependent kinases PKC). These are a well-studied group of kinases conserved in all eukaryotic genomes, with 39 families found in *Arabidopsis*. Evolutionary adaptations of the AGC kinases in plants most probably introduced structural changes that permit an effect on kinase activity by external stimuli such as light (Rademacher and Offringa, 2012). Of the 39 AGC kinases in *Arabidopsis*, 23 have no known homologues outside the plant kingdom and are further subdivided into subfamily VIII (Galván-Ampudia and Offringa, 2007). *Arabidopsis* contains two blue light photoreceptors, the phototropins, which are members of the subfamily VIII, named phototropin 1 (phot1) and phototropin 2 (phot2) and they share approximately 60% amino acid sequence identity (Christie et al., 2002).

The proteins are divided into two parts, at the N-terminus of the phototropins there are two photosensory domains termed Light, Oxygen or Voltage (LOV) domains and at the C-terminus there is a Serine/Threonine kinase domain.

Between the LOV2 domain and kinase domain there is a flexible linker region termed the Jα helix. The LOV domains (designated LOV1 and LOV2) themselves are part of a domain superfamily termed Per Arnt Sim (PAS). PAS domains are sensors that detect an extensive range of external stimuli and regulate the function of effector domains (Möglich, Ayers and Moffat, 2009). Each LOV domain non-covalently binds one molecule of the cofactor Flavin Mononucleotide (FMN) and upon illumination with blue light a covalent linkage is formed between the FMN and a conserved cysteine residue within the LOV domains (Christie et al., 1998, Salomon et al., 2000). This results in the phototropins undergoing autophosphorylation at a number of serine residues including upstream of the LOV1 domain and between the LOV1 and LOV2 linker region, as well as within the kinase activation loop and the extreme C-terminus (Inoue et al. 2008; Sullivan et al. 2008). The kinase domain of the phototropins, although part of the larger AGC kinase family, contains a 36-118 amino acid insertion domain between the conserved magnesium binding loop and the activation loop, which is absent in other eukaryotic AGC kinases (Zegzouti et al., 2006, Rademacher and Offringa, 2012). This variable insertion domain has been found to direct protein localisation to specific subcellular locations (Zegzouti et al., 2006) suggesting it may play a similar role in the membrane association of *Arabidopsis* phototropins.

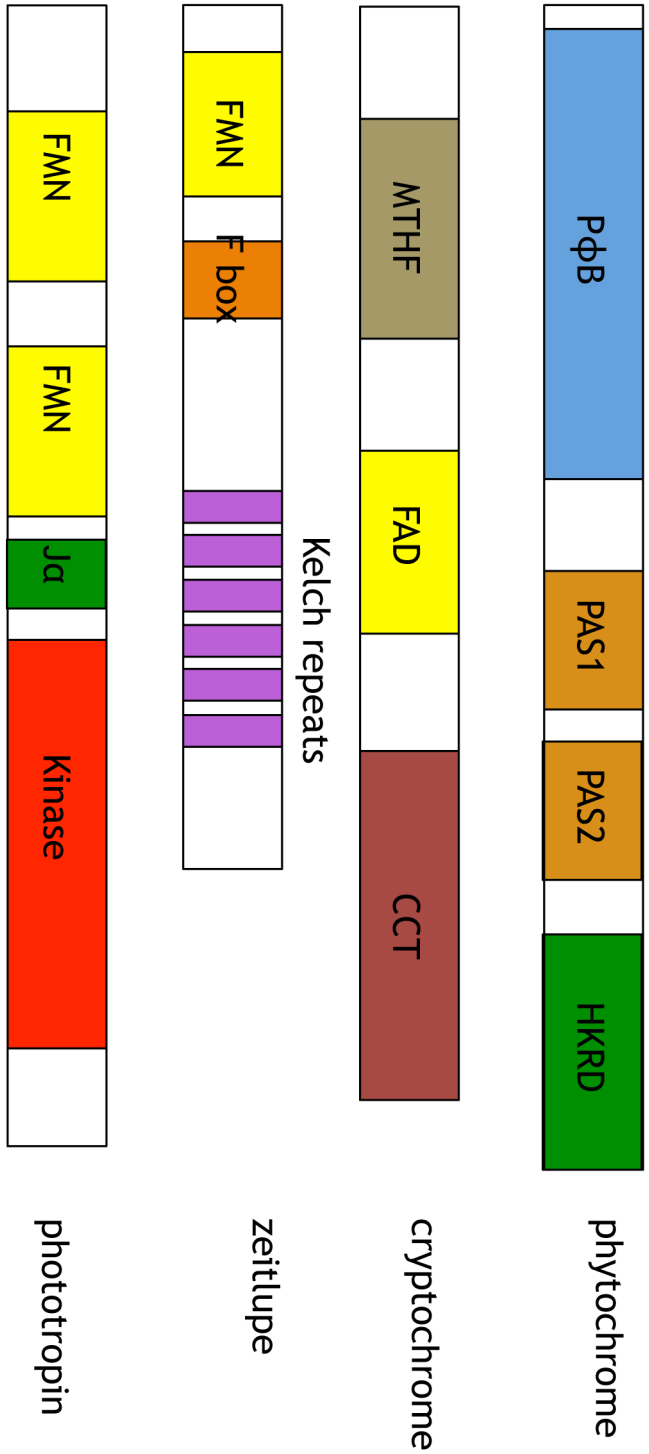


Figure 1.1 Photoreceptor domain structure

Schematic diagrams illustrating the major domains found in the structure of plant photoreceptors. PAS: Per, ARNT, Sim domain; HKRD: Histidine kinase-related domain; LOV: Light, Oxygen or Voltage-sensing domain; CCT: Cryptochrome C terminus. KINASE: serine/threonine kinase domain. The chromophores bound by the photoreceptors are also indicated. PΦB: phytochromobilin; MTHF: methenyltetrahydrofolate; FAD: flavin adenine dinucleotide; FMN: flavin mononucleotide.

1.5.2 LOV domains and phototropin photocycle

LOV domains from various phototropins can be expressed and purified from *E. coli* either as single domains or together (Kasahara et al. 2002). The large quantity of protein allows analysis of the domains and their photochemistry upon blue light illumination. Purified LOV domains are a yellow colour and are highly fluorescent when exposed to UV-light due to the bound FMN molecule (Christie, 2007). Spectral analysis of the phototropins reveals a distinctive mode of photochemistry that underlies the mechanisms associated with light sensing by the phototropins. Computer modelling and subsequent crystallography of the LOV2 core revealed that the domain contains five ß-sheets and two α-helices (Salomon et al., 2000, Crosson and Moffat, 2002). This structure is very similar to other PAS domains despite the amino acid sequences of these proteins being different, suggesting that they form part of an important signal-transduction pathway (Crosson and Moffat, 2002). In the darkness, the FMN is non-covalently associated with the LOV domain from phototropins and is held in place by hydrogen binding and van der Waals forces (Crosson and Moffat, 2002). This species is designated LOV_{447} as it absorbs maximally at 447 nm. Absorption of blue light by the FMN chromophore results in the formation of an excited singlet state which then decays into a flavin triplet state (LOV_{660}) that absorbs maximally within the red region of the spectrum (Swartz et al., 2001, Kennis et al., 2003, Kottke et al., 2003). FMN triplet state is a primary photoproduct of the LOV domain photocycle and occurs within nanoseconds of the blue light absorption. The decay of this triplet state results in the formation of a covalent adduct between the C(4a) carbon of the FMN chromophore and the conserved cysteine residue at position 39 in the LOV domain. This occurs within microseconds and absorbs maximally at 390 nm. The formation of the LOV_{390} species is fully reversible in the darkness (at a rate of tens to hundreds of seconds) back to the initial ground state of LOV_{447}. Therefore the LOV domains undergo a photocycle between the FMN being non-covalently bound in the inactive state LOV_{447} and the FMN covalently binding the cysteine adduct in the active state LOV_{390} (Swartz et al. 2001; Figure 1.2).

The cysteine residue at amino acid position 39 in both the LOV domains is important for the phototcycle of the phototropins. LOV2 also contains this

conserved cysteine residue within the NCRLFQ motif. The side chains of the cysteine are found buried deep inside the molecule in close proximity to the hydrophobic side chains of amino acids where the C(4a) atom of the FMN can bind (Salomon et al., 2000, Harper, Christie and Gardner, 2004). Mutation of this conserved cysteine residue results in a loss of the photocycle, although not a loss of FMN binding (Salomon et al., 2000). The photocycle is well conserved in phototropins from a number of different species including oat (*Avena sativa*) as well as the phototropin from the green algae *Chlamydomonas reinhardtii*. This suggests the photocycle is an important evolutionary mechanism for phototropin activation.

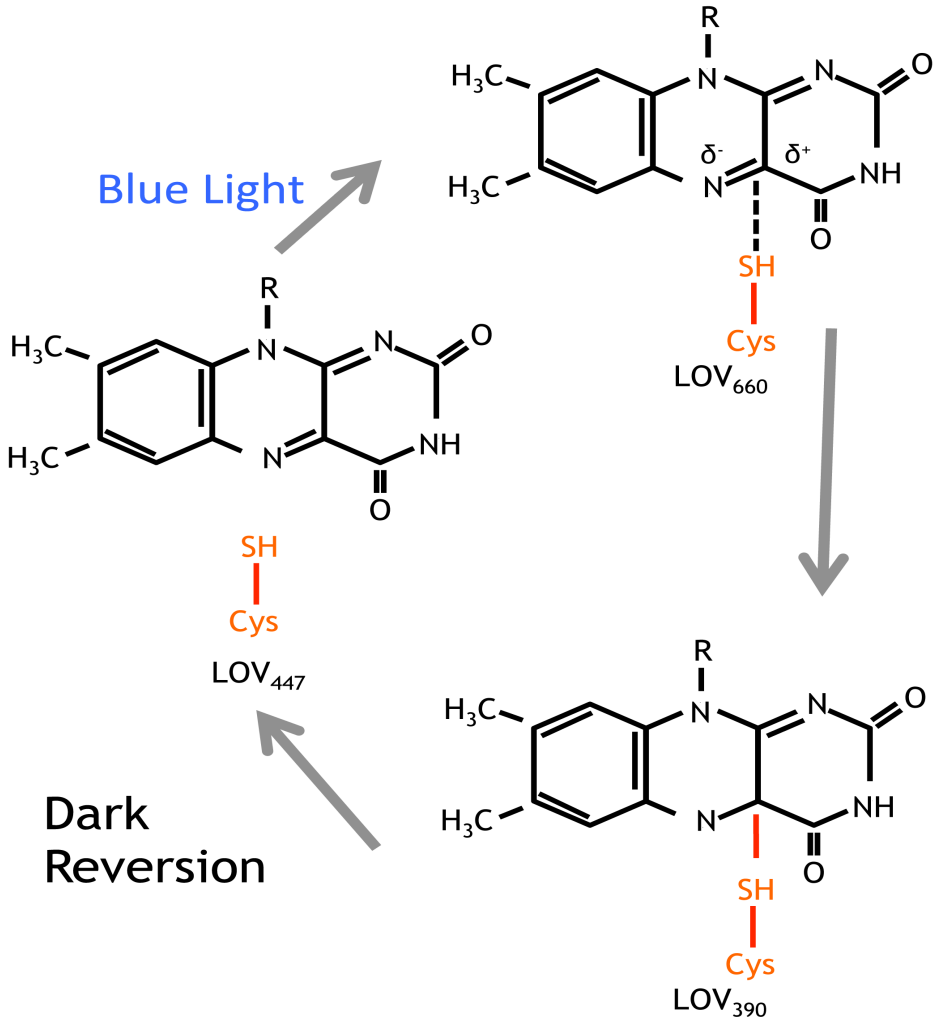


Figure 1.2: LOV domain photochemistry

Schematic diagram of the proposed mechanism of adduct formation between the conserved cysteine residue of the LOV domain and dark recovery back to the ground state. In the dark the FMN is noncovalently bound within the LOV domain (LOV_{447}). Upon blue light absorption an excited singlet state decays to a flavin triplet state (LOV_{660}) species leading to the flavin-cysteinyl adduct between FMN and LOV_{390}. This then decays back to the LOV_{447} in the darkness.

1.5.3 Functional roles of the LOV domains

The crystal structures of LOV1 and LOV2 revealed that they are very similar and each is able to bind one molecule of FMN. There are however differences in the quantum efficiencies of the two LOV domains, with LOV1 exhibiting a lower photosensitivity than LOV2 (Salomon et al., 2000, Kasahara et al., 2002). For phot1, the relative quantum efficiency for photobleaching of LOV1 is $1/10^{th}$ that of LOV2 while the dark regeneration of LOV1 is twice that of LOV2 (Salomon et al., 2000). These findings would suggest that the LOV domains have different functional roles within the plant. Recent analysis has revealed that mutations within the LOV domain can change the photocycle making it faster or slower (Circolone et al., 2012, Kawano et al., 2013, Zayner and Sosnick, 2014). This allows the opportunity of perhaps impacting upon the photosynthetic efficiency of plants and may reveal more about the fine-tuning of the photocycle itself.

Phototropins appear to be the only proteins that contain two LOV domains and the functional relevance of this is still not fully understood (Christie et al., 2014). The LOV domains are only around 40% identical so it is not surprising that they are probably involved in different physiological roles (Christie et al., 1999). Within *Arabidopsis* the role of the LOV domains of phot1 was investigated by employing transgenic plants expressing phototropin where the conserved cysteine residue involved in LOV photosensitivity had been mutated to alanine. Expression in the *phot1-5* mutant background showed that the seedlings were still able to undergo hypocotyl phototropism at low light intensities (<1 μmol m^{-2} s^{-1}) when the LOV1 domain was mutated. However when the LOV2 domain was mutated the seedlings were unable to undergo phototropism (Christie et al., 2002). The use of the single *phot1-5* mutant meant the response to higher light intensities could not be investigated. For this reason Cho et al. (2007) transformed the same constructs into the *phot1-5 phot2-1* double mutant. This allowed the response to higher light intensities to be investigated. As before, it was found that the LOV2 domain of phot1 was required for hypocotyl phototropism. However for phot2, when the LOV1 domain alone was mutated, the seedlings were still phototropic albeit not to the same degree of curvature as the wild type. The authors attributed this to a leaky *phot2-1* allele, but this

has not been shown since suggesting that perhaps the LOV1 domain of phot2 has a role to play in its function. Suetsugu et al. (2013) analysed a number of *phot2* mutants and there results indicated that the LOV1 domain of phot2 showed a slight but significant function in hypocotyl phototropism and chloroplast movements indicating that LOV1 can perhaps function when LOV2 is absent. It has also been shown that the LOV2 domain from the fern *Adiantum capillus-veneris* is essential for the chloroplast avoidance response since deletion of the LOV1 domain did not affect the avoidance response in the fern (Kagawa et al., 2004).

The LOV1 domain has been proposed to be involved in dimerization of the protein. Expression of LOV1 and LOV2 of phototropin from *Avena sativa* fused to a calmodulin binding peptide (CBP) resulted in dimerization of the protein in solution. A CBP-LOV2 fusion was found to be only a monomer in solution, conversely a CBP-LOV1 fusion was found to be both a monomer and a dimer in solution with the dimer increasing upon prolonged incubation (Salomon, Lempert and Rüdiger, 2004, Eitoku et al., 2007). Full-length phototropin would need to be examined to investigate if the same occurs in the native protein as the kinase domain is a large structure and may affect interactions between LOV1 domains from different molecules of phototropin.

1.5.4 Phototropin activation and phosphorylation activity

There is no crystal structure available for full-length phototropin. Recently FTIR difference spectroscopy (Pfeifer et al., 2010) and small angle X-ray scattering (SAXS) (Okajima et al., 2014) has provided greater information on the domain positions within the protein as well as further structural information upon photoreceptor excitation. SAXS analysis indicates that the domains are arranged LOV1, LOV2 and then the kinase domain and that the LOV1 domain alters its position relative to LOV2 and the kinase domain (Okajima et al., 2014). This is in agreement with the role of LOV2 functioning as the main regulator of phototropin activity. Previous investigations have shown that the LOV2 domain and the kinase domain have a “closed” or inactive conformation in the dark, whereby the LOV2 domain acts as a repressor of the kinase activity of the photoreceptor (Harper, Neil and Gardner, 2003). Blue light sensing by the LOV2 domain results in an a conformational change in the protein which is propagated

along the length of the protein to the Jα-helix and results in the repression being lifted (Harper, Christie and Gardner, 2004) and the catalytic cleft between the N- and C-terminal lobes of the kinase domain is able to bind ATP and receptor autophosphorylation occurs.

The activation of the phototropin photoreceptor leads to receptor autophosphorylation (Christie et al., 1998). This autophosophorylation occurs mainly on serine residues within the protein and identification of the phosphorylation sites of the phototropins has been elucidated using a number of techniques resulting in 21 sites identified to date in *Arabidopsis* phot1 and 28 sites identified in *Arabidopsis* phot2 (Salomon et al. 2003; Inoue et al. 2008; Sullivan et al. 2008; Deng et al. 2014). Within phot1, serine residues close to LOV1 appear to be phosphorylated at lower fluence rates of light than the residues close to LOV2 (Salomon et al., 2003). This is most likely the reason for the photosensitivity of autophosphorylation *in vitro* being an order of 1 or 2 magnitudes greater than that required for phototropism (Briggs, 1996). Salomon et al. (2003) also identified that the recovery of phosphorylated phot1 in the dark after saturating light pulse is caused by dephosphorylation of the protein rather than degradation or synthesis of new protein. This implies that there is a phosphatase involved in the dephosphorylation of phototropins in the dark, although this remains to be identified (Kinoshita et al., 2003, Tseng and Briggs, 2010).

The major phosophorylation sites of phot1 and phot2 are found in the kinase activation loop and have an impact on the function of the proteins (Inoue et al. 2008). Ser-851, and to a lesser degree Ser-849, in phot1 are required for phototropin signalling. Mutation of these residues to alanine and subsequent transformation into the *phot1-5 phot2-1* double mutant does not complement the physiological responses controlled by the phototropins. The conserved residues mutated in phot2, Ser-761 and Ser 763, also did not complement the physiological responses when transformed into *phot1-5 phot2-1* (Inoue et al., 2011). However, these mutations did not affect the blue light induced electrophoretic mobility shift of the proteins indicating that phosphorylation of other residues is still able to occur.

1.5.5 Substrate phosphorylation

Although autophosphorylation of the phototropins is well documented in both *Arabidopsis* and heterologous systems, until recently no downstream phosphorylation targets of phot1 had been identified. One of the physiological responses controlled by the phototropins is stomatal opening (Christie, 2007). Blue light perception by the phototropins activates plasma membrane H^+-ATPase causing stomata to open (Kinoshita & Shimazaki 1999; Kinoshita et al. 2001). Takemiya et al. (2013) recently identified a novel protein involved in stomatal opening via a screen for *Arabdiopsis* defective in blue light dependent stomatal opening. *BLUE LIGHT SIGNALING1* (*BLUS1*) encodes a Ser/Thr kinase that is directly phosphorylated by phot1 at the C-terminus of BLUS1. Phosphorylation of the BLUS1 protein occurred in the *phot1-5* and *phot2-1* single mutants, although the signal was reduced in the *phot1-5* mutant, but was completely absent in the *phot1-5 phot2-1* double mutant. The phosphorylated BLUS1 appeared after 15 seconds of blue light illumination, reached a maximum within 1 minute and returned to the ground state within 5 minutes. This is much more rapid than the phosphorylation of H^+ ATPase that is required for stomatal opening (Kinoshita and Shimazaki, 1999, Kinoshita et al., 2003) and reflects more closely the phosphorylation of phot1 (Inoue et al. 2008). This was confirmed by the co-immunoprecipitation between blue light activated phot1-GFP and BLUS1 (Takemiya et al., 2013). The phosphorylation of BLUS1 by phot1 confirmed the role of the LOV domains as repressors of kinase activity since the phosphorylation of BLUS1 occurred much more when the kinase domain alone was expressed than either the photosensory domains alone or the full-length protein. This signifies that *in vitro* work is relevant to phototropin functions *in vivo*. The phosphorylation status of phot1 and phot2 also show a clear difference between the two proteins with the findings of Boex-Fontvieille et al. (2014) which demonstrate that Ser 58 and Ser 170 in phot1 are less phosphorylated in the light, whereas the equivalent sites in phot2 (Ser 22 and Ser 105) are more phosphorylated in the same light conditions. This again highlights the difference between the two photoreceptors that may help to explain the different roles they play in plants.

Another protein recently identified that is phosphorylated by phot1 is the ATP-BINDING CASSETTE B19 (ABCB19) (Christie et al., 2011). ABCB19 belongs to a superfamily of proteins involved in a broad range of processes (Verrier et al., 2008) and within plants ABCB19 is an auxin efflux carrier (Spalding, 2013). *abcb19* mutants are impaired in auxin transport in the hypocotyls (Noh, Murphy and Spalding, 2001), the site of phototropism within *Arabidopsis*. Interestingly mutants from the same family as ABCB19, the multidrug resistance genes encoding P-glycoproteins show enhanced phototropic curvature compared to the wild-type seedlings due to mislocalisation of the auxin efflux carrier PIN1 (Noh et al., 2003). This suggests that the ABC proteins could be involved in phototropism by controlling the auxin flow required for movement. Expression of ABCB19 in HeLa cells and expression of phot1 in insect cells revealed that phot1 could phosphorylate ABCB19 only when the kinase domain was active (Christie et al., 2011). The protein was also found to co-immunoprecipitate with phot1-GFP in the dark. Phot1 was subsequently found to inhibit ABCB19 activity resulting in a localised pool of auxin above the hypocotyl apex that halts vertical growth and primes lateral auxin fluxes that are subsequently channelled to the elongation zone by PIN-FORMED 3 (PIN3) (Christie et al., 2011). This interaction between phot1 and auxin flow suggests a more complicated model for phototropism than was originally thought.

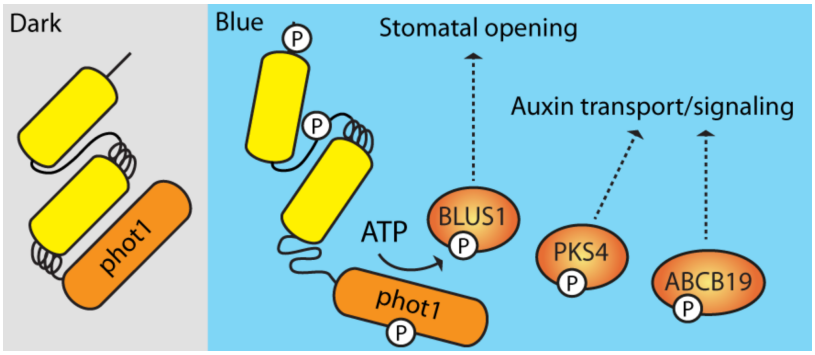


Figure 1.3 Downstream phosphorylation targets of phot1

In the dark state the kinase domain is docked underneath the LOV2 domain resulting in inhibition of kinase activity. Upon blue light absorption a conformational change occurs resulting in autophosphorylation of phot1 within the kinase domain, between LOV1 and LOV2 as well as upstream of the LOV1 domain. Phot1 is also able to then phosphorylate substrates downstream including PKS4, ABCB19 and the recently identified BLUS1 (adapted from Christie et al., 2014).

1.5.6 Phot1-interacting proteins

The phototropins control a large number of responses in *Arabidopsis* but the proteins they interact with to perform these varied responses remains largely unknown. To date NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3), root phototropism 2 (RPT2), phytochrome kinase substrate (PKS) and 14-3-3 proteins have been identified as interacting with phot1 (Motchoulski and Liscum, 1999, Inada et al., 2004, Lariguet et al., 2006, Sullivan et al., 2009). Protein phosphatase 2A (PP2A) has been shown to interact with phot2 in yeast as well as an *in vitro* binding assay (Tseng and Briggs, 2010) and the AUXIN RESPONSE FACTOR (ARFs) have also been shown to interact with the phototropins and the *arf7-1* mutant is impaired in phototropism (Okushima et al., 2005, Hohm, Preuten and Fankhauser, 2013).

1.5.6.1 Non-phototropic hypocotyl 3

NPH3 was the first protein to be identified that interacted with phot1. NPH3 contains three domains, a Broad-complex Tramtrack and Bric-á-Brack (BTB) domain at the N-terminus; a central NPH3 domain and a C-terminal coiled coil domain (Motchoulski and Liscum, 1999). NPH3 belongs to the NPH3/RPT2 family of which there are 33 members in *Arabidopsis* (Pedmale, Celaya and Liscum, 2010). NPH3 was first identified as it is aphototropic but still retains other response that optimise photosynthetic efficiency, such as chloroplast movement (Liscum and Briggs, 1995, 1996, de Carbonnel et al., 2010). Similar to phot1, NPH3 is a hydrophilic protein and is α-helical in nature (Motchoulski and Liscum, 1999, Christie and Murphy, 2013) as well as being found associated with the membrane similar to phot1 (Liscum et al., 2014), this means they are within the same physical location for interaction (Motchoulski and Liscum, 1999, Inada et al., 2004). The interaction between phot1 and NPH3 occurs between the N-terminal LOV domains of phot1 and the coiled-coil region of NPH3 (Motchoulski and Liscum, 1999). NPH3 is found to be phosphorylated in the darkness in wild type seedlings and undergoes dephosphorylation in the light, a response that can be seen by the increase in electrophoretic mobility in SDS-PAGE over a wide range of fluence rates suggesting that it is not a fluence rate dependent response (Pedmale and Liscum, 2007). This dephosphorylation requires phot1 to be present as the electromobility shift does not occur in the *phot1-5* and *phot1-*

5 phot2-1 mutants (Pedmale and Liscum, 2007), however there must be another protein that phosphorylates NPH3 as it is found only in the phosphorylated form in the mutants lacking an active phot1 indicating that phot1 itself is only involved in the dephosphorylation of NPH3. Pharmacological evidence suggests that this dephosphorylation is important for phototropic signalling (Liscum et al., 2014).

1.5.6.2 Root Phototropism 2

Another member of the NPH3 family involved in phototropin signalling is ROOT PHOTOTROPISM 2 (RPT2). This mutant was first isolated in a screen for mutants lacking root phototropism (Okada and Shimura, 1994). The expression of RPT2 is different from that of NPH3 whereby its expression is induced in a light dependent manner whereas NPH3 is highly expressed in dark grown seeds and is not light inducible (Sakai et al., 2000). Yeast-2-hybrid studies showed that RPT2 also physically interacts with phot1 at the N-terminal LOV domains through the N-terminus of RPT2. The protein also interacts with the N-terminus of NPH3 suggesting the three proteins form a complex *in vivo* to direct downstream signals from phot1. The RPT2 protein is also found to localise to microsomal membranes similar to phot1 and NPH3 (Inada et al., 2004). There has been no interaction detected between RPT2 and phot2 suggesting it does not work in the phot2 dependent pathway. *rpt2* mutants are found to lack the phototropic response under high intensity blue light but have a normal response under low intensity wavelengths (Sakai et al., 2000). One explanation for this is possibly that phot2 bypasses the phot1 and RPT2 pathways under high intensity blue light. Harada et al. (2013) found that for petiole and cotyledon positioning under high intensity blue light (70 µmol m^{-2} s^{-1}) a *phot1-5 rpt2-2* responded similar to wild type whereas a *phot1-5 rpt2-2 phot2-1* triple mutant lost this response. The authors suggested that phot1 inhibits phot2 under low intensity blue light and that RPT2 then inhibits this. There is however some conflicting data about the response of RPT2 in phototropin mediated functions as the *rpt2-1* mutant does not show the petiole and cotyledon positioning (Harada et al., 2013). This mutant was also postulated to have a role in phot1 stomatal opening (Inada et al., 2004). However a subsequent study identified that the proton pumping required for guard cell movements was not affected by the *rpt2* mutation

(Tsutsumi et al., 2013). The authors do note that the studies looked at different growth conditions as well as ecotpyes as the *rpt2-1* mutant is in the Ler background whereas *rpt2-2* is in the Col-0 background. Since the phot1 and phot2 mutants most often investigated are in the Col-0 *gl-1* background it would seem most likely that RPT2 does not function in stomatal opening but does have a role to play in leaf flattening and petiole positioning of plants.

1.5.6.3 Phytochrome kinase substrate

NPH3 and RPT2 belong to the same family in *Arabidopsis* whereas the third phot1-interacting proteins do not. The PHYTOCHROME KINASE SUBSTRATE (PKS) proteins were first identified as interacting with the phytochrome photoreceptors (Fankhauser et al., 1999, Lariguet et al., 2003). There are four family members PKS1-4, and subsequently PKS1, PKS2 and PKS4 were found to interact with phot1 and to a certain degree phot2 (Lariguet et al., 2006, de Carbonnel et al., 2010, Demarsy et al., 2012, Kami et al., 2014). The proteins also localise to the plasma membrane providing the first evidence that they interact with phot1. PKS1 is expressed in the elongation zone of the hypocotyl and localises to the plasma membrane in *Arabidopsis*, which can only be solubilised by treatment with 1% Triton X-100 (Lariguet et al., 2006). PKS1 also interacts with NPH3 *in vivo* and is co-immunoprecipitated in a phot1/NPH3/PKS1 complex from *Arabidopsis* seedlings (Lariguet et al., 2006). Triple *pks1pks2pks4* mutants do not bend in response to pulses of unilateral blue light (Lariguet et al., 2006) but their leaves are similar to wild type with a ratio of unflattened to flattened leaf around 1, indicating a flat leaf (de Carbonnel et al., 2010). However the petioles of the *pks2* mutant are more epinsatic than wild type indicating that it has a role to play in this response alongside phot1 since petiole positioning in a *phot1pks2* double mutant is reduced when compared to the singe *phot1* and *pks2* mutants (de Carbonnel et al., 2010). The expression of PKS2 colocalises with the auxin reporter gene *DR5:GUS* (Guilfoyle, 1999) indicating that it is involved in growth regulation. This interplay between PKS, phot1 and auxin is confirmed by the finding that the *pks1pks2pks4* mutant has an altered DR5 gradient. This indicates that it is auxin that is preventing the PKS mutants from performing phototropism (Kami et al., 2014). PKS4 is known to work upstream of auxin as the phosophorylated form of the protein is still found in

Arabidopsis seedlings altered in the auxin pathway (Demarsy et al., 2012). It is phot1 itself that phosphorylates PKS4 in response to blue light treatment. This is a response that is specific to phot1 as kinase inactive phot1 seedlings as well as the *phot2* mutant do not show the phosphorylated form of PKS4 (Demarsy et al., 2012). It was also noted that phot1-GFP is still localised to the membrane in the *pks*1pks4 mutant background (Kami et al., 2014), illustrating that PKS proteins cannot be affecting the subcellular localisation of phot1 and most likely interacts with the phototropins to regulate other processes.

1.5.6.4 The 14-3-3 proteins

The other major family of proteins that have been shown to interact with the phototropins in *Arabidopsis* is the 14-3-3 family. These proteins comprise 13 members in *Arabidopsis* (DeLille, Sehnke and Ferl, 2001) and in eukaryotes bind a large number of diverse signalling proteins allowing 14-3-3 proteins to be involved a wide variety of responses (Fu, Subramanian and Masters, 2000). The interaction of 14-3-3 proteins and phototropins was first identified in the broad bean *Vicia faba* for phot1a and phot1b. It was found that a 14-3-3 protein bound to phot1a and phot1b following illumination and subsequently phosphorylation of the phototropins occurred (Kinoshita et al., 2003). The authors then determined that Ser 358 (phot1a) and Ser 344 (phot1b) became phosphorylated in response to light. These serine residues lie between the LOV1 and LOV2 domains. Inoue et al. (2008) subsequently identified these Ser residues in *Arabidopsis* phot1 between the LOV1 and LOV2 domains, which become phosphorylated in response to light (Ser 350 and Ser 376) and bound 14-3-3 proteins. Phot1 and 14-3-3λ can interact in yeast but this only occurs when the LOV2 linker region is present which contains the serine residues. Mutation of these two serine residues to alanine resulted in residual 14-3-3λ binding to phot1 in yeast-2-hybrid assays (Sullivan et al., 2009). The 14-3-3λ protein from *Arabidopsis* was found to fractionate in the soluble fraction with some detected in the membrane indicating that it is in the same subcellular localisation as phot1 and phot2 (Sullivan et al., 2009). However the authors could not detect binding of phot2 to 14-3-3λ suggesting that they do not interact. Recently Tseng et al. (2012) determined that phot2 and 14-3-3λ did interact but through the C-terminal

domain of phot2 which is different to the interaction with phot1. This difference may play a role in the differing functions that phot1 and phot2 perform.

1.6 Physiological responses mediated by phototropins

Phototropins function redundantly controlling a wide variety of physiological processes that serves to optimise the photosynthetic efficiency of plants by increasing light capture in the aerial portions whilst increasing water and nutrient uptake in the roots (Takemiya et al., 2005). *PHOT1* mutations result in an increase in the random nature of roots resulting in inefficient penetration into the soil and therefore nutrient and water uptake is affected (Galen, Rabenold and Liscum, 2007). The responses controlled by phot1 and phot2 is varied and both photoreceptors function redundantly to control the processes. The main responses controlled by the phototropins are discussed in further detail below.

1.6.1 Phototropism

Phototropism is the unidirectional curvature of a plant, usually the hypocotyl or stem, in response to unilateral illumination (Christie, 2007), a response that was first noticed by Darwin when studying grass coleoptiles leading to his publication The Power of Movement in Plants (Darwin, 1880). Generally aerial sections of the plant undergo positive phototropism which is controlled by phot1 at low light intensities and phot2 at higher light intensities (Sakai et al., 2001), whilst the roots undergo negative phototropism, controlled by phot1 (Kutschera and Briggs, 2012). Phot1 also enhances plant functions under drought conditions by controlling plastic increases in root growth near the surface of soil (Galen, Rabenold and Liscum, 2007). The movement of plants towards a light stimulus also relies on gravitropism, nevertheless for the purposes of phototropism here the gravitropic effect can be considered negligible.

1.6.1.1 First and Second positive phototropism

Phototropism can be divided into two main responses. First positive phototropism obeys the Bussen-Roscoe law that states that the magnitude of the response is directly proportional to the total energy regardless of the time administered (Busen and Roscoe, 1855). Plants will therefore bend towards light in the same manner whether they receive 10 µmol of light for 10 minutes or 100 µmol of light for 1 minute. This first positive phototropism occurs at very low fluence rates pulses of blue light. However second positive phototropism does not follow this law as the degree of total curvature increases and happens in a time-dependent manner in response to longer term exposure to light (Christie, 2007, Briggs, 2014). The phototropic response is controlled redundantly by phot1 and phot2 under high intensities of blue light (Sakai et al., 2001), whilst phot1 controls the response under low intensities of blue light (Liscum and Briggs, 1995, Sakai et al., 2000, 2001). These differing responses to light intensities could be attributed to the different expression patterns of the two proteins. Phot1 is found at higher levels in dark grown seedlings and at a lower abundance in light treated seedlings, whilst phot2 expression is induced by blue light and is found at higher levels in light grown seedlings compared to dark grown seedlings (Sakamoto and Briggs, 2002, Kong et al., 2006, Aihara et al., 2008, Kang et al., 2008). The different quantum efficiencies of the LOV domains may also play a role in the differences between the two photoreceptors. NPH3 is one of the proteins required for phototropism mediated by phot1 and phot2 (Motchoulski & Liscum 1999; Inada et al. 2004) with the NPH3 protein becoming rapidly dephosphorylated in response to blue light. Further indications of the important function of NPH3 in phototropism is the finding that the rice NPH3 homolog COLEOPTILE PHOTOTROPISM1 (CPT1) is required for phototropism in the coleoptiles as well as lateral translocation of auxin (Haga et al., 2005).

1.6.1.2 Role of auxin in phototropism

The bending of plants towards the unidirectional light source requires increased growth on the shaded side of the hypocotyl. This process occurs due to the redistribution of auxin from the lit to the shaded side of the plant. This is the mobile signal first noted by Darwin (Darwin, 1880) but was not identified until 1926 by Went (Went, 1926). The Chlondy-Went hypothesis has been accepted as

central to the phototropic response and states that light from one direction drives lateral movement of the phytohormone auxin from the irradiated to the shaded side of the stem. It is this greater accumulation of auxin on the shaded side that stimulates the differential growth of the seedling towards light (Christie and Murphy, 2013). This differential growth results from increased cell elongation on the shaded side compared to the lit side (Orbovic and Poff, 1993).

In etiolated seedlings, the resulting region of curvature and light sensing is within the upper hypocotyl, however in de-etiolated, or light grown, seedlings elongation of the hypocotyl occurs in lower parts (Christie et al., 2011, Preuten et al., 2013, Yamamoto et al., 2014). These modifications in growth during phototropism have been shown to correlate with the redistribution of the auxin indole-3-acetic acid (IAA) in *Brassica oleracea* seedlings (Esmon et al., 2006). By using sections it was discovered that around 20% more free IAA was found in the hypocotyl furthest from the light source than the hypocotyl closest to the light. Investigation of this is more difficult in *Arabidopsis* due to the small size of the seedlings however Hohm et al. (2014) showed that the auxin reporter DII-Venus, which is degraded by auxin, is homogenously distributed throughout seedlings before illumination. One hour after unilateral blue light exposure there is a weaker signal at the shaded side of the hypocotyl than the lit side indicating greater auxin accumulation. Under physiological conditions, the protonated form of IAA can freely enter cells due to the difference in electrochemical gradient. This is increased by auxin importers, the AUXIN(AUX)/LIKE AUX1(AUX/LAX) family (Spalding, 2013). Once inside the cell, the pH is neutral and IAA is unable to flow back out the cell and requires auxin efflux carriers to pump it out and allow transport from cell to cell (Spalding, 2013).

1.6.1.3 Auxin Transporters

The two main families of transporters in *Arabidopsis* that are most likely involved in phototropsim are the pin-formed (PIN) efflux carriers and the ATP-Binding Cassette B (ABCB), particularly ABCB19 which has been shown to interact with phot1 (Christie et al. 2011; Ding et al. 2011; Sakai & Haga 2012; Rakusová et al. 2015). Blue light illumination results in auxin being redistributed from the vasculature to the epidermis in the upper hypocotyl due to ABCB19 phosphoryation by phot1 that results in inhibitions of ABCB19 activity (Christie et

al., 2011). As a consequence auxin is directed by PIN3 through the epidermis to the elongation zone (Friml et al., 2002). The role of PIN3 in the phototropic response was first notice when *pin3* mutants showed reduced phototropism when compared to wild type seedlings and its role as an auxin efflux carrier was confirmed by treatment of wild type seedlings with efflux inhibitors resulting in mimicking of the *pin3* mutant (Friml et al., 2002). The localisation of PIN3 also has a part to play in its function. In the dark PIN3-GFP shows apolar localisation and is present in both the inner and outer lateral membranes of the endodermal cells. Upon illumination PIN3-GFP is depleted from the outer membranes but remains in the inner membranes (Ding et al., 2011). Under unidirectional light there is polarisation of *PIN3* away from the light, towards the shaded side. This results in the perfect position to divert basipetal auxin flow laterally towards the shaded side of the hypocotyl (Rakusová, Fendrych and Friml, 2015). There is no PIN3 polarisation in phot1 mutants indicating that PIN3 works downstream of phot1. However the explanation for auxin redistribution and phototropism is not as simple, as the phototropic curvature can result from polar as well as lateral transport of auxin as well as the involvement of PIN proteins in other tropic responses (Rakusová, Fendrych and Friml, 2015). The redundancy of the PIN proteins also complicates phototropism. The *pin3-3* mutants show phototropism whereas the *pin3-3 pin4-101 pin7-102* triple mutant is aphototropic (Willige et al., 2013). This makes defining the role of the PIN proteins in phototropism more complicated.

1.6.1.4 Photoreceptor integration

The phototropism response is also complicated by the involvement of other photoreceptors in phototropism. The cryptochrome family of blue light photoreceptors are also involved in the phototropic response. *phot1, phot2* and *cry1* single mutants all showed enhanced phototropism at high fluence rates, a response that was also seen in the *phot1cry1* and *cry1cry2* double mutants (Whippo and Hangarter, 2003). This indicates that the cryptochromes decrease phototropism at high light intensities. Recently Vandenbussche et al. (2014) showed that the *phot1-5 phot2-1* mutant was phototropic towards UV-B light, and this was dependent on the UVR8 phototreceptor. This is intriguing as it is another indication of the integration of the photoreceptor networks but it is

probable that UV-B mediated phototropism is a different pathway to blue light mediated phototropism (Vandenbussche and Van Der Straeten, 2014). Phototropism in the fern *Adiantium capillis-veneris* is regulated by blue and red light, through the red-blue light absorbing phytochrome-phototropin chimera receptor called neochrome (Kawai et al., 2003). The neochrome is also responsible for the chloroplast response in the fern, again illustrating that the photoreceptors interact to ensure photosynthetic efficiency. Although higher plants are aphototropic in red light, phyA action on phot1 results in an enhanced phototropic response due to retention of phot1 at the plasma membrane (Parks, Quail and Hangarter, 1996, Stowe-Evans, Luesse and Liscum, 2001, Han et al., 2008). However this enhanced response is also seen in seedlings expressing constitutively localised phot1 suggesting that another mechanism is involved, which is most likely to be auxin flow (Preuten et al., 2015).

1.6.2 Stomatal opening

Stomata regulate gas exchange between plants and the atmosphere as well as helping to minimise transpiration loss (Shimazaki et al., 2007). CO_2 is taken in by the plants to synthesise the carbohydrates needed for growth and development. In exchange water and oxygen are released through the stomatal pore. The opening of stomata is driven by the accumulation of K^+ salts in the guard cells, a response that is induced by blue light through the voltage gated K^+ channels, resulting in a swelling of the guard cells and subsequent opening of the stomata (Dietrich, Sanders and Hedrich, 2001). In *Vicia faba,* blue light activates the membrane localised H^+ pump H^+-ATPase and results in its subsequent phosphorylation in a fluence rate dependent manner (Kinoshita and Shimazaki, 1999). The phosphorylation of H^+-ATPase occurs on serine and threonine resiudes in the C-terminus of the protein and leads to binding of a 14-3-3 protein. Endogenous 14-3-3 is found to co-precipitate with H^+-ATPase and specifically binds to the phosphorylated version. This binding is required for protein activation as dissociation of the 14-3-3 protein using inhibitors resulted in decreased activity of H^+-ATPase (Kinoshita and Shimazaki, 2002). H^+-ATPase can also be activated by the fungal toxin fusicoccin through the accumulation of the complexes of phosphorylated H^+-ATPase and 14-3-3 in the guard cells (Kinoshita and Shimazaki, 2001, 2002).

Both phot1 and phot2 act redundantly in stomatal opening in response to blue light over a range of intensities (Kinoshita, Doi and Suetsugu, 2001). The stomata were found to open in the *phot1-5* and *phot2-1* single mutants but did not open in the double mutant. Despite having normal membrane localised H^+-ATPase there was no proton extrusion in response to blue light (Kinoshita, Doi and Suetsugu, 2001). Subsequently it was shown the blue light phosphorylated forms of the *Vicia faba* phototropins bind to a 14-3-3 protein (Kinoshita et al., 2003). The sites were determined to be Ser-358 for Vfphot1a and Ser-344 for Vfphot1b, sites which map to the region between the LOV1 and LOV2 domains and that are phosphorylated in response to intermediate light intensities in *Avena Sativa* (Salomon et al., 2003). In *Arabidopsis* the 14-3-3λ isoform was found to interact specifically with phot2 and a 14-3-3λ mutant in *Arabidopsis* does not exhibit normal stomatal opening (Tseng et al., 2012). A *phot1-5 14-3-3 λ* double mutant, which contains functional phot2, had stomata which were significantly less open than the *phot1-5* single mutant after 2 hours of blue light treatment. The *phot2-1 14-3-3 λ* double mutant however responded normally to blue light treatment. The site of interaction was subsequently mapped to Ser-747 in the kinase domain of *Arabidopsis* phot2 and is required for stomatal opening (Tseng et al., 2012).

The recently identified BLUS1 protein is also involved in stomatal opening, (Takemiya et al., 2013). The *blus1-1* mutants have lost the ability of stomatal opening in response to blue light but open in response to fusicoccin indicating that the H^+-ATPase pump was still active. The *blus1-1* mutants are also phenotypically different from the *phot1-5 phot2-1* double mutant as they are only defective in stomatal opening in response to blue light whereas *phot1-5 phot2-1* double mutant is defective in all other responses (Kinoshita, Doi and Suetsugu, 2001). The *phot1-5 blus1-1* and *phot2-1 blus1-1* showed the same stomatal response as the *blus1-1* mutant indicating that the signal is passed from the phototropins to BLUS1. Indeed the phosphorylation of BLUS1 by the phototropins is required for the activation of H^+-ATPase and subsequent stomatal opening (Takemiya et al., 2013).

1.6.3 Chloroplast movement

Chloroplasts are the organ in the plant cell where photosynthesis occurs and they orientate themselves depending on the light quality and intensity. In the

dark, chloroplasts are found at the cell bottom or anticlinal walls, during low light levels the chloroplasts position themselves towards the source of light to maximise the light available for photosynthesis and during high light levels the chloroplasts move away from the light to prevent photobleaching (Kasahara et al., 2002, Suetsugu and Wada, 2012, Wada, 2013). The correct positioning of chloroplasts is mediated redundantly by both phot1 and phot2 under low light conditions (Sakai et al., 2001), with phot1 being more sensitive to lower light conditions than phot2. However it is phot2 alone that controls the response of chloroplasts to high blue light above 16 μmol m^{-2} s^{-2}, the avoidance response, to prevent photobleaching (Jarillo et al., 2001, Kagawa et al., 2001). Dark-adapted *Adiantum* prothallus cells irradiated with a high intensity microbeam of blue light show chloroplast movement towards the light but they never enter the area of the microbeam, a response which remains in the darkness suggesting that the avoidance signal is retained by cells (Kagawa and Wada, 2000, 2002). This also indicates that the accumulation and avoidance responses occur within the same area but the avoidance response has precedence over accumulation (Kagawa and Wada, 2002). The leaves of *phot2-1* mutants show photobleaching and are necrotic under high blue light and although the wild-type and *phot1-5* mutant show signs of stress they recover under low blue light, which the *phot2-1* mutant is unable to do (Kasahara et al. 2002).

This avoidance response mediated by phot2 is dependent on the LOV2 domain of the protein as chloroplast movement is unaffected in *Adiantum* expressing phot2 without the LOV1 domain whereas removal of the LOV2 domain by deletion or truncation results in no function (Kagawa et al., 2004). The same has also been shown for *Arabidopsis* phot1 and phot2 where the LOV1 domain is dispensable for the chloroplast response under both low and high light conditions (Han et al., 2013). Further analysis has identified that the extreme C-terminus of phot2 was also required for the correct localisation of the protein to the membrane and for the chloroplast avoidance in both the *Adiantum* transient expression system and in transgenic *Arabidopsis* under the control of the native *PHOT2* promoter (Kagawa et al. 2004; Kong et al. 2013). Addition of a nuclear localisation signal (NLS) or a nuclear export signal (NES) to phot2 showed that cytosolic localised phot2 is not required for chloroplast accumulation (Kong et al. 2013). The same study also revealed that phot2 localises to the chloroplast outer membrane, with

phot2 constitutively being detected in the chloroplasts isolated from wild type plants whereas phot1 localisation to the outer membrane was more variable. This may provide a explanation for the seemingly long distance signalling of the accumulation response as opposed to the avoidance response (Kagawa and Wada, 1996, 1999, Suetsugu and Wada, 2012).

It is well established that chloroplasts move in response to light, nevertheless how the chloroplasts physically move is still not fully resolved. Recently it has been discovered that short actin filaments play role in this movement. In *Arabidopsis* these short actin filament termed chloroplast-actin filaments (cp-actin filaments) associate with the chloroplast periphery (Kadota et al., 2009). When irradiated with blue light the cp-actin filament relocalise to the leading edge of the chloroplasts, a response that is mediated by the phototropins. The cp-actin filament dynamics were similar in the *phot1* mutants and wild type, whereas the cp-actin filaments in the *phot2* mutants did not disappear under high intensity light (Kadota et al., 2009). Phot2 was also found to be the photoreceptor responsible for the rapid reorganisation of cp-actin filaments to allow chloroplasts to change direction rapidly and control the velocity of the response (Kong et al. 2013).

Phosphoinositides have also been implicated in regulating chloroplast movement controlled by phot1 and phot2. Treatment of *phot1* or *phot2* mutants with phospholipase C (PLC) inhibitors supressed chloroplast movements in the *phot1* mutant but not the *phot2* mutant (Aggarwal et al. 2013). This is proposed to be due to the activation of the PLC pathway by phot2 in wild type (and *phot1* mutants) plants leading to Ca^{2+} release from internal stores and subsequent chloroplast movement (Aggarwal et al. 2013). However the role of phosphoinositides, phototropins and chloroplast movement remains to be studied in more detail.

1.6.4 Other responses mediated by phototropin

There are other responses that the phototropins mediate in addition to the major ones explained above. Inhibition of hypocotyl elongation is an important response of seedlings upon reaching the soil surface to ensure they become auxotrophic and blue light is the predominant effector of this response (Liscum

and Hangarter, 1991). It was discovered that although cry1 and cry2 mediate the response it is phot1 that controls the rapid inhibition of the seedlings upon exposure to blue light (Folta and Spalding, 2001). This response allows the seedlings to respond to the environmental surroundings and increase photosynthetic efficiency. Another response of the phototropins that helps the seedlings become efficient plants is the petiole positioning in response to low intensities of blue light. The petioles of wild type *Arabidopsis* position themselves perpendicular to the incoming light to ensure there is enough surface area for light capture, thereby increasing photosynthetic efficiency (van Zanten et al., 2010). Under low light conditions the petioles of the wild type plants position themselves upwards to allow this light capture to continue, whilst the leaves of the *phot1-5 phot2-1* double mutant point downwards decreasing the amount of surface area available for light capture (Takemiya et al., 2005). The phototropins also contribute to the fresh weight of *Arabidopsis* plants. There is not much difference in green tissue growth between wild type and *phot* mutants in older plants (Kasahara et al. 2002; Sakamoto & Briggs 2002) whilst plants grown under photosynthetically active radiation (PAR), which is close to natural sunlight in terms of light quality, illustrate the differences between wild type and the *phot1-5 phot2-1* double mutant. The wild type plants and single phot mutants grew noticeably quicker than the double mutant illustrating the redundant role that phot1 and phot2 play in plant growth. The final contribution of the phototropins to overall plant growth is leaf flattening. *Arabidopsis* plants grown under red light have leaves that curl downwards whereas leaves grown under blue light have flat, fully expanded leaves (Inoue et al. 2008; Kozuka et al. 2011). It is phot1 and phot2 that mediate this response redundantly; hence the *phot1-5 phot2-1* mutant has a curled leaf phenotype (epinastic) that can be used to visually distinguish the mutant plants from wild type *Arabidopsis*. (Sakai et al., 2001, Sakamoto and Briggs, 2002, de Carbonnel et al., 2010).

Phot1 has been shown to be involved in transcript stability of the nulear localised light-harvesting, chlorophyll-binding (*Lhcb*) under high fluence rates of light, indicating that the phototropins are able to influence other protein expression levels in response to light to optimise photosynthesis (Folta and Kaufman, 2003). In the fern *Adiantum capillus-veneris* the phototropins have been shown to be responsible for nuclear movements in response to blue light,

which is similar to the chloroplast movements in *Arabidopsis* (Tsuboi et al., 2007). This indicates the evolutionary conservation of the phototropins in response to blue light as the light-mediated nuclear movement may be for efficient light perception under low light, to switch on gene expression, and for avoiding nuclear damage under strong light (Tsuboi et al., 2007). Recently phot1 was also shown to be involved in lateral root suppression with the *phot1* mutant having a greater lateral root number and density compared to both wild-type and *phot2* mutants (Moni et al., 2014). The responses the phototropins control are varied but come together to mediate seedling establishment and maximise photosynthetic efficiency to increase plant growth.

1.6.5 Algal plant phototropins

It is not just plants that require photoreceptors to respond to light, algae also require machinery to respond to the quality, intensity, duration and direction of light. Algae, similar to higher plants like *Arabidopsis*, also possess the blue light photoreceptors phototropins and cryptochromes but neither phytochromes nor the Zeitlupe family have been identified in algae (Kianianmomeni and Hallmann, 2014). Similar to *Arabidopsis* the algae *Mougeotia scalaris* contains two phototropins, MsPHOTA and MsPHOTB which mediates the blue light induced chloroplast photorelocation (Suetsugu et al., 2005). A single phototropin has also been identified in the algae *Chlamydomonas reinhardtii* (Crphot) (Huang, Merkle and Beck, 2002) where it controls the sexual life cycle (Huang and Beck, 2003). It was also discovered that Crphot was involved in controlling eyespot size in the algae and the kinase domain alone of Crphot was sufficient to rescue a knockout mutant, whereas the photosensory domains alone were only sufficient for the light response (Trippens et al., 2012). Importantly the transformation of Crphot into the *phot1-5 phot2-1* double mutant is able to complement the physiological functions of *Arabidopsis* (Onodera et al., 2005). High-expressing Crphot lines showed normal phototropism, chloroplast movement, petiole positioning and leaf expansion. This suggests the basic mechanism of phototropin action is conserved between higher and lower plants.

Ostreococcus tauri is another algae that has been shown to contain a single phototropin (Otphot) (Derelle et al., 2006, Veetil et al., 2011) as well as containing a LOV-histidine kinase, which controls the circadian clock in the algae

(Djouani-Tahri et al., 2011). The LOV1 domain of Otphot has been show to have the same spectral properties as the LOV1 domains from higher plants, albeit lacking the characteristic 361 nm absorption peak of other LOV1 domains (Veetil et al., 2011). The purified protein was also found to exist as a dimer in solution and isoleucine at position 43 in the protein was responsible for modulating the dark state recovery of the LOV domain. This provides more key evidence that lower plant phototropins are as functional as those from higher plants although more investigation remains to fully understand the physiological role of *Ostreococcus* phototropin.

1.7 Membrane association of phototropin

Since the identification of phot1 as the blue light photoreceptor responsible for phototropism in 1998 (Christie et al., 1998) one major puzzle has remained unsolved. Phototropins are mostly hydrophilic proteins yet are found to associate with the membrane in darkness and even more puzzling, they partially internalise from the membrane in response to light (Sakamoto and Briggs, 2002, Kong et al., 2006, Aggarwal, Banaś, Kasprowicz-Maluśki, Borghetti, Łabuz, et al., 2014). The localisation pattern of *Arabidopsis* phot1 was investigated by a GFP fusion and assessed in stable transgenic lines expressing *PHOT1* under the control of its native promoter to ensure the spatial expression of the GFP fusion protein was a reflection of native phot1 localisation (Sakamoto and Briggs, 2002). In etiolated seedlings the protein is localised to the plasma membrane and a fraction internalises to the cytoplasm in response to blue light stimulus, usually from the laser used to excite GFP. Transient expression of phot1-GFP in *N. benthamiana* epidermal cells also results in localisation of the GFP fusion protein to the plasma membrane in darkness and phot1 internalises to cytosolic structures upon blue light illumination (Kaiserli et al., 2009). Similarly investigation of phot2 localisation revealed that the protein internalises from the membrane in response to light. However unlike phot1, a phot2-GFP fusion internalises to punctate structures that were found to colocalise with the Golgi marker KAM1ΔC:mRFP indicating that the two *Arabidopsis* phototropins relocalise to different subcellular locations (Kong et al., 2006). It has been shown that *Chlamydomonas* phototropin also localises to the membrane in the algae suggesting that the mechanism of attachment in the phototropins is

conserved in lower plants (Huang, Merkle and Beck, 2002). Phot1-GFP internalisation occurs outside the region of the hypocotyl involved in phototropism, indicating that relocalisation from the plasma membrane is most likely also involved in other functions (Yamamoto et al., 2014, Preuten et al., 2015). The internalisation from the membrane is attenuated by prior treatment of seedlings with red light, a response that is controlled by phyA as the *phyB-9* mutant continued to show a retention of phot1-GFP at the plasma membrane whereas the *phyA-211* mutant showed normal redistribution of phot1-GFP (Han et al., 2008).

To investigate further the membrane association of phot2, Kong et al. (2007) used GFP fusion proteins to determine which region of the protein interacted with the plasma membrane. The authors divided the protein in half, expressing the C-terminal kinase domain or the N-terminal photosensory region containing the LOV domains. A phot2 C-terminal GFP fusion localised to the plasma membrane in darkness and upon illumination with blue light, relocalised to the Golgi apparatus. Conversely the N-terminal GFP fusion, lacking the kinase domain, could only be visualised in the cytoplasm both before and after blue light illumination (Kong et al., 2007). Further investigation revealed that the extreme C-terminal 42 amino acids of phot2 are sufficient for Golgi association. However the region required for membrane association was more difficult to fully define as the results obtained from the yeast interactions did not fully translate when the same constructs were expressed in *Arabidopsis* (Kong et al. 2013). Recently phot1 and phot2 were also found to localise to the chloroplast membrane indicating that membrane localisation can be closely linked with function (Kong et al. 2013).

A similar analysis has not been published for phot1, however autophosphorylation of phot1 is known to be required for correct function and localisation (Inoue et al. 2008). Aspartate at position 806 in phot1 is required for chelating Mg^{2+} and subsequent ATP binding (Rademacher and Offringa, 2012). Mutation of this residue to asparagine removes the kinase activity of phot1 (Christie et al., 2002) and was subsequently found to prevent the internalisation of phot1-GFP in *N. benthamiana* cells (Kaiserli et al., 2009). Conversely, mutation of Ser-851 within the kinase activation loop, which is required for

correct function of phot1, to aspartate mimics phosphorylation and results in internalisation of a phot1-GFP fusion protein internalising in the absence of a blue light stimulus (Kaiserli et al., 2009). This suggests that kinase activation is important for instigating internalisation from the plasma membrane. However the mechanism of membrane association and function of this internalisation response remains to be fully understood.

1.8 Aims of this project

The physiological responses controlled by the phototropins are well understood. However the function of the membrane association of phototropins and subsequent internalisation in response to light is currently not known. Since the kinase domain is reported to be involved in directing phot2 to the membrane, mutational analysis of a region conserved from lower to higher plants was carried out. Subsequently examination of the localisation in insect cells, *N. benthamiana* as well as functionality in *Arabidopsis* was performed to address the role of the kinase domain in directing phot1 localisation. Following a similar approach to Kong et al. (2013), truncation analysis of the phot1 kinase domain was also performed to address if there is a specific region of phot1 that is required for membrane interaction. The insect cell system and transient expression in *N. benthamiana* were employed for this purpose. Finally the role of internalisation of phot1 from the membrane was also investigated using a constitutively plasma membrane localised version of phot1-GFP to assess the effect of this modification on the physiological responses controlled by phot1 in transgenic *Arabidopsis*. This method has been used successfully to target and retain other plant proteins to the membrane.

Chapter 2: Materials and Methods

2.1 Materials and chemicals

All chemicals were obtained from Sigma-Aldrich (Poole, Dorest, UK), Fisher Scientific (Loughbourgh, UK) and VWR International Ltd (Poole, Dorset, UK) unless otherwise stated. Ampicillin and gentamycin were purchased from Melford Ltd. (Ipswich, Suffolk, UK). Kanamycin was obtained from Sigma-Aldrich and chloramphenicol from Duchefa Biochemie B.V. (Haarlem, The Netherlands). Enzymes used for DNA restriction digests and ligations were obtained from New England Biolabs (Hitchin, Hertfordshire, UK) and Promega (Southampton, Hampshire, UK). KOD Hot Start DNA polymerase was purchased from Merck (California, USA) and Taq polymerase was from New England Biolabs Ltd (NEB). Molecular weight markers were provided by NEB. *E.coli* strains TOP10 and XL-10 gold were purchased from Agilent Technologies (California, USA). QIA-quick PCR purification kit, QIA-quick gel extraction kit, QIA-prep mini prep kit and Qiagen Plasmid Mini kit were obtained from Qiagen Ltd (Manchester, UK). LB broth and agar were provided by Merck (Darmstadt, Germany). BacMagic DNA kit was obtained from Merck Millipore (Hertfordshire, UK) whilst BD BaculoGold transfection kits were supplied by BD Biosciences (California, USA). TC-100 insect cell media and fetal bovine serum were purchased from Invitrogen Ltd (Paisley, UK). Cell culture flasks were provided by Corning (New York, USA). Radiolabelled ATP γ-^{32}P was obtained from Perkin Elmer (Buckinghamshire, UK). 37.5:1 acrylamide:bis-acrylamide, Bradford reagent and nitrocellulose membrane were purchased from BioRad Laboratories (Hertfordshire, UK). Complete protease inhibitor cocktail tablets (EDTA-free) were supplied by Roche (Mannheim, Germany). Ponceau S and BCIP/NBT solution were sourced from Sigma Aldrich. ECL+ was provided by Pierce (Rockford, USA). X-ray film was supplied by Kodak (USA). Square petri dishes were obtained from Fisher.

2.2 Plant material and growth conditions

Arabidopsis thaliana of the ecotype Columbia (Col) were used in this study. The Col-0 *gl1* was used as a control, since *phot* mutants used in this study originated in this background. The phot-deficient mutants *phot1-5, phot2-1* and *phot1-5*

phot2-1 have been described previously (Liscum and Briggs, 1995; Kagawa *et al.*, 2001; Kinoshita *et al.*, 2001). *Phot1-5 phot2-1* double mutants were transformed with a variety of phot-containing constructs to confirm function. Previously characterized lines created in this manner which were used in this study include *pPHOT1::PHOT1-GFP* (Sakamoto and Briggs, 2002). Transgenic plants expressing farnesyl-tagged phot1-GFP, mutated farnesyl-tagged phot1-GFP, phot1 6xK-N-GFP, phot1-GFP and *Ostreococcus* phototropin (Otphot) were generated for use in this study. Seeds were surface sterilised and grown on 0.5x Murashige and Skoog (MS) salts containing 0.8% (w/v) agar, supplemented with kanamycin (50 μg mL^{-1}) where required. Alternatively seeds were sown on soil and grown in controlled environmental conditions (Fititron, Weiss-Gallenkamp, Loughborough, UK) under 16/8 hour light-dark cycle at 70 μmol m^{-2} s^{-1}, unless otherwise stated. To induce uniform germination, seeds were given a cold treatment at 4°C for three days prior to being placed in a growth room. For phototropism assays germination was induced by a 6 hour white light treatment before the plates were wrapped in aluminium foil and grown vertically in the dark for 2.5 days. Light intensities used are described in the text. Fluence rates of the light sources used were measured using a Li-250A and quantum sensor (LI-COR, Lincoln, NE).

2.3 Surface sterilisation of *Arabidopsis* seeds

Seeds were surface sterilised for 5 mins in 1 mL of a solution containing 50% (v/v) sodium hypochlorite (>6% (w/v) available chlorine), 0.1% Triton X-100 before being washed three times in sterile water and resuspended in the appropriate volume of sterile water for sowing onto 0.5 X MS/0.8% agar plates.

2.4 Protein extraction from *Arabidopsis*

Arabidopsis tissue was ground in a mortar and pestle on ice or at 4°C in the cold room with the aid of a red safe light in the appropriate volume of extraction buffer (50mM Tris-MES pH 7.5, 300 mM sucrose, 150 mM NaCl, 10 mM potassium acetate, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and a protease inhibitor mixture (1 tablet/10 mL; complete EDTA free). All further manipulations were carried out at 4°C. Total protein extracted was clarified by

centrifugation at 16,000*g* for 10 mins at 4°C. Microsomal membranes were prepared by ultra-centrifugation of the total protein extract at 100,000*g* for 75 mins at 4°C. The supernatant from this stage was used as the soluble fraction and the pellet resuspended in extraction buffer to be used as the membrane fraction. Quantification of protein concentration was determined by Bradford colourimetric method (Bio-Rad, UK) using bovine serum albumin as the standard.

2.5 DNA cloning and manipulation

2.5.1 PCR

Polymerase chain reactions (PCR) were completed using a MJ Research DNA Engine PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation, Essex, UK). Primers used in this study are listed in Table 2.4. PCR reactions were completed to a final volume of 25 µL. 50 ng of template DNA was added to Taq polymerase buffer in combination with 10 µM of each primer, 200 µM of dNTPs and 0.625 U of Taq polymerase. Amplification was performed using a suitable number of cycles following a denaturation step for 2 mins at 94°C. A typical cycle consisted of an additional denaturation step at 94°C for 30 secs, annealing step at 58°C for 1 min and extension at 72°C for 1 min per Kb of template DNA. Modifications to this basic program were made when required by specific template DNA or primers.

2.5.2 Site-directed mutagenesis

Site directed mutagenesis was performed using KOD Hot Start DNA Polymerase. Primers were designed visually to contain 10-15 bases either side of the mutated codon with care taken to ensure the GC content was kept to a minimum. PCR reactions were carried out to a final volume of 50 µL. 500 ng of template DNA was added to 10 X KOD buffer, 2 mM $MgSO_4$, 0.8 mM dNTPs, 0.3 µM forward primer and 0.3 µM reverse primer and 1 U KOD Hot Start DNA Polymerase. The basic cycle was denaturation at 95°C for 2 mins, followed by a further denaturation at 95°C for 20 secs, annealing at 68°C for 10 secs and extension at 70°C for 25 secs/Kb for 20 cycles. Following mutagenesis DpnI digests were performed to remove template DNA. 20 U were added to PCR product and

incubated at 37°C for 1 hour before 2 µL of the reaction was transformed into XL-10 Gold Ultra-competent *E. coli* cells.

2.5.3 Restriction digest

DNA to be digested (0.5 µg plasmid DNA) was prepared in a total reaction volume of 50 µL containing 1 x CutSmart buffer (NEB) and 10 U of restriction enzyme. Reactions were incubated in a 37°C heat block for 1 hour before DNA was purified using PCR purification column (Qiagen, UK). The restrictions were monitored by running an aliquot of each reaction on an agarose gel.

2.5.4 Plasmid DNA ligation

DNA was ligated into plasmids after digestion using T4 ligase (Promega, UK). The ratio of insert:plasmid was 3:1. 10 X ligase buffer was added to DNA at a final concentration of 1X and 400 U of ligase was used. Total reaction volume was 20 µL. The reaction was incubated at 4°C overnight before being transformed into XL-10 Gold Ultra-Competent *E. coli* cells.

2.5.5 DNA Gel electrophoresis

Purified plasmid DNA or PCR products to be visualised were mixed with 5 X loading dye (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) to a final concentration of 1 X and resolved by electrophoresis on a 0.8% agarose gel. Agarose gels contained SYBR safe DNA gel stain (Life Technologies, UK) to a final concentration of 1 X and were run in 1 X TAE (40mM Tris, 2mM EDTA, 1.15% acetic acid) at 100V for the required time. A 1 kb ladder or a 100 bp ladder (NEB) was used to estimate the molecular mass of the DNA. Staining was visualised using a Fusion FX7 Imager (Peqlab).

2.5.6 Construction of vectors expressing farnesyl-tagged phot1-GFP

Transformation vectors for generating farnesylated phot1 lines and the respective mutated farnesyl controls were constructed using the modified binary expression vector pEZR(K)-LN as described previously (Kaiserli et al., 2009) to obtain a C-terminal GFP fusion. The *CaMV-35S* promoter was removed using

restriction sites SacI and HindIII and replaced with the native *PHOT1* promoter region to generate the plasmid pEZR-prPHOT1. To create the farnesyl tag, the C-terminal GFP from pEZR(K)-LN was removed using restriction sites BamHI and XbaI. pEZR(K)-LN was used as a template to amplify a modified GFP using the primers farnesyl F and farnesyl R to generate a GFP coding sequence with a C-terminal farnesylation sequence. This was cloned into pEZR-prPHOT1 using the restriction sites BamHI and XbaI to generate the plasmid pEZR-prPHOT1-GFPfarn. Amino acid changes in the mutated farnesyl construct were introduced by site-directed mutagenesis using KOD Hot-Start DNA Polymerase (Novagen) and the primers farnesyl C-A F and farnesyl C-A R. For primer sequences see table 2.4.

2.6 Bacterial Transformation and Growth

2.6.1 E.coli transformation and growth

Chemically competent *E. coli* cells were transformed with plasmid DNA according to Sambrook and Russell, (2001). XL-10 Gold cells were used for transformation of plasmids mutated by site directed mutagenesis as well as newly ligated plasmids. TOP10 cells were used for routine DNA transformations. 2 µL of plasmid DNA was incubated with 50 µL of chemically competent cells on ice for 20 mins before cells were heat shocked at 42°C for 45 secs. Treated cells were then placed back on ice for 2 mins before LB broth was added and cells incubated at 37°C for one hour. Transformed cells were plated onto LB-agar plates (1% (w/v trytone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) bactoagar pH7.5) containing antibiotics where appropriate (which were added once LB agar had cooled) and grown at 37°C overnight. Positive transformants were rescued from the plates using sterile pipette tip and inoculated in 10 mL LB broth as above (without bactoagar) containing the appropriate antibiotics. Cultures were grown overnight at 37°C in a shaking incubator (200rpm). Plasmid DNA was isolated using the Qiagen® Miniprep Kit and bacterial colonies containing the plasmid of interest were screened by digestion.

2.6.2 Preparation of DNA from E. coli

Transformed *E. coli* cells were selected using LB-agar plates containing the appropriate antibiotic. Transformed *E. coli* cells were rescued from the plate

using a sterile pipette tip and grown in 10 mL cultures overnight at 37°C with shaking at 200 rpm. Plasmid DNA was isolated using the Qiagen® Miniprep Kit. DNA required for insect cell transfection was purified using Qiagen® Plasmid Mini Kit. To quantify DNA 2 µL of plasmid was diluted in 68 µL of water and absorbance at 260 nm and 280 nm was measured (Eppendorf Bio Photometer) against a water blank sample. An OD_{260} of 1 was taken to equal 50 µg mL^{-1}. DNA integrity was determined by 1% (w/v) agarose gel electrophoresis as described. The ratio of the absorbance 260/280 was used to indicate the purity of the sample.

2.6.3 DNA sequencing

Sequencing of DNA was carried out by the Dundee Sequencing Service (University of Dundee, UK) or by GATC Biotech (Konstanz, Germany) according to the service's instructions. Sequencing was always performed after a series of sub-cloning reactions to verify the sequence of the DNA insert in every vector used in this study.

2.6.4 Preparation of competent Agrobacterium tumefaciens for electroporation

Agrobacterium tumefaciens strain GV3101 was used in this study. An aliquot of *A. tumefaciens* was inoculated in a 10 mL starter culture of LB containing 30 µg mL^{-1} gentamycin and grown for 20-24 h at 28°C with constant shaking at 200 rpm. The following day, 1 L of LB medium with rifampicin and gentamycin was inoculated with the starter culture and grown at 28°C with shaking at 200 rpm until the culture reached an OD_{600} of 0.5 - 0.8. Cells were then pelleted at 2,000 g for 10 min at 4°C. The supernatant was discarded and the pellet was gently resuspended in 100 ml cold sterile 10% (v/v) glycerol. Centrifugation and resuspension was repeated twice, changing the volume of 10% glycerol used to 10 mL and finally 1 mL. 50 µL aliquots were frozen on dry ice and stored at -80°C.

2.6.5 Agrobacterium-mediated transformation

0.5 µL of plasmid DNA of interest was added to 100 µL of *A. tumefaciens* electrocompetent cells and incubated on ice for 20-30 min. Cells were then transferred to an electroporation cuvette (Bio-Rad) and pulsed with 2.2kV using a MicroPulser™ Electroporator (Bio-Rad). Immediately after electroporation 900 µL of LB was added to the cells. The solution was then incubated at 28°C for 3 hours. Serial dilutions were spread on LB-agar plates containing appropriate antibiotics. Plates were incubated at 28°C for 2 days.

2.7 Protein expression

2.7.1 Insect cell expression

Expression of phototropin proteins using the insect cell system was carried out as described previously (Christie et al., 1998) with modifications. Recombinant baculovirus encoding *Arabidopsis* phot1 or phot2 or any constructs containing specific mutations was generated using BD Baculogold Transfection Kit (BD Biosciences) or Bac-Magic™ DNA kit (Millipore, Cat#71545) in accordance with the manufacturers instructions. This recombinant virus was used to infect *Spodoptera frugiperda* (*Sf9*) insect cells. Cells were grown in serum-free medium (Invitrogen) supplemented with 10% (v/v) foetal bovine serum (Invitrogen) at 27°C for three days in either 25 cm^2 or 75 cm^2 culture flasks (Corning) wrapped in aluminium foil to exclude light. Cells were then harvested under a red safe light by washing the cells from the wall of the flask and centrifuging at 1,000*g* for 5 mins. The culture medium was removed and the cell pellet resuspended in either 100 µL or 400 µL of phosphorylation buffer (37.5 mM Tris-HCl pH 7.5, 5.3 mM $MgSO_4$, 150 mM NaCl, 1mM EGTA, 1 mM dithiothreitol, 1 mM PMSF and 1 X EDTA free complete protease inhibitor cocktail (Roche). Cells were lysed by sonication and cell debris pelleted by centrifugation at 16,000*g* for 3 mins in a microfuge. The supernatant was designated as the crude soluble fraction and was removed to a fresh tube. An aliquot was taken for determination of protein concentration. For investigation of membrane association the crude soluble fraction was ultra-centrifuged at 100,000*g* for 75 mins, an aliquot of the supernatant was taken as the soluble fraction and the pellet washed in 1x

phosphorylation buffer before being centrifuged at 100,000*g* for a further 75 mins. The pellet was then resuspended in the same volume and designated as the membrane fraction.

2.8 Protein Gel Electrophoresis

2.8.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were denatured by addition of the correct volume of 2x SDS loading buffer (BioRad, UK) and subsequent boiling of the samples for 5 mins at 95°C. Proteins were separated using either a 7.5% or 10% gel with a 5% stacking gel. Proteins were separated in SDS running buffer (25 mM Tris-Hcl pH8.5, 190 mM glycine and 0.1% (w/v) SDS) at 200 V until the loading dye front reached the bottom of the gel. Protein molecular weights were determined using a prestained molecular weight marker (P7708, New England Biolabs) for Western blotting and an unstained marker for all other work (P7703, New England Biolabs).

2.8.2 Coomassie Brilliant Blue staining of SDS-PAGE Gels

Gels were submerged for approximately 30 mins in 0.1% Coomassie Brilliant Blue R250 (BioRad), 45% methanol and 10% acetic acid. They were subsequently destained in 45% methanol and 10% acetic acid until most background staining was removed. Gels were then left in rehydration buffer (10% ethanol and 5% acetic acid) overnight to remove any remaining background staining before being dried onto Whatmann 3 mm chromatography paper using a Scie Plas Gel Drier (model GD4535) connected to a Vacuubrand MZ C2 vacuum pump. Dried gels were scanned whilst radioactive dried gels were exposed to X-Ray film (Kodak) before being developed.

2.8.3 Western blotting

Proteins were electro-transferred at 400 mA for 45 mins from the SDS-PAGE gel to a nitrocellulose membrane (162-0115, BioRad) in transfer buffer (25mM Tris-HCl pH 8.5, 190 mM glycine, 20% (v/v) methanol. Ponceau S staining (0.1% (v/v) in 1% acetic acid) was carried out to ensure protein transfer and equal loading

by visualisation of the Rubisco large subunit for plant-derived samples. After removal of the stain, the membrane was blocked at room temperature for at least an hour or overnight at 4°C using 8% (w/v) milk powder (Marvel) dissolved in 1 X TBS (25 mM Tris-HCl pH 8, 150 mM NaCl, 2.7 mM KCl) containing 0.1% (v/v) Triton X-100 (TBS-T) to prevent non-specific protein binding. Primary antibodies were then incubated with the membrane at room temperature for 1 hour or overnight at 4°C in 1 X TBS-T in the presence of 8% (w/v) milk powder at the dilutions indicated in section 2.16. The membrane was washed three times with 1 X TBS-T containing 0.05% Tween-20 (v/v) (TBS-TT) before secondary antibodies were added (section 2.16) for 1 hour at room temperature. The membrane was then washed 5 times with TBS-TT and once with TBS before developing. For chemiluminescent detection of the protein bands the ECL Plus Western Blotting Detection Reagent (Pierce Fisher) was used according to the manufacturers instructions. After incubation with the ECL reagents the proteins on the membrane were detected using a Fusion FX7 Imager (Peqlab, UK). For alkaline phosphatase (AP) detection of proteins the blots were developed using BCIP/NBT solution.

2.9 Detergent Treatment of Membranes

Insect cells expressing phot1 constructs were harvested in darkness with the aid of a red safe light and resuspended in 1 X phosphorylation buffer (section 1.7.1) before being sonicated and centrifuged at 14,000*g* for 3 mins to pellet cell debris. The resulting supernatant was then subjected to ultra-centrifugation at 100,000*g* for 90 mins. The pellet from this step was resuspended in the appropriate volume of 1 X phosphorylation buffer. NaCl was added to the membrane samples at various concentrations and the detergents mentioned in the main text were added to the membrane samples to a final concentration of 1%. Samples were incubated on ice in the darkness for 30 mins. Membranes subsequently underwent a further round of ultra-centrifugation at 100,000*g* for 90 mins. The supernatant from this spin was designated the soluble fraction whilst the pellet was resuspended in 1 X phosphorylation buffer and designated the membrane fraction.

Three-day-old *Arabidopsis* seedlings grown in darkness were ground in a mortar and pestle on ice or at 4°C in the cold room with the aid of a red safe light in the appropriate volume of extraction buffer (Section 2.4) before being treated as described above for insect cell membranes. Kinase assays were then carried out on the soluble and membrane fractions as described in section 2.10.

2.10 *In vitro* Kinase Assays

Phototropin kinase activity *in vitro* was monitored as previously described (Christie et al., 1998). All manipulations were carried out under dim red light. 20 µg of protein extract prepared from Sf9 insect cells as described in 2.7.1 was suspended in 1 x phosphorylation buffer. Easytides adenosine 5'γ-^{32}P triphosphate (^{32}P-ATP; Perkin Elmer, UK) was diluted 1:5 with 10 µM unlabelled ATP. 1 µL of this diluted ^{32}P-ATP was used per reaction. Samples were either kept in the dark or exposed to 30,000 µmol m^{-2} white light for 10 secs before being incubated at room temperature for a total of 2 mins. Reactions were stopped by the addition of an equal volume of 2 x SDS Loading Dye (BioRad, UK). Samples were separated by a 7.5% SDS-PAGE gel, Coomassie stained and dried. Dried radioactive gels were exposed onto X-ray film for at least three hours at room temperature. The X-ray film was developed using a Kodax X-OMAT processor (model ME-3).

2.11 Transient Expression in *Nicotiana benthamiana*

Nicotiana benthamiana plants were grown for 3 weeks in individual pots at 26°C in a 16/8 hour light dark cycle. Plants with healthy green leaves were used for transient expression. Colonies from freshly transformed *Agrobacterium* cells with the desired plasmid DNA were scraped from the plate and inoculated into 20 mM $MgCl_2$, 10 mM MES pH 6.5 until the OD_{600} was 0.4-0.5. The culture was left at room temperature for 3 hours before being infiltrated into the under side of *N. benthamiana* leaves using a syringe (adapted from Li et al., 2009). Plants were returned to the growth room at 26°C for 2 days before being moved to dim lighting 12-18 hours before being imaged.

2.12 Confocal Microscopy

Subcellular localization of GFP-tagged proteins in *Arabidopsis* seedlings and in leaves of *N. benthamiana* were visualized by a confocal laser-scanning microscope (Zeiss LSM 510) under water in dark conditions. GFP was excited using an argon laser at 488 nm, and emission was collected between 505 and 530 nm. To induce blue light activation of phot1, plant samples mounted on a slide were irradiated with an argon laser (488 nm) for a period of 30 secs after the initial scan. Subsequent images were taken after a period of 3 to 9 mins as indicated. Images were processed using ImageJ software (http://rsb.info.nih.gov/ij) and Adobe Photoshop (http://www.adobe.co.uk).

2.13 Generation of Stable Transgenic Lines

2.13.1 Floral dipping

The transgenic lines in this study were generated in the *phot1-5 phot2-1* mutant background by *Agrobacterium*-mediated transformation (Clough and Bent, 1998). *Arabidopsis* plants for transformation were grown until flowers developed (4-5 weeks). A single colony of *Agrobacterium* containing the plasmid of interest was inoculated in 500 mL of LB media with appropriate antibiotics and grown at 28°C with constant shaking overnight. The cells were then pelleted by centrifugation at 4,000*g* for 10 mins and resuspended to an OD_{600} of 0.6-0.8 in 5% sucrose containing 500 U/L of Silwet L-77. Upper sections of the plants containing the bolting stems with flowers were then submerged in the *Agrobacterium* solution for 30 secs with gentle agitation. Plants were then kept under humid conditions overnight and returned to the growth room at 70 µmol m^{-2} s^{-1} white light with a 16/8 hour light-dark cycle. Two days later the plants were again immersed in the *Agrobacterium* solution and returned to these growth conditions until seeds were set.

2.13.2 Selection of Arabidopsis transgenic lines

Transgenic seeds were surface sterilised and grown on 0.8% agar plates containing 1/2 MS and 75 mg mL^{-1} kanamycin for selection. 30-40 surviving T1

seedlings were transferred to soil and allowed to set seed. T2 generation plants showing a 3:1 segregation on selective plates were carried forward. Homozygous T3 lines were selected based on 100% resistance to kanamycin and satisfactory GFP expression levels (assessed by confocal microscopy).

2.14 Physiological Characterisation of Transgenic *Arabidopsis*

2.14.1 Phototropism

Second positive hypocotyl curvature was measured as described in Lascève et al., (1999). Seedlings were surface sterilised and spotted individually onto square Petri dishes containing 0.5 X MS salts pH 5.7 and 0.8% agar. After vernalisation at 4°C for three days plates were given a 6 hour light treatment to induce germination before being wrapped in foil and grown vertically for 2.5 days. Seedlings were then exposed to unidirectional blue light at differing light intensities and times as described in the main text. Blue light source up to 10 µmol m^{-2} s^{-1} was provided by a white fluorescent lamp filtered through blue plexiglass. Above 10 µmol m^{-2} s^{-1} light was provided by a projector (Optoma DS211 DLP, Texas Instruments) in combination with a water filter and blue pexiglass. Following blue light treatment plates were scanned and angles measured using ImageJ software.

2.14.2 Petiole positioning

Seeds were sown on soil and stratified for three days at 4°C before being transferred to a controlled growth room for one week under 70 µmol m^{-2} s^{-1} white light in a 16/8 hour light dark cycle. The plants were then transferred to 10 µmol m^{-2} s^{-1} white light 16/8 hour light dark cycle for a further week. Representative seedlings were photographed and petiole angles of the first true leaves were measured using ImageJ software.

2.14.3 Leaf expansion

Seeds were sown on soil and stratified as described in section 2.14.2 before being grown for three weeks under 70 µmol m^{-2} s^{-1} white light in a 16/8 hour

light dark cycle. The fifth rosette leaves of representative plants were detached and photographed. Leaves were then manually flattened and photographed again. Leaf area before and after was measured using Image J software. Leaf expansion index (LEI) was designated as the ratio of unflattened leaf area to flattened leaf area.

2.14.4 Chloroplast accumulation

Arabidopsis seeds were sown on soil and given a 3 day treatment at 4°C before being placed in a growth room on a 16 hour/8 hour light dark cycle for 3 weeks at 70 µmol m^{-2} s^{-1}. Leaves were then detached and placed on 0.5X MS salts with 0.8% agar under either 1.5 µmol m^{-2} s^{-1} of blue light or kept in darkness for 3 hours. Blue light was provided by fluorescent white lamps in combination with a blue filter. The chloroplasts in the palisade mesophyll cells were viewed using confocal microscopy with an excitation wavelength of 488nm and emission at 560nm as described previously (Kaiserli et al., 2009). Images were processed using ImageJ software and Adobe Photoshop.

2.15 Sequence Alignments

Amino acid alignments were performed using the ClustalW algorithm supplied by www.ebi.ac.uk/Tools/msa/clustalw2, with all parameters set to default (Protein Gap Open Penalty = 10.0, Protein Gap Extension Penalty = 0.2, Protein matrix = Gonnet; Chenna et al., 2003). The protein sequences were taken from NCBI (www.ncbi.nlm.nih.gov/protein) with the following accession numbers used:

Arabidopsis phot1 NP_190164; *Arabidopsis* phot2 NP_851210.1; Otphot XP_003083739; Zea mays phot1 NP_001104886.1; *Vicia faba* phot1 BAC23099; *Physcomitrella patens* photB2 XP_001785726; *Medicago truncatula* phot2 XP_003597291; *Phaseolus vulgaris* phot1a XP_007132147; *Solanum lycopersicum* phot1 NP_001234214; *Adiantum capillus-veneris* phot1 BAA95669; *Avena sativa* phot1AAC05083; *Chlamydomonas reinhardtii* phot XP_001693387.

2.16 Antibodies

Table 2.1 Antibodies used in this study

Antibody	Host	Dilution	Source
Anti-C terminal phot1 (polyclonal)	Rabbit	1/5,000	Cho et al. 2007
Anti-C terminal-phot2 (polyclonal)	Rabbit	1/5,000	Cho et al. 2007
Anti-GFP (monoclonal)	Mouse	1/5,000	Clontech
Anti-UGPase (polyclonal)	Rabbit	1/5,000	Komatsu et al. 2014
Anti-GP-64 (monoclonal)	Mouse	1/10,000	Abcam
Anti-α-tubulin (polyclonal)	Rabbit	1/10,000	Abcam
Anti-His (monoclonal)	Mouse	1/5,000	Sigma Aldrich
Anti-GST (monoclonal)	Mouse	1/10,000	Novagen
Anti-Rabbit-AP	Rabbit	1/10,000	Promega
Anti-Mouse-HRP	Rabbit	1/10,000	Promega
Anti-Rabbit-HRP	Mouse	1/10,000	Promega

Table 2.2: Generation of constructs used for insect cell expression in this study

Construct name	Tag	Protein encoding	Source/primers used
pAcHLTA-phot1	6xHis (N-term)	phot1 (7-996)	(Christie et al., 1998)
pAcHLTA-phot1 6xK-N	6xHis (N-term)	phot1 (7-996)	phot1 K-N F phot1 K-N R
pAcHLTA-phot1 6xK-A	6xHis (N-term)	phot1 (7-996)	phot1 K-A F phot1 K-A R
pAcHLTA-phot1 Δkinase	6xHis (N-term)	phot1 (7-661)	phot1 NcoI F phot1 ΔK NotI R
pAcHLTA-phot1 ΔC-terminus	6xHis (N-term)	phot1 (7-952)	phot1 NcoI F phot1 ΔC NotI R
pAcHLTA-phot1 726	6xHis (N-term)	phot1 (7-726)	phot1 726 stop F phot1 726 stop R
pAcHLTA-phot1 814	6xHis (N-term)	phot1 (7-814)	phot1 814 stop F phot1 814 stop R
pAcHLTA-phot1 907	6xHis (N-term)	phot1 (7-907)	phot1 907 stop F phot1 907 stop R
pAcHLTA-phot1 964	6xHis (N-term)	Phot1 (7-964)	phot1 964 stop F phot1 964 stop R
pAcHLTA-phot1 972	6xHis (N-term)	phot1 (7-972)	phot1 972 stop F phot1 972 stop R
pAcG3X-phot1	GST (N-term)	phot1 (1-996)	phot1 BamHI F phot1 EcoRI R
pAcG3X-phot1 663-726	GST (N-term)	phot1 (663-726)	phot1 663 BamHI F phot1 726 EcoRI R
pAcHLTA-phot2	6xHis (N-term)	phot2 (1-916)	(Sakai et al., 2001)
pAcHLTA-phot2 640	6xHis (N-term)	phot2 (1-640)	phot2 640 stop F phot2 640 stop R

pAcHLTA-phot2 Δkinase	6xHis (N-term)	Phot2 (1-576)	phot2 ΔK stop F phot2 ΔK stop R
pAcHLTA-Otphot	6xHis (N-term)	Otphot (1-738)	Otphot NcoI F Otphot NotI R
pAcHLTA-Otphot K-N	6xHis (N-term)	Otphot (1-738)	Otphot K-N F Otphot K-N R

Table 2.3: Generation of constructs used for *Arabidopsis* and *N. benthamiana* expression in this study

Construct name	Tag	Protein encoding	Source/primers used
pEZR(K)-LN-phot1	GFP (N-term)	phot1 (7-996)	(Kaiserli et al., 2009)
pEZR(K)-LN-phot1 6xK-N	GFP (N-term)	phot1 (7-996)	phot1 K-N F phot1 K-N R
pEZR(K)-LN-prphot1-phot1-farnesyl	GFP (N-term)	phot1 (7-996)	prphot1 SacI F prphot1 HindIII F pEZRLN-farnesyl F pEZRLN-farnesyl R
pEZR(K)-LN-prphot1-phot1-farnesyl C-A	GFP (N-term)	phot1 (7-996)	Farnesyl C-A F Farnesyl C-A R
pEZR(K)-LN-phot1 663-726	GFP (N-term)	phot1 (663-726)	phot1 663 EcoRI F phot1 726 BamHI R
pEZR(K)-LN-phot1 663-814	GFP (N-term)	phot1 (663-814)	phot1 663 EcoRI F phot1 814 BamHI R
pEZR(K)-LN-phot1 726-996	GFP (N-term)	phot1 (726-996)	phot1 726 EcoRI F phot1 BamHI R
pEZR(K)-LN-phot1 Δkinase	GFP (N-term)	phot1 (1-662)	phot1 EcoRI F phot1 663 BamHI R
pEZR(K)-LN-phot1 ΔC-terminus	GFP (N-term)	phot1 (1-950)	phot1 EcoRI F phot1 ΔC BamHI R
pEZR(K)-LN-phot1	GFP (N-term)	phot1 (613-996)	phot1 663 EcoRI F

kinase			phot1 BamHI R
pEZR(K)-LN-phot1 607-726	GFP (N-term)	phot1 (607-726)	phot1 607 EcoRI F phot1 726 BamHI R
pEZR(K)-LN-Otphot	GFP (N-term)	Otphot (1-738)	Otphot EcoRI F Otphot BamHI R
pEZR(K)-LN-Otphot 5xK-N	GFP (N-term)	Otphot (1-738)	Otphot K-N F Otphot K-N R
pEZR(K)-LC	GFP (C-term)	GFP	Dr. Gert-Jan de Boer

Table 2.4: Primers used in this study

Primer name	Sequence
phot1 K-N F	ATAGACGAGAACAACAACAACAACCAACAAAACAGTCAACAAACT
phot1 K-N R	AGTTTGTTGACTGTTTTGTTGGTTGTTGTTGTTCTCGTCTAT
phot1 K-A F	ATAGACGAGGCGGCGGCGGCGGCGCAACAAAACAGTCAACAAACT
phot1 K-A R	AGTTTGTTGACTGTTTTGTTGCGCCGCCGCCGCCGCCTCGTCTAT
phot1 NcoI F	(Christie et al., 1998)
phot1 ΔK NotI R	TCGAGCGGCCGCCTTCAAACCAATCGGTTCACCACT
phot1 NcoI F	(Christie et al., 1998)
phot1 ΔC NotI R	TCGAGCGGCCGCGAAGAAAGAATGTTGCTT
phot1 726 stop F	CATCCTTTTCTTCCTGCATAGTACGCTTCTTTTCAGACA
phot1 726 stop R	TGTCTGAAAAGAAGCGTACTATGCAGGAAGAAAAGGATG
phot1 814 stop F	TTTGATTCTTGCTTGACATAATGCAAACCTCAGCTGTTG
phot1 814 stop R	CAACAGCTGAGGTTTGCATTATGTCAAGCAAGAATCAAA
phot1 907 stop F	TTCACCAATGTTCTTCAATAAGATCTCAAGTTTCCAGCT
phot1 907 stop R	AGCTGGAAACTTGAGATCTTATTGAAGAACATTGGTGAA
phot1 964 stop F	GCTCTGATTCGATGCACGTAACCTCCAGAGCTCGAGACT
phot1 964 stop R	AGTCTCGAGCTCTGGAGGTTACGTGCATCGAATCAGAGC
phot1 972 stop F	CCAGAGCTCGAGACTCCGTAATTTTCTGGTGAAGCTGA
phot1 972 stop R	TCAGCTTCACCAGAAAATTACGGAGTCTCGAGCTCTGG
phot1 BamHI F	GGATTCATGGAACCAACAGAAAAACCATCGACC
phot1 EcoRI R	GAATTCTCAAAAAACATTTGTTTGCAG

phot1 663 BamHIF phot1 726 EcoRI R	ATAGGGATCCACTTCAAACCGGTGAAACCTTTG TAATGAATTCGGGAGTGCAGGAAGAAAAGGATGG
phot2 640 stop F phot2 640 stop R	CATCCATTCCTTCCTACTTAATACGCTTCTTTTCAGACC GGTCTGAAAAGAAGCGTATTAAGTAGGAAGGAATGGATG
phot2 ΔK stop F phot2 ΔK stop R	ACAGTGGGACTACATCATTAGAAACCAATAAAACCGTTG CAACGGTTTTATTGGTTTCTAATGATGTAGTCCCACTGT
Otphot NcoI F Otphot NotI R	AATAATCCATGGGTGATGGCGTCGCATAGGATCTCCGAG TCGAGCGGCCGCCCTAAATCTCCATCTCGAAGATCTC
Otphot K-N F Otphot K-N R	GCGACGGCGCACGCGAACAACAACAACTTGAACCCGCCTCGGTTA TAACCGAGGCGGGTTCAAGTTGTTGTTGTTCGCGTGCGCCGTCGC
prphot1 SacI F prphot1 HindIII F	AAAAGAGCTCTGAACTTTTTTATGGTAGGGTTT AAAAAAGCTTCTTTGTGCTCTCTCTATACACG
farnesyl F farnesyl R	GATGTGACATCTCCACTGACG GGCATGGACGAGCTGTACAAGTCTAAGGATGGAAAGAAGAAGAAG AAGAAGTCTAAGACTAAGTGTGTTATTATGTAATCTAGATATAT
Farnesyl C-A F Farnesyl C-A R	AAGAAGAAGAAGAAGTCTAAGACTAAGGCTGTTATTATGTAATCTA GAGTCC GGACTCTAGATTACATAATAACAGCCTTAGTCTTAGACTTCTTCTTC TTCTT
phot1 663 EcoRI F phot1 726 BamHI R	TAAGAATTCAATTCAAACCGGTGAAACCTTTGGGT TATGGATCCTTGTAGAGTGCAGGAAGAAAAGG
phot1 663 EcoRIF phot1 814 BamHI R	TAAGAATTCAATTCAAACCGGTGAAACCTTTGGGT TATATGGATCCTTAGATGTCAAGCAAGACAGATC
phot1 726 EcoRI F phot1 BamHI R	AATATAGAATTCAAATGGACCATCCTTTTCTTCCTGCACTC (Christie et al., 1998)
phot1 EcoRI F phot1 ΔC BamHI R	(Christie et al., 1998) AAAAGGATCCTAGAAGAAAGAATGTTGCTT
phot1 EcoRI F phot1 ΔKBamHI R	(Christie et al., 1998) AAAAGGATCCATATGCTTCAAACCAATCGGT
Otphot EcoRI F Otphot BamHI R	TATATGAATTCACCATGGCGTCGCATAGGATCTC ATATAGGATCCGCAATCTCCATCTCGAAGATCTCCTC

Chapter 3: Analysis of the Membrane Association of *Arabidopsis* Phototropin 1

3.1 Introduction

Arabidopsis contains two closely related phototropins phot1 and phot2 responsible for plant responses to blue light such as phototropism, leaf expansion, petiole positioning and chloroplast positioning (Christie, 2007). Both proteins contain two N-terminal Light Oxygen or Voltage sensing (LOV) domains and a C-terminal kinase domain that mediates light dependent autophosphorylation of the receptor protein upon blue light irradiation (Christie et al., 1998). Much work has been done to elucidate the biochemical properties of plant phototropins (Briggs, Christie and Salomon, 2001) and the photochemical properties of the LOV domains and how these photosensors regulate the activity of the C-terminal kinase domain (Christie et al., 1999, 2002). In darkness both phot1 and phot2 localise exclusively to the plasma membrane (Sakamoto and Briggs, 2002, Kong et al., 2006). Upon blue light irradiation phot1 and phot2 have been shown to partially change their subcellular localisation and internalise to cytosolic strands (Sakamoto and Briggs, 2002, Wan et al., 2008, Kaiserli et al., 2009) or the Golgi, respectively (Kong et al. 2006; Kong et al. 2007; Kong et al. 2013; Aggarwal et al. 2014) . A loss of phototropin kinase activity has been shown to prevent this partial internalisation and trafficking for phot1 (Kaiserli et al., 2009) and phot2 (Kong et al. 2006; Aggarwal et al. 2014) in response to blue light. Kinase inactive versions of these receptor proteins therefore show constitutive plasma membrane localisation. Although first identified at the biochemical level in 1988 (Gallagher et al., 1988), it is still not fully understood how phototropins associate with the plasma membrane, despite being mostly hydrophilic proteins. In this chapter the mode of membrane association of *Arabidopsis* phot1 is examined through biochemical analysis, mutagenesis of targeted sequences that could play a role in membrane association combined with functional characterisation of these mutations in transgenic *Arabidopsis*.

3.2 Results

3.2.1 Arabidopsis phot1 is associated with the membrane in insect cells

Arabidopsis phot1 and phot2 associate with the plasma membrane in darkness in transgenic *Arabidopsis* expressing a phot1-GFP fusion in the *phot1-5* null mutant (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008) or a phot2-GFP fusion in the *phot1-5 phot2-1* double mutant background (Kong et al., 2006) as well as when transiently expressed in *Nicotiana benthamiana* cells (Kaiserli et al. 2009; Aggarwal et al. 2014). Another method that provides information on phototropin biochemical function and could be useful for assessing the membrane associating properties of phot1 is the *Baculovirus*/insect cell expression system (Christie et al., 1998, Sakai et al., 2001). In this system, phot1 can be expressed in an active form (Christie et al. 1998; Sakai et al. 2001; Jones et al. 2007; Jones & Christie 2008). There is a short time scale from transfection of cells to virus production (≈2 weeks) providing a convenient method to potentially examine the membrane association properties of the receptor and a rapid means to study the effect of any mutations on this process.

Previous work has been carried out to clone full-length *Arabidopsis PHOT1* and *PHOT2* into the appropriate transfer vectors for co-transfection in *Spodoptera frugiperda (Sf9)* insect cells (Christie et al., 1998, 2002, Sakai et al., 2001) (Fig. 3.1A). This transfer vector contains *PHOT1* or *PHOT2* fused to an N-terminal 6XHis-tag that when used for co-transfection alongside linearised *Baculovirus* DNA produces recombinant viral stocks via homologous recombination that can be used for subsequent infection of the insect cells. The production of His-tagged phot1 or phot2 can then be detected by Western blotting using antibodies against phot1, phot2 or the 6XHis-tag. To assess the localisation of phot1 and phot2 in insect cells, the His-tagged phot proteins were expressed in *Sf9* cells and total protein extracted. Ultra-centrifugation was then performed to separate this protein extraction into membrane and soluble fractions. As shown in Figure 3.1B, when expressed in insect cells, both phot1 and phot2 are detected in the soluble fraction as well as the membrane fraction.

The purity of the membrane and soluble fractions was examined by monitoring the levels of two proteins that are produced in insect cells infected with virus. Glycoprotein-64 (GP-64) is a *Baculovirus* protein that is produced on the plasma membrane of infected cells (Blissard and Wenz, 1992). GP-64 was only detected in the membrane fraction, demonstrating an absence of membrane proteins in the soluble fraction (Fig. 3.1B). An antibody which was raised against the cytosolic marker protein α-tubulin was used to confirm the purity of the membrane fraction. No α-tubulin protein was detected in the membrane fraction (Figure 3.1B). Thus, the presence of phot1 and phot2 in both soluble and membrane fractions is not a result of contamination and a lack of purity. These findings therefore indicate that when expressed in insect cells, *Arabidopsis* phot1 and phot2 are both targeted to the membrane fractions.

To further test the membrane attachment mechanism of lower plant phototropins, we also expressed the phototropin from the marine algae *Ostreococcus tauri* (Otphot) in the insect cell system. The LOV1 domain from Otphot has previously been shown to have similar spectral properties to that of LOV domains from higher plants (Veetil et al., 2011). Therefore it was of interest to assess if the membrane attachment properties of Otphot are also similar to higher plants. The full-length *Ostreococcus PHOT* cDNA was cloned into the appropriate transfer vectors for co-transfection in *Sf9* cells as previously described above for phot1 and phot2 (Figure 3.2A). This His-Otphot fusion was used alongside linearised *Baculovirus* DNA to produce recombinant viral stocks for subsequent infection of the insect cells. The His-tagged Otphot protein was expressed in the *Sf9* cells and total protein extracted. Ultra-centrifugation was then performed to separate this protein extraction into membrane and soluble fractions. As shown in Figure 3.2B, Otphot is also detected in the soluble fraction as well as the membrane fraction similar to *Arabidopsis* phototropins expressed in the insect cell system. The GP-64 membrane marker shows that there is no membrane protein detectable in the soluble fraction and the soluble α-tubulin marker also shows that there is no soluble protein detectable in the soluble fraction. Therefore the presence of Otphot in the soluble and membrane fractions is not a consequence of impurity of the fractions and is likely due to overexpression of phot1 at the membrane within the insect cell system. Thus the mechanism of attachment of the phototropins, from lower to higher plants, to

the membrane appears to be conserved in insect cells. The insect cell system therefore provides a convenient means to study the membrane association properties of phototropin proteins from higher and lower plants.

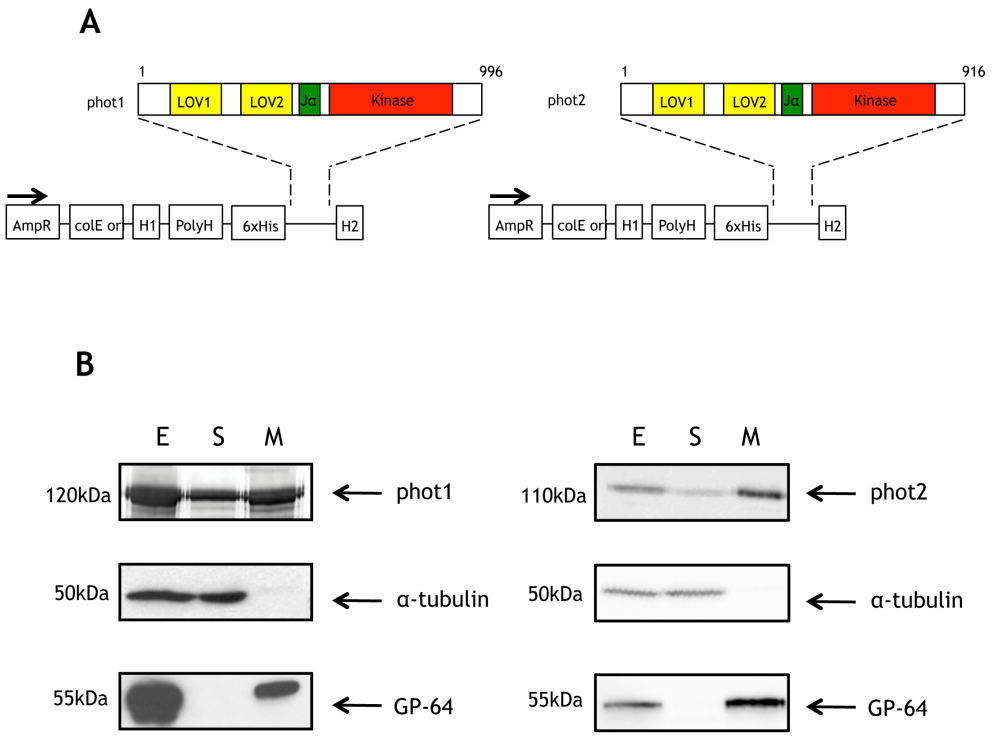


Figure 3.1 Expression and localisation of phot1 and phot2 in insect cells.

A) Diagram illustrating the transfer vector containing *Arabidopsis PHOT1* or *PHOT2* cDNA used to create recombinant *Baculovirus* producing His-tagged phot1 or His-tagged phot2. H1 and H2 are homologous regions that allow recombination with linearised *Baculovirus* DNA to produce recombinant viral stocks for subsequent infection and protein production.

B) Western blot analysis on protein fractions isolated from virally infected insect cells. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. C-terminal phot1 antibody was used to detect phot1 whilst anti-His antibody was used to detect phot2 in each fraction. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64.

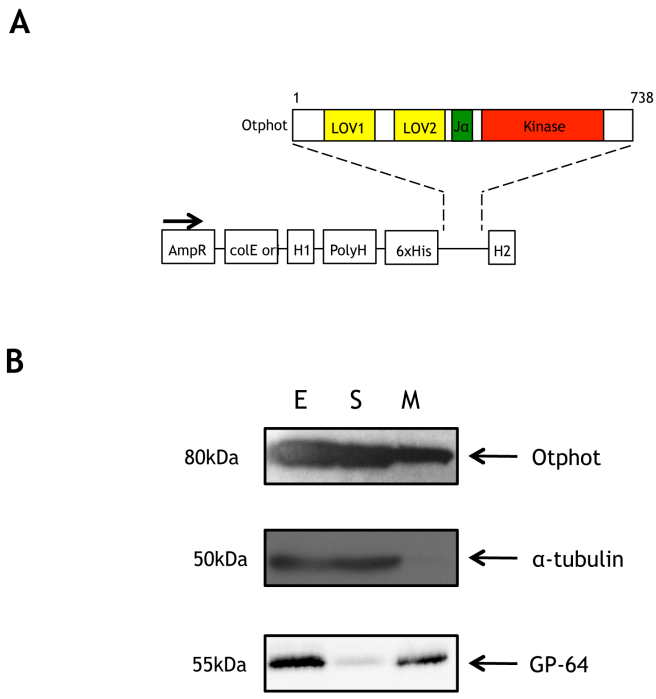


Figure 3.2: Expression and localisation of *Ostreococcus tauri* phototropin expressed in insect cells.

A) Diagram illustrating creation of transfer vectors containing *Ostreococcus tauri PHOT* (Otphot) cDNA. The vector was used to create recombinant baculovirus containing His-tagged Otphot. H1 and H2 are homologous regions that allow recombination with linearised baculovirus to produce recombinant viral stocks for subsequent transfection.

B) Western blots of protein fractions iolated from virally infected insect cells expressing Otphot. Ultra-centrifugation was used to separate the soluble (S) and membrane (M) fractions. Anti-His antibody detects Otphot in each fraction. Purity of the fractions was detected using α-tubulin antibody against the cytoplasm and GP-64 antibody against the membrane.

3.2.2 Phot1 only undergoes light-dependent autophosphorylation at the membrane in insect cells

To date there have been no kinases identified which phosphorylate phot1. However, phototropins are known to undergo autophosphorylation in response to blue light (Christie et al. 1998; Briggs et al. 2001; Christie 2007). This can be readily measured *in vitro* using the *Baculovirus*/insect cell expression system following the increased incorporation of radiolabelled phosphate into light-

treated phot1. The kinase activity of each of the fractions obtained from insect cells expressing phot1 was therefore tested using this method. Ultra-centrifugation was again used to separate soluble and membrane fractions from virally infected cells. These were performed under red safe light conditions that do not activate the recombinant phot1 protein. Samples were then subjected to *in vitro* phosphorylation analysis in the presence or absence of light. As shown previously by Christie et al. (1998) and Christie et al. (2002), an increase in phot1 autophosphorylation could be detected in protein extracts following exposure to light compared to dark controls. No signal was detected for phot1 autophosphorylation in the soluble fraction despite protein being detected there (Fig. 3.1B). There is however a large increase in phot1 autophosphorylation following irradiation within the membrane fraction, indicating that this is the only fraction that contains kinase active phot1 (Figure 3.3A). These findings provide evidence that only the membrane-localised form of phot1 is in the active state when expressed in insect cells. The autophosphorylation activity of Otphot and phot2 was also assessed in the protein extract. Both Otphot and phot2 undergo light dependent autophosphorylation in insect cells. There is slightly higher background in the dark sample in phot2 but this may be caused by overexposure as there is still an increase of signal in the light-treated sample compared to the dark sample. These findings show that phototropins are able to undergo light-dependent autophosphorylation in insect cells and that Otphot retains light-induced activity.

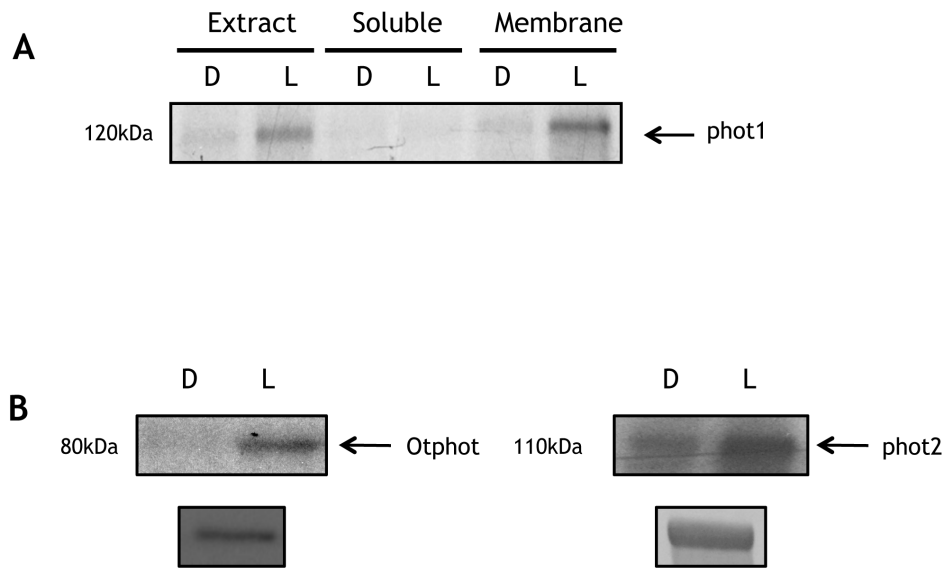


Figure 3.3 Autoradiograph of phot1, phot2 and Otphot autophosphorylation expressed in insect cells

A) Soluble and membrane fractions were separated by ultra-centrifugation. Radiolabelled ATP was added to the protein samples before they were given a mock irradiation (D) or illuminated with white light for 10 secs (L) with a total fluence of 30,000 µmol m^{-2}. Samples were then subjected to SDS-PAGE and autoradiography.

B) Autoradiograph showing light-induced autophosphorylation of Otphot and phot2 from insect cell extract. Radiolabelled ATP was added to the protein samples before they were given a mock irradiation (D) or illuminated with white light for 10 secs (L) with a total fluence of 30,000 µmol m^{-2}. Protein levels are shown in the panel below detected with anti-His antibody.

3.2.3 Solubilisation of phot1 from the membrane

Despite being tightly associated with the membrane, phot1 has no transmembrane spanning domains and is overall a hydrophilic protein (Sakamoto and Briggs, 2002). The detergent Triton X-100 has been used previously to solubilise phot1 from plant membranes (Short, Reymond and Briggs, 1993). It has been suggested that solubilisation from plant membrane extracts with Triton X-100 results in rapid degradation of the phototropin, possibly by activation of proteases (Knieb, Salomon and Rudiger, 2004). To gain a greater understanding of the membrane association properties of phot1 a range of different detergents were examined for their ability to solubilize phot1 from both insect cell membranes and *Arabidopsis* microsomal membranes without impairing

autophosphorylation activity. The impact upon autophosphorylation was assessed first to see if phot1 could retain any activity in the presence of detergents.

Firstly, membranes were isolated from insect cells expressing phot1 and from wild-type *Arabidopsis* seedlings, then incubated in the presence of a range of detergents for 30 minutes before the addition of radiolabelled ATP and subsequent irradiation of the sample. Autophosphorylation reactions were allowed to proceed for 2 minutes before the addition of SDS loading buffer to terminate the assay. As shown in Figure 3.4A insect cell expressed phot1 retains activity equal to an untreated sample in the presence of the non-ionic detergents Triton X-100, n-dodecyl-ß-D-Maltoside (DDM) and n-decyl-ß-D-Maltopyranoside (DM). The other non-ionic detergent n-octyl-ß-D-glucoside (BOG) caused a reduction in phot1 activity compared to the untreated sample. The anionic detergent Na Cholate as well as the zwitterionic detergents Fos choline 9 (Fos-9) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) also resulted in decreased autophosphorylation activity of phot1 compared to the control. As shown in Figure 3.4B the impact of the above detergents on phot1 activity in *Arabidopsis* microsomal membranes is slightly different to that observed for their effect on phot1 expressed in insect cell membranes. Treatment with all of the detergents had little impact on phot1 autophosphorylation activity compared to the untreated sample, suggesting phot1 activity in plant membranes is more resistant to detergent treatment.

The ability of detergents to solubilize phot1 from membranes was then assessed alongside increasing salt concentrations, which can be used to solubilise peripheral membrane proteins without the use of detergents (Lodish et al., 2000). Triton X-100 and DDM were chosen as they retained similar levels of activity to the untreated sample (Fig. 3.4A and 3.4B); a zwitterionic detergent (CHAPS) and an anionic detergent (Na Cholate) were also tested to see if these could help in the understanding of the mechanism of attachment even though there is a loss of phot1 activity in the presence of these detergents, at least in phot1 expressed in insect cells. Triton X-100 is a non-ionic detergent commonly used for the solubilisation of membrane proteins since it has little effect on protein:protein interactions, instead interrupting lipid:lipid and lipid:protein interactions (Seddon, Curnow and Booth, 2004). This allows proteins to be

solubilised without affecting protein structure. The alkylglucoside n-dodecyl-ß-D-Maltoside (DDM) is a milder non-ionic detergent, which has the propensity to solubilise membrane associated proteins such that they retain their functional properties (Seddon, Curnow and Booth, 2004). Membranes isolated from insect cells expressing phot1 or *Arabidopsis* microsomal membranes were incubated, on ice, in the presence of 1% detergent or various concentrations of NaCl for 30 minutes before being subjected to a further round of ultra-centrifugation. Phot1 expressed in insect cells is solubilised using Triton X-100 (Fig. 3.4C), as is phot1 from *Arabidopsis* microsomal membranes (Fig. 3.4D). These common properties provide initial evidence to suggest that the mechanism of membrane attachment may be conserved between plant cells and insect cells and involve non-ionic interactions.

As shown in Figure 3.4C, the detergent DDM also solubilises phot1 from insect cell membranes, with the protein being detected in the soluble fraction following a second round of ultra-centrifugation, as well as *Arabidopsis* microsomal membranes treated with the same detergent (Figure 3.4D). Anionic detergents have a net negative charge and contain a hydrophobic hydrocarbon chain or a steroidal backbone (Seddon, Curnow and Booth, 2004). Sodium cholate is an anionic detergent that has a net negative charge and like other ionic detergents is very good at solubilising membrane proteins but they are also harsh and often result in denaturation of the protein. This generally makes them unsuitable for downstream applications such as purifying proteins in the active form. Some phot1 was detected in the soluble fraction after treatment with sodium cholate from both insect cells and *Arabidopsis* (Figure 3.4C and D), however most protein is retained on the membrane unlike treatment with Triton X-100 and DDM. A third class of detergent is zwitterionic detergents, which have the properties of being both ionic and non-ionic detergents (Seddon, Curnow and Booth, 2004). They are more denaturing than non-ionic detergents but they can be used for structural studies since they keep protein:protein interactions stable. CHAPS is a zwitterionic detergent that does not result in solubilisation of phot1 from insect cell membranes (Fig. 3.4C) nor from *Arabidopsis* microsomal membranes (Figure 3.4D). The use of increasing concentrations of sodium chloride also does not result in detectable release of phot1 from either insect cell membranes (Figure 3.4C) nor *Arabidopsis* microsomal membranes (Figure

3.4D). These findings indicate that phot1 is probably associated with the membrane by non-ionic interactions as the effect of the salt treatment indicates there are no ionic interactions involved.

The results from phot1 are also confirmed with phot2 solubilisation from *Arabidopsis* microsomal membranes given the same detergent and salt treatments. As shown in Fig. 3.5A, phot2 is solubilised by both Triton X-100 and DDM. Again there is also some phot2 detected in the soluble fraction after sodium cholate treatment similar to phot1 but most appears to be localised to the microsomal membranes. Treatment with various concentrations of salt also did not solubilise phot2 from the membrane. This suggests that the mechanism of attachment of phot2 to the membrane is similar to that of phot1 and is also unlikely to be a peripheral membrane protein or an ionic interaction with the membrane. The signal for phot2 protein is reduced compared to the signal for phot1 protein, which is most likely due to etiolated *Arabidopsis* seedlings being used. Transcript levels of *PHOT1* are reduced upon seedling exposure to blue light whereas transcript levels of *PHOT2* is increased (Jarillo et al., 2001, Kagawa et al., 2001). Therefore etiolated seedlings contain lower levels of phot2 protein (Christie and Murphy, 2013).

The ability of the detergents to solubilise Otphot expressed in insect cells was then evaluated to compare the membrane attachment of lower plant phototropins to the *Arabidopsis* phot1 and phot2. Membrane samples were extracted from Otphot by ultra-centrifugation and incubated on ice for 30 mins in the presence of 2% detergent before a further round of ultra-centrifugation. Figure 3.5A shows that most of the detergents have the ability to solubilise Otphot from the membrane, with the non-ionic detergents, DM, DDM and Triton X-100 showing protein being detected in these samples. The other non-ionic detergent BOG did not appear to solubilise Otphot from the membrane consistent with a reduced autophosphorylation signal seen for phot1 expressed in insect cells (Fig 3.4A). Sodium cholate appears to solubilise Otphot, which is unlike the result from both phot1 and phot2, where sodium cholate did not solubilise these proteins. There is however consistency between Otphot and *Arabidopsis* phototropins since non-ionic detergents are able to solubilise all three phototropins from the membrane (Figs 3.4A, 3.5A, 3.5B). This suggests

that the mechanism of attachment of phototropins to the membrane is via non-ionic interactions and is conserved between insect cells and *Arabidopsis*, as well as between lower and higher plants.

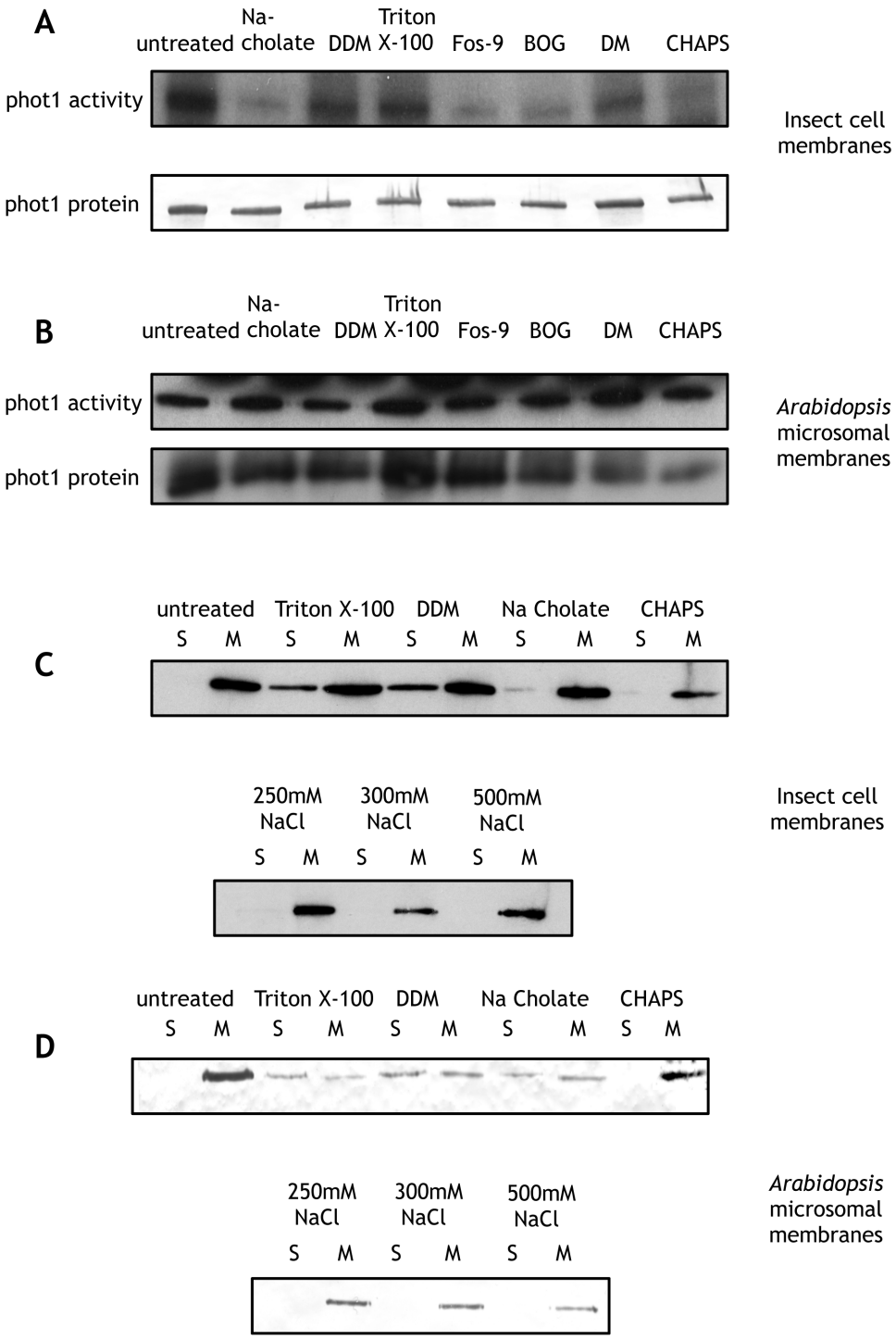


Figure 3.4 Analysis of phot1 solubilisation from insect cell membranes and *Arabidopsis* microsomal membranes

A) Autoradiograph showing the impact of detergents on phot1 autophosphorylation. Membranes isolated from insect cells expressing phot1 were incubated in the presence of detergent before addition of radiolabelled ATP. Western blot analysis is shown below,

using C-terminal phot1 antibody is shown to demonstrate that a loss of activity is not due adverse effects on protein stability.

B) Autoradiograph showing autophosphorylation of phot1 from *Arabidopsis* microsomal membranes in the presence of detergent. Western blot analysis, shown below, of protein levels in each sample using anti-C-terminal phot1 antibody as a loading control.

C) Western blot analysis using anti-C-terminal phot1 antibody to examine detergent and salt solubilisation of phot1 from insect cell membranes. After detergent or salt treatment, soluble (S) and membrane (M) fractions were separated by ultra-centrifugation (DDM n-dodecyl-β-D-maltopyranoside, CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate).

D) Western blot analysis using anti-C-terminal phot1 antibody showing detergent and salt solubilisation of phot1 from *Arabidopsis* microsomal membranes. After detergent or salt treatment, soluble (S) and membrane (M) fractions were separated ultra-centrifugation as in C.

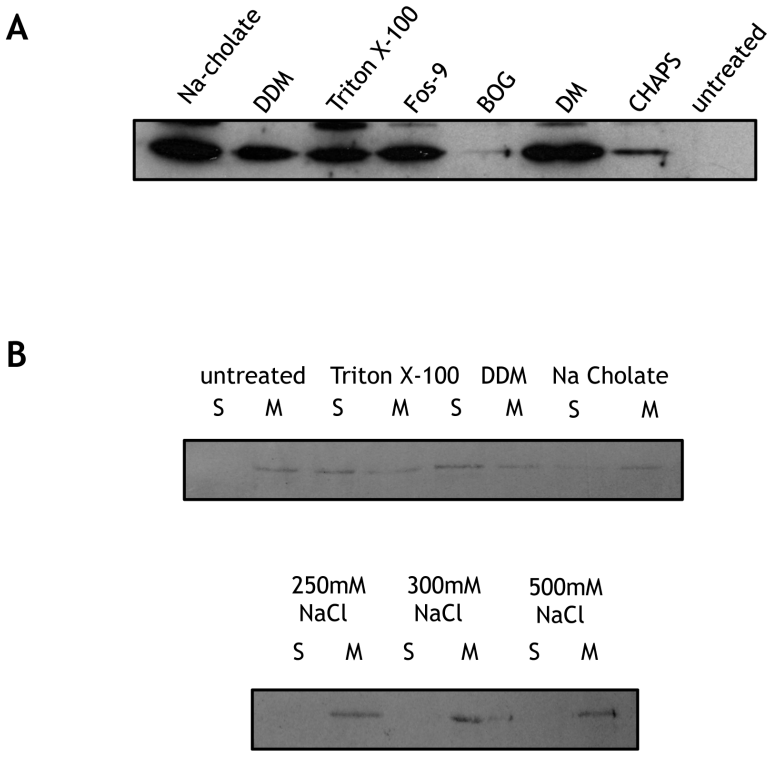


Figure 3.5: Analysis of phot2 solubilisation from *Arabidopsis* microsomal membranes and Otphot from insect cell membranes

A) Western blot analysis using His antibody to examine detergent solubilisation of Otphot from insect cell membranes. After detergent or salt treatment, soluble (S) and membrane (M) fractions were separated by ultra-centrifugation. Only the soluble fraction is shown. (DDM n-dodecyl-β-D-maltopyranoside, CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate).

B) Western blot analysis using anti-phot2 antibody to examine detergent and salt solubilisation of phot2 from *Arabidopsis* microsomal membranes. After detergent or salt treatment, soluble (S) and membrane (M) fractions were separated by ultra-centrifugation (DDM n-dodecyl-β-D-maltopyranoside.

3.2.4 A conserved lysine rich motif is present in phototropin kinases

The analysis in section 3.2.3 suggests that the mechanism of phot1 attachment to the membrane in insect cells and *Arabidopsis* could possibly be via interaction with a lipid via non-ionic interactions. Since the identification of *Arabidopsis* phot1 and phot2 there have been a number of phototropins identified in monocotyledonous and dicotyledonous higher plants such as oat, rice, pea in addition to ferns (Briggs et al. 2001; Christie & Murphy 2013). There has also been a phototropin identified and studied from the lower plant *Chlamydomonas reinhardtii* (Huang, Merkle and Beck, 2002) where it controls the sexual life cycle of the algae (Huang and Beck, 2003). Despite having a different function to that of the higher plant phototropins, *Chlamydomonas reinhardtii* phototropin (Crphot) complements some higher plant functions when it is expressed in the *phot1-5 phot2-1* double mutant, such as petiole positioning, phototropism and chloroplast positioning (Onodera et al., 2005). Crphot was also found to localise to the plasma membrane in the algae suggesting the mechanism of attachment is conserved from lower to higher plant species.

Studies on phot2 indicate that the C-terminal kinase domain is necessary for localising to the plasma membrane in *Arabidopsis* (Kong et al. 2006; Kong et al. 2007; Aggarwal et al. 2014). To determine if any regions within the C-terminus of phot1 were conserved from lower to higher plants, amino acid sequences from both higher and lower plant phototropins were aligned using the Clustal Omega program (Sievers et al., 2011) and visualised using Jalview (Waterhouse et al., 2009). Within the kinase domain there is a large insertion between the magnesium-binding loop and the activation loop which is only observed in plant AGC kinases (Rademacher and Offringa, 2012). This insertion domain in the AGC kinase PINOID (PID) from *Arabidopsis* is sufficient to drive PID expression to the peripheral membrane in yeast cells and BY-2 tobacco cells (Zegzouti et al., 2006). Interestingly the amino acid sequence alignments of the C-terminal region of phototropins from lower to higher plants shows a highly conserved stretch of lysine residues called a lysine rich motif (LRM) (Figure 3.6). The number of lysine residues varies within this region from one to nine in the minimal alignment shown. Another protein that contains a LRM, termed the K segment, is the dehydrin proteins that are involved in plant responses to abiotic stress. The

protein is highly hydrophilic and most likely binds the membrane by a peripheral membrane protein (Graether and Boddington, 2014). The K-segment of the maize dehydrin 1 (DHN1) is suggested to be involved in the binding of the protein to lipid vesicles and subsequently the membrane (Koag et al., 2003). The presence of the LRM in both lower plant phototropins and the involvement of the insertion loop in directing other proteins to their respective subcellular locations suggests that this region may be involved in phot1 membrane localisation.

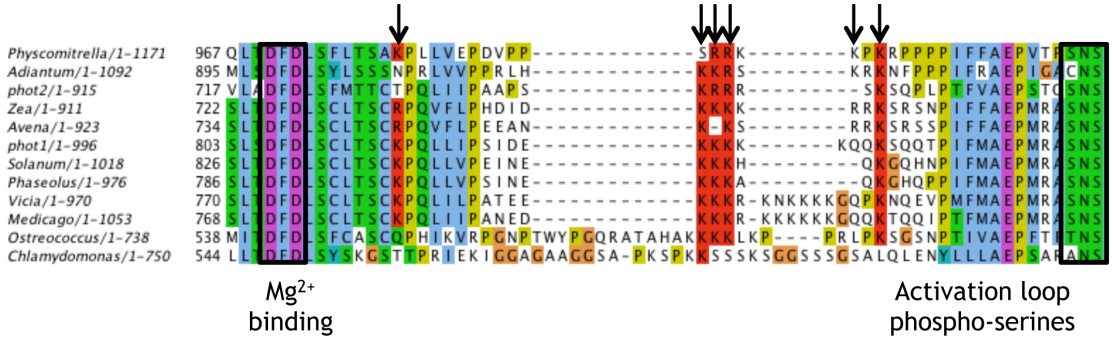


Figure 3.6 Amino acid sequence alignment of phototropins from lower and higher plants indicates the presence of a conserved lysine rich motif (LRM)

Section of an alignment of phototropin kinases taken from the NCBI database of lower to higher plants highlighting the LRM within the kinase domain. The insertion domain between the magnesium binding loop and conserved phospho-serines is unique in plant AGC kinases. Arrows indicate the six lysine residues present in *Arabidopsis* phot1. Sequences are derived from *Physcomitrella patens* photB2, *Adiantum capillus-veneris* phot1, *Arabidopsis* phot2, *Zea mays* phot1, *Avena sativa* phot1, *Arabidopsis* phot1, *Solanum lycopersicum* phot1, *Phaseolus vulgaris* phot1a, *Vicia faba* phot1, *Medicago truncatula* phot2, *Ostreococcus tauri* phot, *Chlamydomonas reinhardtii* phot.

3.2.5 Mutation of the LRM does not affect phot1 autophosphorylation or membrane association in insect cells

Identification of a LRM within phototropins illustrates a highly polar region which may interact with the negatively charged regions of the membrane. The cytoskeleton-membrane linker protein ezrin belongs to a family of proteins, which bind the plasma membrane. Ezrin specifically binds to phosphatidyl-inositol 4,5-bisphosphate (PIP_2) containing liposomes (Barret et al., 2000). Ezrin contains a LRM that directs interaction with PIP_2 liposomes. It has been shown that mutation of the LRM to asparagine in the protein results in a change to its

interaction with PIP_2. The mutation of LRM motifs to asparagine has also been shown previously to have an effect on the interaction of the stress protein CDeT11-24 with phosphatidic acid (Petersen et al., 2012). Both proteins interact with phospholipids and this interaction is lost by either mutation to asparagine or truncation of the entire region containing the LRM. We therefore rationalised that mutation of the LRM could also change the membrane localisation of phot1 if it displays a similar interaction with phospholipids such as PIP_2. Therefore it was of interest to examine whether the equivalent mutation in phot1 could change its ability to attach to the membrane in insect cells.

The only other change in localisation of phot1 observed previously has been due to a change in the autophosphorylation status. The phosphomimic mutation S851D potentiates re-localisation from the membrane in the absence of light, whereas the kinase inactive D806N mutation prevents blue light-induced internalisation (Kaiserli et al., 2009). The LRM of *Arabidopsis* phot1 contains six lysine residues that were mutated simultaneously to asparagine resulting in the mutation phot1 6xK-N. This mutated sequence was used to transfect *Sf9* insect cells as described in section 3.2.1. Protein extracts obtained from insect cells expressing phot1 6xK-N were subjected to ultra-centrifugation to separate the soluble and membrane fractions. Figure 3.7A shows that phot1 6xK-N is still detected in the soluble fraction and membrane fraction of insect cells similar to wild-type phot1 (Figure 3.1B), however expression appears to be reduced at the membrane in the phot1 6xK-N. Fraction purity was confirmed using antibodies raised against the viral membrane marker GP-64 and the soluble marker α-tubulin. Thus, these results suggests that the mutation of the LRM to asparagine does not affect the ability of phot1 to bind to the plasma membrane and that this is not the region of interaction, at least in insect cells.

We therefore decided to test another mutation to assess if this had any effect on the ability of phot1 to associate with the membrane. The six lysine residues were again simultaneously mutated this time to alanine and used to transfect *Sf9* cells. Figure 3.7B shows that mutation of the LRM to alanine (phot1 6XK-A) does not alter the ability of phot1 to localise to the membrane in insect cells similar to what was observed for phot1 6XK-N (Fig. 3.7A). Mutation of the LRM within the kinase domain may have an impact upon the autophosphorylation of

phot1 since it is positioned close to the magnesium-binding loop and the activation loop containing the conserved phosphoserines, S849 and S851 (Inoue et al. 2008). Within AGC kinases a conserved serine needs to be phosphorylated for activation of the kinase (Rademacher and Offringa, 2012) and this corresponds to S851 in phot1. S851 is phosphorylated in response to light and does so in a fluence dependent manner (Inoue et al. 2008). The LRM mutated to asparagine was chosen to assess the impact of this mutation on phot1 autophosphorylation activity. Crude protein extract containing either wild-type phot1 or phot1 6xK-N was extracted under a dim red safe light and incubated with radiolabelled ATP and given a light treatment or kept in the dark. Figure 3.7C shows that the phot1 6xK-N exhibits light-induced autophosphorylation comparable to that of wild-type phot1. Western analysis of phot1 protein levels also showed that these were comparable between the two extracts. Incorporation of the 6xK-N mutation into phot1 therefore does not appear to impact its autophosphorylation activity or its ability to attach to the membrane, at least when expressed in insect cells.

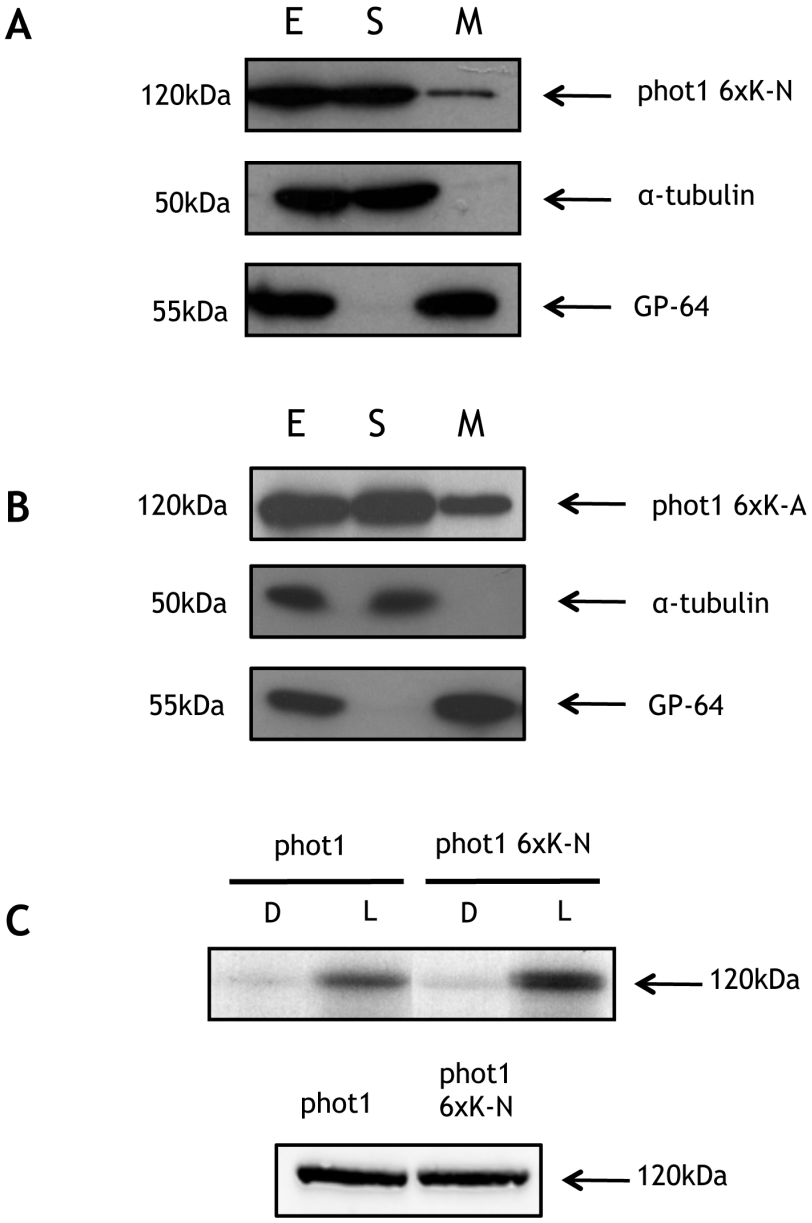


Figure 3.7 Mutation of the LRM does not alter membrane attachment or affect light-dependent phot1 autophosphorylation when expressed in insect cells

A) Western blot analysis on protein fractions isolated from virally infected insect cells expressing phot1 6xK-N. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. C-terminal phot1 antibody was used to detect phot1. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64.

B) Western blot analysis on protein fractions isolated from virally infected insect cells expressing phot1 6xK-A. Protein extracts were fractionated and subjected to analysis as in A.

C) Autoradiograph showing the autophosphorylation activity of wild-type phot1 and phot1 6xK-N expressed in the crude protein extracts from insect cells. Radiolabelled ATP was added to the protein samples before they were given a mock irradiation (D) or illuminated for 10 secs (L) with a total fluence of 30,000 μmol m^{-2}. Western blot analysis of phot1 levels using C-terminal phot1 antibody is shown below.

3.2.6 Transient expression of phot1 6xK-N-GFP in Nicotiana benthamiana cells

The above findings showed that the LRM was not required for phot1 membrane attachment in insect cells. Despite this, it was important to establish whether this was also the case for plant cells. The localisation of *Arabidopsis* phot1 has been studied in depth by the transformation of a functional *PHOT1-GFP* construct into the *phot1-5* null mutant under the control of the native *PHOT1* promoter (Sakamoto and Briggs, 2002). Phot1-GFP localises exclusively to the plasma membrane in darkness and partially internalises in response to blue light illumination (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008). The native *PHOT1* promoter was used in this study to ensure that the localisation pattern observed was similar to endogenous phot1 with the authors using a genomic *PHOT1* clone. Since the use of the native promoter gave the expected localisation pattern of wild-type phot1, this promoter was also chosen to drive the expression of the phot1 6xK-N construct to establish if this mutated form of phot1 still localises to the membrane in plant cells as in insect cells. A C-terminal GFP tag was incorporated into our constructs to allow the use of confocal microscopy to visualise localisation as shown schematically in Figure 3.8A. Another method, in addition to generating transgenic *Arabidopsis* that has proved to be a reliable and rapid means of examining phot1 localisation is transient expression in *Nicotiana benthamiana* (Kaiserli et al. 2009; Aggarwal et al. 2014). Phot1-GFP expressed in this system localises to the membrane in darkness and internalises in response to light (Kaiserli et al., 2009). Transient expression of phot1 6xK-N-GFP in this system resulted in localisation of the GFP fusion protein to the membrane in darkness similar to the pattern seen for wild-type phot1 (Fig. 3.8B). This suggests that the LRM is not involved in the membrane attachment of phot1 in plant cells similar to what was observed in insect cells.

Since Otphot also contains conserved lysine residues (Fig 3.6), the five residues present in Otphot were also mutated simultaneously to asparagine. The resulting Otphot 5xK-N was fused to a C-terminal GFP tag for localisation in plant cells. Since it is unknown if the native *PHOT1* promoter would drive expression of the Otphot protein correctly, the Cauliflower mosaic virus 35S (*CaMV 35S*) promoter

was used instead. Transient expression of Otphot-GFP in the *N. benthamiana* system results in localisation to the plasma membrane in darkness (Fig 3.8B) similar to phot1-GFP. Otphot 5xK-N-GFP also localises to the plasma membrane in darkness. There is perhaps localisation in the nucleus, which may have arisen from overexpression of the protein in *N. benthamiana*. These findings show that Otphot wild type also localises to the plasma membrane *in planta* in darkness like *Arabidopsis* phot1 and phot2 and that mutation of the LRM has no effect on membrane localisation in the darkness.

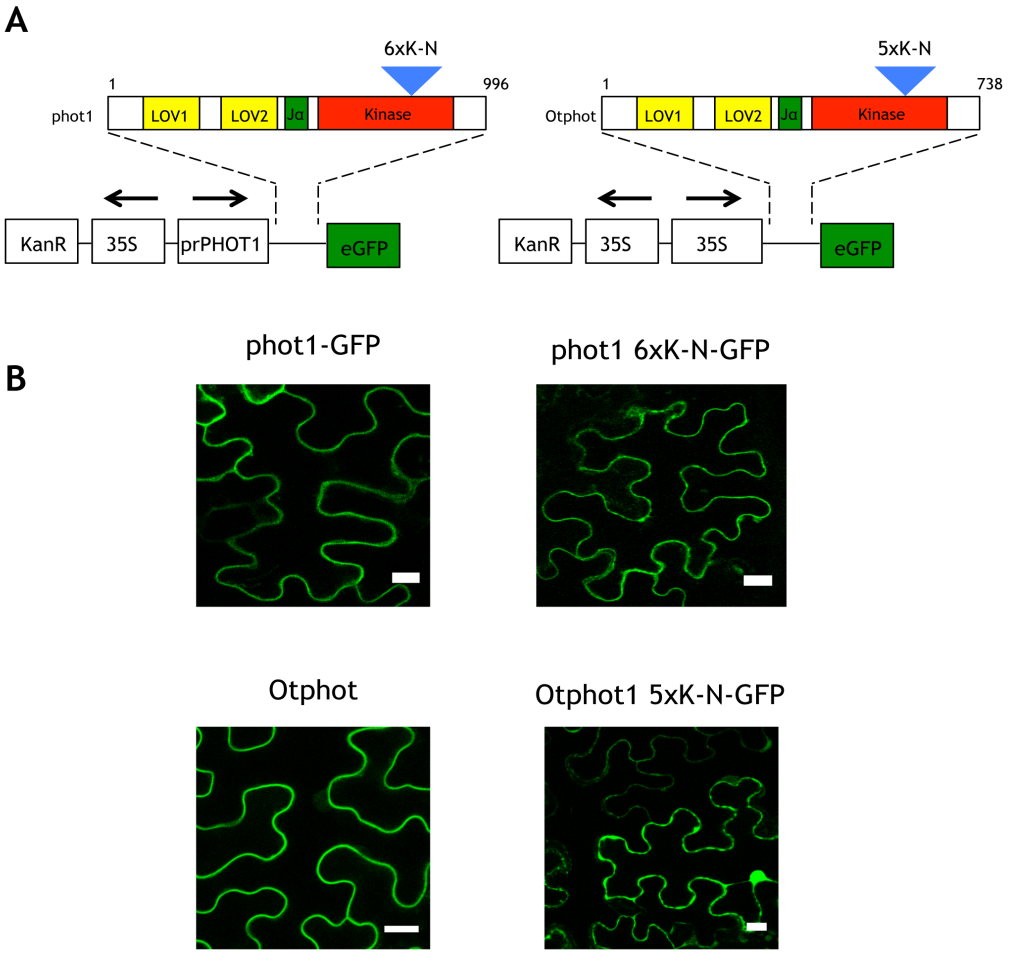


Figure 3.8 phot1 6x K-N-GFP and Otphot 5x K-N-GFP localise to the membrane in *Nicotiana benthamiana* in darkness

A) Diagram illustrating creation of the vector containing *PHOT1* cDNA or *OTPHOT* cDNA. Vectors were used to transform *Argrobacterium tumefaciens* for transient expression in *N. benthamiana* . The *CaMV 35S* promoter was used to drive kanamycin resistance for selection of stable transgenics.

B) Transient expression of phot1 6xK-N-GFP in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Mutation of the LRM does not change the membrane localisation of phot1 or Otphot in the darkness. Scale bar represents 20 µm.

3.2.7 Expression of phot1 6xK-N-GFP in the Arabidopsis phot1-5 phot2-1 double mutant

The above results indicate that the LRM does not appear to be involved in attaching phot1 to the plasma membrane both in insect cells (Fig 3.7A) and tobacco epidermal cells (Fig. 3.8B). Further, mutation of the LRM did not appear to affect phot1 activity in insect cells (Fig 3.7C). However, it was important to assess if the LRM could be important for phot1 signalling. There are a variety of physiological responses that are controlled by phot1, which can only be investigated using stable transgenic lines. Therefore stable transgenic lines were generated for this purpose and wild-type phot1-GFP expressing lines were used as a control. Firstly the subcellular localisation pattern of phot1 6xK-N-GFP in transgenic *Arabidopsis* was examined when transformed into the double mutant *phot1-5 phot2-1* (Kagawa and Wada, 2000). The double mutant was chosen to ensure that there was no influence from the presence of phot2 on any responses tested since phot2 is still present in the *phot1-5* null mutant and the two proteins function redundantly (Christie, 2007).

Transformants were selected by kanamycin resistance and resulted in the generation of three independent homozygous T3 lines expressing the fusion protein phot1 6xK-N-GFP designated K-N 18-7, K-N 20-2 and K-N 23-7. Protein levels in the three lines were first examined by western blot analysis. As shown in Figure 3.9A there are comparable protein levels between native phot1, phot1-GFP and the K-N 18-7 line. The other two lines, phot1 K-N 20-2 and phot1 K-N 23-7 have lower protein expression levels but it was decided that the levels are detectable and these were analysed in further detail alongside the line 18-7. The localisation pattern of phot1 in three-day-old etiolated seedlings was then examined by confocal microscopy in the dark state. The phot1-GFP line (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008) previously described was used as a control. As shown in Figure 3.9B, phot1 6xK-N-GFP in the seedlings examined from all three lines showed GFP localisation to the plasma membrane in darkness consistent with the *N. benthamiana* experiments above. These findings confirm that *in planta* there is no effect on phot1 attachment to the plasma membrane when the LRM is mutated similar to what was observed for the insect cell expressed protein (Fig. 3.7A).

Internalisation of phot1-GFP from the membrane can be visualised as the blue light from the laser used to excite GFP also excites phot1. Seedlings are scanned for 30 secs and imaged in the dark then rescanned 3 minutes later to observe partial internalisation of phot1. As shown in Figure 3.9B, phot1-GFP partially internalises to cytosolic strands as described previously (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008). The three lines expressing phot1 6xK-N-GFP also show internalisation in response to the blue light illumination. These results show that the LRM within the phot1 kinase domain is not responsible for the internalisation processes of phot1 from the membrane.

Another response that can be investigated in the transgenic lines generated is the autophosphorylation status of phot1 6xK-N. This can be measured indirectly by examining the decrease in electrophoretic mobility of phot1 in protein extracts by SDS-PAGE and western blotting following its illumination *in vivo*. Figure 3.10 shows that, like wild-type phot1, the three phot1 6xK-N-GFP lines exhibit a decrease in electrophoretic mobility in light treated samples. Shown below is western blot analysis of protein levels using the soluble marker, UDP-glucose pyrophosphorylase (UGPase) (Komatsu et al., 2014). Fig. 3.10 indicates that there is more protein in the K-N 20-2 and K-N 23-7 lines than K-N 18-7, contrary to the protein levels detected by the phot1 antibody (Fig. 3.9). However there are also reduced phot1-GFP protein levels in Fig. 3.10 compared to the K-N 20-2 and K-N 23-7. This would seem to suggest that the GFP antibody used to detect the electrophoretic mobility shifts (Fig. 3.10) is more suited to accurate recognition of the protein levels in the transgenic lines than the phot1 antibody since the GFP fluorescence visualised in the K-N 23-7 line is similar to that for phot1-GFP. The reason for this is unknown and would require further investigation. Nonetheless the data clearly demonstrates that phot1 6xK-N protein becomes phosphorylated in response to irradiation in all lines tested showing that mutation of the LRM does not perturb phot1 autophosphorylation activity in *Arabidopsis*.

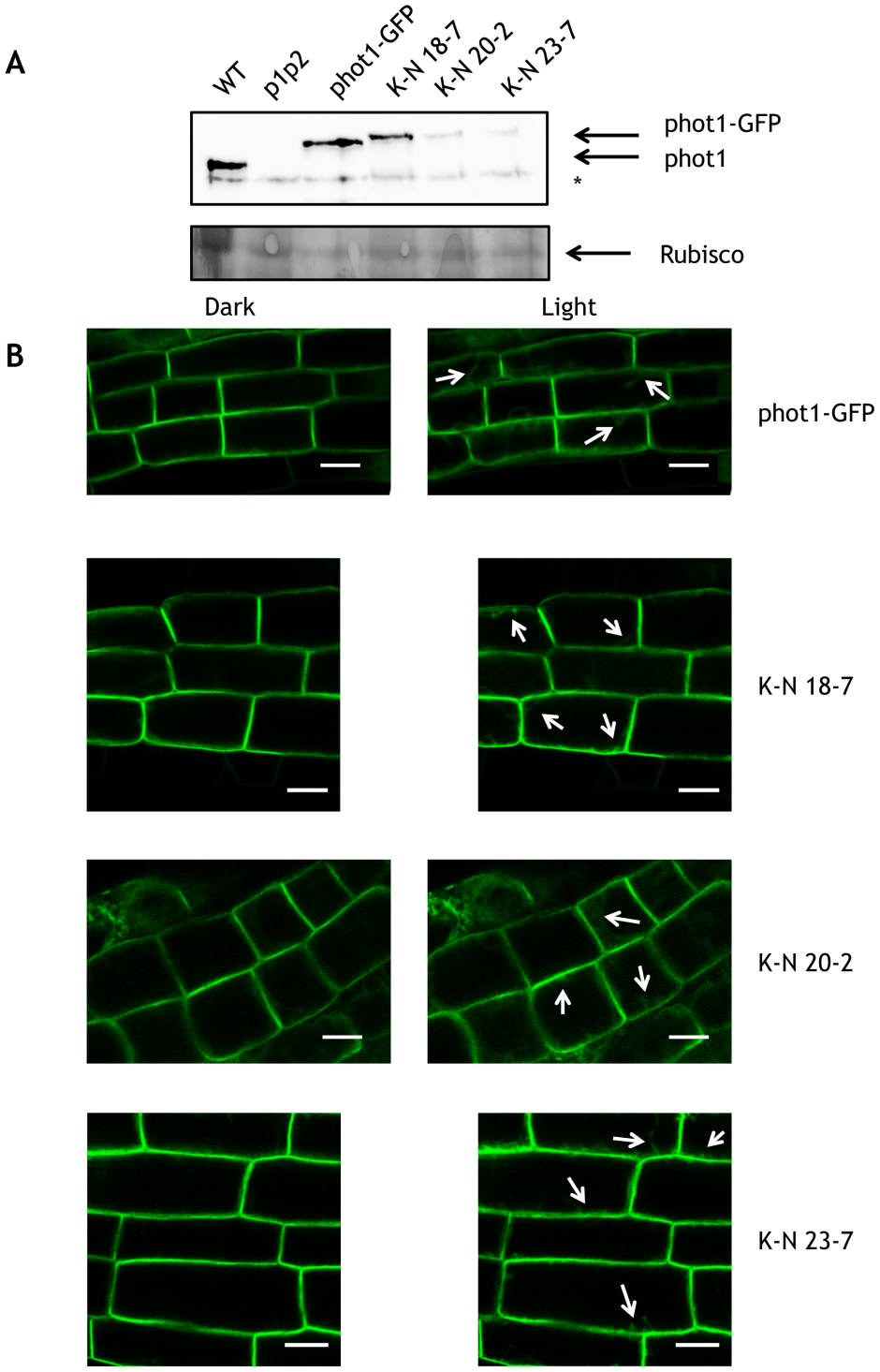


Figure 3.9 phot1 6xK-N-GFP localisation and activity in *Arabidopsis*

A) Western blot analysis of protein levels in phot1-GFP and each of the phot1 6xK-N lines wild-type (WT), *phot1-5 phot2-1* (p1p2), phot1-GFP, K-N 18-7, K-N 20-2, K-N 23-7) probed with C-terminal phot1 antibody. *denotes an non-specific reactivity. Ponceau S staining of the Rubisco large subunit is shown below as a loading control.

B) Analysis of phot1-GFP and phot1 6xK-N-GFP localisation in homozygous T3 seedlings. Confocal images of hypocotyl cells from three-day-old etiolated *Arabidopsis* seedlings. Scale bar represents 20 µm.

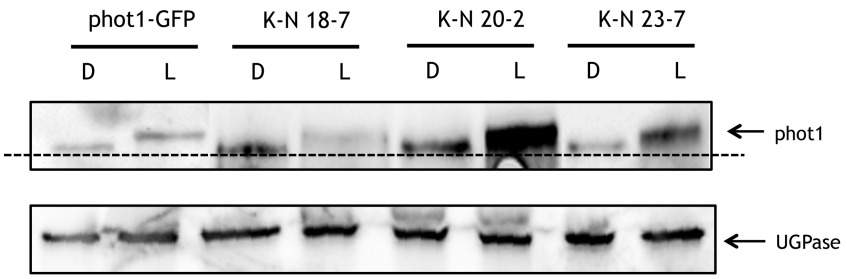


Figure 3.10 Light-induced shift in electrophoretic mobility of phot1 in phot1-GFP and phot1 6xK-N lines from *Arabidopsis*

Three-day-old etiolated *Arabidopsis* seedlings expressing phot1-GFP and phot1 6xK-N lines were kept in darkness (D) or given a light treatment of 10 µmol m^{-2} s^{-1} blue light for 10 minutes (L). Total protein was extracted and analysed by western blotting with anti-GFP antibody. Blots were then probed with anti-UDP-glucose pyrophosphorylase (UGPase) antibody as a loading control (lower panel).

3.2.8 Kinetics of hypocotyl curvature is restored in phot1-6xK-N-GFP expressing lines

One of the main responses controlled by the phototropins is phototropism, which is the directional movement of plants toward light, a response that serves to optimise photosynthetic efficiency by increasing the amount of light available for capture (Liscum et al., 2014). To investigate the phototropic response over time in the phot1 6xK-N-GFP transgenic lines, etiolated seedlings were exposed to 1 µmol m^{-2} s^{-1} unilateral blue light for between one and eight hours. The *phot1-5 phot2-1* double mutant does not bend in response to the unilateral stimulus whilst wild-type seedlings begin to respond after 4 hours (Figure 3.11A). The three independent phot1 6xK-N lines also start to bend after 4 hours and the degree of curvature increases to reach a maximum of around 80 degrees at 6 hours. Bending does not begin until 2 hours, which is likely a result of the complexities of the signalling required for phototropism. The similarities in the curves for phototropism response kinetics in the wild type and lines expressing phot1 6xK-N-GFP show that neither the degree of phototropic curvature nor the kinetics of the response is affected by the LRM mutation in phot1. These finding show that mutation of the phot1 LRM does not affect phototropic responsiveness. It has been shown previously that undetectable levels of phot1

protein in transgenic lines created in the *phot1-5 phot2-1* double mutant are still able to complement phot1 function to a level comparable to wild type (Doi et al., 2004) and are consistent with the results observed here for lines expressing different levels of phot1 6XK-N.

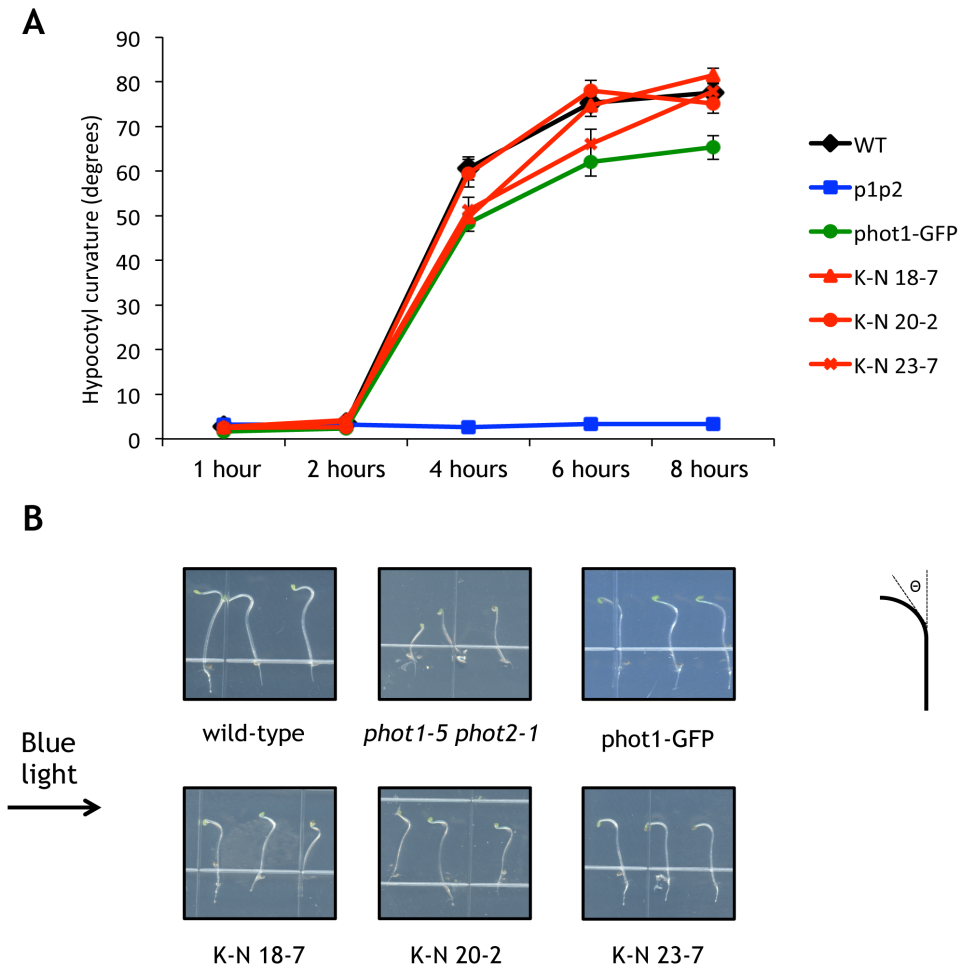


Figure 3.11 Kinetics of hypocotyl phototropism in *Arabidopsis* seedlings expressing phot1 6xK-N-GFP.

A) Phototropism fluence response curves for wild type (WT), *phot1-5 phot2-1* (p1p2), phot1-GFP and phot1 6xK-N-GFP (K-N) lines in three-day-old etiolated seedlings. Seedlings were exposed to 1 µmol m^{-2} s^{-1} unilateral blue light for the times indicated before curvature was measured. Error bars indicate standard error (n>20).

B) Images of representative seedlings of wild type, *phot1-5 phot2-1,* phot1-GFP and phot1 6xK-N-GFP lines 8 hours after exposure to 1 µmol m^{-2} s^{-1} unilateral blue light. Θ indicates the angle that was measured.

3.2.9 Fluence-rate response curves of hypocotyl phototropism are restored in the phot1 6xK-N-GFP expressing lines

Although phot1 K-N expressing lines showed no difference in kinetics of curvature to low intensities of blue light when compared to wild type it was important to establish whether the fluence-rate responsiveness was altered in these seedlings. The benefit of using the *phot1-5 phot2-1* double mutant is that there are no phot2 present in these seedlings so that the effect of mutations on curvature at higher fluence rates can be measured (Kagawa et al., 2001). Etiolated seedlings were exposed to an increasing fluence rate of blue light from 0.1 µmol m^{-2} s^{-1} to 10 µmol m^{-2} s^{-1} for eight hours. At a fluence rate of 0.1 µmol m^{-2} s^{-1} both wild type and the phot1 6x K-N-GFP expressing lines responded to unilateral blue light as shown in Figure 3.12A. However, the magnitude of the response is reduced compared to that seen for 1 µmol m^{-2} s^{-1}. As the fluence rate increases to 0.5 µmol m^{-2} s^{-1}, the magnitude of curvature is similar to that of 1 µmol m^{-2} s^{-1} at around 80 degrees. Under the conditions examined, the fluence rate response is similar between both the wild type and the phot1 6xK-N-GFP expressing lines indicating that the mutation does not have an affect on phototropism at higher fluence of blue light. These findings indicate that the LRM mutation does affect phot1 photosensitivity.

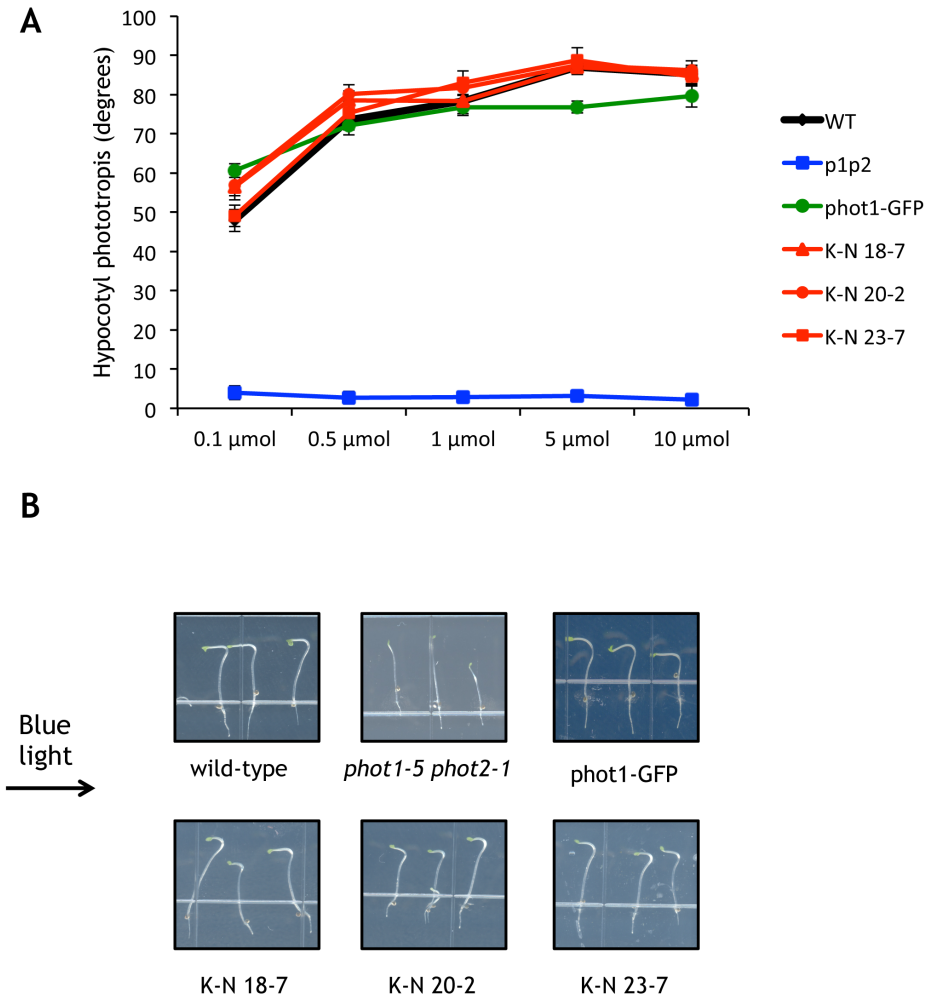


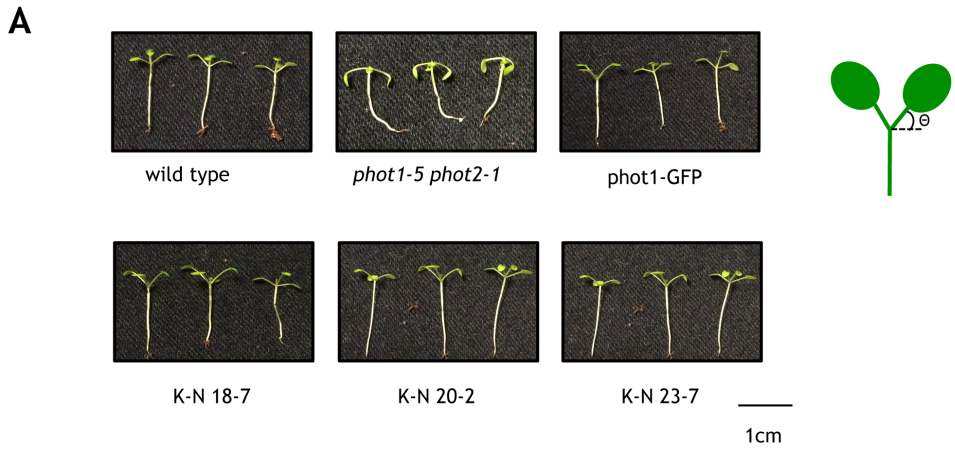
Figure 3.12 Hypocotyl phototropism fluence-rate response curves in *Arabidopsis* seedlings expressing phot1 6xK-N-GFP.

A) Phototropism fluence-rate response curves for wild type (WT), *phot1-5 phot2-1* (p1p2), phot1-GFP and phot1 6xK-N-GFP (K-N) in three-day-old etiolated *Arabidopsis* seedlings. Seedlings were exposed to unilateral blue light for 8 hours at the indicated fluence rates before curvature was measured. Error bars indicate standard error (n>20).

B) Images of representative seedlings from A at 10 µmol m^{-2} s^{-1} unilateral blue light.

3.2.10 Petiole positioning is restored in the phot1-6xK-N-GFP lines

The LRM does not appear to have an impact upon the fluence-rate response or the kinetics of phototropism over a long time scale. It is important, however, to examine other responses that phot1 controls in addition to phototropism that are also involved in maximising photosynthetic efficiency. The petioles of wild-type *Arabidopsis* position themselves to maximise light capture for photosynthesis, a response that is controlled by the phototropins and NPH3 in response to blue light (Inoue et al. 2008). Wild-type seedlings grown under 70 µmol m^{-2} s^{-1} for one week before being transferred to 10 µmol m^{-2} s^{-1} for a further week position their petioles upwards to allow a large surface area to capture light and the leaves are perpendicular to the incident light. However, the *phot1-5 phot2-1* double mutant has petioles that are flat and the leaves point downwards. The phot1 6xK-N-GFP expressing lines grown under the same conditions have petioles orientated in a similar manner to wild type. Petioles are positioned such that they are pointing upwards with leaves perpendicular to the light (Figure 3.13A). These finding indicate that each of the phot1 6xK-N-GFP functions similar to wild type for petiole positioning with the lines 20-2 and 23-7 responding slightly less. However the phot1 6xK-N-GFP lines do demonstrate complementation of the *phot1-5 phot2-1* mutant phenotype.



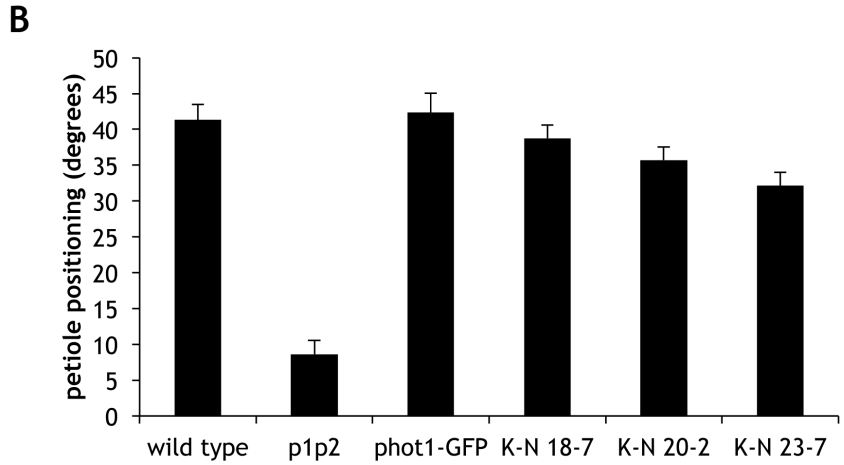


Figure 3.13 Petiole positioning of phot1 6xK-N-GFP expressing lines under low light conditions

A) Petiole positioning of wild type, *phot1-5 phot2-1* (p1p2), phot1-GFP and phot1 6xK-N-GFP expressing seedlings grown on soil for 1 week under 70 µmol^{-2} s^{-1} white light in a 16/8 hour light-dark cycle then transferred to 10 µmol^{-2} s^{-1} in a 16/8 hour light-dark cycle for a further week. Representative images of three experiments are shown.

B) Quantification of petiole angles from the horizontal as indicated in A. Error bars indicate standard deviation (n=9).

3.2.11 Leaf expansion is restored in phot1-6xK-N-GFP expressing lines

Another response controlled by phototropins to increase photosynthetic activity is leaf expansion. The leaves of the *phot1-5 phot2-1* double mutant are epinastic and curl underneath resulting in long, thin shaped leaves when compared to the large flat shape of leaves from wild type plants (Van Volkenburgh, 1999). It is known that either phot1 or phot2 can function to promote leaf expansion in *Arabidopsis* (Takemiya et al., 2005). Leaves of phot1 6xK-N-GFP expressing seedlings grown for two weeks under 70 µmol m^{-2} s^{-1} white light have large flat

leaves similar to wild type plants. This response can be measured using the leaf expansion index (LEI), the ratio of unflattened and artificially flattened leaf. A LEI of 1 would indicate a totally flat leaf. Leaves of wild type and phot1 6xK-N-GFP expressing plants have a LEI of around 0.8-0.9 whilst the *phot1-5 phot2-1* double mutant has a value of around 0.4. These results combined with those obtained for the other responses tested indicate that phot1 6xK-N-GFP expressing lines appear to completely restore the growth responses that are controlled by phot1.

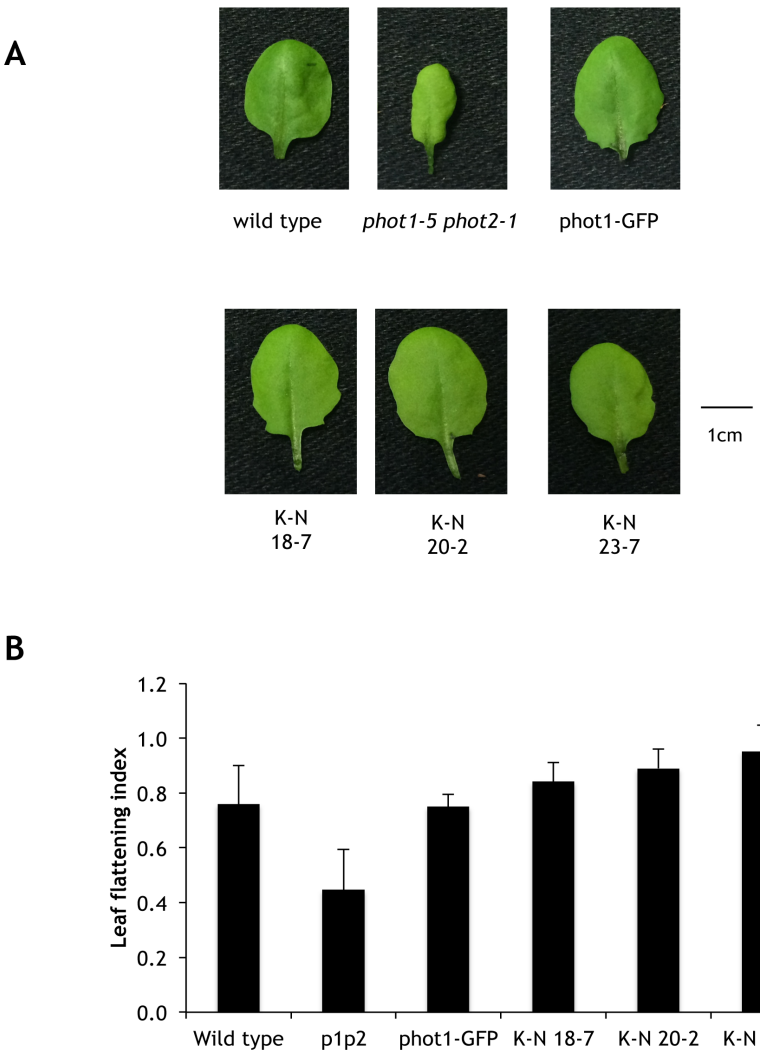


Figure 3.14 : Leaf expansion measurements of phot1 6xK-N-GFP expressing lines

A) Representative images of the fifth rosette leaves taken from wild type, *phot1-5 phot2-1* (p1p2), phot1-GFP and phot1 6xK-N-GFP expressing lines. Plants were grown on soil for 3 weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Scale bar represents 1cm.

B) The leaf expansion index of the fifth rosette leaves described in A. The leaf expansion index is expressed as the ratio before and after artificial flattening. Error bars indicate standard error (n=10).

3.2.12 Chloroplast accumulation movement is restored in phot1-6xK-N-GFP expressing lines

Under low light conditions, phot1 and phot2 mediate the movement of chloroplasts to the upper face of the palisade mesophyll cells to enable maximum light capture for photosynthesis (Kagawa et al., 2001). Since light capture is important for photosynthetic efficiency this response was also assessed in the lines expressing phot1 6xK-N-GFP. Three-week-old plants were grown under 70 µmol m^{-2} s^{-1} white light before the rosette leaves were detached and either kept in the dark or given a blue light treatment of 1.5 µmol m^{-2} s^{-1} before being imaged by confocal microscopy. The number of chloroplasts at the upper face of the mesophyll cells was counted in the leaves kept in darkness and compared to the number of chloroplasts at the upper surface in the blue light treated leaves. Both the wild type and phot1 6xK-N GFP expressing lines show accumulation of chloroplasts to the upper face of the mesophyll cells compared to the dark controls and the *phot1-5 phot2-1* double mutant (Figure 3.15A). Hence, under these low light conditions the positioning of the chloroplasts is restored in the phot1 6xK-N-GFP expressing lines.

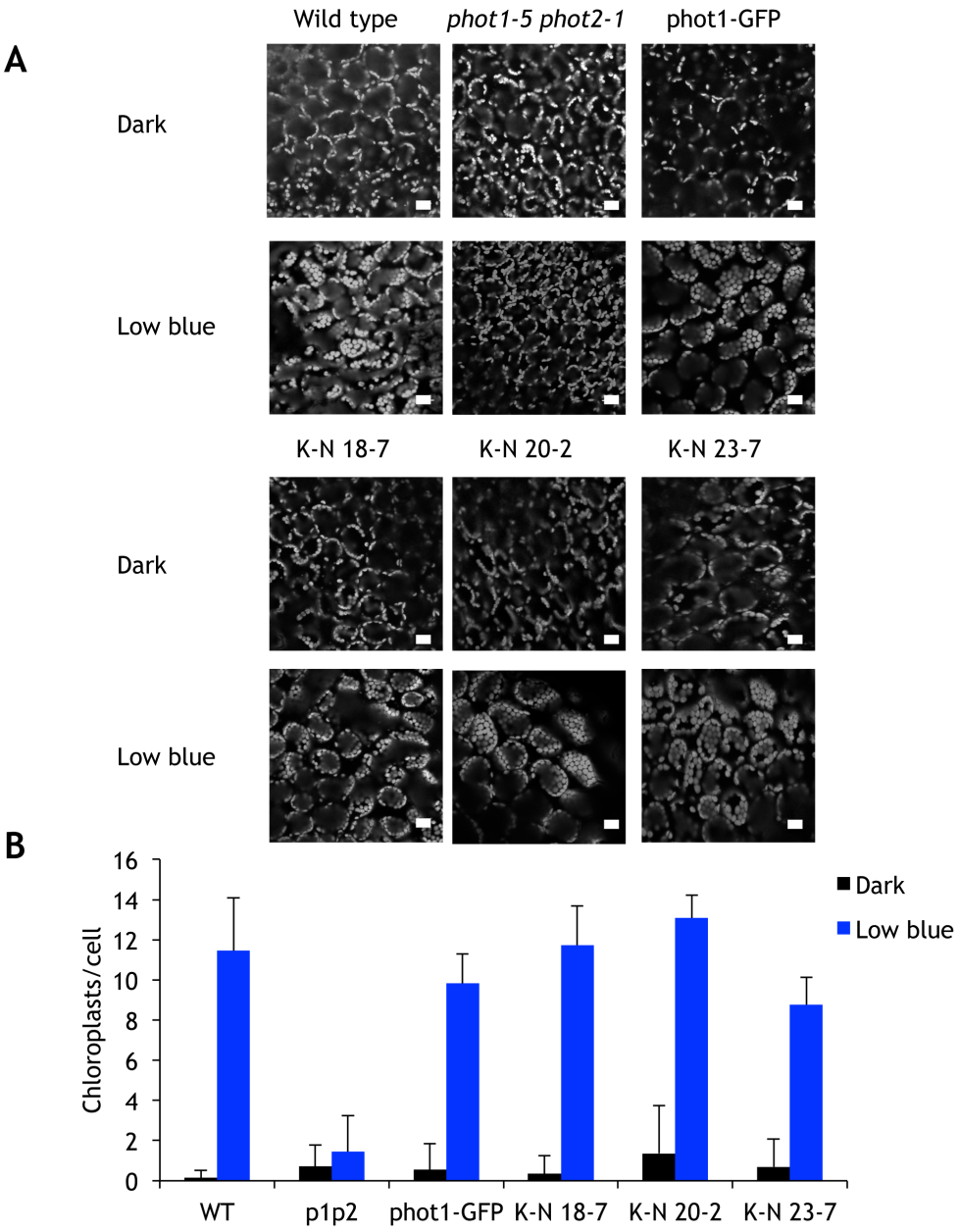


Figure 3.15 Chloroplast accumulation movement in phot1 6xK-N GFP expressing lines

A) Chloroplast accumulation response of wild type (WT), *phot1-5 phot2-1* (p1p2), phot1-GFP and phot1 6xK-N-GFP expressing plants grown on soil for 3 weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Rosette leaves were detached and treated with low intensity blue light (1.5 µmol m^{-2} s^{-1}) for 3 hours or kept in the dark for 3 hours before observation of chloroplast autofluorescence by confocal microscopy. Scale bar represents 20 µm.

B) Quantification of the number of chloroplasts at the upper face of palisade mesophyll cells of the plants described in A. Error bars indicate standard error of the mean of >12 cells per line.

3.3 Discussion

3.3.1 Attachment of phot1 to the membrane appears to be conserved in insect cells and Arabidopsis

Despite being mostly hydrophilic proteins, both phot1 and phot2 associate with the membrane in darkness in *Arabidopsis* (Sakamoto and Briggs, 2002, Kong et al., 2006). The mechanism of attachment is still not fully understood 15 years after phot1 was first identified as the photoreceptor for blue light responses (Christie et al., 1998). Mutational analysis in *Arabidopsis* can be time consuming as the creation of stable transgenic lines takes a large amount of time. The ease of use of the insect cell system provided a good method in which to monitor the properties associated with how phot1 attaches to the membrane. Wild-type phot1 and phot2 proteins are still found localised to the membrane in insect cells, with some protein also being detected in the soluble fraction (Figure 3.1B). Further analysis of phot1, using autophosphorylation assays, demonstrated that the soluble fraction is not active in insect cells (Figure 3.2). There is however a large increase in activity in the light-treated sample of the membrane fraction compared to the dark sample as shown in Figure 3.2 therefore indicating that phot1 is required to be in a membrane/lipid environment to be active. This has been observed for other kinases such as the G protein-coupled receptor kinases (GRKs) in humans, a group of kinases belonging to the AGC kinase family (Mushegian, Gurevich and Gurevich, 2012), which are required to be in a lipid environment to be active. GRKs are involved in the regulation of G protein-coupled receptors (GPCRs), which are found in eukaryotes with conserved members in *Arabidopsis* (Jaffé et al., 2012) and are activated by extracellular stimuli resulting in the exchange of GDP for GTP (Kobilka, 2007). To avoid abnormal signalling, activated GPCRs are turned off via phosphorylation by GRKs (Gurevich et al., 2012). This phosphorylation of GPCRs by GRKs is dependent upon the presence of phospholipids (Homan, Glukhova and Tesmer, 2013). The kinase GRK5 is recruited to the plasma membrane by electrostatic interactions between anionic membrane phospholipids and positively charged patches on the protein itself (Homan, Glukhova and Tesmer, 2013). Mutation or deletion of these charged patches results in inhibition of phospholipid binding and

consequently a reduction in autophosophorylation (Kunapuli, Gurevich and Benovic, 1994).

The requirement of phot1 to be in a lipid environment is further supported by the solubilisation of both insect cell and *Arabidopsis* microsomal membranes by detergents. It has long been known that phot1 could be solubilised using Triton X-100 (Short, Reymond and Briggs, 1993), but there may be a deleterious impact of the detergent upon the autophosphorylation activity of phot1 (Knieb, Salomon and Rudiger, 2004). As shown in Figure 3.4A, phot1 protein from insect cell membranes has the greatest activity in the presence of non-ionic detergents. The activity of *Arabidopsis* microsomal membranes are slightly different as all the detergents tested showed similar levels of activity to the untreated control. Nevertheless it is interesting to note that the non-ionic detergents, Triton X-100 and DDM, can solubilise phot1 from both insect cell membranes as well as from *Arabidopsis* microsomal membranes (Figures 3.4C and 3.4D), suggesting the phot1 in these different membrane preparations exhibit similar properties and that the mechanism of phot1 attachment to the membrane is conserved. The non-ionic detergents Triton X-100 and DDM can also solubilise phot2 from *Arabidopsis* microsomal membranes and Otphot from insect cell membranes. This, coupled with all three phototropins showing light-dependent autophosphorylation, suggests the mechanism of attachment to the membrane is conserved in phototropins from lower to higher plants and is likely to be due to lipid:protein interactions.

Recent publications have shown that it is possible to produce active phototropins in *E. coli* cells and that they are able to undergo autophosphorylation (Okajima, Matsuoka and Tokutomi, 2011, Aihara et al., 2012, Okajima et al., 2014). This is obviously advantageous as large-scale quantities of protein could be produced to investigate the properties of phototropins, for structural studies and biophysical studies of the full-length protein. However the reaction times for autophsophorylation are 15 times longer than that of both insect cell expressed phototropins and phototropin from wild type *Arabidopsis* seedlings (Reymond et al. 1992; Christie 1998). Phosphorylation usually reaches saturation point at around 2 minutes at room temperature for *Arabidopsis* phot1 (Reymond et al., 1992, Kaiserli et al., 2009) compared to around 30 mins for the

autophosphorylation reaction of the *E. coli* expressed protein at a temperature of 30°C. The quantity of irradiation used to measure the incorporation of ATP in the *E. coli* system is also much greater than that conventionally used, suggesting that the reaction is much less efficient. For these reasons, expressing phototropins in *E. coli* to investigate function should perhaps be approached with some caution.

Kong et al. (2013) recently reported that treatment with NaCl could not solubilise phot1 or phot2 from *Arabidopsis* microsomal membranes similar to what was observed for phot1 in this study for insect cell membranes as well as *Arabidopsis* microsomal membranes (Fig. 3.4C). High salt concentrations reduce non-covalent protein:protein interactions (Lodish et al., 2000) suggesting that the mechanism of phot1 attachment to the membrane is not via a non-covalent linkage with another protein. Indeed, such a protein would need to be present in both plant membrane and insect cell membranes to achieve such an attachment as well as being conserved in algae for Otphot to associate with the membrane. Triton X-100 and DDM are routinely used for the solubilisation of membrane proteins as they do not interrupt lipid:lipid and lipid:protein interactions (Seddon, Curnow and Booth, 2004) allowing proteins to retain their native structure. Taken together, the findings obtained in this study suggest that phot1 associates with the membrane via a lipid binding mechanism, which could be conserved between plant and insect cell membranes. Further investigation could utilise the DDM-solubilised phot1 to purify the protein from insect cell membranes using nickel affinity chromatography. Purifying phot1 in such an active form (since DDM does not impair its activity) could help identify new phosphorylation substrates targets for the receptor. Furthermore, lipid chip analysis on purified phot1 could be used to identify specific phospholipids which phot1 may be binding to. Such an approach has been used successfully to identify protein and lipid interactions in *Arabidopsis* as well as other plant species (De Silva et al., 2011, Petersen et al., 2012) and would allow identification of any lipids that may interact with phot1. This has not been possible since phot1 could not be purified in an active form previously. The membrane association and internalisation of phot1 requires active protein. Further evidence for the binding of phot1 to lipids is the visualisation of cytosolic strands in phot1-GFP seedlings treated with the phosphatidic acid (PA)

inhibitor 1-butanol (Sullivan et al., 2010). Interaction with PA usually occurs on positively charged residues such as lysine (Shin and Loewen, 2011). This can usually be verified by mutation of the conserved residues that results in a loss of membrane interaction as the electrostatic charges between the positively charged amino acid residues and negatively charged PA is lost.

3.3.2 A conserved lysine rich motif is found within the kinase domain of phototropins

Sequence alignment of the kinase domain of phototropin proteins identified a lysine rich motif (LRM) in the kinase domain (Fig. 3.6). This was within the insertion domain of plant AGC kinases between the magnesium binding loop and the conserved phosphoserines in the kinase domain (Rademacher and Offringa, 2012). Each phototropin contains a different number of lysine residues, with the surrounding region overall positively charged (Fig. 3.6). The high conservation of this region from lower to higher plants suggested that this motif might be involved in membrane association as this region has also been shown to direct subcellular localisation in other proteins (Zegzouti et al., 2006, Ek-Ramos et al., 2010). The protein Ezrin also contains a conserved LRM involved in binding to PIP_2 (Barret et al., 2000), which when mutated to asparagine causes a loss of binding. As observed in Figure 3.7A, mutation of the six lysine residues of phot1 resulted in the phot1 6xK-N protein still localising to the membrane indicating that the LRM is not involved in membrane localisation. Consequently we rationalised that mutating the same region to alanine may cause a change in localisation of phot1 since alanine is a small, non-polar amino acid unlike asparagine which has a longer uncharged side chain. However, as shown in Figure 3.7B, the phot1 6xK-A protein also still localises to the membrane in insect cells. This suggests that the LRM does not play a role in localisation of phot1. It was still important however to check the autophosphorylation activity of the phot1 protein harbouring this mutation as the region mutated is close in vicinity to the highly conserved aspartate and phosphoserines involved in phosphorylation (Fig. 3.4). As seen in Figure 3.7C, the phot1 6xK-N mutant protein is still active when compared to the activity of the phot1 wild-type protein.

Another protein that localises to the membrane and is important for plant growth is the brassinosteroids (BRs), which are plant hormones that are required for a number of plant responses (Fariduddin et al., 2014), with the pathway being one of the best studied in the plant field (Vert et al., 2005). BRs are recognised by plasma membrane localised receptor kinases, two of which undergo autophosphorylatation on tyrosine residues (Oh et al., 2010). The BR receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) changes localisation between the plasma membrane and endosomes thus it can influence different signalling mechanisms (Geldner et al., 2007). An important first step in the change of BRI1 localisation is the dissociation of BRI1 KINASE INHIBITOR 1 (BKI1) from the plasma membrane (Wang and Chory, 2006). BKI1 is an unstructured protein and has been proposed to function via linear motifs that are involved in protein interactions and/or modifications (Diella et al., 2008). Interestingly BKI1 N-terminus contains four [KR] [KR] repeats that direct localisation to the membrane in *Arabidopsis* root cells (Jaillais et al., 2011). The authors showed that when this motif was mutated to alanine the BKI1 protein no longer localised to the plasma membrane. This [KR][KR] motif of the BKI1 protein is highly cationic and is hypothesised to associate with the membrane via phospholipids (Jaillais, Université Lyon, unpublished communication). The evidence from membrane solubilisation suggests that phot1 may interact with lipids (section 3.2.3). There are a number of phospholipids present in cells and the interaction of phot1 with the membrane may not have been affected by the lysine to asparagine mutation. Many proteins and effectors bind to phospholipids in the cell, however it is not always known if these proteins recognise and bind specific phospholipids or the negatively charged environment itself (Stace and Ktistakis, 2006).

Identification of short amino acid sequence that can interact with phospholipids does not always translate into binding of the phospholipids when the full-length protein is tested (Nakai et al., 2005, Stace and Ktistakis, 2006). A Drosophila screen identified a short sequence with conserved arginine residues that when mutated to alanine no longer interacted with phospholipids. However when the full-length clone was mutated it was still able to bind the phospholipid albeit to a reduced level suggesting that there may be other interactions occurring in proteins that allow interaction with lipids. Protein Kinase C (PKC) another AGC

kinase has been shown to bind phospholipids (Rosse et al., 2010) and this interaction is mediated in two distinct ways, electrostatic interactions and specific binding to lipids (Orr and Newton, 1992, Stace and Ktistakis, 2006). This shows that there are multiple interacting sites between proteins and lipids therefore mutation of amino acid residues may not provide the full story of interaction of the phot1 protein with the membrane.

3.3.3 The LRM of phot1 is not required for signalling

Despite not affecting localisation in insect cells the localisation of phot1 6xK-N *in planta* was examined by transient expression of a GFP fusion protein in *N. benthamiana*, which is a convenient model to study mutations and both phot1 and phot2 localise the plasma membrane and internalise in this system (Kaiserli et al. 2009; Aggarwal et al. 2014). Phot1 6xK-N-GFP is still localised to the membrane in *N. benthamiana* (Figure 3.8B) cells confirming that the LRM does not direct membrane localisation. Despite not associating with the membrane in insect cells or in *N. benthamiana*, the high conservation of this region from lower to higher plants lead us to rationalising that there may be an effect on the other functions of phot1 that would effect the photosynthetic efficiency of the plant. In *Arabidopsis*, the phot1 harbouring the LRM mutation still localises to the membrane and internalises in response to light (Figure 3.9B). This is consistent with what has been seen previously with phot1 whereby mutations that affect phot1 protein activity have also been shown to cause a change in the localisation and internalisation pattern compared to the wild type protein (Cho et al. 2007; Inoue et al. 2008; Jones & Christie 2008; Kaiserli et al. 2009). However phosphorylation of the phot1 6xK-N is not affected as the light induced electrophoretic mobility shift, which indicates autophosphorylation and is a known trigger for movement, is still seen in all three lines (Figure 3.10).

All three phot1 6xK-N-GFP expressing lines complement the kinetics and fluence rate responses for phototropism (Fig. 3.11 and 3.12) showing that mutation of the LRM does not impact upon phototropism. Petiole positioning and leaf expansion are also complemented in the three lines (Fig. 3.1 and 3.14). The final response investigated was chloroplast accumulation, which was also complemented in all three phot1 6xK-N-GFP expressing lines (Fig. 3.13). Taken together the data presented in this chapter shows that the LRM is not required

for membrane attachment or involved in signalling to bring about the physiological responses tested. Further investigation, however, would be required to eliminate fully, a role for the LRM in phot1 function. Experimental limitations of live imaging mesophyll cells prevent the kinetics of both chloroplast accumulation and the petiole positioning response, which would require measurements over a long time period which is not possible at this stage. These are more sensitive measurements that may be altered in the transgenic lines. Another response that could be tested is NPH3 dephsophorylation, which occurs in response to blue light and is phot1 dependent (Matsuoka and Tokutomi, 2005, Pedmale and Liscum, 2007). A third response is the avoidance movement of chloroplasts which prevents photodamage (Suetsugu & Wada, 2012), a response that is controlled by phot2. By mutating the LRM in phot2 this response could be measured to assess if the LRM affects this function. Further investigation of the Otphot LRM may also give further indication if membrane association of phot1 in lower plants is also conserved.

Chapter 4: The role of the kinase domain of phototropin1 in membrane association

4.1 Introduction

Phot1 and phot2 share a common feature of being membrane associated hydrophilic proteins (Sakamoto and Briggs, 2002, Kong et al., 2006) albeit relocalising to different subcellular regions (Sakamoto & Briggs 2002; Wan et al. 2008; Kaiserli et al. 2009; Kong et al. 2006; Kong et al. 2007; Kong et al. 2013; Aggarwal et al. 2014). The C-terminal kinase domain of phot2 has been shown to direct localisation of the protein to the plasma membrane and internalise to the Golgi in response to blue light in three-day-old *Arabidopsis* seedlings (Kong et al., 2007), as well as the production of punctate staining of the Golgi when transiently expressed in mesophyll protoplasts (Kong et al., 2006). Abolishing the kinase activity by mutating the conserved aspartate (D720N), which is required for magnesium chelating for ATP binding, resulted in the mutated protein being detected mainly in the plasma membrane region indicating that loss of phosphorylation does not result in a loss of membrane localisation. The punctate staining normally visualised after illumination was not observed indicating that phosphorylation is required for enhanced association with the Golgi (Kong et al., 2007). Similarly, for phot1, abolishing the kinase activity by mutating the conserved aspartate (D806N) resulted in constitutive membrane association of the protein when transiently expressed in *Nicotiana benthamiana* (Kaiserli et al., 2009). This above data suggests that kinase activity is not required for localisation to the plasma membrane. Kong et al., (2013) showed that the C-terminus of the kinase domain of phot2 was required for membrane association both in yeast and in *Arabidopsis*. We therefore rationalised that the membrane association of phot1 may be within the kinase domain, similar to phot2. In this chapter the membrane association of the phot1 kinase domain is examined through biochemical analysis in insect cells as well as transient expression of these truncations of phot1 proteins in *N. benthamiana*.

4.2 Results

4.2.1 Deletion of the kinase domain of phot1 causes loss of membrane localisation

The C-terminal kinase domain of phot2 has been shown to be sufficient to direct membrane association of the protein with a GFP fusion localising to the membrane whilst the N-terminal photosensory domain, including LOV1 and LOV2, localises to the cytosol (Kong et al., 2006; Kong et al., 2007). These truncation analyses indicate that the photosensory domain alone localises to the cytosol whilst the kinase domain alone is sufficient to direct membrane localisation. To investigate if phot1 behaves in a similar manner, the entire C-terminal region including the kinase domain was removed from the protein and the N-terminal photosensory domains alone were expressed in insect cells as well as *N. benthamiana*. Fig. 4.1A illustrates the amino acid positions and the domains expressed in the His-tagged fusion protein used for insect cell expression and the GFP fusion protein used for transient expression in *N. benthamiana*. The localisation pattern of phot1 when the kinase domain is deleted was first assessed in *Sf9* insect cells. The His-tagged phot1 kinase deletion (phot1 ΔK; Fig. 4.1A) protein was expressed in the insect cells and total protein extracted. Ultra-centrifugation was then performed to separate this protein extraction into membrane and soluble fractions. As shown in Fig. 4.1B, when expressed in insect cells, phot1 ΔK is detected in the soluble fraction but not in the membrane fraction where wild type phot1 is localised (Fig. 3.1B). The purity of the membrane and soluble fractions was examined by monitoring the levels of the plasma membrane marker glycoprotein-64 (GP-64) and the cytosolic marker protein α-tubulin. GP-64 was only detected in the membrane fraction demonstrating an absence of membrane proteins in the soluble fraction (Figure 4.1B). There is some α-tubulin detected in the membrane fraction, however there is no phot1 ΔK protein detected at the membrane. Therefore, the contamination of soluble protein in the membrane fraction does not affect the finding that the absence of phot1 ΔK protein detected in this fraction is due to the deletion of the kinase domain and suggests that this region is involved in targeting phot1 to the membrane, similar to what has been found for phot2 in *Arabidopsis* (Kong et al., 2007), at least in insect cells.

To assess the localisation *in planta* the transient *Nicotiana benthamiana* system was used. Phot1-GFP localises to the plasma membrane in darkness in this system (Fig. 4.1C) similar to what has been shown previously for both transient expression of phototropins fused to GFP (Kaiserli et al. 2009; Aggarwal et al. 2014) and stable transgenic lines (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008). As shown in Fig. 4.1C, there is some evidence of the phot1 ΔK protein localising to cytosolic strands usually seen in wild type phot1 after blue light illumination (Sakamoto and Briggs, 2002, Wan et al., 2008, Kaiserli et al., 2009). There is some punctate GFP fluorescence in the cytosol suggesting phot1 ΔK is no longer exclusively localised to the plasma membrane in darkness. However the use of the GFP alone as a control shows that there is still a portion of phot1 ΔK localised to the plasma membrane as GFP expressed alone is visualised throughout the cell including the nucleus. A maximal projection of a Z-stack shows that phot1 ΔK also appears to localise to the nucleus as well as the visualisation of a diffuse GFP pattern in the cytosol (Fig 4.1D). This suggests that although the photosensory domains do not directly target phot1 localisation to the membrane, there may be interactions that influence the localisation and that the protein is not entirely soluble.

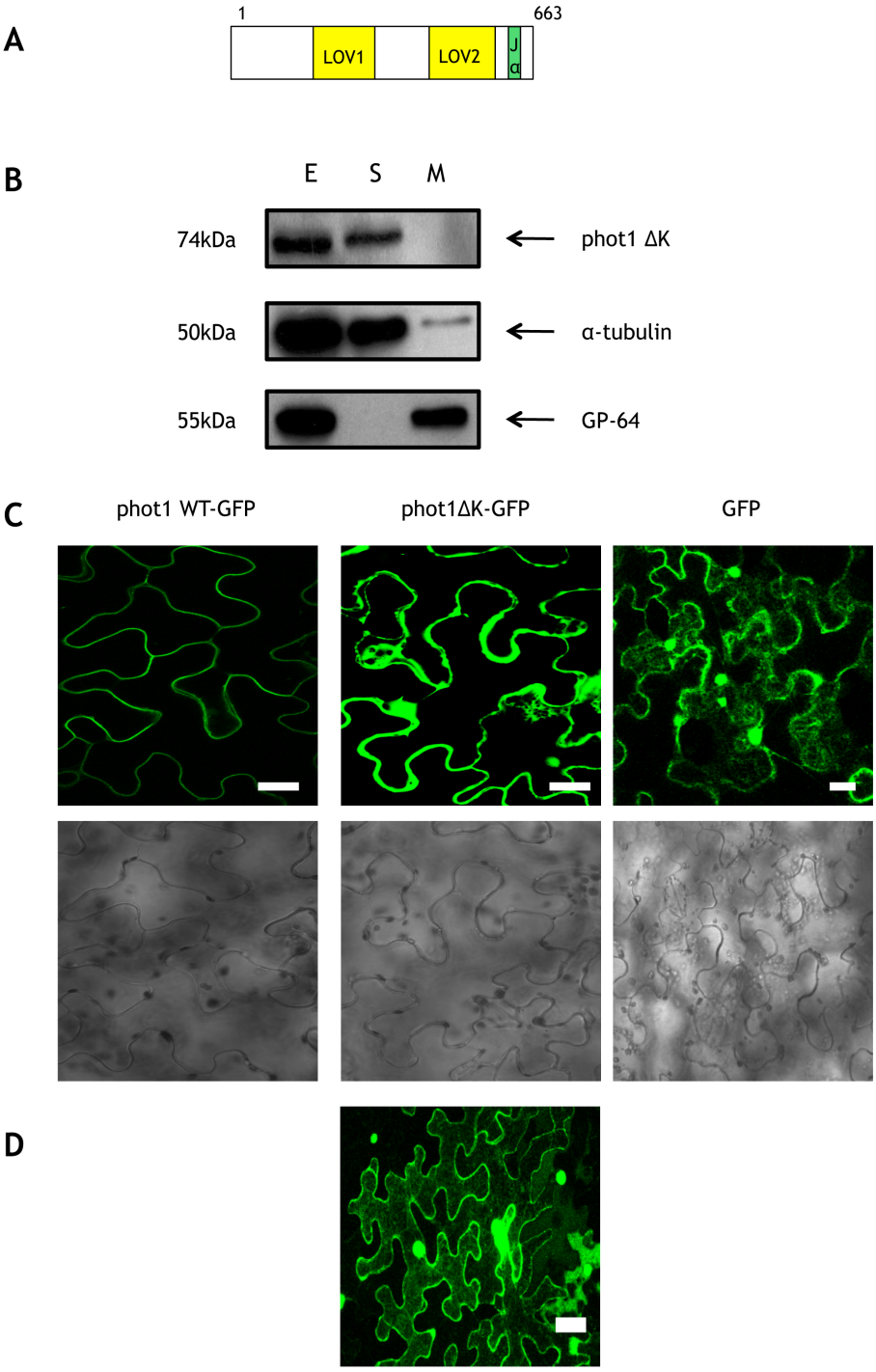


Figure 4.1: Deletion of the phot1 kinase domain prevents membrane association of phot1 in insect cells and *Nicotiana benthamiana*

A) Schematic diagram illustrating the amino acid positions of the phot1 kinase deletion (ΔK) construct used to assess localisation in insect cells and *N. benthamiana*.

B) Western blot analysis on protein fractions isolated from virally infected insect cells. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. Anti-His antibody was used to detect phot1 in each fraction. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64.

C) Transient expression of wild-type phot1-GFP (phot1 WT-GFP), phot1 ΔK (phot1ΔK-GFP) and free GFP in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-

18 hours before imaging. Phot1 WT is localised to the plasma membrane in darkness whilst phot1 ΔK is cytosolic (shows internalisation in the absence of a light stimulus). Free GFP is found expressed throughout the cell. Scale bar represents 20 μm.

D) Maximal Z-stack projection of phot1 ΔK-GFP in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Scale bar represents 50 μm.

4.2.2 The kinase domain alone is sufficient for membrane localisation of phot1

Deletion of the kinase domain of phot1 appears to result in soluble protein, compared to wild-type phot1, in both insect cells and *N. benthamiana*. These studies indicate that there may be a region of membrane interaction within the kinase domain of phot1. Deletion of the kinase domain of phot2 results in punctate staining in the cytoplasm of protoplasts (Kong et al., 2006) whilst the expression of the phot2 kinase domain alone was sufficient for membrane localisation in both protoplasts and *Arabidopsis* (Kong et al., 2006, 2007). We therefore rationalised that expression of the kinase domain together with the extreme C terminus (amino acids 607-996) but lacking the photosensory domains, would direct phot1 to the plasma membrane. A similar method as before was applied with the use of both the insect cell system and *N. benthamiana*. Figure 4.2A schematically illustrates the amino acid positions and the domain expressed in the fusion protein used for both insect cell expression and for transient expression in *N. benthamiana*. Figure 4.2B shows that the phot1 kinase protein is detected in the membrane fraction following ultra-centrifugation but not the soluble fraction. This is in contrast to phot1 wild type, which is detected in both the soluble and membrane fraction following ultra-centrifugation (Fig. 3.1B). The membrane marker GP-64 is not detected in the soluble fraction demonstrating the purity of the fraction. There is also no detection of the soluble marker α-tubulin in the membrane fraction indicating that this fraction is also free from contamination. This suggests that the kinase domain of phot1 alone is sufficient for membrane localisation in insect cells.

To assess its localisation *in planta,* the phot1 kinase protein was expressed transiently in *N. benthamiana* as a GFP fusion. As shown in Fig 4.2C, both phot1 wild type and phot1 kinase localise to the plasma membrane in darkness. There is no visualisation of GFP in the nucleus as is seen for GFP expressed alone nor is

the punctate staining visualised when phot1 ΔK is expressed in the same system. As indicated by the white arrows in Fig 4.2C, there is visualisation of some cytosolic strands when phot1 kinase is expressed. This could possibly be due to overexpression, as the construct contains no photosensory domains, the protein will not be light sensitive. Despite the cytosolic strands, the results still suggest that the phot1 kinase domain alone is sufficient for membrane localisation similar to what has been shown for phot2.

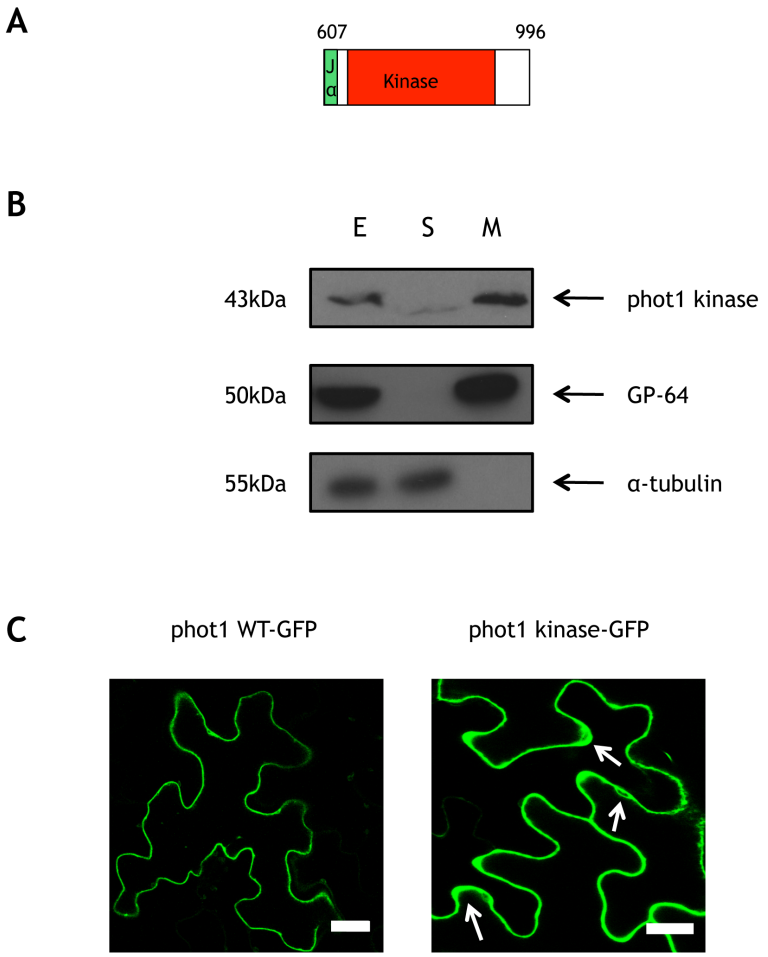


Figure 4.2: Expression of the phot1 kinase domain alone results in membrane association of phot1 in insect cells and *Nicotiana benthamiana*

A) Schematic diagram illustrating the amino acid positions of the phot1 kinase construct used to assess localisation in insect cells and *N. benthamiana*.

B) Western blot analysis on protein fractions isolated from virally infected insect cells. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. Anti-His antibody was used to detect phot1 in each fraction. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64.

C) Transient expression of phot1 WT (phot1 WT-GFP) and phot1 kinase (phot1 kinase-GFP) in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Phot1 kinase is localised to the plasma membrane in darkness similar to phot1 WT. White arrows indicate cytosolic strands. Scale bar represents 20 μm.

4.2.3 Deletion of the extreme C-terminus of phot1 has no effect on membrane localisation

The C-terminus including the kinase domain has been shown to be sufficient for membrane localisation for both phot1 (Fig. 4.2) and phot2 (Kong, Kagawa, et al., 2013). In all cases studied, expression constructs spanned the full-length of the C-terminus of the protein including a region at the extreme C-terminus which is not part of the kinase domain (Kong et al., 2007; Fig 4.2A). To further investigate the region of phototropins that is required for membrane localisation, Kong et al. (2013) employed the yeast CytoTrap system. This system can be used to detect protein-protein interactions in the cytosol as opposed to the nucleus. Fusion of the human Sos protein (hSos) to a bait protein can only complement the cdc25-induced growth defect of the yeast cells when it is recruited to the plasma membrane through cytosolic interaction with a membrane-anchored prey protein or through direct interaction with the plasma membrane. If this interaction occurs, the yeast are able to grow at the restrictive temperature of 37°C. The authors fused full-length phot1 or phot2 from *Arabidopsis* or the phototropin from the algae *Chlamydomonas* to the hSos protein and showed that a small region of around 78 amino acids for phot1 and 84 amino acids for phot2, at the C terminus of the proteins was responsible for targeting phototropin to the membrane in yeast. Deletion of 35 amino acids from the C terminus of phot1 resulted in no yeast growth at the restrictive temperature suggesting that this small region is required for membrane association.

We therefore employed a similar analysis to investigate the role of the extreme C-terminus of phot1 in membrane association using the insect cell system. To investigate this we expressed phot1 lacking the last 44 amino acids, designating the deletion as phot1 ΔC (Fig 4.3A). As shown in Fig. 4.3B phot1 with the C-terminal deletion (phot1 ΔC) still localises to the plasma membrane in insect cells. The use of the membrane marker GP-64 and the soluble marker α-tubulin, show that there is no contamination in the membrane or soluble fractions and that phot1 ΔC does localise to the membrane in insect cells. This is different to the results shown by Kong et al. 2013 where a smaller deletion prevented yeast

growth at a restrictive temperature, indicating that phot1 was no longer localised to the membrane in this experimental system.

To further confirm our results from the insect cell system, the phot1 ΔC protein was transiently expressed in *N. benthamiana* as a GFP fusion, where it localised to the plasma membrane in darkness (Fig 4.3C). There was however visualisation of cytosolic strands, as indicated by the white arrows. This is slightly different from the result obtained with the insect cell system where the protein was membrane localised. Kong et al. (2013) also demonstrated similar results. When the authors moved from the yeast system to expressing the constructs in the protoplasts, the phot2 Δ34 construct showed membrane localisation in yeast but in protoplasts there was visualisation of punctate GFP staining. This would suggest that there might be other factors influencing membrane localisation *in planta*. Although there may be differences between the insect cell system and *N. benthamiana,* overall the extreme C-terminus of phot1 does retain membrane localisation as the phot1 ΔC protein is detected in the membrane fraction when expressed in insect cell and when transiently expressed in *N. benthamiana*.

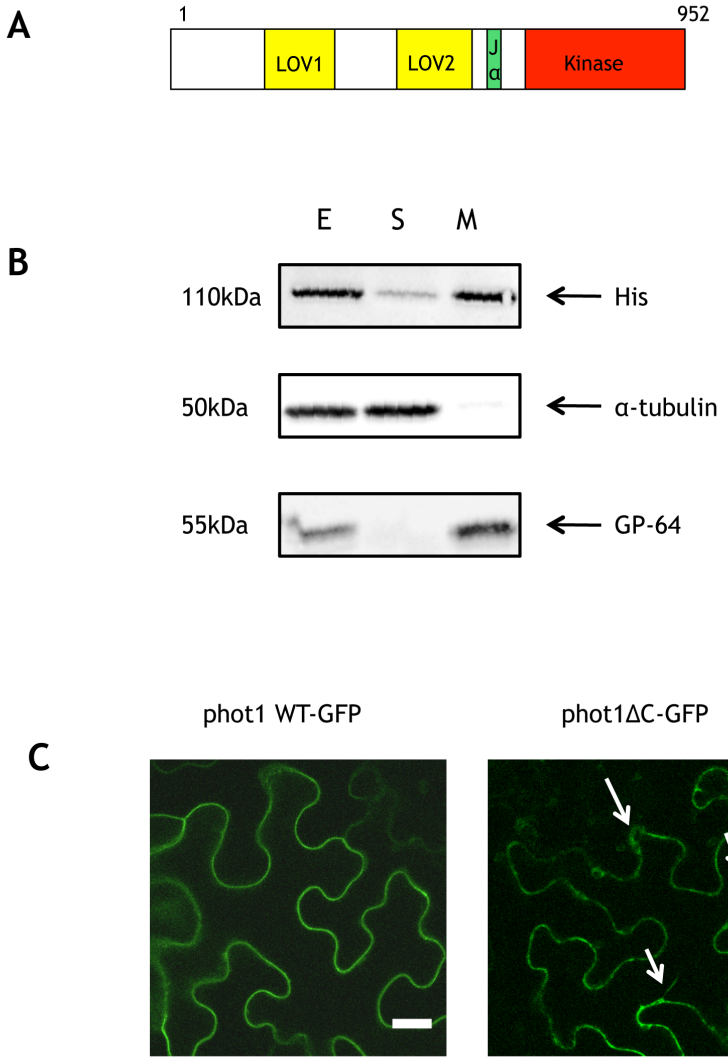


Figure 4.3: Deletion of the extreme C-terminus of phot1 does not have a major impact on membrane localisation in insect cells and *Nicotiana benthamiana*

A) Schematic diagram illustrating the amino acid positions of the phot1 C-terminal deletion (ΔC) construct used to assess localisation in insect cells and *N. benthamiana*.

B) Western blot analysis on protein fractions isolated from virally infected insect cells. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. His antibody was used to detect phot1 in each fraction. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64.

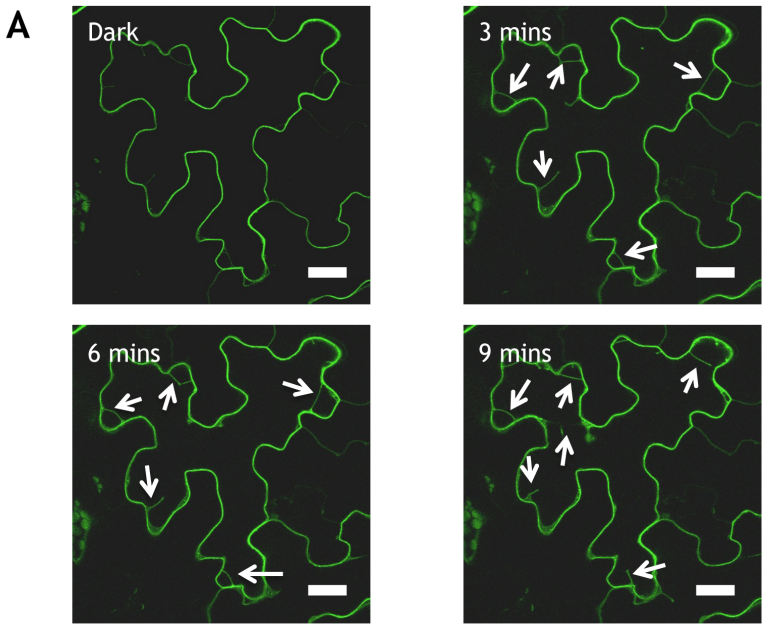
C) Transient expression of phot1 WT (phot1 WT-GFP) and phot1 ΔC (phot1 ΔC-GFP) in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Phot1 ΔC is localised to the plasma membrane in darkness similar to phot1 WT. Scale bar represents 20 μm.

4.2.4 Truncation of the extreme C-terminus of phot1 results in a loss of autophosphorylation activity

Localisation of *Arabidopsis* phot1 to the plasma membrane in darkness and internalisation in response to light (Sakamoto and Briggs, 2002, Wan et al., 2008, Kaiserli et al., 2009) to cytosolic strands is lost when the conserved aspartate responsible for chelating Mg^{2+} and subsequent ATP binding (Rademacher and Offringa, 2012) is mutated to asparagine (D806N) (Kaiserli et al., 2009). This kinase inactive version of phot1 has been shown to lack the physiological responses controlled by phototropin (Inoue et al., 2008). Therefore it was important to assess if phot1 ΔC would internalise from the membrane in response to blue light in *N. benthamiana*. As shown in Fig. 4.4A, phot1 wild-type localises to the plasma membrane in darkness and 3 mins following blue light illumination with the laser used to excite GFP, internalises from the membrane to cytosolic strands as indicated by the white arrows. The phot1 ΔC protein although localised to the plasma membrane, is slightly different to wild type phot1 in the same system. There is visualisation of punctate staining around the membrane, which is likely caused by autofluorescence of the chloroplasts (Fig. 4.4B). There is no relocalisation of phot1 ΔC 9 mins following excitation with the blue light. The bright field images are shown below the GFP images to show that there are chloroplasts localised around the membrane where the punctate staining is seen. The lack of relocalisation of phot1 ΔC suggests that the kinase activity of the protein has been affected by this truncation and can no longer undergo autophosphorylation.

To assess the autophosphorylation activity crude protein extract from insect cells expressing either His-tagged phot1, phot1 ΔC or phot1 ΔK was extracted under a dim red safe light, incubated with radiolabelled ATP and given a light treatment or kept in the dark. The amino acid positions of each truncations is shown in Fig 4.5A. Phot1 wild type undergoes light dependent autophosphorylation whilst phot1 ΔK does not, which is as expected since the kinase domain has been removed from this protein. The phot1 ΔC protein also does not undergo light dependent autophosphorylation in insect cells. This is consistent with the protein remaining localised to the membrane when transiently expressed in *N. benthamiana* (Fig 4.4B). The protein levels in each of

the insect cell samples examined are comparable (Fig. 4.5C) illustrating that the loss of signal for both phot1 ΔC and phot1 ΔK is not the result of lack of protein in the samples. This shows that the constitutive membrane attachment of the phot1 ΔC protein is likely caused by a loss of autophosphorylation activity of the protein.



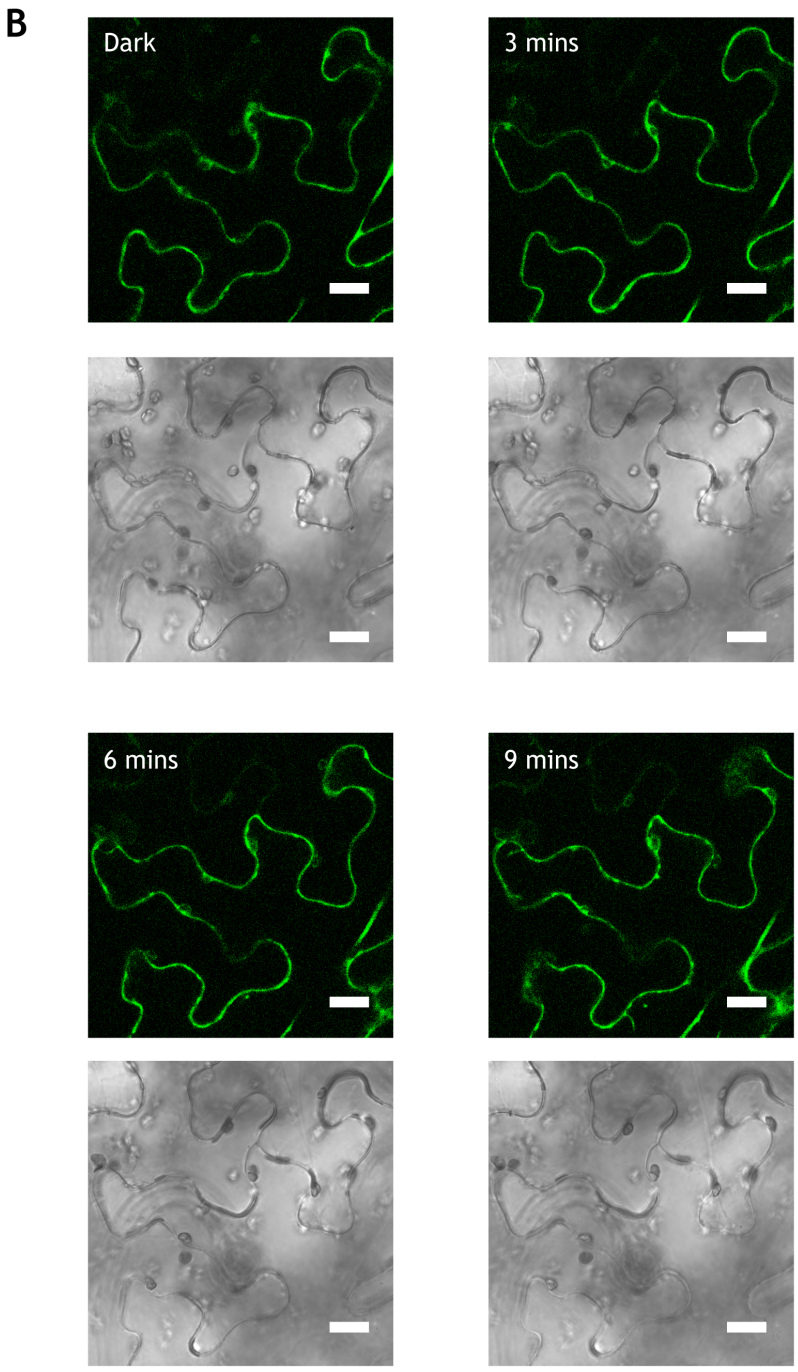


Figure 4.4: Deletion of the extreme C-terminus of phot1 prevents internalisation from the membrane in *Nicotiana benthamiana*

A) Transient expression of phot1 WT in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Arrows indicate internalisation from the membrane in response to blue light illumination. Scale bar represents 20 μm.

B) Transient expression of phot1 ΔC in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Phot1 ΔC remains localised to the membrane 9 mins after illumination with blue light. Scale bar represents 20 μm.

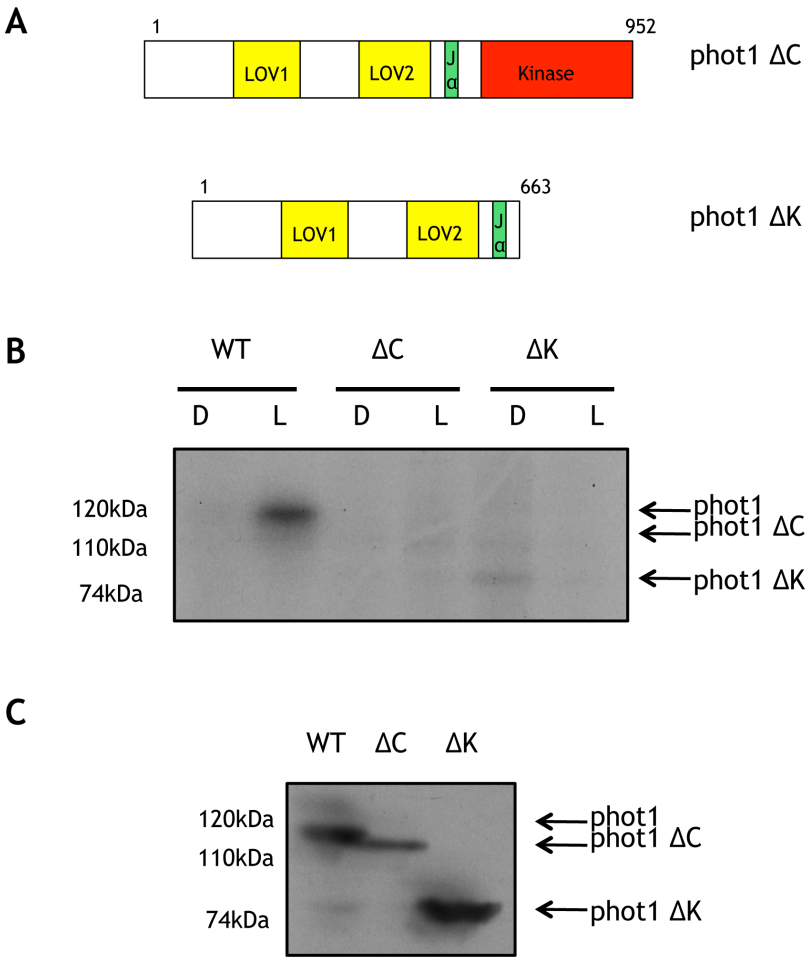


Figure 4.5 Effect of C-terminal truncations on phot1 autophosphorylation activity in insect cells

A) Schematic diagram illustrating the amino acid positions of the phot1 C-terminal truncations used for insect cell expression.

B) Protein was extracted from virally infected insect cells under dim red light. Radiolabelled ATP was added to the protein samples before they were given a mock irradiation (D) or illuminated with white light for 10 secs (L) with a total fluence of 30,000 μmol m^{-2}. Samples were then subjected to SDS-PAGE and autoradiography.

C) Western analysis of protein levels in each sample using anti-His antibody.

4.2.5 The effect of multiple C-terminal truncations on phot1 autophosphorylation

The lack of autophosphorylation activity in the phot1 ΔC protein suggests that the activity of the kinase domain has been impacted by this truncation. Our definition of the C-terminal border of the kinase domain was based on sequence alignments with multiple phototropins as shown by the black arrow in Fig 4.6. The C-tail of kinases has been defined from the tryptophan of the conserved FxxxxW motif (Hanks and Hunter, 1995, Rademacher and Offringa, 2012). The red arrow in Fig 4.6 indicates the position of this tryptophan residue, which is found in the phototropins as well as the other AGC kinases from humans and mouse. Therefore according to this definition the phot1 ΔC construct may have removed part of the kinase domain itself as opposed to only the C-terminus. This would explain the loss of autophosphorylation activity as a result of the truncation. To address this, two further truncations of the C-terminus were performed. These truncations were performed outside the kinase domain defined by sequence alignments with other AGC kinases, beginning at amino acid position 964. This includes a short number of amino acids after the conserved tryptophan residue to help prevent impacting the structure of the kinase domain. An additional truncation was also performed at amino acid 972 as a further comparison of the extreme C-terminus of phot1. These two truncations (Fig 4.7A) were used to transfect insect cells. Crude soluble protein was extracted from virally infected cells under the aid of a red safe light and subjected to either 30,000 μmol m^{-2} s^{-1} of white light or kept in the dark. As shown in Fig 4.7B both truncations retain light induced autophosphorylation, however there is a small decrease in signal compared to phot1 wild-type which does not appear to be a consequence of differing protein levels which are comparable between all three samples (Fig 4.7C). These findings suggest that phot1 does not require the C-terminus of the protein to be active, and that there may be further phosphorylation sites in the C-terminus in addition to the sites identified by Inoue et al., (2008). However this would require quantification of the signals generated as well as other experiments to be fully concluded.

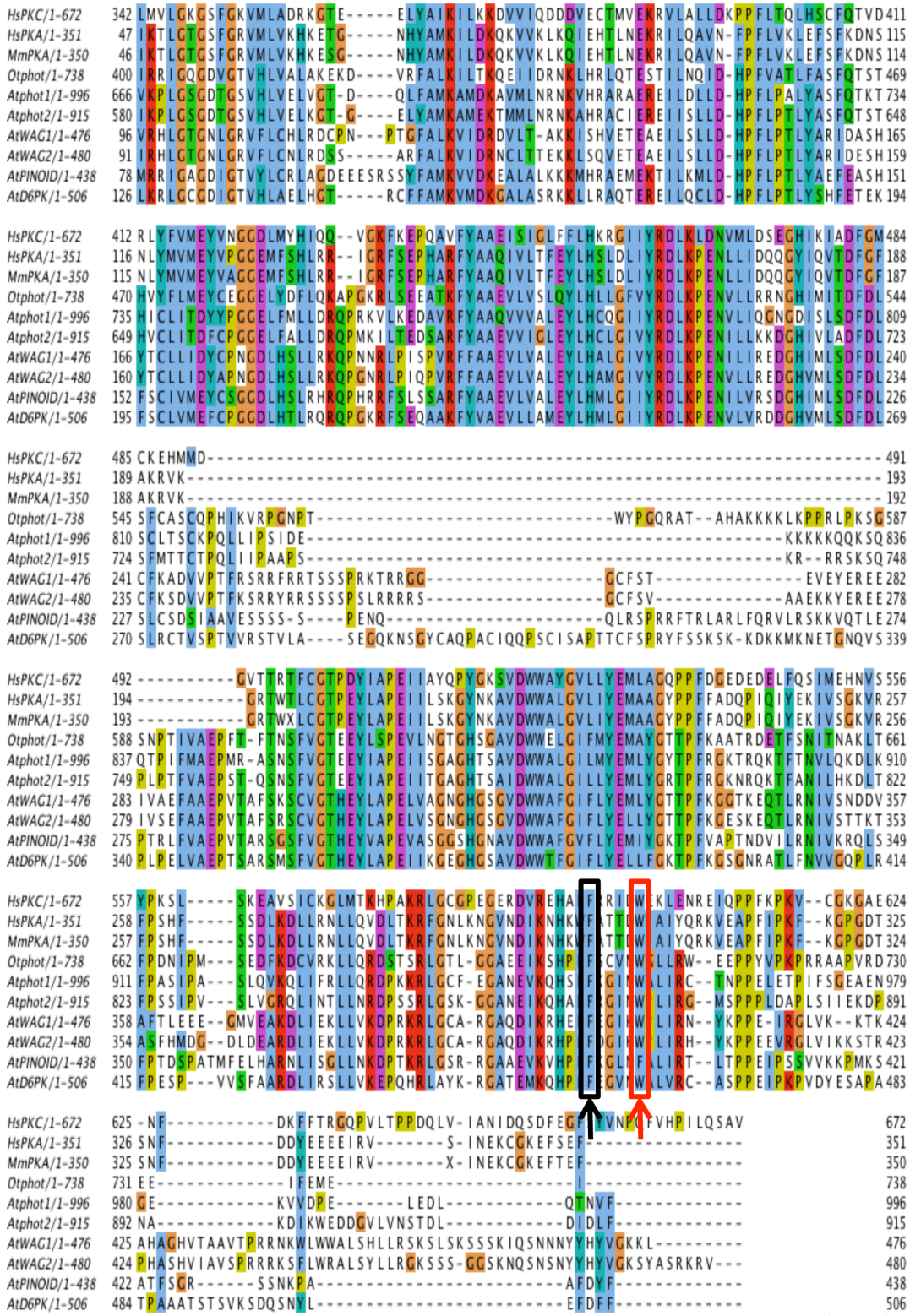


Figure 4.6: Amino acid sequence alignment of various AGC kinases from *Arabidopsis*, *Mus musculus* and *Homo sapiens*

Alignment of various AGC kinases from *Arabidopsis*, *Mus musculus* and *Homo sapiens* taken from the NCBI database showing the positioning of the kinase domain. The black arrow indicates the original ΔC defined in this study whilst the red arrow indicate the end of phototropin kinase domain as described by Rademacher & Offringa (2012) where the C-terminus is defined by the tryptophan of a conserved FxxxxW motif. Sequences are derived from *Homo sapiens* Protein Kinase C (PKC) α-type, *Homo sapiens* Protein Kinase A (PKA) α-type, *Mus musculus* PKA, *Ostreococcus tauri* phot, *Arabidopsis* phot1, *Arabidopsis* phot2, *Arabidopsis* WAG1, *Arabidopsis* WAG2, *Arabidopsis* PINOID, *Arabidopsis* D6PK.

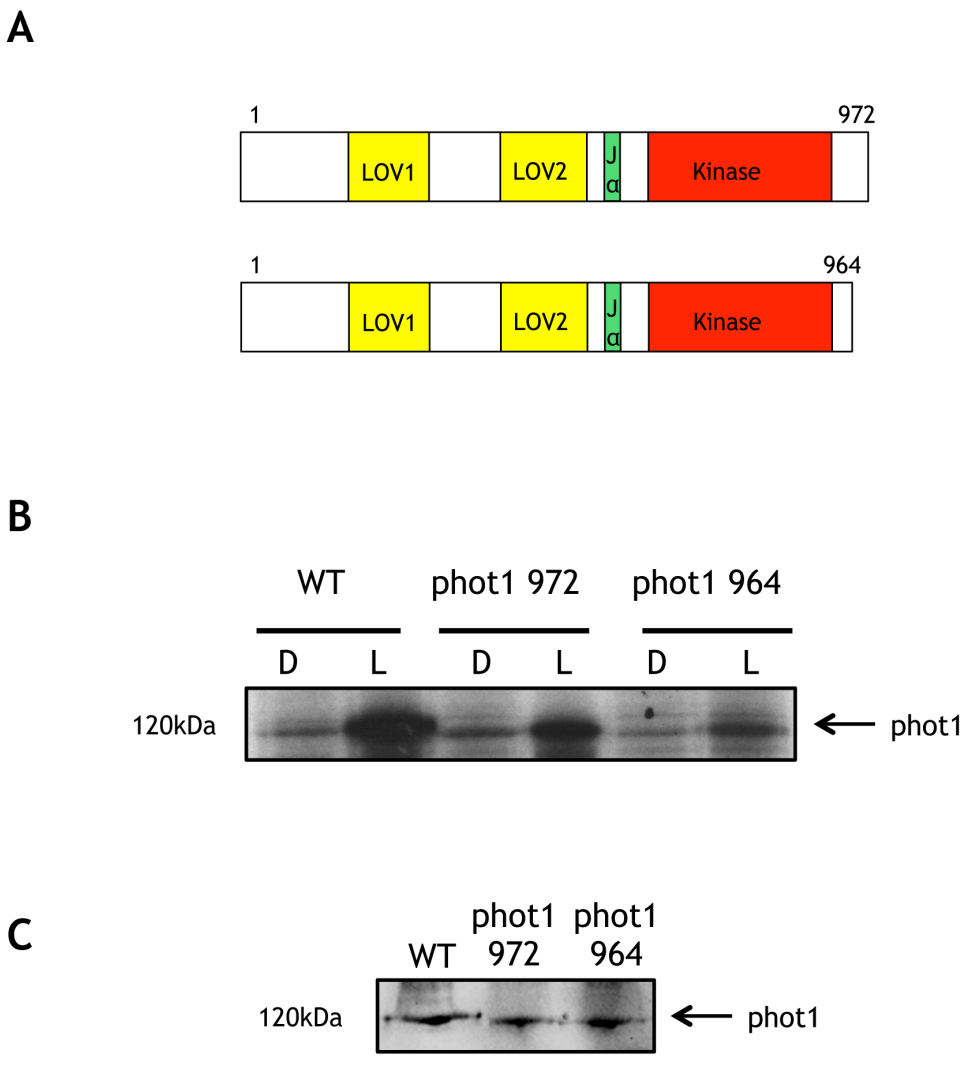


Figure 4.7: Effect of further C-terminal truncations on phot1 autophosphorylation activity in insect cells

A) Schematic diagram illustrating the amino acid positions of the phot1 972 and phot1 964 truncations used to assess autophosphorylation in insect cells.

B) Protein was extracted from virally infected insect cells under dim red light. Radiolabelled ATP was added to the protein samples before they were given a mock irradiation (D) or illuminated with white light for 10 secs (L) with a total fluence of 30,000 µmol m^{-2}. Samples were then subjected to SDS-PAGE and autoradiography.

C) Western analysis of phot1 protein levels in each sample using anti-His antibody.

4.2.6 Truncation analysis of the phot1 kinase domain identifies a small region that retains membrane association in insect cells

Truncation of the extreme C-terminus demonstrates that it does not appear to be involved in membrane association of phot1, which was of particular interest given the difference in the membrane association for phot1 and phot2 in the yeast CytoTrap system (Kong, Kagawa, et al., 2013). In order to determine if a different region of membrane association could be identified for phot1 using the insect cell system, further truncations of the kinase domain were investigated. To determine the predicted secondary structures of the kinase domain the phot1 protein sequence was analysed using the PSIPRED server (Buchan et al., 2013), allowing truncations to be performed outside these secondary structures (Fig 4.8). Three further truncations of the phot1 kinase domain were then performed based on this prediction at amino acid position 907, one at amino acid position 814 and a further at amino acid position 726. These truncations were performed outside the α-helix and ß-sheets identified by the prediction server (Fig 4.8).

The above phot1 truncation constructs were used to transfect *Sf9* insect cells. Protein extracts from the virally infected insect cells were subjected to ultra-centrifugation to separate the soluble and membrane fractions. As shown in Fig. 4.9A wild-type phot1 localises to the membrane fraction, with no contamination of the soluble and membrane fractions as shown by the membrane marker GP-64 only being detected in the membrane fraction and the soluble marker α-tubulin only detected in the soluble fractions. Fig 4.9B shows that phot1 truncated at amino acid position 907 retains membrane association. Similarly the phot1 truncations at amino acid position 814 and position 726 also retained membrane association with the protein being detected in membrane fractions (Fig 4.9C and Fig. 4.9D). In each case there is no contamination in the fractions as the GP-64 membrane marker is only detected in the membrane fraction whilst the soluble marker α-tubulin is only detected in the soluble fraction. These results, together with the results in Fig 4.2, suggest that the region from the beginning of the kinase domain at amino acid position 663 to amino acid 726 is responsible for the membrane association of phot1 since the kinase domain alone is sufficient for membrane association (Fig 4.2B). It also suggests that regions beyond amino acid 726 are not required for membrane localisation. We therefore rationalised that

expressing a fragment of phot1 from amino acid position 663 to amino acid 726 would result in membrane association of phot1 in insect cells.

The region of phot1 from amino acid 663 to 726 is approximately 7kDa, so therefore the corresponding coding sequence was cloned as glutathione S-transferase (GST) fusion to aid better detection of the protein in insect cells. Previous work in the lab has successfully shown that phot1 wild type fused to the C-terminus of GST can be expressed in an active form in insect cells (Kaiserli et al., 2009). Protein extracted from virally infected insect cells expressing wild-type phot1 was subjected to ultra-centrifugation. As expected, GST-phot1 localises to the plasma membrane (Fig. 4.10A) similar to what has been shown previously for phot1 fused to a His tag (Fig. 4.9A). The membrane marker GP-64 and the soluble marker α-tubulin do not detect contamination in either fraction. However, expression of phot1 from amino acid 663 to amino acid 726 in insect cells results in only soluble protein being detected (Fig 4.10B) when protein was extracted from the virally infected cells and subjected to ultra-centrifugation. This is an unexpected result as the earlier truncation analysis of the phot1 kinase domain indicated that truncation of phot1 protein at amino acid position 726 retained membrane association (Fig 4.7D). These findings indicate that expression of the small region from amino acid 663 to 726 is not sufficient to target GST to the membrane.

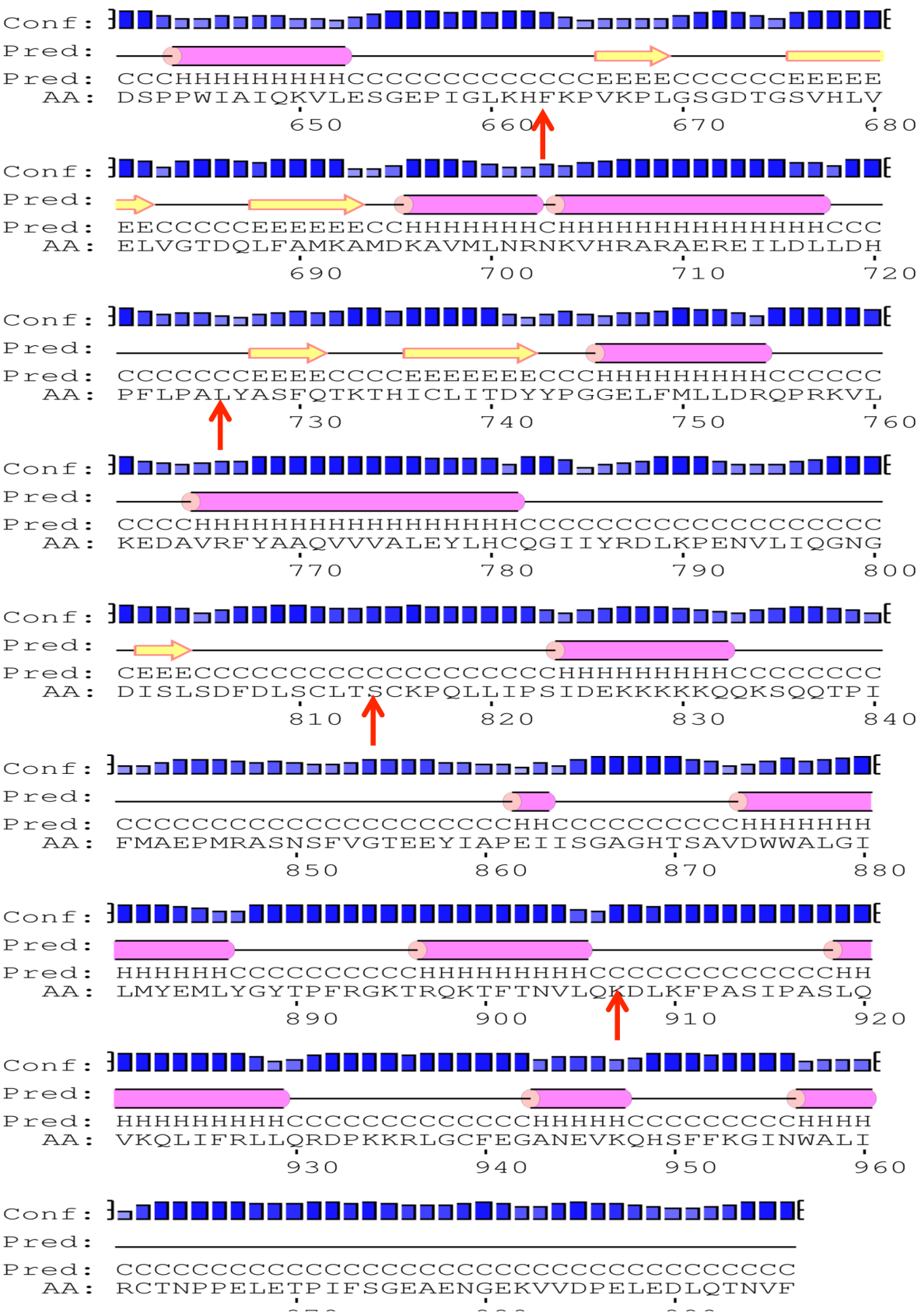


Figure 4.8: Predicted secondary structure of the phot1 kinase domain

Amino acid sequence of phot1 kinase domain was run through the PSIPRED software to generate a secondary structure prediction. α-helices are indicated in pink, ß-strands are indicated in yellow and the black lines indicate coil structure. The red arrows indicate the position of the phot1 truncations used to analyse membrane localisation.

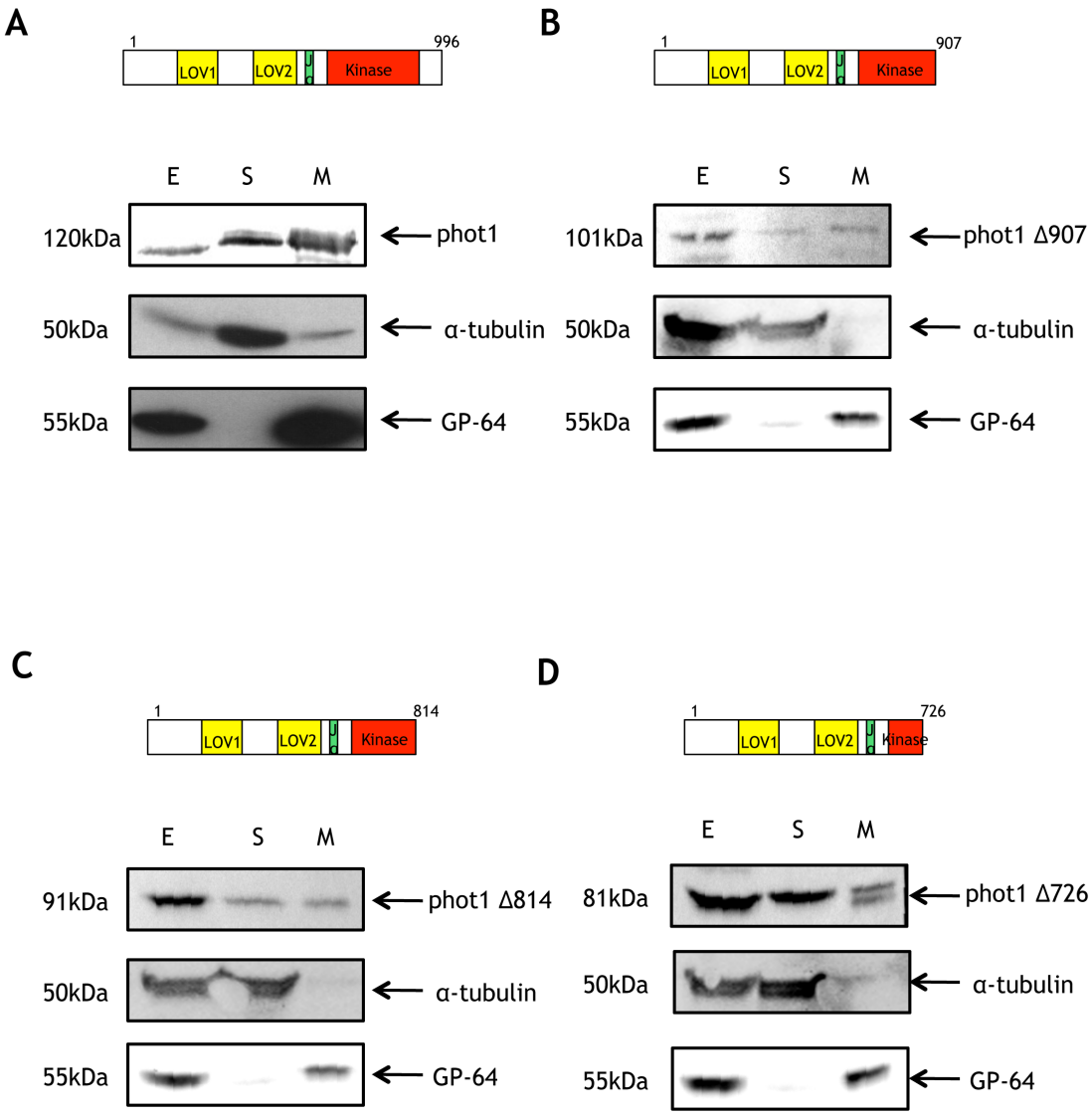


Figure 4.9: Truncation analysis of the phot1 kinase domain

A) Western blot analysis of phot1 wild-type protein fractions isolated from virally infected insect cells. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. Anti-His antibody was used to detect phot1 in each fraction. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64. Schematic diagram illustrating the amino acid positions of the full length phot1 construct is shown above.

B) Western blot analysis of phot1 truncated at amino acid position 907 protein fractions isolated from virally infected insect cells as performed as in A.

C) Western blot analysis of phot1 truncated at amino acid position 814 protein fractions isolated from virally infected insect cells as performed as in A.

D) Western blot analysis of phot1 truncated at amino acid position 726 protein fractions isolated from virally infected insect cells as performed as in A.

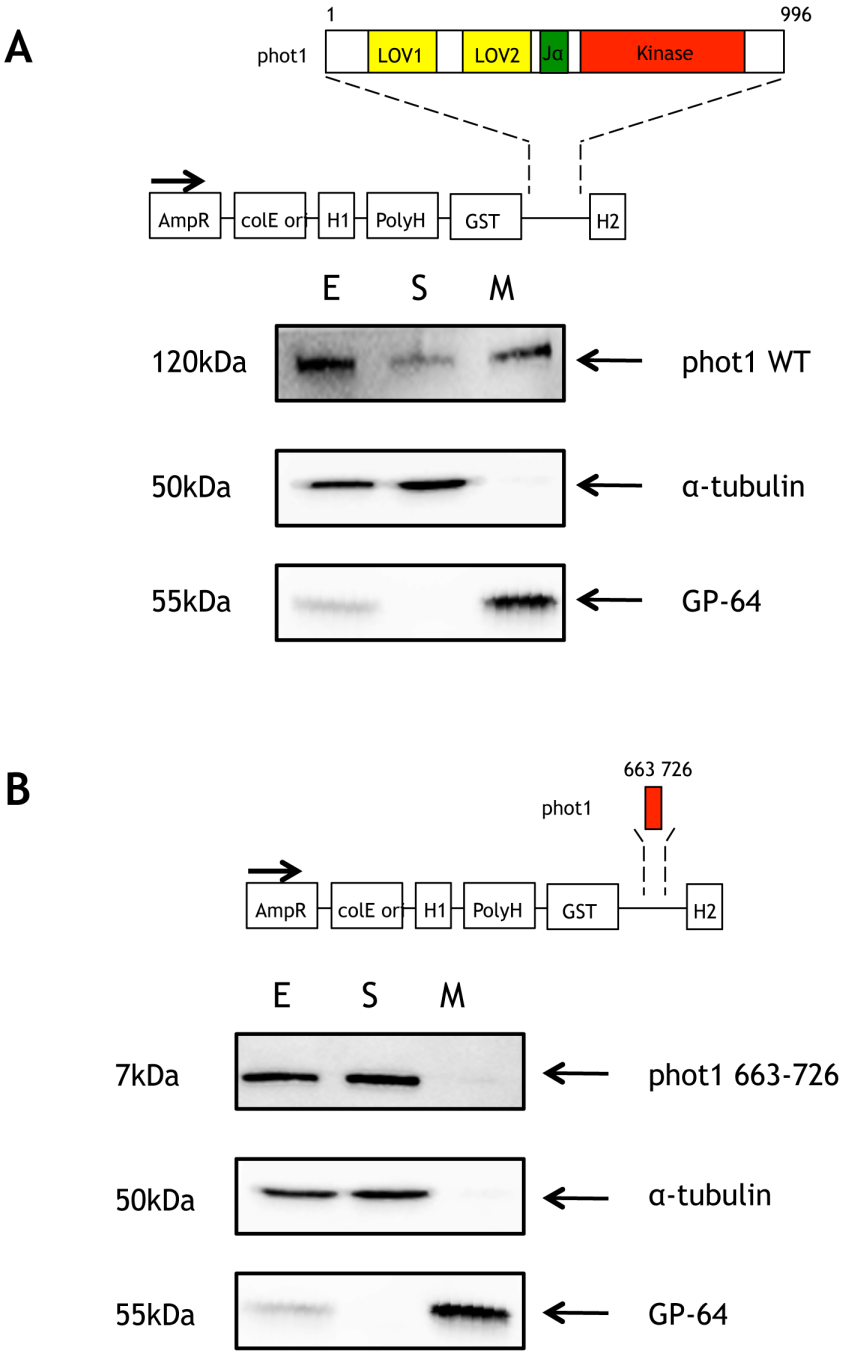


Figure 4.10: Localisation of GST-phot1 and GST-663-726 in insect cells

A) Western blot analysis on protein fractions isolated from virally infected insect cells expressing a GST-phot1 fusion. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. Anti-GST antibody was used to detect phot1 in each fraction. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64.

B) Western blot analysis on protein fractions isolated from virally infected insect cells expressing a GST-663-726 fusion. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. Anti-GST antibody was used to detect phot1 in each fraction. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64.

4.2.7 Truncation analysis of the phot1 kinase domain in Nicotiana benthamiana

Truncation analysis of phot1 suggested that regions upstream of amino acid position 726 were important for membrane localisation. However 663-726 is not sufficient to drive phot1 to associate with the membrane. We therefore rationalised that localisation of phot1 would perhaps be different in *N. benthamiana* since Kong et al. (2013) showed that localisation of their proposed membrane association region was different in *Arabidopsis* compared to yeast. As shown in Fig 4.11A (and Fig 4.1C), wild-type phot1 fused to GFP localises only to the membrane in darkness. In contrast, GFP expressed alone localises to the nucleus as well as the cytoplasm as indicated by the arrows (Fig 4.11B). This acts as a control for a nuclear protein as wild-type phot1 fused to GFP has only been detected at the membrane and never in the nucleus (Sakamoto & Briggs 2002; Wan et al. 2008; Kaiserli et al. 2009; Aggarwal et al. 2014). Detection of phot1 truncations in the nucleus of *N. benthamiana* would indicate different subcellular localisation from wild-type phot1 and therefore suggest that the truncated protein had lost the ability to target to the plasma membrane. Alternatively the GFP may have been cleaved from the phot1 protein, which could be measured by western blotting. However difficulties were experienced when extracting phot1 proteins from *N. benthamiana* due to the presence of high concentrations of phenolic compounds when the tissue sample was extracted.

Transient expression of phot1 663-726 in the epidermal cells of *N. benthamiana* shows localisation to the nucleus but also the appearance of some punctate GFP in the cytoplasm (Fig 4.11C). To investigate if phot1 663-726-GFP localised elsewhere in the cell, a Z stack image of the cell was taken. A Z-projection of the cell shows that phot1 663-726-GFP is found at the membrane but also within the nucleus, as well as cytosolic strands (Fig 4.11D). This confirms the result from the insect cells that 663-726 is not sufficient to target phot1 to the membrane. Therefore a longer region of phot1 was investigated for its ability to target the protein to the membrane. The region from amino acid 663 to 814 was fused to GFP and transiently expressed in the epidermal cells of *N. benthamiana*. This shows localisation to the plasma membrane but there is also

visualisation of cytosolic strands that are usually seen after illumination with blue light (Fig. 4.11E). A Z-projection of a cell expressing phot1 663-814-GFP shows that, similar to phot1 663-726-GFP, the protein is found localised within the nucleus as well as the visualisation of cytosolic strands (Fig. 4.11F). Again this shows that phot1 cannot be exclusively targeted to the membrane by the region from amino acid 663 to 814.

This lead to us rationalising that truncations from the N terminus of the kinase domain of phot1 may provide further information as to the membrane-targeting region of phot1. Therefore the region from amino acid 726 to amino acid 996, which is the end of the protein including the extreme C-terminus, was fused to GFP and used for transient expression in the epidermal cells of *N. benthamiana*. Fig 4.11G shows the protein localises mostly to the plasma membrane, however there is also punctate staining of around the membrane and some GFP staining in the cytoplasm. A Z-projection of cells expressing phot1 726-996-GFP shows that the protein localises to the nucleus as well as the plasma membrane. This suggests that the entire kinase domain is required for targeting phot1 to the membrane as expression of the kinase domain alone shows membrane localisation (Fig 4.2C) with no fluorescence in the nucleus.

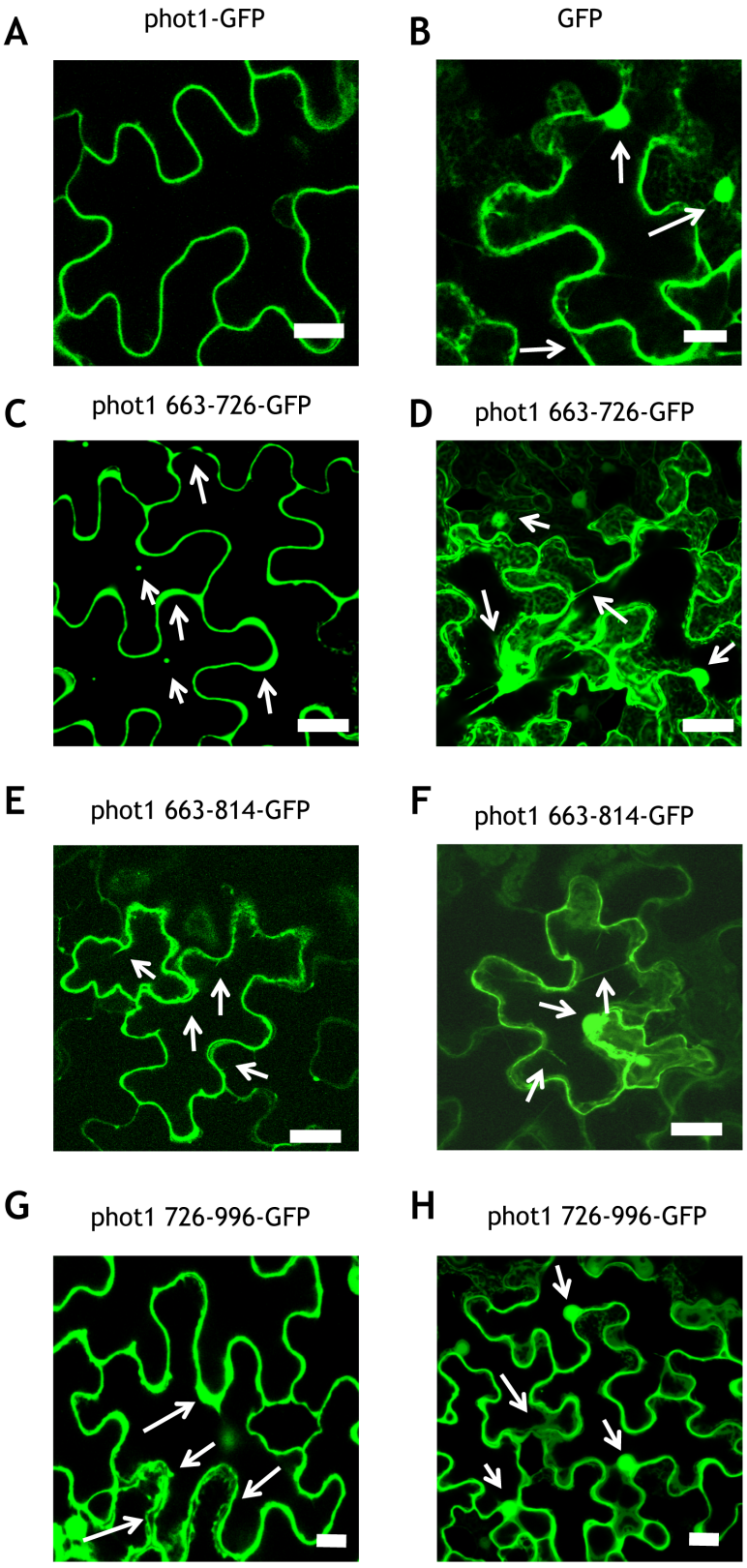

A
phot1-GFP
B
GFP
C
phot1 663-726-GFP
D
phot1 663-726-GFP
E
phot1 663-814-GFP
F
phot1 663-814-GFP
G
phot1 726-996-GFP
H
phot1 726-996-GFP

Figure 4.11: Localisation of phot1 kinase truncations in *Nicotiana benthamiana* in darkness

A) Transient expression of phot1 WT in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. phot1 WT is localised to the plasma membrane in darkness. Scale bar represents 20 µm.

B) Transient expression of GFP in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Free GFP is found in the nucleus as well as the cytoplasm in darkness. Arrows indicate GFP in the nucleus and in the cytoplasm. Scale bar represents 20 µm.

C) Transient expression of phot1 663-726 in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Phot1 663-726 localises to the plasma membrane and as well as GFP visualised in the cytoplasm as shown by the arrows. Scale bar represents 20 µm.

D) Transient expression of phot1 663-726-GFP in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Image shows a projection of the sum of a Z stack. Arrows indicate nucleus and cytosolic protein. Scale bar represents 20 µm.

E) Transient expression of phot1 663-814 in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Phot1 663-814 localises to the plasma membrane and as well as cytosolic strands visualised in darkness as shown by the arrows. Scale bar represents 20 µm.

F) Transient expression of phot1 663-814-GFP in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Image shows a projection of the sum of a Z stack. Arrows indicate nucleus and cytosolic protein. Scale bar represents 20 µm.

G) Transient expression of phot1 726-996 in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Phot1 726-996 localises to the plasma membrane and as well as cytosolic strands visualised in darkness as shown by the arrows. Scale bar represents 20 µm.

H) Transient expression of phot1 726-996 in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Image shows a projection of the sum of a Z stack. Arrows indicate nucleus and cytosolic protein. Scale bar represents 20 µm.

4.2.8 Truncation analysis of phot2 truncations on membrane localisation in insect cells

Since the truncation analysis of phot1 in insect cells and *N. benthamiana* resulted in different membrane localisation patterns from the results obtained in a recent study using the yeast CytoTrap system (Kong, Kagawa, et al., 2013), truncation analysis of the phot2 kinase domain was performed to assess its localisation pattern in insect cells in comparison to phot1. Protein extracts of wild-type phot2 expressed in insect cells shows localisation to the membrane as well as the soluble fraction (Fig 4.12A) similar to the localisation of phot1 (Fig. 4.9A) illustrating that the insect cell system is also suitable for assessing membrane localisation of phot2. The membrane marker GP-64 and soluble marker α-tubulin confirm that there is no contamination in either fraction. The

first truncation investigated was at amino acid position 640, which is comparable with the truncation of phot1 at amino acid 726 (Fig 4.9D). Protein extracts from insect cells infected with phot2 640 also localise to the membrane similar to wild-type phot2 (Fig. 4.12B). Again there is no contamination of the membrane or soluble fractions as shown by the use of the membrane marker GP-64 which is only detected in the membrane fraction and α-tubulin, the soluble marker, which is only detected in the soluble fraction. This shows that the truncation of phot2 at amino acid 640 retains membrane association. The signal detected at the membrane is lower than that of the soluble fraction, which may be due to less of the protein localising to the membrane. This suggests that the kinase domain including the C-terminus does have a role to play in targeting phot2 to the plasma membrane in insect cells.

The second truncation was a deletion of the entire kinase domain, which is at amino acid position 534 (phot2 ΔK), as defined by sequence alignments (Fig. 4.6). Protein extracts from insect cells expressing the phot2 ΔK show localisation to the membrane (Fig 4.12C). This is in contrast to the phot1 ΔK deletion that results in only soluble protein being detected (Fig 4.1B) and is also different to the membrane localisation found for phot2 expressed in yeast (Kong, Kagawa, et al., 2013). The finding that phot2 lacking the kinase domain still localises to the membrane cannot be attributed to contamination as the membrane marker GP-64 is only detected in the membrane fraction whilst α-tubulin, the soluble marker, is only detected in the soluble fraction. These findings suggest that at least in the insect cell system, deletion of the kinase domain of phot2 does not exclusively abolish the ability of the protein to attach to the membrane.

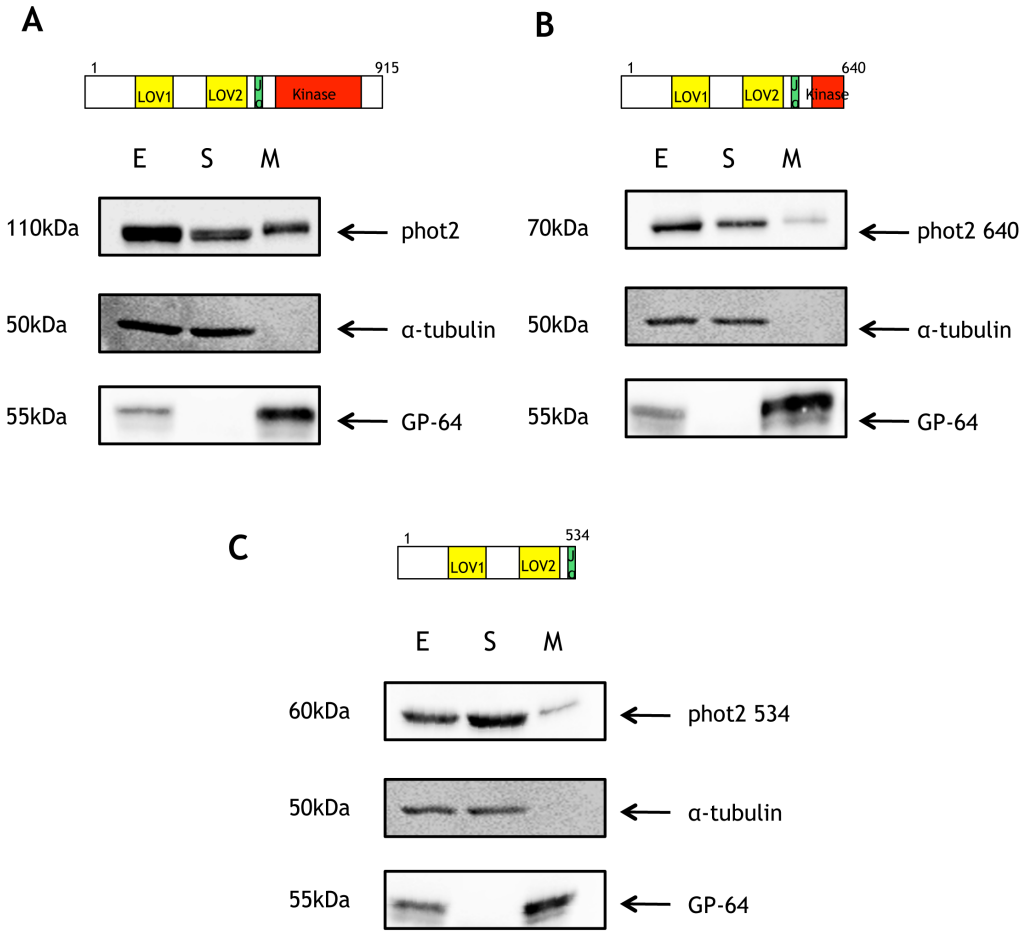


Figure 4.12: Truncation analysis of the phot2 kinase domain

A) Western blot analysis of phot2 wild-type protein fractions isolated from virally infected insect cells. Protein extracts (E) were fractionated into soluble (S) and membrane (M) fractions by ultra-centrifugation. Anti-His antibody was used to detect phot2 in each fraction. Purity of each fraction was measured by using antibodies against the soluble marker α-tubulin and membrane associated GP-64. Schematic diagram illustrating the amino acid positions of the phot2 construct are shown above.

B) Western blot analysis of phot2 truncated at amino acid position 640 protein fractions isolated from virally infected insect cells. Western analysis was carried out as described in A.

C) Western blot analysis of phot2 truncated at amino acid position 534 (ΔK) protein fractions isolated from virally infected insect cells. Western analysis was carried out as described in A.

4.2.9 Treatment with 1-butanol affects membrane association of phototropins

The truncation analysis of both phot1 and phot2 revealed the membrane association of the phototropins to be more complex than a single region of interaction. To further investigate the membrane association of the phototropins, the phospholipase D (PLD) inhibitor 1-butanol was used to treat etiolated *Arabidopsis* seedlings before visualisation of the GFP tagged protein by confocal microscopy. Three-day-old etiolated seedlings were submerged in 0.8% 1-butanol for one hour prior to imaging. 1-butanol affects the ability of PLD to generate the important secondary messenger phosphatidic acid (PA) by competing with water as a substrate to form phosphatidylbutanol rather than PA, whilst 2-butanol does not interfere with this pathway (Gardiner et al., 2003). PLD is involved in a number of different processes within the plant including seedling development, pollen tube growth and abscisic acid induced stomatal closure (Wang, 2005). Since the solubilisation analysis in Section 3.2.3 indicated that phot1 might interact with lipids we investigated this further to see if the phot1 6xK-N-GFP and Otphot-GFP lines also exhibited sensitivity to 1-butanol.

Phot1-GFP is known to internalise in the absence of a blue light stimulus when seedlings are exposed to 1-butanol (Sullivan et al., 2010) and was therefore used as a control for the localisation pattern of phot1 6xK-N-GFP and Otphot-GFP seedlings treated with 1-butanol. As expected phot1-GFP shows cytosolic strands in the absence of a blue light stimulus whereas when seedlings are treated with 2-butanol, phot1-GFP remains localised to the plasma membrane in darkness (Fig. 4.13A and B). Treatment of phot1 6xK-N also results in cytosolic strands being visualised in darkness, line 18-7 is shown as representative of the phot1 6xK-N lines (Fig 4.13C). However it appears to be more severe than phot1-GFP as there is GFP visualised within the entire cell suggesting that although the LRM is not responsible for membrane association of phot1 (Fig. 3.7A), it may have another role to play in the interaction. 2-butanol does not show this response with phot1 6xK-N-GFP remaining localised to the plasma membrane in darkness (Fig. 4.13D). The same is seen for Otphot *Arabidopsis* seedlings treated with the 1-butanol inhibitor. Treatment with 1-butanol results in relocalisation of Otphot

from the membrane in darkness as well as visualisation of GFP throughout the cell (Fig 4.13E) a response that is absent when seedlings are treated with the control 2-butanol (Fig. 4.13F). This adds more evidence that lower plant phototropins associate with the plasma membrane with the same mechanism as that of higher plants. It also suggests that although both phot1 6xK-N-GFP and Otphot are associated with the plasma membrane, the interaction is weaker than wild-type *Arabidopsis* phot1.

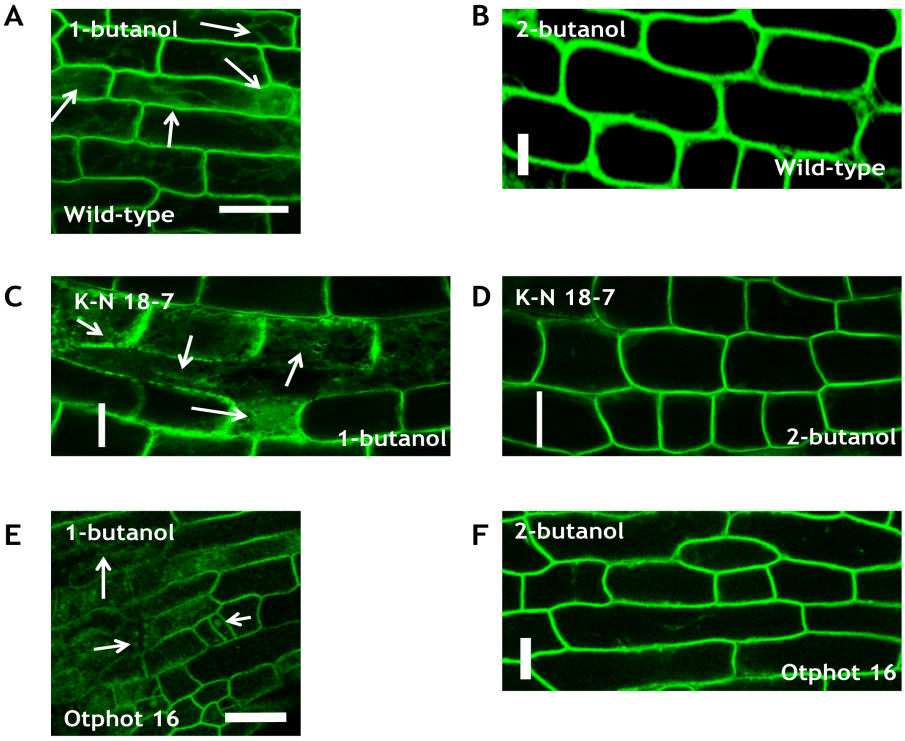


Figure 4.13: Localisation of GFP tagged phototropins treated in etiolated *Arabidopsis* seedlings treated with inhibitors

A) Three-day-old phot1-GFP seedlings were exposed to 1-butanol (0.8%) for one hour prior to imaging. Phot1-GFP is induced to relocalise in darkness with cytosolic strands visualised prior to illumination.

B) Three-day-old phot1-GFP seedlings exposed to 2-butanol (0.8%) for one hour do not relocalise in darkness with phot1-GFP localising to the membrane and no cytosolic strands visualised.

C) Three-day-old phot1 6xK-N-GFP seedlings were treated as in A. Phot1 6xK-N-GFP is induced to relocalise in darkness with cytosolic strands visualised prior to illumination.

D) Three-day-old phot1 6xK-N-GFP seedlings treated as in B do not relocalise in darkness with phot1-GFP localising to the membrane and no cytosolic strands visualised.

E) Three-day-old Otphot-GFP seedlings were treated as in A. Phot1 6xK-N-GFP is induced to relocalise in darkness with cytosolic strands visualised prior to illumination.

F) Three-day-old Otphot-GFP seedlings treated as in B do not relocalise in darkness with phot1-GFP localising to the membrane and no cytosolic strands visualised.

4.3 Discussion

4.3.1 Kinase domain of phot1 is sufficient for membrane localisation

Phototropins are a member of the large cyclic AMP dependent kinases (PKA), cGMP-dependent kinases, and the diacylglycerol- activated/phospholipid-dependent kinase PKC (AGC) kinase family, which are involved in a number of responses in plants (Rademacher and Offringa, 2012). This family of proteins is known to localise to the plasma membrane, with the C-terminus of the AGC kinases PINOID, WAG1 and WAG2 localising to the plasma membrane in *Arabidopsis* protoplasts (Galván-Ampudia and Offringa, 2007). This suggests that the highly conserved C-terminus of AGC kinases is involved in directing subcellular localisation (Anthony et al., 2004; Lee and Cho, 2006; Zhang et al., 2009). Expression of the C-terminal kinase domain of phot2 is known to direct localisation to the plasma membrane in *Arabidopsis* protoplasts as well as transgenic lines expressing a GFP fusion (Kong et al., 2006, Kong et. al, 2007). The autophosphorylation activity of the phot1 kinase domain also has a role to play in regulating the subcellular localisation of the receptor, with the kinase inactive version of phot1 remaining localised to the membrane following illumination (Inoue et al., 2008, Kaiserli et al., 2009). We first examined localisation of the phot1 kinase domain alone, which resulted in membrane localisation in insect cells (Fig 4.2B) whilst expression of the photosensory domain alone resulted in soluble protein being detected (Fig 4.1B). This localisation pattern is consistent with the localisation of the phot2 kinase domain to the membrane of transgenic *Arabidopsis* (Kong et al., 2007). Further evidence for a role of the kinase domain in directing membrane localisation comes from yeast and the CytoTrap system where the kinase domain of both phot1 and phot2 allow growth at restrictive temperatures indicating that they are sufficient to target to the plasma membrane (Kong, Kagawa, et al., 2013). This confirms that within the kinase domain there is likely a membrane localisation signal for both phot1 and phot2. Interestingly the first truncation of phot1 at amino acid 952 results in the phot1 protein retaining membrane association in insect cells (Fig 4.3B). However assessment of the autophosphorylation activity showed that the protein is no longer active (Fig 4.5B). The C-terminus of the kinase domain has so far only had one

phosphorylation site identified at amino acid position 993 (Inoue et al., 2008). It would seem unlikely that losing one phosphorylation site would cause a loss of activity. Kagawa et al. (2004) showed that deletion of a 20 amino acid sequence at the C-terminus of phot2 from the fern *Adiantum capillus-veneris* L. did not change the chloroplast accumulation response, whereas deletion of 40 amino acids resulted in no accumulation of the chloroplast under high light conditions. Alignment of the proteins shows that this region is close to the end of phot1ΔC, suggesting that this region although not controlling membrane localisation is important for function of the photoreceptor. This loss in activity observed for phot1 952 could be attributed to the truncation being within the kinase domain itself. Further truncations at the C-terminus illustrate that the truncation at amino acid 952 is indeed most likely to be within the kinase domain itself. The two truncations (amino acids 972 and 964) retain autophosphorylation activity, albeit at a reduced level. This could be attributed to the phosphorylation site at amino acid 993 being absent from the protein, resulting in slight reduction in activity (Inoue et al., 2008) although the sites in the C-terminus may not be involved in the function of phot1 as it appears to be serine-851 in the activation loop that is the significant phosphorylation site for function of the phot1 protein (Inoue et al., 2008).

Kong et al. (2013) used the yeast CytoTrap system to narrow down the region of membrane interaction of phot1 to the C-terminus of the protein, specifically to a small region of phot1 from amino acid 920 to amino acid 996. However expression of phot1 with a truncation at amino acid 952 in insect cells results in the protein being detected in the membrane fraction (Fig. 4.3B), which was confirmed with detection at the membrane in *N. benthamiana* (Fig 4.3). The difference in the localisation pattern between the two studies could be attributed to a number of factors. Kong et al. (2013) found that the results from the yeast CytoTrap system were not directly comparable to the localisation pattern when the same constructs were expressed in *Arabidopsis* mesophyll protoplasts or in transgenic *Arabidopsis*. Deletion of an 84 amino acid sequence from the C-terminus of phot2 resulted in membrane localisation in yeast, however upon moving to the protoplast system the same deletion was found to localise in the cytosol similar to the GFP alone control. The authors also found that fractionation placed the Δ84 construct in the membrane fraction which they

attribute to the activity of the protein not being sufficient to solely localise to the membrane. This suggests that the proteins are behaving differently *in vitro* when compared to *in vivo* and should be interpreted carefully. Another possible explanation for the difference in results between the insect cell system and yeast is that they are different eukaryotic cells. Whilst both yeast and insect cells are eukaryotes, insect cells are a higher system than yeast and are able to carry out more complex post-translational modification whilst the insect cell system also has the best machinery for protein folding (Fernandez and Hoeffler, 1999) compared to yeast. Insect cells are the only known heterologous system where full-length *Arabidopsis* phototropins are active (Christie et al., 1998, Jones et al., 2007). The differences between yeast and insect cells may result in phot1 protein folding differently in yeast and changing the localisation pattern compared to native protein. In their study Kong et al. (2013) only expressed phot2 truncations in *Arabidopsis*. The truncations did not behave as expected from the yeast results with phot2 Δ42 not localising to the membrane in yeast and retaining residual membrane association in *Arabidopsis*. The reason for this is not commented on other than to suggest that this region is required for the chloroplast response of phot2, but the protein is unlikely to be functional as the kinase domain will be inactive. The difference in localisation patterns of phot1 and phot2 may also be a consequence of the different signals required for internalisation of phot1 and phot2 to different subcellular compartments, namely cytosolic strands for phot1 (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008, Kaiserli et al., 2009) and the Golgi apparatus for phot2 (Kong et al. 2006; Kong et al. 2007; Aggarwal et al. 2014).

4.3.2 Truncation analysis of phot1 does not reveal a possible amino acid region involved in membrane association

Investigation of the region of phot1 that interacts with the membrane did not yield conclusive results. Truncation analysis of the kinase domain initially indicated that there is perhaps a 63 amino acid region from the beginning of the kinase domain from amino acid 663 to amino acid 726 that directs phot1 localisation to the plasma membrane in insect cells (Figs. 4.9B-D). However expression of this small region alone resulted in only soluble protein being detected at the membrane in insect cells (Fig. 4.10B) and importantly when

transiently expressed in *N. benthamiana* (Fig 4.11C). There was a difference between the two constructs, with the insect cell construct containing the GST tag at the N-terminus of the protein whereas the *N. benthamiana* construct had a C-terminal tag. This may also have interfered with membrane localisation as the larger tags may have masked such a small sequence of protein. This could be overcome by having a linker region between the phot1 sequence and the tag to prevent any steric hindrance. It was important to examine localisation in both systems as it is essential to ensure that what is seen in the insect cells can be reproduced *in planta*.

Given that the truncation analysis of the phot1 kinase domain did not reveal a specific region of interaction, similar truncations were performed in phot2. Fig 4.12C shows that deletion of the kinase domain results in reduced affinity of phot2 to associate with the plasma membrane. Kong et al., (2007) also found that deletion of the kinase domain resulted in some protein associating with the plasma membrane in *Arabidopsis*. The authors also noted that there is some visualisation of punctate staining under dark conditions when the kinase domain alone was expressed, suggesting that there may be other factors involved in targeting phototropins to the membrane. These are not pure membranes that are examined after fractionation therefore there may be other membranes contaminants in the preparation where the protein could be localising. Purification of the plasma membrane specifically would indicate which membrane compartment the proteins are specifically localising to. The localisation of the phot2 kinase domain alone would need to be examined in the insect cell system and in *N. benthamiana* to fully elucidate the interaction of phot2 and the membrane.

A recent study of another hydrophilic membrane protein, SYMBIOTIC REMORIN 1 (SYMREM1) from *Medicago truncatula*, showed that a C-terminal region was sufficient to direct the protein to the membrane of *M. truncatula* roots (Konrad et al., 2014). The authors then identified a S-acylation site in the C-terminus of the protein. Mutation of the conserved cysteine residue of the S-acylation site to alanine resulted in membrane localisation of the full-length protein, but the same mutation in the C-terminal region resulted in loss of the membrane association. This illustrates that it is not only protein sequence that is important

for directing protein subcellular localisation but also post-translational modifications to the protein itself that can be involved in associating proteins to the membrane. Another protein that requires post-translational modifications for membrane association is the ADP ribosylation factors (Arfs) (Liu, Kahn and Prestegard, 2009). These proteins are found in eukaryotes from yeast to humans where they are involved in vesicle transport (D'Souza-Schorey and Chavrier, 2006) and undergo myristolaytion. Truncation of the protein and a lack of myristolyation results in impaired membrane binding (Liu, Kahn and Prestegard, 2010) suggesting that it is not only one factor that plays a role in the localisation of a protein to the membrane.

To date the only post-translational modification of phot1 other than phosphorylation that has been identified is ubiquitination by Non-Phototropic Hypocotyl 3 (NPH3) at high light intensities (Roberts et al., 2011) and more recently a lysine residue in the LOV2 domain was identified as a putative ubiquitination site (Deng et al., 2014). Post-translational modifications have an important role to play in plant growth, development and environmental responses (Running, 2014) as well as a secondary role in supporting protein-protein interactions between the hydrophobic chain of the protein and a hydrophobic region of an accessory protein (Thompson and Okuyama, 2000), although it is not likely that phot1 associates with the membrane via another protein as discussed in Chapter 3. Plant growth and development are largely controlled by the hormone auxin (Teale, Paponov and Palme, 2006), which is also involved in the phototropism response of the phototropins. Pin-formed proteins (PIN) are auxin efflux carriers (Krecek et al., 2009) found at the plasma membrane which also undergo post-translational modifications (Löfke, Luschnig and Kleine-Vehn, 2013). The localisation of the PIN2 protein specifically has been shown to be dependent on phot1 and NPH3 (Wan et al., 2012). Given the interplay between phototropins and auxin at the membrane and the difficulty with identifying a specific region of phot1 that interacts with the membrane there is a strong indication that phot1 and perhaps phot2 undergo post-translational modifications, other than phosphorylation, to associate with the membrane. There are also the physiological responses that are controlled by the phototropin proteins that serve to increase growth. To further investigate this, any putative post-translational modifications sites would need to be mutated

and the proteins expressed in either the insect cell system or in *N. benthamiana* to assess the localisation pattern before solid conclusions could be drawn.

4.3.3 Treatment with 1-butanol results in internalisation of phot1 in the absence of a light stimulus

Truncation analysis of the kinase domain of phot1 did not reveal a specific region of interaction indicating that the membrane association of phot1 is more complicated than previously thought. The solubilisation analysis in section 3.2.3 indicated that perhaps phot1 interacts with the plasma membrane via a lipid. Sullivan et al., (2010) have shown that treatment with 1-butanol results in internalisation of phot1-GFP from the plasma membrane in the absence of a blue light stimulus. 1-butanol acts by competing with water as a substrate for PLD, resulting in a depletion of the lipid PA providing further evidence that phot1 interacts with the membrane via a lipid. Treatment of the phot1 6xK-N-GFP and Otphot-GFP lines with 1-butanol showed that, similar to phot1-GFP, the proteins internalise to cytosolic strands in the absence of a blue light stimulus (Fig. 4.13C and Fig. 4.13E). The internalisation also appears to be greater than the internalisation observed in the phot1-GFP line (Fig. 4.13A), suggesting that phot1 6xK-N-GFP and Otphot-GFP are more sensitive to 1-butanol. The sensitivity of phot1 6xK-N-GFP and Otphot-GFP to reduced lipid levels in the cells could be measured by reducing the concentration of 1-butanol that the seedlings were treated with or reducing the time of exposure to the inhibitor, before visualisation of the internalisation. Another response that can be measured is phototropism. Treatment of phot1-GFP lines with 1-butanol results in a loss of phototropism (Sullivan et al., 2010), therefore phototropism could be measured in the phot1 6xK-N-GFP seedlings exposed to 1-butanol. By varying the concentration of 1-butanol and measuring hypocotyl curvature we would be able to determine the concentration at which the phot1 6xK-N-GFP seedlings are sensitive to 1-butanol, when compared to phot1-GFP. The proposed interaction of phot1 with a lipid needs further investigation. Now that phot1 can be solubilised from insect cell membranes using detergents (Fig. 3.4), phot1 can hopefully be purified in an active form for lipid chip analysis. Comparison of wild-type phot1 and phot1 6xK-N would be of interest to understand if the LRM is involved in membrane binding affinity.

Chapter 5: Artificial Anchoring of Phototropin 1 to the Plasma Membrane

5.1 Introduction

Phot1 and phot2 are both known to associate with the plasma membrane when expressed in insect cells (Fig. 3.1B), when transiently expressed in *Nicotiana benthamina* and in transgenic *Arabidopsis* (Sakamoto & Briggs 2002; Wan et al. 2008; Kaiserli et al. 2009; Aggarwal et al. 2014; Kong et al. 2006; Kong et al. 2007). After blue light illumination in transgenic *Arabidopsis* there is partial internalisation of phot2-GFP to the Golgi, a response that is controlled by the activity of the C-terminal kinase domain (Kong et al., 2007). The significance of this re-localisation is not known and in addition the functional relevance of the phot1 internalisation from the plasma membrane to cytosolic strands in response to blue light remains unanswered (Wan et al., 2008, Kaiserli et al., 2009). Only a small fraction of phot1 internalises in response to light, around 20% in the case of mustard phot1 (Knieb, Salomon and Rudiger, 2004), but the function of this internalisation to a different subcellular region is still unknown. Phototropism at low fluence rates of blue light has long been known to be enhanced by a red light pretreatment (Janoudi and Poff, 1991, 1992). This enhancement of phototropism was subsequently attributed to the red light photoreceptor phytochrome A (Whippo and Hangarter, 2004). Han et al. (2008) showed that the internalisation of phot1-GFP from the plasma membrane of hypocotyl cells was severely reduced when the *Arabidopsis* seedlings were given a brief pulse of red light 2 hours prior to imaging. This loss of internalisation from the plasma membrane has been suggested to be the reason that phototropism at low fluence rates of blue light is enhanced under red light. In this chapter, to further investigate the role of phot1 relocalisation from the plasma membrane, we constitutively anchored phot1 to the plasma membrane by addition of a lipid modification and examined the physiological responses controlled by phot1 in transgenic *Arabidopsis*.

5.2 Results

5.2.1 Construction of the farnesylated phot1-GFP plasmid

To constitutively anchor phot1 to the plasma membrane we employed the use of post-translational modifications that anchor proteins to the plasma membrane. The post-translational modification of proteins by prenylation, N-myristoylation or S-acylation can target proteins to a variety of subcellular membranes (Sorek, Bloch and Yalovsky, 2009). Prenylation is the addition of a farnesyl or geranylgeranyl molecule to conserved cysteine residues at the C-terminus of proteins (Running 2014). Consequently, prenylation increases hydrophobicity resulting in the membrane association of proteins (Marshall 1993). Geranylgeranyl modification occurs on the conserved sequence CaaX where X is usually leucine (Leung, Baron and Seabra, 2006) whereas farnesyl modification occurs on the sequence CaaX where X can be alanine, cysteine, glutamine, methionine or serine (Running, 2014). Myristoylation has been used as a modification to target BKI1, an interactor of the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1), to the membrane (Wang and Chory, 2006).

In this chapter a farnesylation sequence was added to the C-terminus of a phot1-GFP fusion to attempt to anchor the protein to the plasma membrane and prevent light-dependent internalisation from the plasma membrane. The farnesylation sequence was fused to the C-terminus of GFP to help prevent interference of the farnesyl tag on phot1 protein function. The binary expression vector pER(K)-LN was used previously to express a number of phot1-GFP fusion proteins (Christie et al., 2002, Cho et al., 2007, Kaiserli et al., 2009) however protein expression is driven by the constitutive *CaMV-35S* promoter. Previous work investigating phot1 localisation in *Arabidopsis* was carried out using the native *PHOT1* promoter (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008), resulting in complementation of the *phot1-5* mutant. We therefore decided to express the farnesylated phot1-GFP under the control of the endogenous *Arabidopsis PHOT1* promoter to ensure correct spatial localisation in the seedlings. The previously generated phot1-GFP fusion was cloned using the entire genomic region of phot1 including upstream regions such as the 5'

untranslated region (Sakamoto and Briggs, 2002). Our strategy was to clone the native *PHOT1* promoter sequence alone as a phot1 cDNA was already available in the lab (Kaiserli et al., 2009). A transposable element was also identified in the 5' upstream untranslated region of the *PHOT1* promoter that could be important for controlling gene expression. The region chosen as the promoter was as close to the ATG start codon of phot1 as possible as this region may also be involved in regulating gene expression.

Transformation vectors for farnesylated phot1 lines and the respective mutated farnesyl controls were constructed using the modified binary expression vector pEZR(K)-LN as described previously (Kaiserli et al., 2009) to obtain a C-terminal GFP fusion. After successful amplification of the native *PHOT1* promoter region the *CaMV-35S* promoter was removed using restriction sites SacI and HindIII and replaced with the native *PHOT1* promoter region to generate the plasmid pEZR-pPHOT1. To create the farnesyl tag, the C-terminal GFP from pEZR(K)-LN was removed using restriction sites BamHI and XbaI. pEZR(K)-LN was used as a template to amplify a modified GFP with a farnesyl tag to generate a GFP coding sequence with a C-terminal farnesylation sequence. This was cloned into pEZR-pPHOT1 using the restriction sites BamHI and XbaI to generate the plasmid pEZR-pPHOT1-GFPfarn. Amino acid changes in the mutated farnesyl construct were introduced by site-directed mutagenesis.

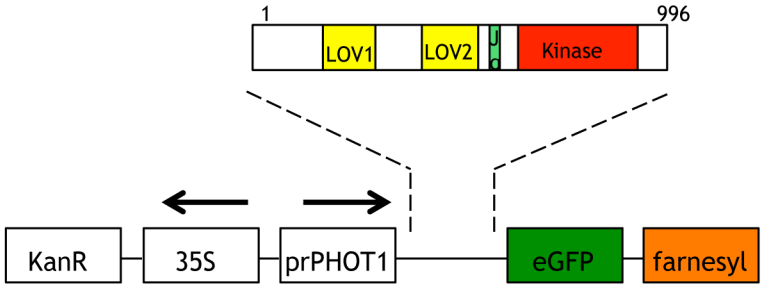


Figure 5.1: Schematic diagram of the farnesyl tagged phot1-GFP construct

Schematic diagram illustrating the construction of farnesyl-tagged phot1-GFP used to assess localisation. The native *PHOT1* promoter is used to drive expression of phot1-GFP, while the farnesyl tag was added to the C-terminus of the GFP to drive constitutive localisation to the plasma membrane.

5.2.2 Addition of a farnesylation sequence to phot1 results in constitutive association with the plasma membrane

The *N. benthamiana* system has been successfully employed to examine localisation of various phototropin constructs prior to generating stable transgenic *Arabidopsis* lines (Kaiserli et al. 2009; Aggarwal et al. 2014; Fig 3.6B; Figure 4.9). Both phot1 and phot2 internalise in response to light treatment in *N. benthamiana* (Kaiserli et al. 2009; Aggarwal et al. 2014) demonstrating that this experimental system is useful for ensuring that the addition of the farnesyl tag results in constitutive localisation of phot1 to the plasma membrane even following blue light illumination. Localisation of farnesyl-tagged phot1-GFP was visualised three days following infiltration. Fig 5.2A shows that the blue light from the laser used to excite GFP causes internalisation of phot1-GFP from the plasma membrane, with cytosolic strands visualised up to nine minutes following initial excitation. Transient expression of farnesyl-tagged phot1-GFP in *N. benthamiana* epidermal cells showed localisation to the plasma membrane in darkness (Fig 5.2B) similar to phot1-GFP (Fig. 5.2A). However nine minutes following illumination with blue light, GFP is still visualised at the plasma membrane with no further increase in the visualisation of cytosolic strands illustrating that the addition of a farnesyl tag onto the phot1-GFP protein results in constitutive localisation to the plasma membrane and prevents the partial internalisation of the receptor following blue light illumination.

The investigation of physiological responses that may be affected by constitutive localisation of phot1 to the plasma membrane can only be investigated using stable *Arabidopsis* transgenic lines. Therefore stable lines expressing farnesyl-tagged phot1-GFP were generated in the *phot1-5 phot2-1* mutant background (Kinoshita, Doi and Suetsugu, 2001) and wild-type phot1-GFP with no modifications as described previously (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008) was used as a control. Homozygous T3 transgenic lines identified by kanamycin selection resulted in three independent lines expressing the fusion protein farnesyl-tagged phot1-GFP. These were designated farnesyl 23-3, farnesyl 24-3 and farnesyl 27-3 and were used to examine the subcellular localisation pattern of farnesyl-tagged phot1-GFP in *Arabidopsis* hypocotyls.

Protein levels in the three lines were first examined by western blot analysis. Fig. 5.3A shows that there is lower expression in the three farnesyl-tagged phot1-GFP lines compared to that in the phot1-GFP line, however the protein levels are still suitable for the transgenic lines to be analysed further. Use of the soluble protein marker UDP-glucose pyrophosphorylase (UGPase) (Komatsu et al., 2014) shows that there are equal protein loading across the samples. The localisation pattern of the phot1-GFP control was examined first in three-day-old etiolated *Arabidopsis* seedlings, both in darkness and 9 mins following blue light illumination. Fig 5.3B shows that phot1-GFP protein localises to the plasma membrane in darkness and subsequently there is partial internalisation in response to light, similar to what has been shown previously (Sakamoto and Briggs, 2002, Wan et al., 2008) and it is consistent with transient expression of phot1-GFP in *N. benthamiana* (Fig 5.2A). As shown in Fig. 5.3C examination of the three independent farnesyl-tagged phot1-GFP lines showed that the protein is localised to the plasma membrane and remains localised 9 mins following blue light illumination. This confirms that the addition of the farnesyl tag to the C-terminus of GFP results in constitutive plasma membrane localisation of phot1 similar to what was observed in *N. benthamiana* (Fig. 5.2B).

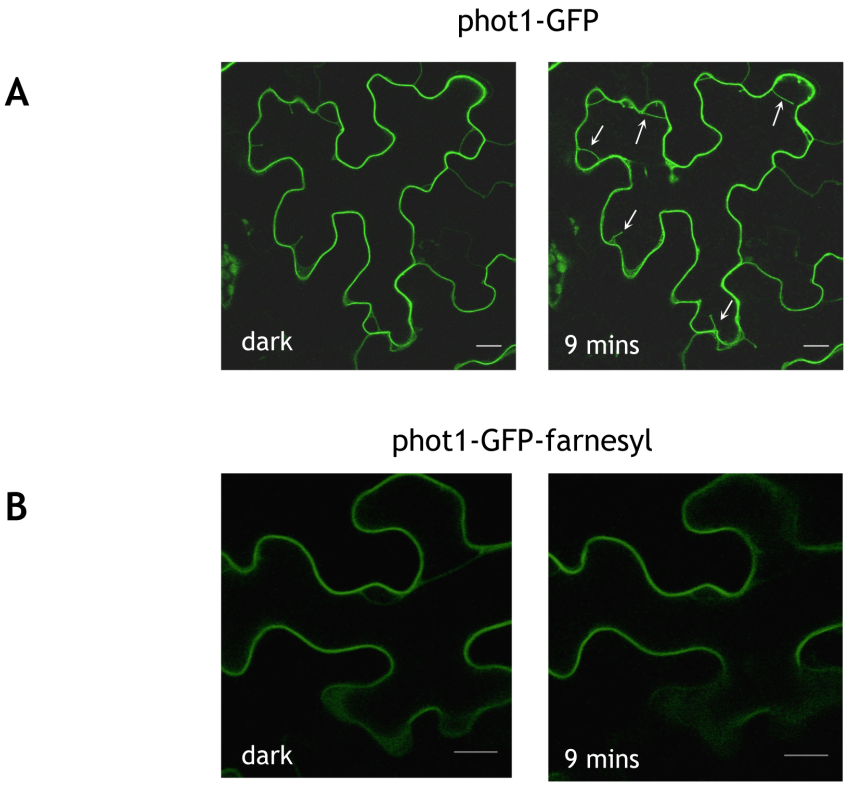


Figure 5.2: Farnesyl-tagged phot1-GFP remains localised to the plasma membrane in *Nicotiana Benthamiana*

A) Transient expression of wild-type phot1-GFP (phot1-GFP) in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Phot1-GFP is localised to the plasma membrane in darkness and the white arrows indicate internalisation in response to the blue light from the laser used to excite GFP. Scale bar represents 20 µm.

B) Confocal images of transiently expressed farnesyl tagged phot1-GFP (phot1-GFP-farnesyl) in *N. benthamiana* epidermal cells. Plants were transferred to darkness 16-18 hours before imaging. Farnesyl-tagged phot1-GFP remains localised to the plasma membrane 9 mins following illumination with blue light. Scale bar represents 20 µm.

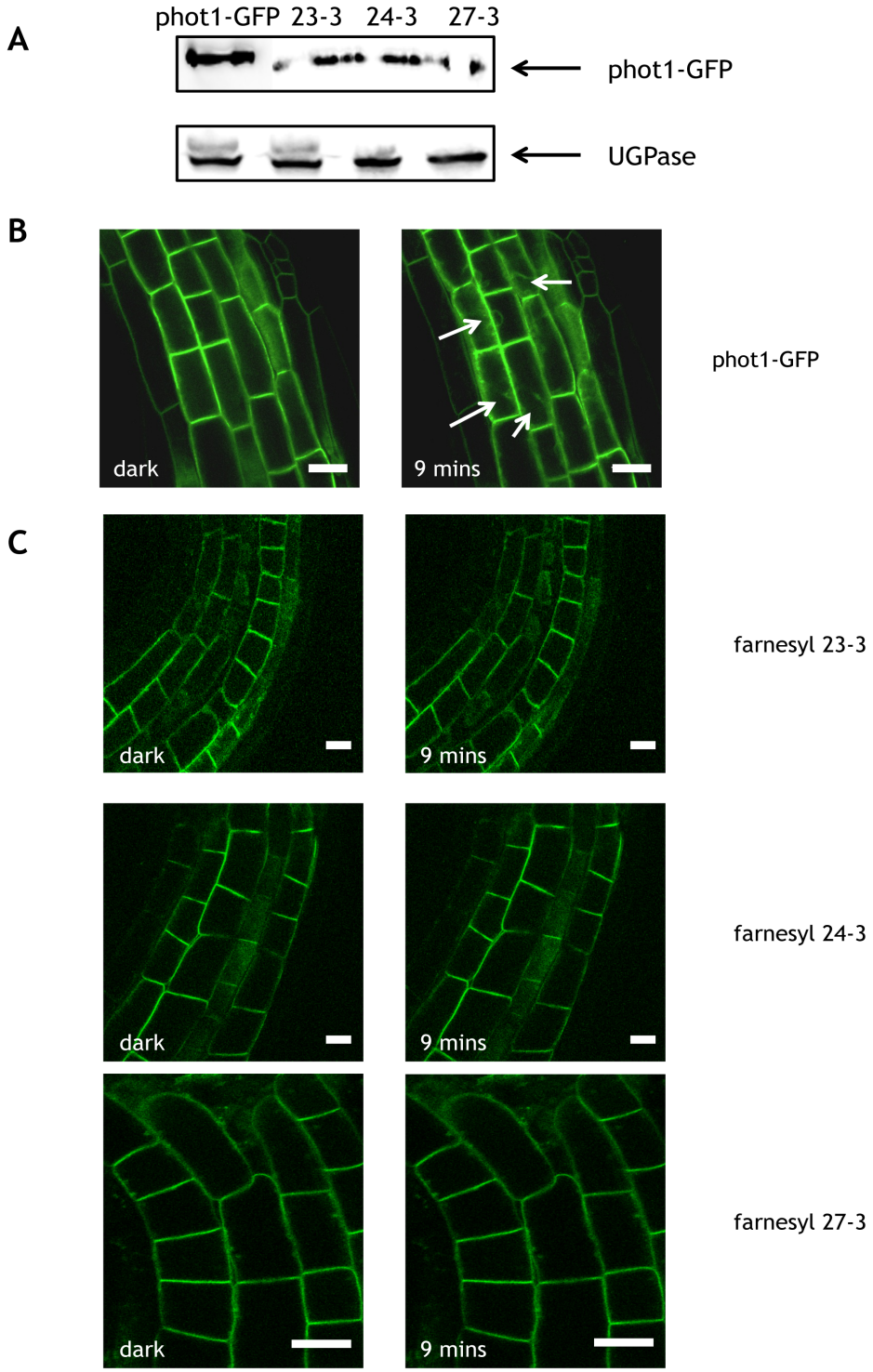


Figure 5.3: Farnesyl-tagged phot1-GFP remains localised to the plasma membrane in *Arabidopsis* seedlings following blue light excitation

A) Western blot analysis of protein levels in phot1-GFP and each of the farnesyl-tagged phot1-GFP lines (line 23-3, line 24-3 and line 27-3) probed with anti-GFP antibody. Western blot analysis using anti-UGPase antibody is shown below as a loading control.

B) Analysis of phot1-GFP localisation in three-day-old etiolated *Arabidopsis* seedlings. Confocal images of hypocotyl cells from the seedlings. Blue light used to excite GFP excites phot1-GFP causing internalisation from the plasma membrane as shown by the white arrows. Scale bar represents 20 μm.

C) Analysis of three independent farnesyl-tagged phot1-GFP localisation in three-day-old etiolated *Arabidopsis* seedlings. Confocal images of hypocotyl cells from the seedlings. Blue light used to excite GFP does not result in internalisation from the plasma membrane. Scale bar represents 20 μm.

5.2.3 Mutation of the conserved cysteine residue in farnesyl-tagged phot1-GFP results in the internalisation response being restored.

Anchoring of proteins to the plasma membrane with a farnesyl tag occurs via a conserved cysteine residue (Thompson and Okuyama, 2000). Mutation of this cysteine to an alanine residue within the conserved CAAX residue causes a loss of farnesylation and consequently a loss of the plasma membrane association. This mutation would act as a control for the farnesyl tag (Benetka et al., 2006) to ensure that the tag is not interfering with phot1-GFP localisation and function. The conserved cysteine residue within farnesyl-tagged phot1-GFP was therefore mutated to alanine by site-directed mutagenesis resulting in a mutated farnesyl-tagged phot1-GFP sequence. To assess the localisation pattern of the mutated farnesyl sequence, stable transgenic lines were created in the *phot1-5 phot2-1* background. Homozygous T3 transgenic lines were identified by kanamycin selection and designated: C-A 2-3, C-A 3-3 and C-A 10-5. Protein levels in these three lines are lower than phot1-GFP (Fig. 5.4A), similar to what was observed for farnesyl-tagged phot1-GFP lines (Fig. 5.3A). These lines were used to examine localisation of the mutated farnesyl-tagged phot1-GFP in three-day-old *Arabidopsis* seedlings by confocal microscopy, as before both in darkness and to assess internalisation from the plasma membrane after light exposure. Mutated farnesyl-tagged phot1-GFP (phot1-GFP farn C-A) localises to the plasma membrane in darkness (Fig 5.4B) similar to phot1-GFP (Fig. 5.3B) The blue light from the laser used to excite GFP also results in partial internalisation of mutated farnesyl-tagged phot1-GFP from the plasma membrane to cytosolic strands (Fig 5.4B). This demonstrates that the farnesyl tag is not interfering with the function of phot1-GFP and that the mutation of the conserved cysteine within the CAAX motif prevents farnesyation by the Farnesyltransferase enzyme and restores partial internalisation of phot1-GFP from the plasma membrane.

To further ensure that the farnesyl tag does interfere with phot1 function the autophosphorylation activity of one of the farnesyl lines and one of the mutated farnesyl lines was assessed indirectly by examining the appearance of a decrease in phot1-GFP electrophoretic mobility in protein extracts after SDS-PAGE and western blotting following seedling illumination. Autophosphorylation at Ser-851 is an important function in initiating internalisation of phot1 from the plasma

membrane whereby a loss of autophosphorylation prevents internalisation in response to light (Kaiserli et al., 2009). Fig 5.5A shows that farnesyl-tagged phot1-GFP exhibits a reduction in electrophoretic mobility in the light treated sample indicative of receptor autophosphorylation. Likewise the mutated farnesyl-tagged phot1-GFP also shows a reduced electrophoretic mobility in the light treated sample (Fig 5.5B). Western blot analysis of protein levels is shown below using the soluble UGPase marker. These experiments indicate that the addition of the farnesyl tag to phot1-GFP and further, mutation of the farnesyl tag does not disrupt phot1 autophosphorylation activity in *Arabidopsis*.

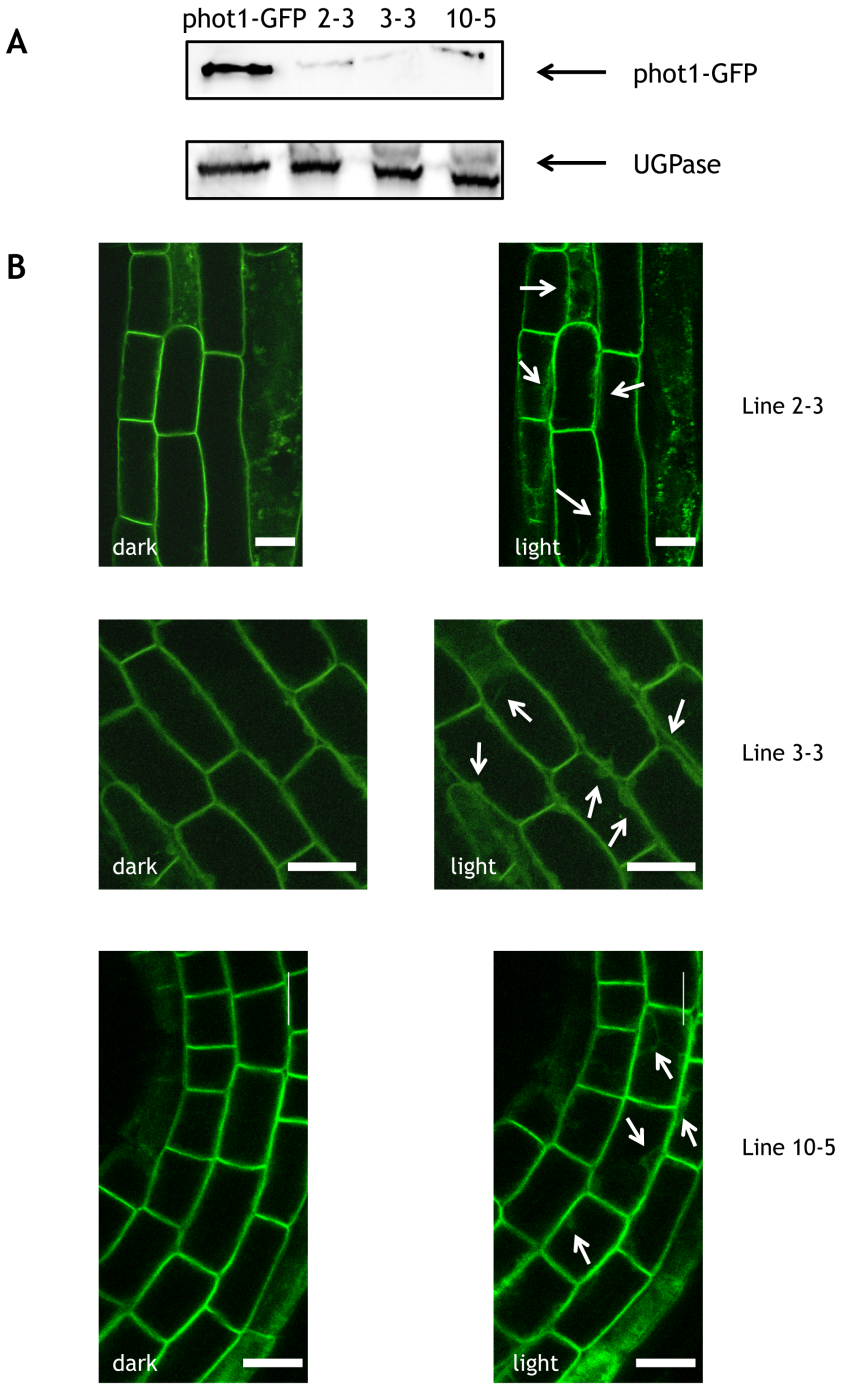


Figure 5.4: Mutation of the farnesyl tag results in internalisation of phot1-GFP from the plasma membrane in *Arabidopsis*

A) Western blot analysis of protein levels in phot1-GFP and each of the mutated farnesyl-tagged phot1-GFP lines (line 2-3, line 3-3 and line 10-5) probed with anti-GFP antibody. Western blot analysis using anti-UGPase is shown as a loading control.

B) Analysis of each of the mutated farnesyl-tagged phot1-GFP T3 homozygous lines. Confocal images of hypocotyl cells from three-day-old etiolated *Arabidopsis* seedlings both in the dark and 9 mins following illumination. Blue light used to excite GFP excites phot1 resulting in internalisation from the plasma membrane as shown by the arrows. Scale bar represents 20 μm.

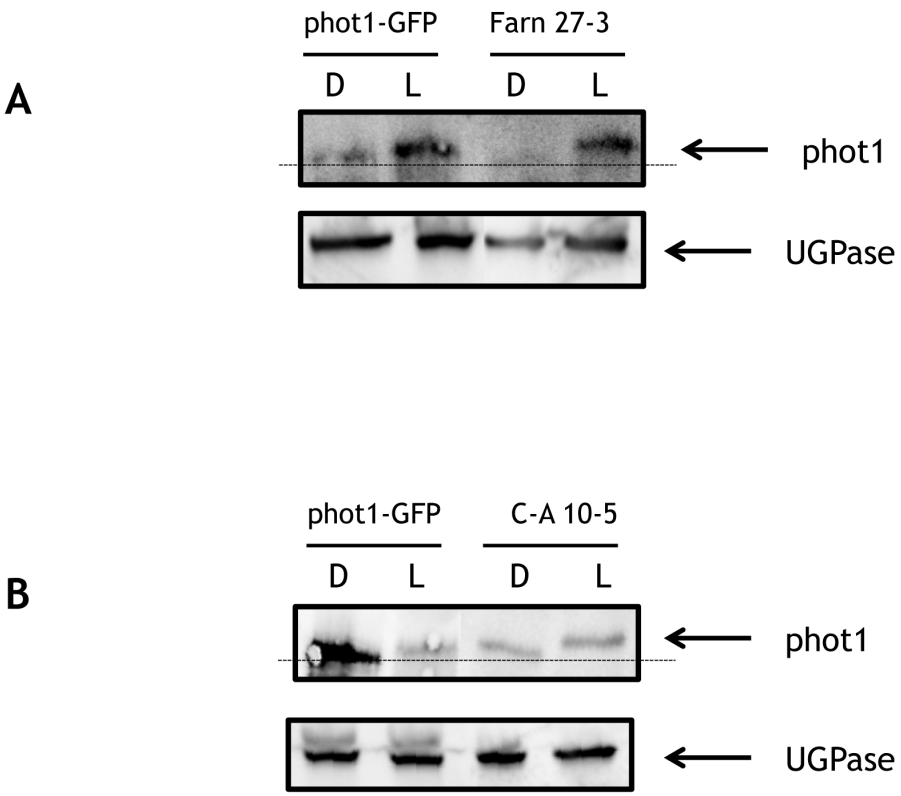


Figure 5.5: Light-induced shift in electrophoretic mobility of phot1-GFP in farnesyl- and mutated farnesyl-tagged phot1-GFP lines.

A) Three-day-old etiolated *Arabidopsis* seedlings expressing phot1-GFP and farnesyl-tagged phot1-GFP line 27-3 (farn 27-3) were kept in darkness (D) or given a light treatment of 10 μmol^{-2} s^{-1} for 10 minutes (L). Crude protein was extracted and analysed by immunoblotting with anti-GFP antibody. The dashed line indicates the lowest mobility edge of phot1. Blots were then probed with anti-UGPase antibody as a loading control (lower panel).

B) Three-day-old etiolated *Arabidopsis* seedlings expressing phot1-GFP and mutated farnesyl-tagged phot1-GFP line 10-5 (C-A 10-5) were kept in darkness (D) or given a light treatment of 10 μmol^{-2} s^{-1} for 10 minutes (L). Crude protein was extracted and analysed by immunoblotting with anti-GFP antibody. The dashed line indicates the lowest mobility edge of phot1. Blots were then probed with anti-UGPase antibody as a loading control (lower panel).

5.2.4 Kinetics of hypocotyl curvature is restored in farnesyl- and mutated farnesyl-tagged phot1-GFP lines.

Farnesyl-tagged phot1-GFP remains constitutively localised to the plasma membrane in *Arabidopsis* regardless of illumination of the seedling. The effect of this constitutive localisation on the physiological responses of the *Arabidopsis* seedlings was first assessed by investigating the kinetics of hypocotyl curvature since phototropism is one of the main physiological responses controlled by the

phototropins, increasing the light available for capture and serves to optimise photosynthetic efficiency (Liscum et al., 2014). Firstly, the phototropic response over time was measured in three-day-old dark grown *Arabidopsis* seedlings expressing either farnesyl-tagged phot1-GFP or mutated farnesyl-tagged phot1-GFP. Etiolated seedlings were exposed to 1 μmol m^{-2} s^{-1} unilateral blue light for between one and eight hours. The *phot1-5 phot2-1* double mutant does not bend in response to unilateral blue light whilst wild type seedlings begin to bend after around 4 hours (Fig 5.6A). The three independent mutated farnesyl-tagged phot1-GFP lines also begin to respond after 4 hours, to a similar degree as wild type (Fig 5.6A). Although the farnesyl-tagged phot1-GFP also begins to respond after 4 hours, the degree of curvature is reduced slightly in all three lines. This remains the case after 8 hours where the degree of curvature for the farnesyl-tagged transgenic lines is reduced. This suggests that constitutive association of phot1-GFP with the plasma membrane using the farnesyl tag results in a reduction in the phototropic response. However there is clearly a large increase in the phototropism response compared to the *phot1-5 phot2-1* double mutant (Fig. 5.6A) and Fig 5.6B illustrates representative seedlings 8 hours after treatment. The overall pattern of curvature of the farnesyl tagged phot1 is similar to wild type albeit at a reduced level and the phototropism response has not been negatively impacted. The complementation of phototropism suggests that internalisation from the plasma membrane is not required for the response but perhaps plays a role in the fine control of phototropism.

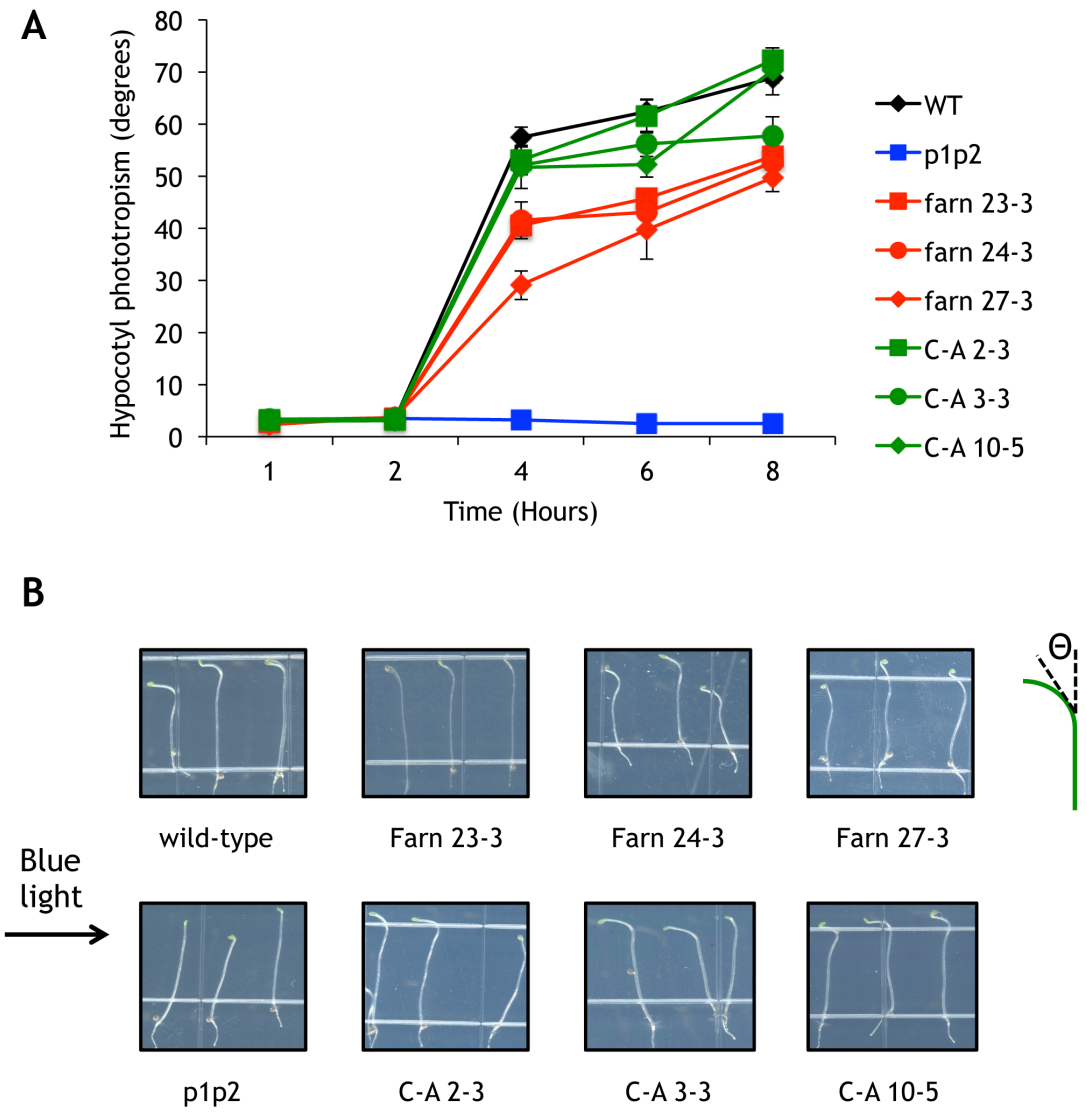


Figure 5.6: Kinetics of hypocotyl phototropism in *Arabidopsis* seedlings expressing farnesyl-tagged phot1-GFP and mutated farnesyl-tagged phot1-GFP lines

A) Phototropism kinetic curves for wild type (WT), *phot1-5 phot2-1* (p1p2), farnesyl-tagged phot1-GFP (farn) and mutated farnesyl-tagged phot1-GFP (C-A) in three-day-old etiolated *Arabidopsis* seedlings. Seedlings were exposed to 1 µmol m^{-2} s^{-1} unilateral blue light for the times indicated before curvature was measured. Error bars indicate standard error (n>20).

B) Images of representative seedlings of wild type (WT), *phot1-5 phot2-1* (p1p2), farnesyl-tagged phot1-GFP and mutated farnesyl-tagged phot1-GFP lines 8 hours after exposure to 1 µmol m^{-2} s^{-1} unilateral blue light. Θ indicates the angle that was measured.

5.2.5 Fluence-rate response curves for hypocotyl phototropism are restored in the farnesyl- and mutated farnesyl-tagged phot1-GFP lines

Since there was a reduction in the maximal phototropic response of the farnesyl-tagged phot1-GFP lines the fluence rate response was investigated in these lines when compared to both wild type and the mutated farnesyl-tagged phot1-GFP lines. The benefit of using the *phot1-5 phot2-1* double mutant is that there is no phot2 present in these seedlings so the effect of mutations on curvature at higher fluence rates can be measured (Kagawa et al., 2001). Three-day-old etiolated *Arabidopsis* seedlings were exposed to an increasing fluence rate of blue light from 0.1 µmol m^{-2} s^{-1} to 10 µmol m^{-2} s^{-1} for eight hours. Fig 5.7A shows that the response of the farnesyl-tagged phot1-GFP lines is reduced at 0.1 µmol m^{-2} s^{-1} blue light when compared to the response at 1 µmol m^{-2} s^{-1} blue light. There is then a trend of increased curvature as the fluence rate increases. This trend is also seen in wild type and mutated farnesyl-tagged phot1-GFP lines where the curvature response at 0.1 µmol m^{-2} s^{-1} is reduced when compared to the response at 10 µmol m^{-2} s^{-1}. Interestingly at 10 µmol m^{-2} s^{-1} blue light illumination the hypocotyl curvature of the farnesyl and mutated farnesyl-tagged phot1-GFP lines reached similar magnitude of curvature to that of wild type, suggesting that the farnesyl-tagged phot1-GFP lines are better able to fully complement phototropism at higher light intensities. The most likely explanation for this is the reduced protein levels of the farnesyl- and mutated farnesyl-tagged phot1-GFP lines when compared to phot1-GFP. The protein levels in wild type plants is likely to be even higher than phot1-GFP and the reduced response of the transgenic lines at lower light intensities can be attributed to this.

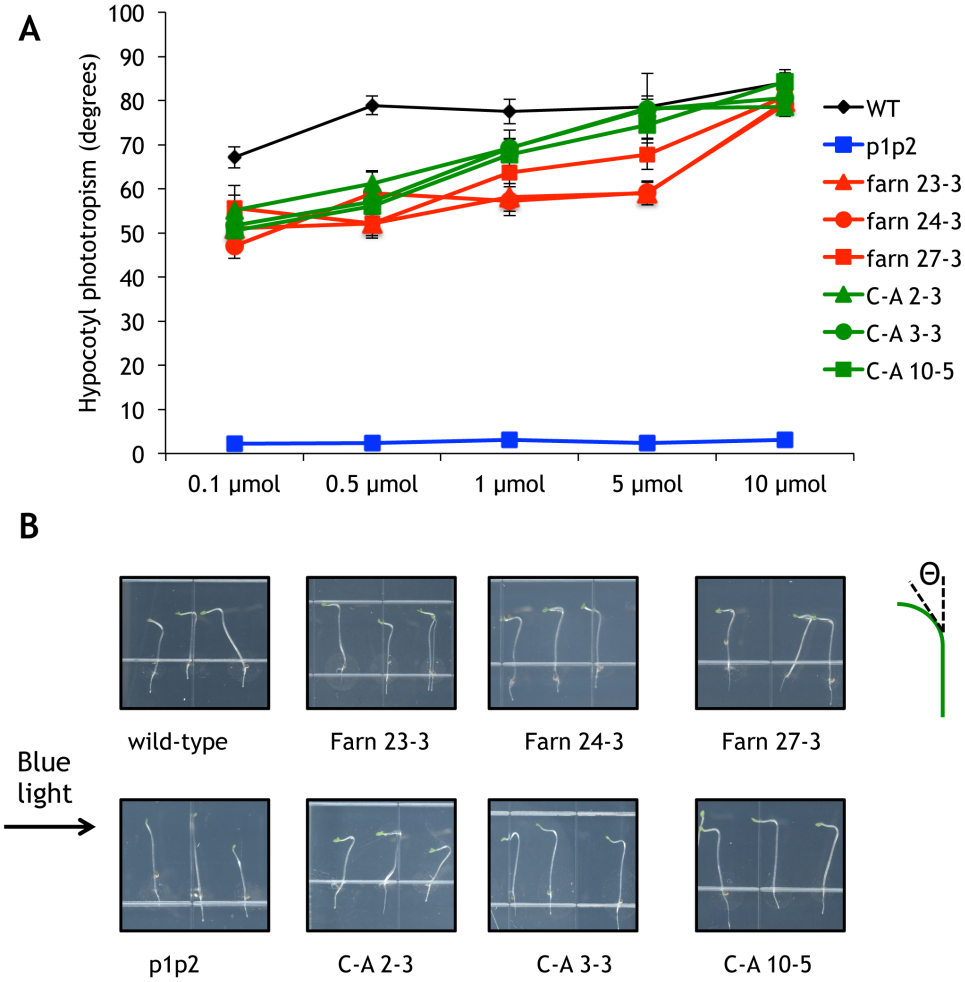


Figure 5.7: Hypocotyl phototropism fluence-rate response curves in *Arabidopsis* seedlings expressing farnesyl-tagged phot1-GFP and mutated farnesyl-tagged phot1-GFP lines

A) Phototropism fluence response curves for wild type (WT), *phot1-5 phot2-1* (p1p2), farnesyl-tagged phot1-GFP (farn) and mutated farnesyl-tagged phot1-GFP (C-A) in three-day-old etiolated *Arabidopsis* seedlings. Seedlings were exposed to unilateral blue light for 8 hours at the indicated fluence rates before curvature was measured. Error bars indicate standard error (n>20).

B) Images of representative seedlings from A at 10 µmol m^{-2} s^{-1} unilateral blue light. Θ indicates the angle which was measured.

5.2.6 Petiole positioning is restored in farnesyl- and mutated farnesyl-tagged phot1-GFP lines

The farnesyl- and mutated farnesyl-tagged phot1-GFP lines do not appear to impact negatively upon phototropism especially at higher light intensities. However, it was important to examine other responses that phot1 controls to increase photosynthetic efficiency in addition to phototropism. The petioles of wild-type *Arabidopsis* position themselves to maximise light capture for photosynthesis, a response that is controlled by the phototropins and NPH3 in response to blue light (Inoue et al. 2008). Wild-type seedlings grown under 70 µmol m^{-2} s^{-1} of white light for one week before being transferred to 10 µmol m^{-2} s^{-1} for a further week position their petioles upwards to allow a large surface area to capture light and the leaves are perpendicular to the incident light. However, the *phot1-5 phot2-1* double mutant has petioles that are flat and the leaves point downwards (Fig. 5.8A). The mutated farnesyl-tagged phot1-GFP expressing lines grown under the same conditions have petioles orientated in a similar manner to wild type with petioles that are positioned such that they are pointing upwards with leaves perpendicular to the light (Fig. 5.8A). There is not a large difference between the measured petiole angles of these mutated lines when compared to the wild type (Fig. 5.8B). The farnesyl-tagged phot1-GFP petioles are also positioned upwards (Fig. 5.8A), however there is a larger difference between these transgenic lines and wild type control (Fig 5.8B). The response however is greater than that seen for the *phot1-5 phot2-1* double mutant indicating that constitutive membrane association of phot1 does not impact upon petiole positioning.

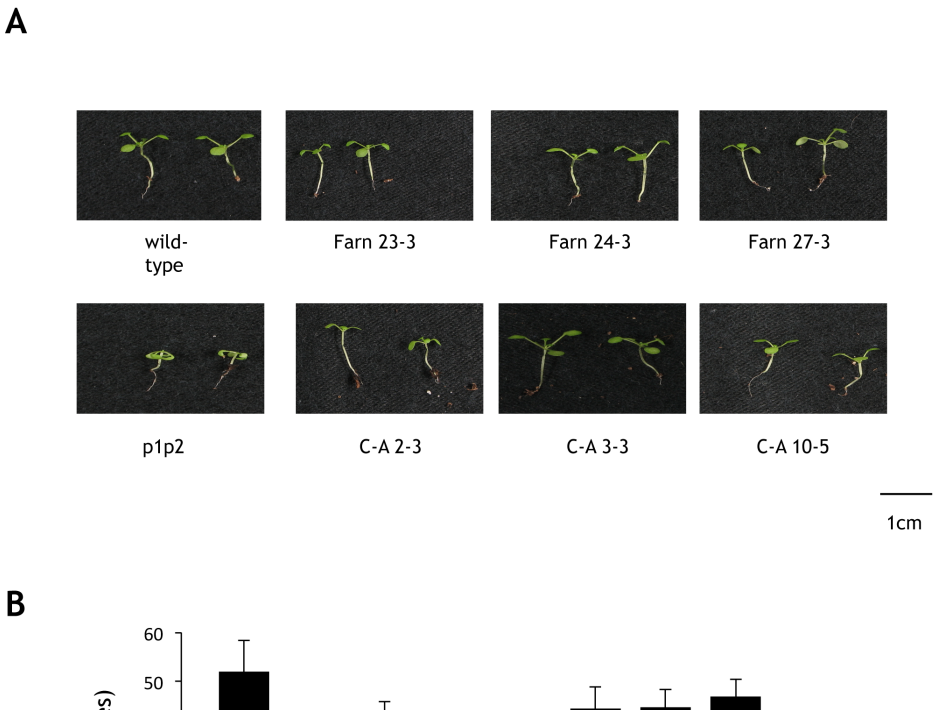


Figure 5.8: Petiole positioning in farnesyl- and mutated farnesyl-tagged phot1-GFP lines under low light conditions

A) Petiole positioning of wild-type (WT), *phot1-5 phot2-1* (p1p2) farnesyl-tagged phot1-GFP (farn) and mutated farnesyl-tagged phot1-GFP (C-A) *Arabidopsis* plants grown on soil for 1 week under 70 µmol m^{-2} s^{-1} white light 16/8 hour light-dark cycle then transferred to 10 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle for a further week. Representative images of three experiments are shown.

B) Quantification of petiole angles from horizontal. Error bars indicate standard deviation (n=9).

5.2.7 Leaf expansion is restored in farnesyl- and mutated farnesyl-tagged phot1-GFP lines.

Leaf expansion is another response that serves to increase photosynthetic activity and is controlled by the phototropins (Doi et al., 2004). The leaves of the *phot1-5 phot2-1* double mutant are epinastic and curl underneath resulting in long, thin shaped leaves when compared to the large flat shape of leaves from wild type plants (Van Volkenburgh, 1999). Either phot1 or phot2 can function to promote leaf expansion in *Arabidopsis* (Takemiya et al., 2005). The leaves of the farnesyl-tagged phot1-GFP plants grown for two weeks under 70 μmol m^{-2} s^{-1} white light have large flat leaves similar to wild type plants with no significant difference between them (Fig 5.9A). This response can be measured using the leaf expansion index (LEI), the ratio of unflattened to the artificially flattened leaf. A LEI of 1 would indicate a totally flat leaf. Both wild type and the farnesyl-tagged phot1-GFP seedlings have a LEI of around 0.9. The mutated farnesyl-tagged phot1-GFP line 2-3 and line 10-5 however, have a LEI of around 0.8 which is reduced compared to wild type and line 3-3 has a LEI of around 0.7, which is a greater difference. However this value is much larger than the LEI of the *phot1-5 phot2-1* double mutant, which has a value of around 0.4. The reduced leaf flattening of the transgenic lines and is most probably due to the reduced protein levels in both the farnesyl-tagged phot1-GFP lines as well as the mutated farnesyl-tagged phot1-GFP lines as the LEI is comparable between these lines. These results show that constitutive association of phot1-GFP with the plasma membrane does not impact upon leaf expansion.

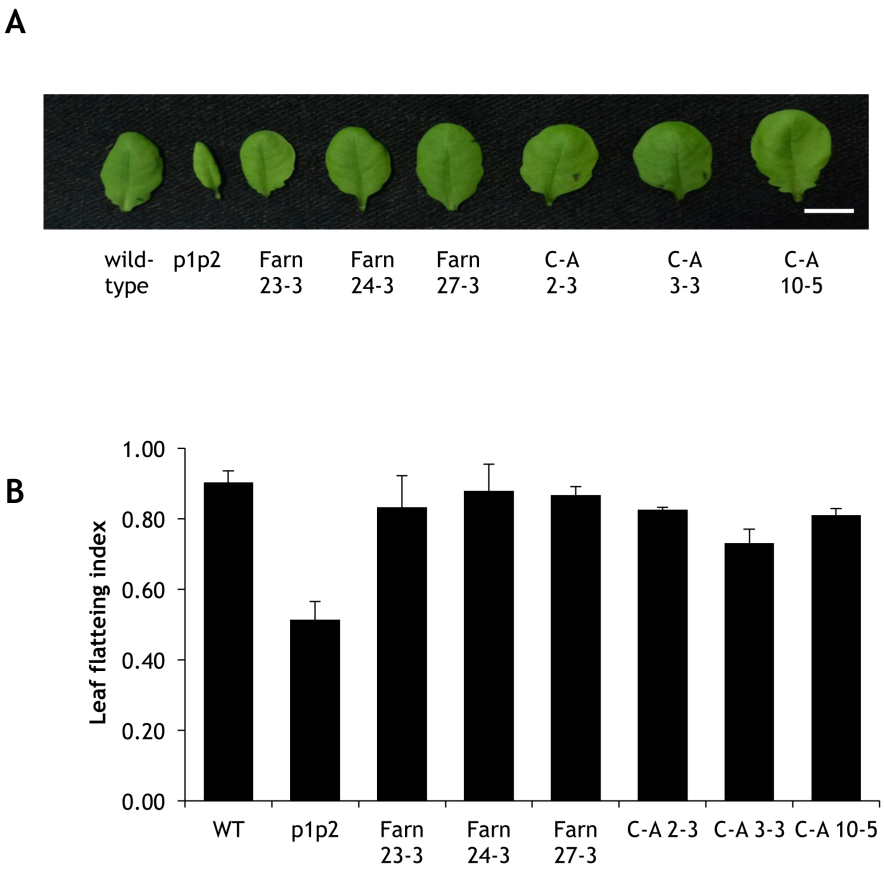


Figure 5.9: Leaf expansion measurements of farnesyl- and mutated farnesyl-tagged phot1-GFP lines

A) Representative images of the fifth rosette leaves from wild-type (WT), *phot1-5 phot2-1* (p1p2), farnesyl-tagged phot1-GFP (farn) and mutated farnesyl-tagged phot1-GFP (C-A) *Arabidopsis* plants. Plants were grown on soil for 3 weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Scale bar represents 1cm.

B) The leaf expansion index of the fifth rosette leaves described in A. The leaf expansion index is expressed as the ratio before and after artificial flattening. Error bars indicate standard error (n=10).

5.2.8 Chloroplast positioning is restored in farnesyl- and mutated farnesyl-tagged phot1-GFP lines

Under low light conditions, phot1 and phot2 mediate the movement of chloroplasts to the upper face of the palisade mesophyll cells to enable maximum light capture for photosynthesis (Kagawa et al., 2001). Since light capture is important for photosynthetic efficiency this response was also assessed in the lines expressing farnesyl- and mutated farnesyl-tagged phot1-GFP. Three-week-old *Arabidopsis* plants were grown under 70 μmol m^{-2} s^{-1} white light before the rosette leaves were detached and either kept in the dark or given a blue light treatment of 1.5 μmol m^{-2} s^{-1} before being imaged by confocal microscopy. The number of chloroplasts at the upper face of the mesophyll cells was counted in the leaves kept in darkness and compared to the number of chloroplasts at the upper surface in the blue light treated leaves. The wild type, farnesyl- and mutated farnesyl-tagged phot1-GFP lines show accumulation of chloroplasts to the upper face of the mesophyll cells compared to the dark controls and the *phot1-5 phot2-1* double mutant (Fig 5.10A). The farnesyl line 23-3 appears to show a bigger accumulation response than wild type (Fig 5.10B), however the response in darkness is also greater in farnesyl line 23-3 suggesting that perhaps the chloroplast positioning is behaving differently in this line. The other two farnesyl-tagged phot1-GFP lines, line 24-3 and line 27-3 do not show a substantial difference being more similar to wild type in both the darkness and after blue light treatment. The mutated farnesyl-tagged phot1-GFP line 2-3 shows a decreased accumulation response when compared to wild type whilst the other two mutated farnesyl-tagged phot1-GFP lines (3-3 and 10-5) behave similar to wild type (Fig 5.10B). Taken together these results show that under low light conditions the accumulation positioning of the chloroplasts is restored in the farnesyl-tagged phot1-GFP lines as well as the mutated farnesyl-tagged phot1-GFP lines. This suggests that phot1 internalisation from the membrane is not required for chloroplast positioning under low light conditions but it may affect the kinetics of chloroplast positioning, which would need to be measured using a different method.

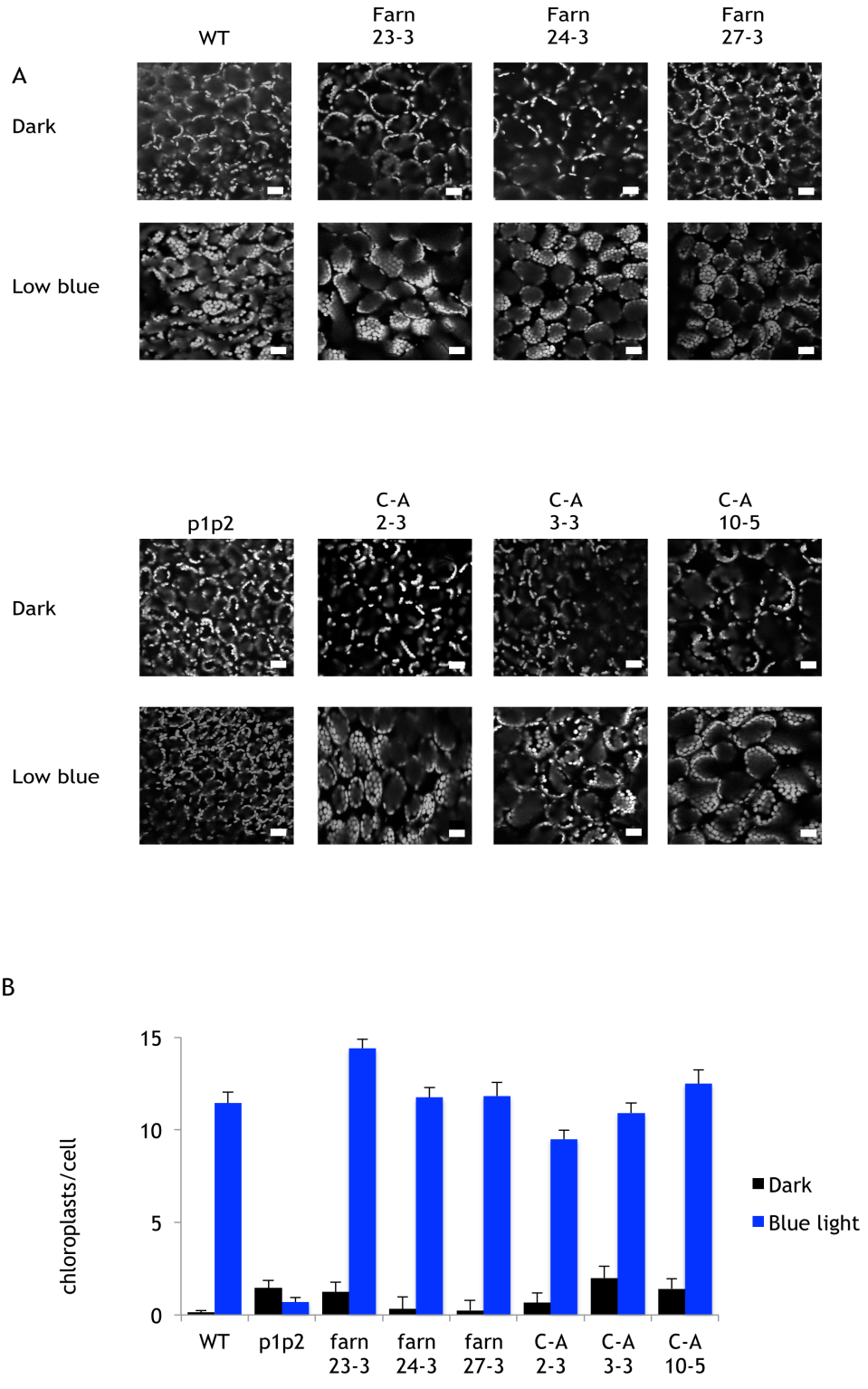


Figure 5.10: Chloroplast movement in farnesyl and mutated farnesyl-tagged phot1-GFP lines

A) Chloroplast positioning of wild type (WT), *phot1-5 phot2-1* (p1p2), farnesyl-tagged phot1-GFP (farn) and mutated farnesyl-tagged phot1-GFP (C-A) plants were grown on soil for 3 weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Rosette leaves were detached and treated with blue light (1.5 µmol m^{-2} s^{-1}) for 3 hours (Low blue; blue) or kept in the dark for 3 hours (Dark; black) before observation of chloroplast autofluorescence by confocal microscopy. Scale bar represents 20 µm.

B) Quantification of the number of chloroplasts at the upper face of palisade mesophyll cells of the plants described in A. Error bars indicate standard error of the mean of >12 cells per line.

5.2.9 Myristoylated phot1 also complements petiole positioning, leaf flattening and chloroplast accumulation movement

The constitutive association of phot1 to the membrane with a farnesyl tag suggests that internalisation from the membrane is not required for the responses which serve to optimise photosynthetic efficiency. To confirm this we also examined petiole positioning, leaf flattening and chloroplast accumulation in *Arabidopsis* seedlings from collaborators at the University of Lausanne that also constitutively anchored phot1 to the plasma membrane using a myristoyl tag. Myristoylation involves the addition of a 14 carbon myristate group to a glycine in target proteins (Gordon et al. 1991; Johnson et al. 1994) by the enzyme N-myristoyltransferase (Running, 2014). The myristoyl tag was added to the N-terminus of a phot1-mCitrine fusion (Fig. 5.11) and following selection three independent T3 homozygous lines were identified that were named myr 11-6, myr 24-3 and myr 36-5. Lines expressing phot1-mCitrine without the myristoyl tag was used as a control (pPHOT1). As shown in Fig. 5.12A the petioles of myristoylated phot1 also position themselves upwards to capture the light similar to wild type but with a slightly smaller angle (Fig. 5.12B). There is a large difference between wild type and myr 26-5 however the response of this myristoyl-tagged line is greater than the positioning of the double mutant showing that myristoylated phot1 complements petiole positioning under low light conditions albeit not to the same degree as wild-type. This suggests that constitutive plasma membrane localisation of phot1 with a myristoyl tag also does not impact upon petiole positioning, similar to the farnesyl-tagged phot1-GFP lines (Fig. 5.8).

The next physiological response tested was leaf flattening. The myristoyl-tagged phot1-mCitrine lines also have large flat leaves with a LEI of around 0.8, which is slightly reduced when compared to wild-type which is around 0.9. However the pPHOT1 control also shows a slight reduction in the LEI compared to wild-type indicating that the constitutive association of phot1 with the plasma membrane is not affecting leaf flattening but is probably due to differences in protein expression (Preuten et al., 2015). The LEI of 0.8 is double the LEI of the double mutant which is only 0.4. Taken together these results indicate that leaf

flattening is complemented despite phot1 being unable to partially internalise in response to light.

The final response investigated in the myristoyl-tagged phot1-mCitrine lines was chloroplast accumulation under low blue light conditions. There is a slight increase in accumulation when compared to wild type in the myristoylated phot1 lines, however this is also seen in the pPHOT1 control where the accumulation of chloroplasts is much greater than the wild type (Fig. 5.15). This may be due to alterations in the kinetics of chloroplast movement, which cannot be measured using this method. The results do however indicate that myristoyl-tagged phot1-mCitrine is able to complement the chloroplast accumulation response similar to what was seen for the farnesyl-tagged phot1-GFP lines (Fig. 5.10) and that internalisation from the plasma membrane is not required for chloroplast accumulation. Taking all the data together for the two complementary methods of artificially anchoring phot1 to the membrane shows that internalisation is not required for the physiological responses controlled by phot1, although relocalisation may be required for fine-tuning the responses.

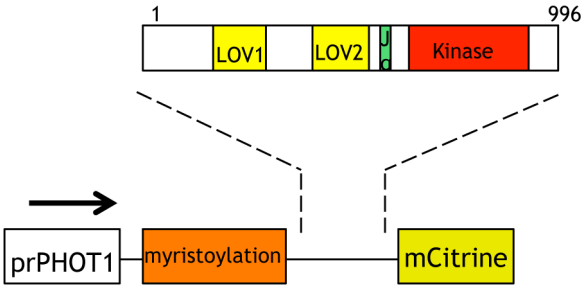


Figure 5.11 Schematic diagram of the myristoylation tagged phot1-mCitrine construct

Schematic diagram illustrating the construction of myristoyl-tagged phot1-mCitrine used to assess localisation. The native phot1 promoter is driving expression of phot1-mCitrine, while the myristoyl tag was added to the N-terminus of phot1 to drive constitutive localisation to the plasma membrane.

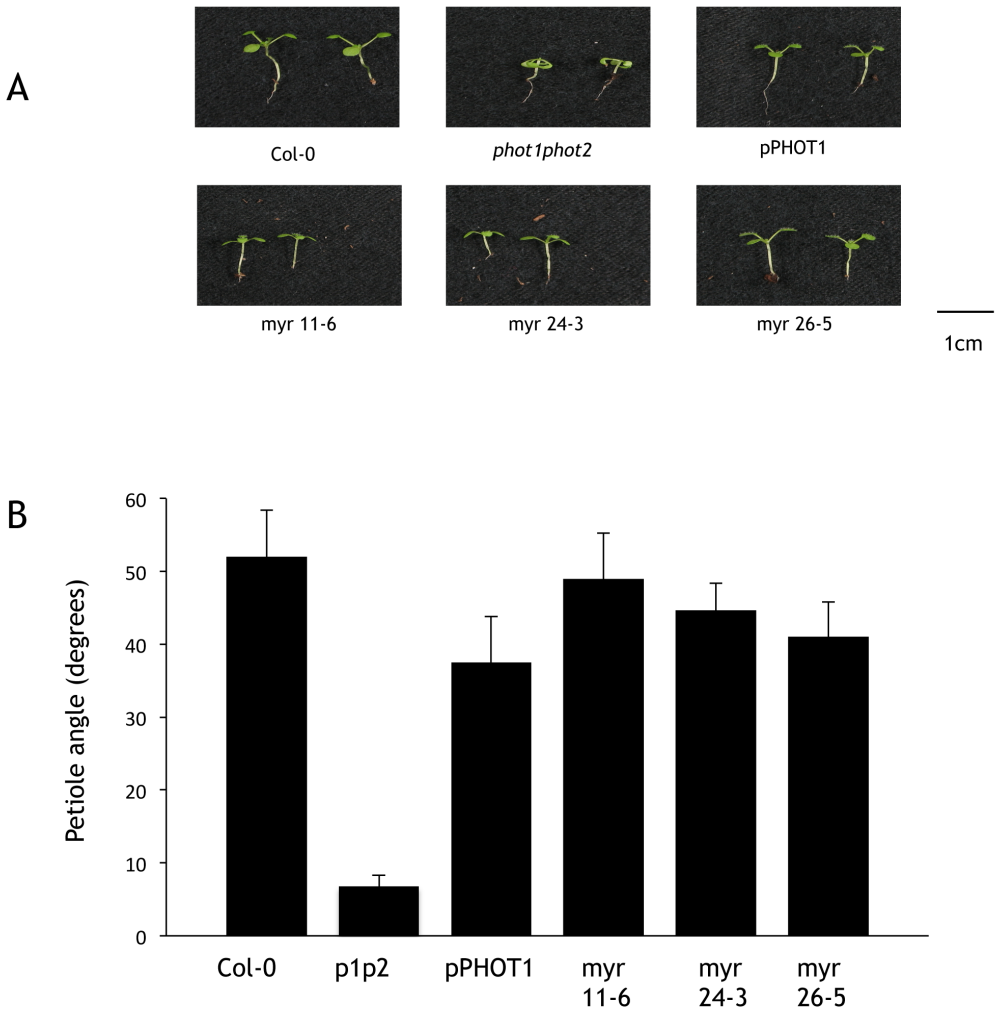


Figure 5.12: Petiole positioning in myristoyl-tagged phot1-mCitrine lines under low light conditions

A) Petiole positioning of wild-type (WT), *phot1-5 phot2-1* (p1p2), myristoylated phot1 (myr) and native promoter phot1 (pPHOT1) *Arabidopsis* seedlings grown on soil for 1 week under 70 µmol m^{-2} s^{-1} white light 16/8 hour light-dark cycle then transferred to 10 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle for a further week. Representative images of three experiments are shown.

B) Quantification of petiole angles from horizontal. Error bars indicate standard deviation (n=9).

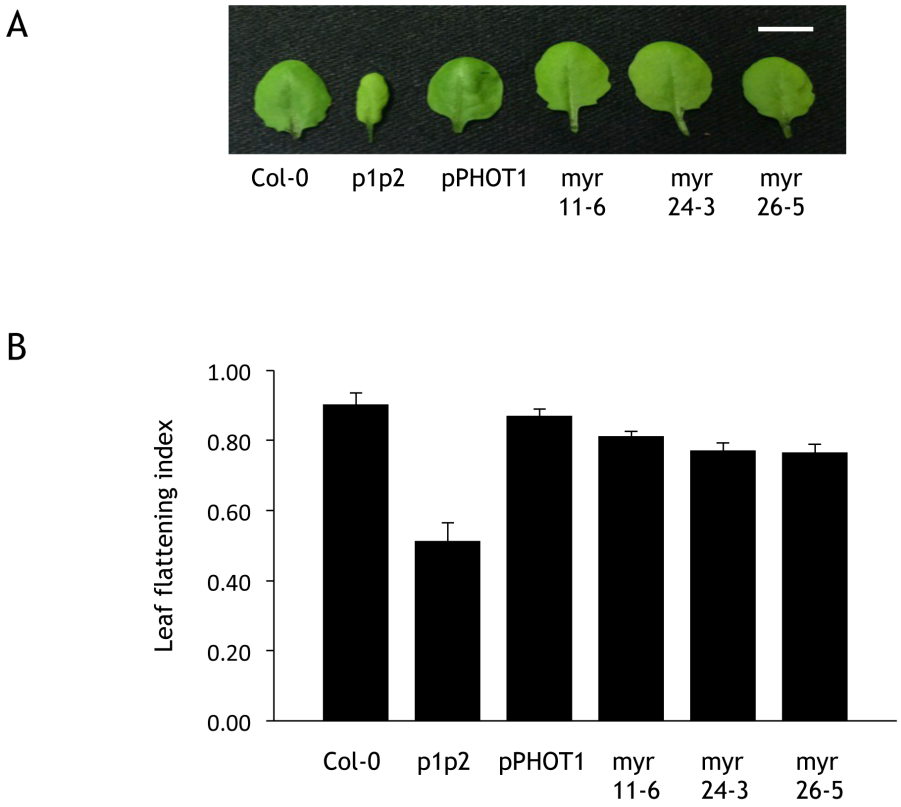


Figure 5.13: Leaf expansion measurements of myristoylated phot1 tagged lines

A) Representative images of the fifth rosette leaves of wild-type (WT), *phot1-5 phot2-1* (p1p2), native promoter phot1 (pPHOT1) and myristoylated tagged phot1 (myr) *Arabidopsis* seedlings. Plants were grown on soil for 3 weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Scale bar represents 1cm.

B) The leaf expansion index of the fifth rosette leaves described in A. The leaf expansion index is expressed as the ratio before and after artificial flattening. Error bars indicate standard error (n=10).

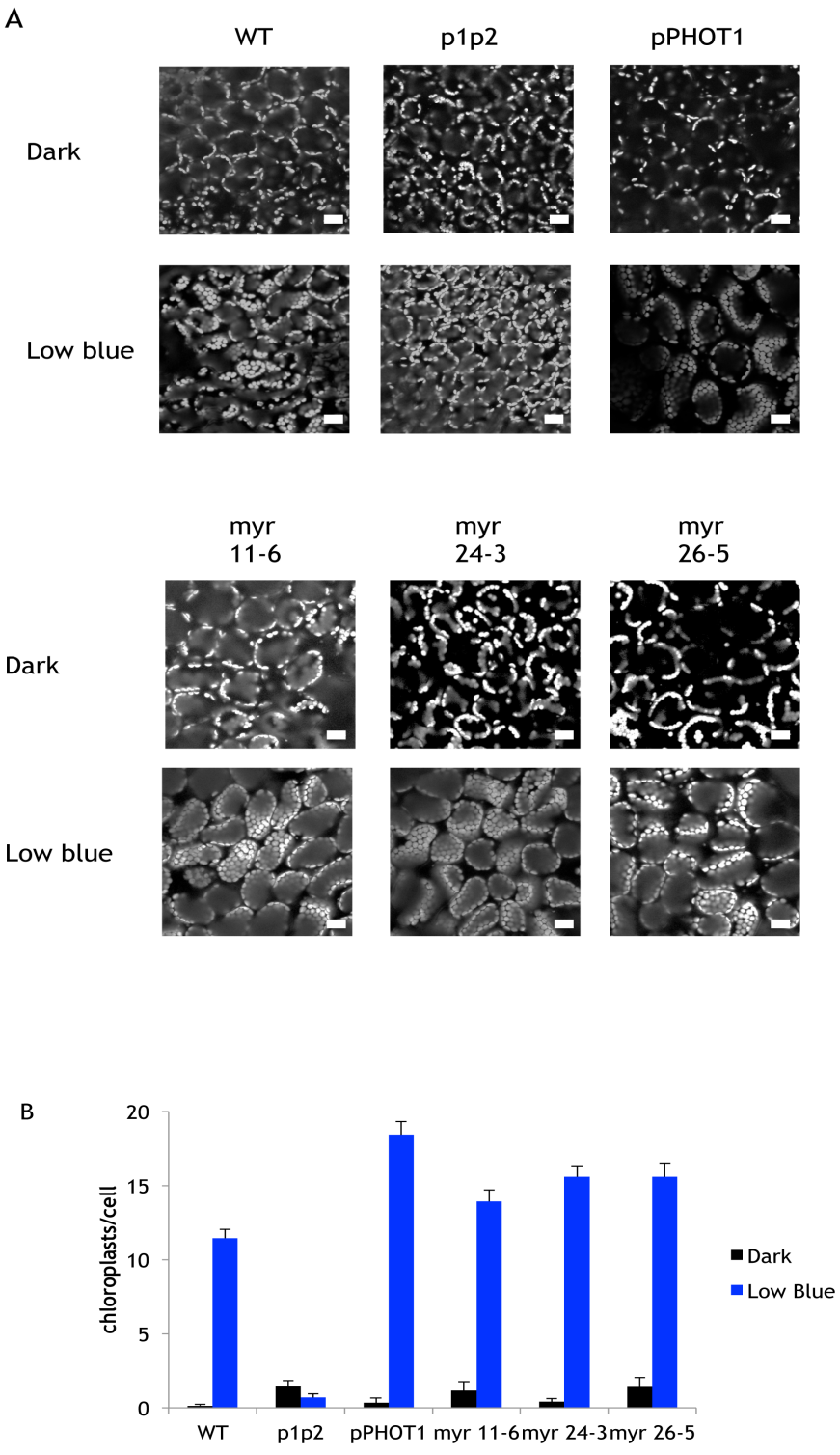


Figure 5.14: Chloroplast movement in myristoylated phot1 lines

A) Chloroplast positioning of wild type (WT), *phot1-5phot2-1* (p1p2) native promoter phot1 (pPHOT1) and myristoylated phot1 (myr) plants were grown on soil for 3 weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Rosette leaves were detached and treated with blue light (1.5 µmol m-2 s-1) for 3 hours (Low blue; blue) or kept in the dark for 3 hours (Dark; black) before observation of chloroplast autofluorescence by confocal microscopy. Scale bar represents 20 µm.

B) Quantification of the number of chloroplasts at the upper face of palisade mesophyll cells of the plants described in A. Error bars indicate standard error of the mean of >12 cells per line.

5.2.10 Ostreococcus phototropin does not appear to internalise from the plasma membrane in response to blue light illumination

Constitutive association of phot1 to the plasma membrane using a farnesyl or myristoyl tag resulted in complementation of the responses analysed in the *phot1-5 phot2-1* double mutant background. However this is an artificial mechanism of attachment and we therefore investigated if any other phototropin homologs showed different relocalisation properties compared to *Arabidopsis* phot1. *Chlamydomonas* phototropin associates with the plasma membrane similar to phototropin from *Arabidopsis* (Huang, Merkle and Beck, 2002). We had already expressed another algal phototropin from *Ostreococcus* (Otphot) in the *phot1-5 phot2-1* mutant background (Fig 4.13E) where it showed membrane localisation as well as internalisation after treatment with 1-butanol. We therefore investigated if Otphot-GFP was able to internalise in response to light since it is able to undergo light dependent autophosphorylation in insect cells (Fig. 3.3B), a step that is required for phot1 internalisation (Inoue et al. 2008; Kaiserli et al. 2009). Protein levels in each of the lines expressing Otphot were analysed to measure protein levels being expressed, as they are T2 generation since we were unable to generate T3 lines, perhaps due to male sterility which was a problem when the *Chlamydomonas* phototropin was transformed into *Arabidopsis* (Onodera et al., 2005). Protein levels are comparable between a line expressing phot1-GFP and the three Otphot-GFP lines examined (Fig. 5.15A). However as shown in the maximal Z stack projections in Fig. 5.15B Otphot appears to remain localised to the plasma membrane after 10 minutes following blue light illumination, similar to farnesyl-tagged phot1-GFP (Fig. 5.3) but very different to phot1-GFP which shows internalisation after 10 mins. The maximal Z stack projections were compiled by Stuart Sullivan. This illustrates that there is a phototropin that does not internalise in response to the same light conditions as phot1 does from *Arabidopsis*. This gave us an additional way to investigate if a naturally constitutively membrane localised phototropin is able to function in complementing physiological responses in *Arabidopsis*.

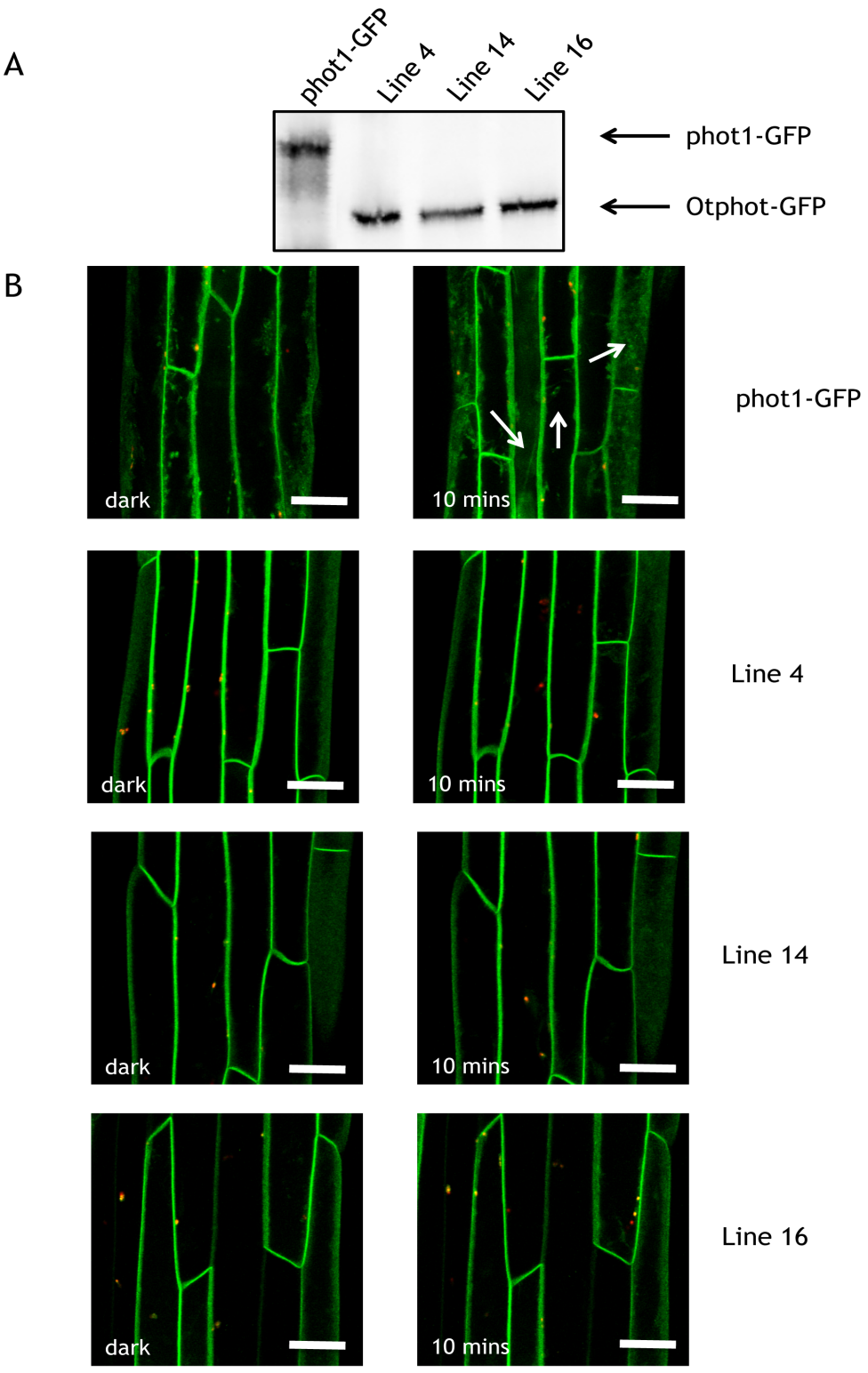


Figure 5.15: Otphot remains localised to the plasma membrane following blue light illumination

A) Western blot analysis of protein levels in phot1-GFP and each of the Otphot expressing lines (line 4, line 14 and line 16) probed with anti-GFP antibody.

B) Analysis of localisation in phot1-GFP and each of the Otphot T2 lines. Maximal projections of Z stacks are shown of the hypocotyl cells from three-day-old etiolated *Arabidopsis* seedlings both in the dark and 10 mins following illumination. Phot1-GFP shows internalisation 10 mins following illumination as indicated by the arrows. Scale bar represents 20 μm.

5.2.11 Ostreoccocus phototropin is unable to fully complement the physiological response in Arabidopsis

Since the farnesyl-tagged and myristoyl-tagged phot1-GFP lines were able to complement phototropism at both high and low fluence rates of blue light (Preuten et al., 2015) this response was tested first in the three Otphot lines. As shown in Fig. 5.16, none of the lines show phototropism at the lower fluence rates tested (1 µmol m^{-2} s^{-1} and 5 µmol m^{-2} s^{-1}), which is perhaps surprising since the Otphot protein undergoes light dependent autophosphorylation (Fig. 3.3B) and internalisation is dispensable for phototropism in the farnesyl-tagged and myristoyl-tagged phot1-GFP lines (Fig. 5.6 and Fig. 5.7, Preuten et al., 2015). Otphot is likely to be more similar to phot2 than phot1 (Briggs et al., 2001), therefore we investigated phototropism at a higher fluence rate of 20 µmol m^{-2} s^{-1} to analyse if Otphot was able to respond to the higher light intensity similar to phot2. However the three lines did not show any response to unilateral blue light at 20 µmol m^{-2} s^{-1} indicating that Otphot is unable to complement phototropism in *Arabidopsis* over a wide variety of fluence rates.

The petiole positioning response was then assessed in the Otphot lines. As shown in Fig 5.17A, the petioles of the Otphot lines are not as curled as the *phot1-5 phot2-1* double mutant in response to low intensity blue light but they are not as perpendicular to the incident light as the wild type. This suggests that the Otphot protein is not as efficient as phot1 in controlling this response similar to phot2, as the *phot1-5* mutant that still has functional phot2 does not show the perpendicular petiole positioning seen in wild type (Inoue et al. 2008). Although the phototropins from *Arabidopsis* control petiole positioning redundantly, phot1 is more efficient. Leaf flattening is another response that optimises photosynthetic efficiency. The leaves of all of the Otphot lines are similar to wild type and are not curled like *phot1-5 phot2-1*. However, Otphot line 14 leaves are much smaller than the wild type and Otphot line 4 and 16. This is most likely due to the differences in transgene insertion as well as these transgenic lines only being T2, therefore the lines may contain more than one copy of the Otphot gene.

The final response analysed was chloroplast positioning. Similar to what was found for all the responses tested, the Otphot lines did not complement this

response. Under low blue light conditions none of the three lines showed any chloroplast accumulation and were more similar to the double mutant than wild type with the same number of chloroplasts in the dark and blue light. Taken together this illustrates that Otphot is only able to partially complement the physiological responses in *Arabidopsis* unlike *Chlamydomonas* where complementation was seen in the high expressing lines for all the responses (Onodera et al., 2005). This suggests there may be differences in the lower algal forms of phototropin which requires further investigations.

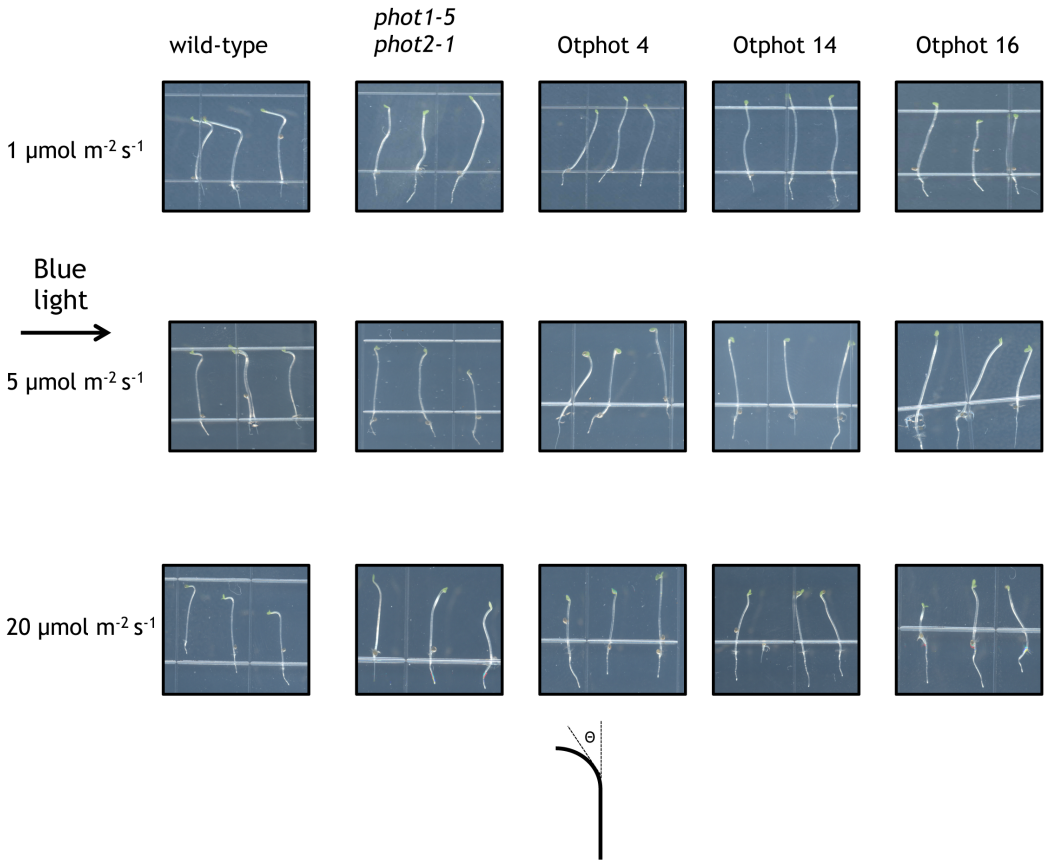
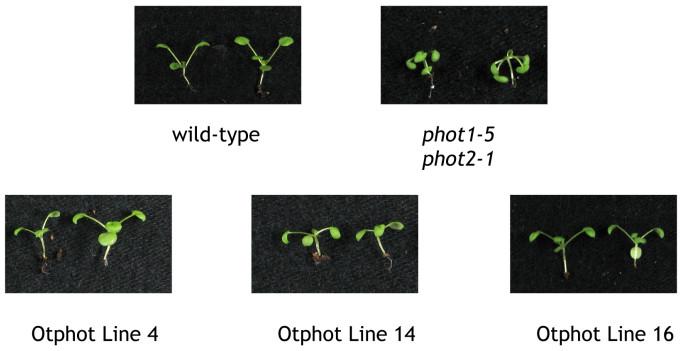


Figure 5.16: Otphot does not complement phototropism at low or high light intensities in *Arabidopsis* seedlings

Images of representative seedlings of wild type, *phot1-5 phot2-1* and Otphot lines 8 hours after exposure to 1 µmol m^{-2} s^{-1}, 5 µmol m^{-2} s^{-1} and 20 µmol m^{-2} s^{-1} unilateral blue light. Θ indicates the angle which was measured. None of the lines expressing Otphot showed the phototropic response over these fluence rates.

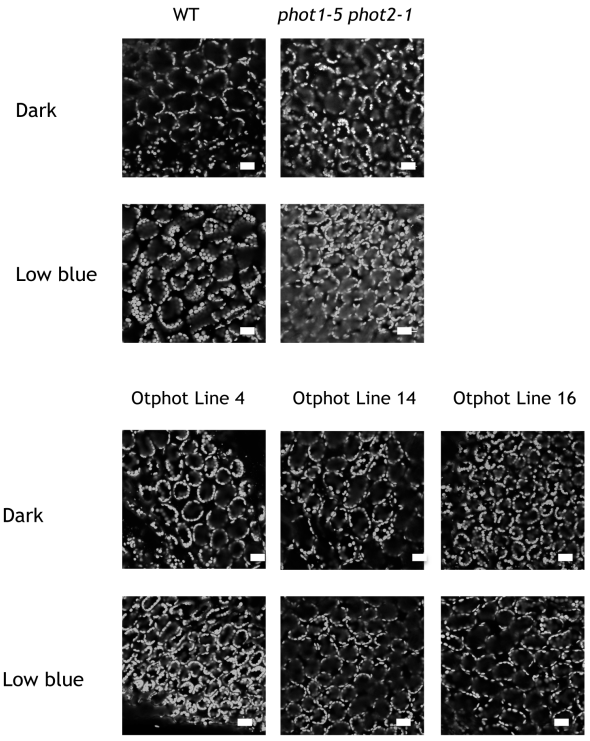
A


wild-type
phot1-5
phot2-1
Otphot Line 4
Otphot Line 14
Otphot Line 16

B


wild-type
phot1-5
phot2-1
Otphot Line 4
Otphot Line 14
Otphot Line 16

C


WT
phot1-5 phot2-1
Dark
Low blue
Otphot Line 4
Otphot Line 14
Otphot Line 16
Dark
Low blue

D

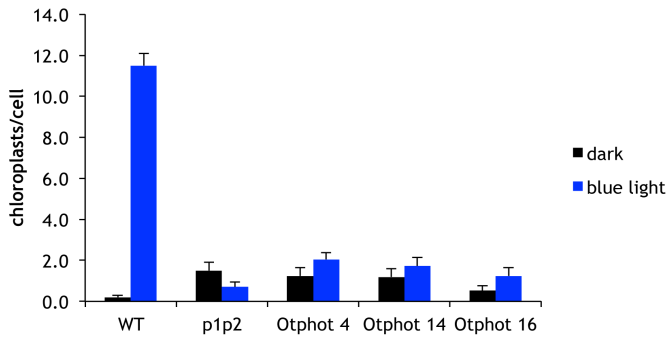

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
chloroplasts/cell
WT
p1p2
Otphot 4
Otphot 14
Otphot 16
dark
blue light

Figure 5.17: Otphot does not complement the physiological responses controlled by phototropin in *Arabidopsis*

A) Petiole positioning of wild-type (WT), *phot1-5 phot2-1* (p1p2) and Otphot expressing *Arabidopsis* seedlings grown on soil for 1 week under 70 µmol m^{-2} s^{-1} white light 16/8 hour light-dark cycle then transferred to 10 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle for a further week. Representative images of three experiments are shown.

B) Representative images of the fifth rosette leaves of wild-type (WT), *phot1-5 phot2-1* (p1p2) and Otphot expressing *Arabidopsis* seedlings. Plants were grown on soil for 3 weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Scale bar represents 1cm.

C) Chloroplast positioning of wild type (WT), *phot1-5phot2-1* (p1p2) and Otphot expressing plants were grown on soil for 3 weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Rosette leaves were detached and treated with blue light (1.5 µmol m-2 s-1) for 3 hours (Low blue; blue) or kept in the dark for 3 hours (Dark; black) before observation of chloroplast autofluorescence by confocal microscopy. Scale bar represents 20 µm.

D) Quantification of the number of chloroplasts at the upper face of palisade mesophyll cells of the plants described in A. Error bars indicate standard error of the mean of >12 cells per line.

5.3 Discussion

5.3.1 Constitutive association of phot1 with the plasma membrane can be achieved by addition of a farnesyl tag.

The localisation of phot1 to the plasma membrane in darkness and partial internalisation after blue light illumination has been known to occur for a number of years, however the mechanism of this re-localisation remains unknown (Sakamoto and Briggs, 2002, Han et al., 2008, Wan et al., 2008). Nevertheless recent research has begun to provide an understanding of how membrane association and re-localisation occur. Kaiserli et al. (2009) showed that the inhibitor of clathrin-mediated endocytosis, Tyrphosin A23, attenuates the constitutive internalisation in *N. benthamiana* of a phot1-GFP fusion expressing two LOV1 domains. The authors also demonstrated that treatment with Brefeldin A (BFA), an inhibitor of endosomal trafficking, resulted in endosomal aggregates in both *N. benthamiana* and *Arabidopsis* expressing phot1-GFP. Co-immunoprecipitation experiments revealed that phot1-GFP as well as phot2-GFP interacts with the clathrin heavy chain (Kaiserli et al., 2009). This was subsequently confirmed by Aggarwal et al. (2014) by co-IPs as well as BiFC. Treatment of phot2-GFP with the inhibitor Tyrphosin A23 did not disrupt formation of punctate phot2-GFP staining. This suggests that phot1 is trafficked through endosomal recycling pathways, whereas phot2 is not.

Despite these indications it has never been determined what the function is of the partial internalisation of phot1 to the cytoplasm in response to illumination (Sakamoto and Briggs, 2002, Kaiserli et al., 2009, Sullivan et al., 2010). Receptor autophosphorylation is required for this internalisation function (Kaiserli et al., 2009) and autophosphorylation is an important first step in the physiological responses controlled by phot1 including phototropism, stomatal opening and chloroplast positioning (Inoue et al. 2008). Ser-851 is an important residue in initiating relocalisation as indicated by the constitutive cytosolic strands visualised in the absence of a blue light stimulus in a phosphomimic, mutation of Ser-851 to aspartate (Kaiserli et al., 2009).

There have been suggestions as to the function of internalisation including attenuation of receptor signalling or receptor desensitisation (Wan et al. 2008; Sullivan et al. 2010; Briggs 2014) and possibly a potential role of clathrin-mediated endocytosis (Kaiserli et al., 2009, Roberts et al., 2011). There is also the enhanced phototropic response of *Arabidopsis* seedlings after a red light pre-treatment, a function which had been noted for a number of years before the identification of phot1 as the blue light photoreceptor for phototropism (Briggs, 1963, Janoudi and Poff, 1992). It is thought that phytochrome A (phyA) retains phot1 at the plasma membrane in specific cells of the hypocotyl (Han et al., 2008), with the hypothesis that it is retained in the active environment and thus is able to interact with auxin transporters required to establish lateral auxin gradients (Han et al., 2008). Therefore the addition of a lipid modification to phot1-GFP provides a good mechanism to investigate if constitutive association with the plasma membrane enhances other responses.

Firstly the localisation pattern following the addition of the farnesyl tag to the C-terminus of phot1-GFP was investigated. Lipid modifications are known to be involved in retaining plant proteins to the membrane (Hemsley and Grierson, 2008, Sorek, Bloch and Yalovsky, 2009). Internalisation of phot1 from the plasma membrane occurs rapidly and is visualised between three and six minutes following blue light illumination from the laser (Sakamoto and Briggs, 2002, Kaiserli et al., 2009). With the addition of a farnesyl tag, the phot1-GFP protein still remains localised to the plasma membrane 9 mins following blue light illumination in both *N. benthamiana* (Fig. 5.2B) and transgenic *Arabidopsis* (Fig

5.3C). This modification can be reversed with the mutation of the conserved cysteine residue, which is the site of lipid modification for the farnesylation sequence, to alanine. As shown in Fig 5.4B, internalisation is visualised in these seedlings following blue light illumination. Importantly, phot1 was still able to undergo light-dependent autophosphorylation in both the farnesyl and C-A lines as measured indirectly through electrophoretic mobility shifts, removing the issue of an inactive phot1 protein affecting physiological responses as the kinase activity that is known to be required for re-localisation could have been affected (Kaiserli et al., 2009).

5.3.2 Lipid modification of phot1 does not affect the physiological responses controlled by phot1

Phototropism is an important physiological response for orientating plants towards light and optimising photosynthesis (Liscum et al., 2014). A red light pre-treatment of seedlings prior to unidirectional blue light results in an enhancement of the phototropic response and is controlled by phyA. It is hypothesised to be due to phot1 remaining in the active membrane environment by inhibiting light induced relocalisation (Han et al., 2008). Since farnesyl-tagged phot1 is constitutively localised to the plasma membrane it would be expected that the same enhancement of phototropism would be seen in the seedlings without red light pretreatment. However the kinetics of phototropism is slightly reduced in the farnesyl-tagged phot1-GFP lines when compared to both wild type and the mutated farnesyl-tagged phot1-GFP control lines (Fig 5.6A). The reduced curvature of the farnesyl and mutated farnesyl lines at lower light intensities could be attributed to lower protein levels since the degree of curvature in the farnesyl-tagged phot1-GFP and mutated farnesyl-tagged phot1-GFP lines reaches the same levels as wild type at higher light intensities. This suggests that association with the plasma membrane initiates phototropism and the internalised cytosolic protein, usually visualised after blue light illumination, does not function in phototropism as the response occurs without internalisation. It would, however, be interesting to investigate the refractory period of phototropism in the farnesyl tagged lines to explore the sensitivity of the plasma membrane localised phot1 (Janoudi and Poff, 1991, 1992). A longer refractory period would suggest that the receptor is in the active form longer for

phototropism compared to wild type and would indicate if localising phot1 to the plasma membrane could affect the phototropic response at a level which has not been measured in this study. Another method to measure this would be the recovery of the electrophoretic mobility shift after saturating light conditions. The electrophoretic mobility of oat phot1 has been shown to be complete 10 mins after a saturating light pulse (Knieb, Salomon and Rüdiger, 2005) and wild type *Arabidopsis* seedlings show a recovery in phosphorylation 20 mins after initial phosphorylation (Reymond et al., 1992).

Phototropism following seedling exposure to blue light pulses could also be investigated to measure first positive phototropism. PIN-FORMED (PIN) proteins are auxin efflux carriers that regulate auxin translocation (Krecek et al., 2009) for plant responses to external stimuli. Of the seven member PIN family, PIN3 is known to be involved in phototropism (Willige et al., 2013) despite there being no negative impact on phototropism in the *pin3* mutant. There is however an impact upon pulse-induced first positive phototropism in the pin3 mutant (Haga and Sakai, 2012) suggesting a difference between seedling exposure to pulses of light and continuous light. Interplay between phot1 and PIN3 occurs when phot1 phosphorylates the auxin efflux carrier ABCB19 inhibiting its activity and resulting in increased auxin levels which are subsequently channelled by PIN3 (Christie et al., 2011). Both PIN3 and ABCB19 localise to the plasma membrane (Titapiwatanakun et al., 2009, Ding et al., 2011) and PIN3 even relocalises to the shaded side of *Arabidopsis* seedlings in response to light. If pulse-induced phototropism was reduced in the farnesyl-tagged phot1-GFP lines it would perhaps indicate that anchoring phot1 at the plasma membrane results in an inability to interact with downstream targets, such as ABCB19, and would suggest a role for the internalisation of phot1 from the plasma membrane. However NPH3 dephosphorylation still occurs in the lipid modified lines indicating that phot1 is still able to interact with this downstream target (Preuten et al., 2015).

Petiole positioning is another important response for optimising photosynthetic efficiency of the plants (Casal, 2012). In the farnesyl-tagged phot1-GFP lines the petioles show a difference in their response compared to the wild type seedlings (Fig 5.8B), which is also seen in the lines from our collaborators that express a

myristoyl-tagged phot1-mCitrine (Fig 5.12B). This suggests that perhaps constitutive association of phot1 to the plasma membrane has an effect on the overall angle of petiole positioning. However the response is also greater than the *phot1-5 phot2-1* double mutant and was not measured over time. The angle of positioning can be measured over time using time lapse imaging, which may show that constitutively associated phot1 may require a longer time scale to reach the same positioning as wild type. There may also be an effect of auxin gradients, which are required for growth of the plant (Leyser, 2010). Auxin is required for differential and nondifferential elongation of hypocotyls under altered light intensity and quality. Additionally auxin is required for high temperature induced hypocotyl elongation and heat induces transcription of the auxin-responsive genes IAA4, which suggests sensitization of the tissues to auxin (van Zanten et al. 2010).

It is unknown if anchoring phot1 to the plasma membrane could have an effect on the interaction with any other proteins downstream and consequently affect responses controlled by this interaction. The role of plasma membrane bound phot1 in leaf expansion was also investigated and the analysis showed that there is no difference in leaf expansion when phot1 was farnesylated or myristoylated compared to wild type (Fig. 5.9B and Fig. 5.13B). This shows that internalisation of phot1 from the plasma membrane is not required for leaf expansion and that cytosolic phot1 found after illumination does not have a role to play in this response either. It is also intriguing that this measure of plant growth is not affected by plasma membrane anchoring of phot1 suggesting that perhaps auxin gradients are not affected. There are however a number of auxin transporters and receptors (Lee, Choi and Cho, 2013, Habets and Offringa, 2014) that are involved in plant growth and development as well as other phytohormones and photosynthesis (Neumann, Imani and Kumar, 2009) that can affect growth and these may play a redundant role if phot1 is unable to interact with downstream effectors.

Similar results were also found with chloroplast positioning where constitutive association of phot1 to the plasma membrane does not affect the chloroplast accumulation response under low light conditions (Fig 5.10A and Fig 5.14A). There are a slightly greater number of chloroplasts accumulating in the lines

expressing farnesyl-tagged phot1-GFP and myrsitoyl-tagged phot1-mCitrine. This would suggest that perhaps there is an enhanced response by the chloroplasts when phot1 remains localised at the plasma membrane, there is also however a greater accumulation of chloroplasts in some of the controls (Fig 5.10B and Fig 5.14B), a response that is also seen in wild-type plants treated with red light. A red-light pre-treatment resulted in greater accumulation of chloroplasts and was mediated by phyA, similar to the mediation of phyA and phototropism (Han et al., 2013). The discrepancy in the farnesyl-tagged phot1-GFP lines could be investigated further by measuring the kinetics of chloroplast accumulation since the measurements in this study are taken at a specific end point and do not take into account any transient movement of chloroplasts within a single cell depending on light conditions. This would require microbeam irradiation to measure the movement of the chloroplasts in different light conditions (Wada, 2013). Another way to monitor chloroplast movement is by measuring any changes in red light transmission through leaves over time (de Carbonnel et al., 2010, Davis and Hangarter, 2012). This would perhaps provide a better indication as to chloroplast accumulation over time.

Interestingly expression of phototropin from the algae *Ostreococcus* (Otphot) in the *phot1-5 phot2-1* mutant background shows that there is a phototropin that is unable to internalise in response to light (Fig. 5.15B). This allowed us to investigate if the physiological responses could be complemented in a similar way to *Chlamydomonas* phototropin (Onodera et al., 2005). We were unable to obtain T3 homozygous lines despite numerous attempts and therefore analysed the best expressing T2 lines. This means that there is more variation in the progeny but experiments were repeated at least three times and representative images are shown. Onodera et al. (2005) also had difficulties obtaining T3 homozygous lines expressing phototropin from *Chlamydomonas* as there was evidence of male sterility. This may be a feature common to algal phototropins as they are functioning in a completely different organism. The *Chlamydomonas* phototropin behaved more similar to phot2 then phot1 which would be expected given the lineage of phot2 which is closer to lower plant phototropins than phot1 (Briggs et al., 2001). However Otphot does not undergo phototropism at higher light intensities where phot2 controls the response in *Arabidopsis*. This may be due to the position of the native phototropin protein in *Ostreococcus* which is

the smallest free living organism (Courties et al., 1994). The lagoon where the algae were discovered is typically exposed to high irradiation (Courties et al., 1994) indicating that the phototropin may have developed to respond to these higher light intensities and would require more light to induce the same response as *Arabidopsis* phototropin. The Otphot may be able to function in petiole positioning and leaf flattening as there are a number of other factors that are involved in these responses, whereas phototropism is largely controlled by phototropins in *Arabidopsis*. Intriguingly the photocycle of the LOV1 domain of Otphot shows that it is much slower than *Arabidopsis* phot1 indicating that it does require more light for the reaction (Veetil et al., 2011). Further work with the Otphot would need to be carried out to assess the effect of the higher light intensities on functions in *Arabidopsis* and to identify if there is a fluence rate where Otphot begins to function in responses other than petiole positioning and leaf expansion.

The complementary methods of tagging phot1 with a farnesyl tag at the C-terminus and independently with a myristoyl tag at the N-terminus illustrate that phot1 can be constitutively targeted to the plasma membrane indicating that trafficking of the protein to the plasma membrane is not affected by the lipid modification. The reasoning for constitutively localising phot1 to the plasma membrane resulted from the findings that a red-light pretreatment enhanced the phototropic response, suggested to be due to phyA dependent retention of phot1 at the plasma membrane (Han et al., 2008). Analysis of red-light enhancement in the lipidated lines shows that this still occurs indicating that it is not the retention of phot1 itself that causes the enhanced phototropic response after red light pretreatment (Preuten et al., 2015). We also analysed NPH3 dephosphorylation and discovered that this process still occurs indicating that phot1 is still able to interact with this target which is absolutely required for phototropism (Motchoulski and Liscum, 1999). Without NPH3, *Arabidopsis* seedlings are aphototropic and dephosphorylation requires active phot1 to present (Pedmale and Liscum, 2007). Since NPH3 localises to the plasma membrane perhaps this interaction occurs in this region, hence constitutively localising phot1 to the plasma membrane does not hinder this. Taken together the results show that plasma membrane localised phot1 retains the ability to regulate physiological responses that are controlled by phot1 in wild type plants.

There may be some effect on functions that have not been tested and on downstream process. There is also the indication that perhaps constitutively localising phot1 to the plasma membrane affects the robustness of the phototropic response and this could be analysed in more detail using time lapse imaging of the seedlings at the point of blue light illumination. The function of partial internalisation of phot1 from the plasma membrane therefore still remains to be fully understood.

Chapter 6: Final Discussion

6.1 Introduction

The phototropins are blue light receptor kinases involved in controlling a wide variety of physiological responses in plants (Christie, 2007; Pedmale, Celaya and Liscum, 2010; Christie and Murphy, 2013; Goyal et al., 2013; Hohm et al., 2013). The photocycle of the LOV domains and the autophosphorylation of phototropins upon blue light illumination is well characterised. The localisation of phot1 and phot2 to the plasma membrane is also well characterised but the mechanism of membrane attachment is not known. There is also a lack of knowledge regarding the mechanism by which internalisation to cytosolic strands occurs for phot1. Therefore the aims of this thesis were to identify if there was specific region of membrane interaction within phot1 and to assess the physiological functions of the internalisation response to blue light illumination. In this final chapter, the data from preceding chapters are summarised and opportunities for future research is discussed.

6.2 Analysis of the kinase domain of phot1 in membrane association

The kinase domain of the phototropins is known to be important for the membrane association of both phot1 and phot2 in *Arabidopsis* and autophosphorylation is an important step required for relocalisation (Kong et al., 2006, Kong et al., 2007; Kaiserli et al., 2009). However the mechanism of attachment to the plasma membrane, and identifying the region within the kinase domain responsible, had not been demonstrated before the beginning of this project. Therefore the kinase domain of phot1 was analysed for regions conserved in other phototropins, in addition to truncation analysis to help identify regions within the kinase domain that were responsible for targeting phot1 to the plasma membrane.

6.2.1 The association of phot1 with the plasma membrane is most likely through a lipid interaction

The insect cell system has been used previously to analyse phot1 and in Chapter 3 this system was demonstrated to be once again useful for the analysis of phot1 membrane association. Firstly, we investigated the localisation and activity of phot1 in the insect cell system. Fractionation analysis revealed that the phot1 protein is localised in the soluble and membrane fraction (Fig. 3.1B). However only the membrane fraction is active, as the soluble fraction does not undergo light dependent autophosphorylation (Fig. 3.3B). This is consistent with the localisation of phot1 in *Arabidopsis*. However ultra-centrifugation does not separate out the membrane compartments therefore phot1 may be associating with a different membrane, such as the endoplasmic reticulum. To test this a sucrose density gradient could be used with insect cell expressed phot1 to separate out the different membrane compartments and western blot analysis could subsequently be used to assess the localisation of phot1. There is also the possibility that the soluble phot1 from insect cells is not folded correctly. However the insect cell system is an overexpression system, therefore it is more likely that the soluble protein detected is unable to localise to the membrane.

Solubilisation analysis and detergent treatment of phot1 from insect cell membranes as well as *Arabidopsis* microsomal membranes was then performed to assess if there are electrostatic interactions that play a role in the membrane association of phot1 (section 3.2.3). The non-ionic detergents Triton X-100 and DDM appear to be the best at solubilising phot1 from the membrane whilst retaining kinase activity. The absence of soluble protein after treatment with salt, which reduces protein:protein interactions, indicates that phot1 does not interact with another protein to associate with the membrane (Lodish et al., 2000). Indeed, such a protein would also have to be conserved in insect cells, where *Arabidopsis* phot1 and phot2 are membrane associated, in *Arabidopsis* itself as well as *Chlamydomonas* where the phototropin is also found associated with the membrane in this alga (Huang et al., 2002). It is highly unlikely that such an interacting protein would be conserved in both the insect cell system, *in planta* and in algae.

The solubilisation of proteins using Triton X-100 and DDM is routinely used for proteins that interact with lipids as it does not disrupt protein:lipid interactions (Seddon et al., 2004). Since these two detergents are both able to solubilise *Arabidopsis* phot1 and phot2 from insect cells as well as plant microsomal membranes, in addition to Otphot expressed in insect cells, it would suggest that there is an interaction between phototropins and lipids at the plasma membrane. Indeed there is conserved lipid content between *Arabidopsis* and *Sf9* insect cells. Both organisms contain a more complex mixture of lipids compared to *E. coli* cells where only the lipds phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and cardiolipin (CL) are identified at the membrane (Marheineke et al., 1998, Opekarová and Tanner, 2003, Zhou et al., 2014). This may help to explain the difficulties experienced when expressing phot1 in an active form in *E. coli*. Although it has been recently shown that active phototropins can be produced in *E. coli* the reaction requires a greater quantity of radiolabelled ATP and the reaction occurs at higher temperatures than for the same reaction when phot1 is expressed in insect cells or from *Arabidopsis* (Matsuoka & Tokutomi 2005; Okajima et al. 2011; Aihara et al. 2012; Okajima et al. 2014). We have been unable to produce active phototropins in *E. coli,* including Otphot, and expression of the LOV2 and kinase region from phot1, which is able to phosphorylate PKS4 (Demarsy et al., 2012), was unable to phosphorylate Otphot as a substrate. This is an obvious discrepancy and requires further investigation to fully understand how we could produce active phot1 in *E. coli* for future experiments such as structural studies of the full-length protein.

The analysis of phot1 and lipids has been difficult to examine until now due to the difficulties in purifying active phot1. The use of the non-ionic detergents to solubilise the protein in an active form from insect cells and *Arabidopsis* microsomal membranes now makes this easier and the phot1 protein can be used to help identify if phot1 does interact with lipids and which lipids this interactions occurs with. A lipid overlay assay could be performed using various lipids spotted onto nitrocellulose membrane and incubated with the purified phot1 (Dowler, et al., 2002; Simon et al., 2014) to narrow the identity of the lipids. Solubilised phot1 from insect cells could also be used alongside phot1

expressed in *E. coli* cells to provide the lipid environment that phot1 is required to be present in to be active and functional.

Analysis of the lipid content of *Arabidopsis* and *Sf9* cell revealed that there are lipids belonging to the phosphoinositides found in small abundance in the organisms (Opekarová and Tanner, 2003). Phosphoinositides are negatively charged phospholipids and intriguingly have the innate capacity to bind proteins and alter their cellular localisation and activity (DeWald et al., 2001). One member of the family is phosphatidylinositol 4,5-bisphosphate (PIP_2) which has an important role to play in the regulation of clathrin-mediated membrane trafficking in *Arabidopsis* and contributes to the subcellular polarisation of the PIN proteins, therefore directing auxin transport (Ischebeck et al., 2013). An *Arabidopsis* mutant with reduced PIP_2 levels displayed abnormal auxin levels which was consequently attributed to apolar localisation of PIN1 and PIN2 (Ischebeck et al., 2013). Overexpression of PIP_2 also lead to abnormal auxin levels due to reduced membrane association of PIN1 and PIN2. The cycling of the PIN proteins between subcellular compartments is important for their function in auxin transport, a process that is partly regulated by clathrin mediated endocytosis (Habets and Offringa, 2014). Therefore the downregulation of PIP_2 prevents the lipid regulating clathrin mediated endocytosis and consequently PIN cycling (Boucrot et al., 2006, Ischebeck et al., 2013). Furthermore the production of PIP_2 was also shown to be connected to the auxin dependent gravitropic curvature in maize (Perera et al., 1999) indicating the importance of the lipid in controlling responses in plants.

PIP_2 clearly has an important role to play in clathrin mediated endocytosis as well as changing the subcellular localisation of proteins and therefore it is not unreasonable to suggest that phot1 association with the membrane and subsequent internalisation involves PIP_2. It has been shown that the internalisation of phot1 from the membrane occurs by clathrin mediated endocytosis (Kaiserli et al., 2009) and treatment of seedlings with 1-butanol lead to internalisation of phot1-GFP in the absence of a blue light stimulus (Sullivan *et al.*, 2010; Figure 4.13). Although the authors attribute this internalisation to the loss of phosphatidic acid (PA) production, 1-butanol can also affect the production of PIP_2 since the loss of PA reduces stimulation of the kinase

phosphatidylinositol 5-kinase (PI5K), involved in the reaction to produce PIP_2, hence decreased formation of PIP_2 (Boucrot et al., 2006). The internalisation of phot1-GFP in the absence of blue light within seedlings treated with 1-butanol may be due to reduced PIP_2 content within the hypocotyl cells resulting in a loss of interaction between the plasma membrane and phot1-GFP. It would therefore be interesting to look at the localisation of phot1 in the *Arabidopsis* mutant lines with reduced PIP_2 content. This could firstly be performed by simple fractionation experiments of the seedlings followed by western blotting, probing with anti-phot1 antibody. Transformation of the phot1-GFP construct into the *Arabidopsis* PIP_2 mutant or crossing of the phot1-GFP line with the PIP_2 mutant line and assessing the localisation by confocal microscopy would also be of interest to also monitor the effects of blue light treatment. This experiment however would need to take into consideration that the seedlings also contain native phot1 that may affect the localisation of the tagged protein.

6.2.2 The role of the LRM within the insertion loop of phot1

Identification of the conserved Lysine Rich Motif (LRM) in phototropins from lower to higher plants within the kinase insertion loop, only found in plant AGC kinases (Rademacher and Offringa, 2012), suggested that this may direct the localisation of phototropins to the plasma membrane. Lysine motifs have been identified in other proteins such as the dehydrin protein, which is involved in plant responses to abiotic stress and it directs membrane localisation by interaction with lipids (Koag et al., 2003, Graether and Boddington, 2014). Lysine residues are positively charged therefore have the potential to interact with the negatively charged lipids such as phosphatidic acid. Mutation of the LRM to asparagine did not affect membrane association in insect cells nor in *Arabidopsis* (Sections 3.2.5 and 3.2.7). Whilst this illustrates that the LRM is not involved in membrane association it does not rule out the possibility of other positively charged patches on the surface of phot1 that may be interacting with lipids and subsequently the membrane.

The role of the insertion loop in directing other proteins to the correct subcellular locations suggests that it may possibly have a role in directing membrane localisation of phot1. Mutation of the LRM within the insertion loop clearly does not affect the ability of phot1 to localise to the plasma membrane

(Fig. 3.7A and Fig. 3.8A). However other sequences around the LRM may influence the localisation as the insertion loop sequence is 40 amino acids long in *Arabidopsis* phot1. Expression of this region alone, fused to either a His-tag for expression in insect cells or a GFP-tag for transient expression in *N. benthamiana,* could be used to discover if this region could target to the plasma membrane. Another approach could be to remove the loop from phot1 and assess if the protein is now localised to the cytosol. Indeed this may be a better approach as the activity of phot1 could then also be measured.

It is interesting to note that mutation of a large section of the activation loop containing multiple lysine residues that is conserved from lower to higher plants does not affect the ability of phot1 to mediate the physiological responses controlled by the wild type protein. Phototropism is complemented both over a wide range of fluence rates as well as the kinetics of this response (Fig. 3.11 and Fig. 3.12). The other responses tested were also complemented in the *phot1-5 phot2-1* double mutant. This illustrates that phot1 is able to function correctly despite a mutation of multiple lysine residues within the insertion loop and suggests that these residues are not functionally important. The sequence alignment in Fig. 3.6 shows that other amino acids that are closer to the Mg^{2+} binding region as well as closer to the phosphoserines involved in phot1 activation are well conserved from lower to higher plants. Another approach to assessing membrane localisation of phot1 would be to mutate these conserved amino acids and assess localisation of the protein in insect cells or *N. benthamiana*.

6.2.3 Truncation analysis of the phot1 kinase domain does not indicate a specific region of interaction

The mutation of the LRM in phot1 did not prevent the protein localising to the plasma membrane nor impact its kinase activity or function, at least for the responses studied. Therefore we rationalised that there may be another region within the kinase domain that targets phot1 to the plasma membrane. Indeed the kinase domain alone is sufficient for membrane localisation in both insect cells and *N. benthamiana* (Fig. 4.2). Truncation analysis was performed on the kinase domain of phot1, which indicated that perhaps the region from amino acid 663 to amino acid 726 was involved in membrane association (Section

4.2.6). However upon expression of this small region alone the protein was soluble, both in insect cells and *N. benthamiana* transient expression system (Section 4.2.7). This indicates that this region is not solely responsible for membrane attachment. Alternatively the association of phot1 with the plasma membrane may occur on hydrophobic patches on the surface of the protein interacting with the membrane. A hydrophobicity plot of phot1 shows that there are indeed regions of phot1 along the length of the protein that are highly hydrophobic (Figure A.1). Taking into account the possibility that phot1 interacts with a negatively charged lipid at the membrane there are also regions of phot1 that are highly hydrophilic. Mutation of these residues could possibly allow the identification of a region of membrane interaction.

A further complication that may need to be taken into consideration when analysing truncated proteins is the findings from the analysis of the hydrophilic membrane protein, SYMBIOTIC REMORIN 1 (SYMREM1) from *Medicago truncatula*. The protein has a S-acylation site in the C-terminus of the protein which is lost upon mutation to alanine but this only occurs in the full length protein, not when a truncated region is expressed (Konrad et al., 2014). This illustrates that although protein sequence is important for directing subcellular localisation other modifications to proteins can also help direct proteins to associate with the plasma membrane. This may help explain the discrepancy between the expression of phot1 726 and phot1 663-726 (Fig. 4.9 and Fig. 4.11). Post-translational modifications play an important role in plant growth, development and environmental responses (Running, 2014). Phototropins undergo phosphorylation in response to light and recently proposed to be undergo ubiquitination (Christie et al., 1998, Roberts et al., 2011, Deng et al., 2014). Expression of the small region from amino acid 663 to amino acid 726 alone may have lost sites outwith this region that may be required for post-translational modifications. Indeed the putative ubiquitination site identified by Deng et al. (2014) is upstream at amino acid 526. It would be interesting to mutate this residue in the phot1 Δ726 construct and assess membrane localisation in insect cells.

The analysis of membrane localisation in *N. benthamiana* is a quick and convenient method to analyse the phot1 truncations. However there are

limitations of the method. The vacuole of the plant cells pushes outwards against the membrane resulting in difficulty in determining definitively if a protein is membrane or soluble. With full-length phot1 this is easier as the protein is clearly defined at the membrane and internalises to cytosolic strands (Fig. 4.4A). For the truncations however there were difficulties in deciding if the protein was truly membrane localised or not. Transient expression of the free GFP provided a useful soluble control but co-localisation of the truncated proteins with a membrane marker such as FM4-64 would have allowed us to determine definitively the localisation pattern. Another method that would also be useful would be to transform the *Arabidopsis phot1-5 phot2-1* double mutant with the truncated proteins and assess localisation in the T1 generation. Since the T1 generation is not homozygous there may be more than one copy of the gene, which would need to be taken in to consideration in the analysis. Alternatively membrane localisation could be visualised in root cells where the vacuole doesn't take up as much space. It would however make the definition of which is membrane-localised protein and which is soluble protein easier thereby allowing the region of interaction to be better defined.

Another limitation of the truncation analysis is the folding of the protein. Use of the predicted secondary structure (Fig. 4.8) allowed us to avoid truncating phot1 within any secondary structures such as α-helices or ß-sheets. However this is only a predicted structure and since there is no crystal structure we cannot be sure that the truncations have not impacted the structure. The truncated protein may also have misfolded, which would result in mislocalisation in general. However truncation analysis is still a valuable method to help identify the region of membrane association and has been used for various other proteins to identify regions of interaction and regions required for function (Dodds et al. 2001; Lin et al. 2005; Sullivan et al. 2009; Jaillais et al. 2011; Kong et al. 2013).

6.2.4 Constitutively membrane localised phot1 is still functional in Arabidopsis

The membrane localisation and subsequent internalisation of both phot1 and phot2 is well documented (Sakamoto and Briggs, 2002; Kong et al., 2006, Kong et al., 2007; Han et al., 2008; Kaiserli et al., 2009). However the role the relocalised proteins play in the physiological functions controlled by the

phototropins remains unanswered. We therefore tagged phot1-GFP with a farnesylation sequence to constitutively localise the protein to the plasma membrane. Expression in *N. benthamiana* and *Arabidopsis* resulted in no internalisation of phot1-GFP after blue light illumination (Fig. 5.2 and Fig. 5.3). Interestingly analysis of the physiological responses of the farnesyl-tagged phot1-GFP revealed that constitutive membrane localisation of the protein does not affect the physiological responses controlled by the phototropins such as phototropism, petiole positioning, leaf expansion or chloroplast accumulation (Fig. 5.7, Fig. 5.8, Fig. 5.9 and Fig. 5.10). This suggests that internalisation of phot1 from the plasma membrane is not required for these responses. Indeed myristoyl-tagged phot1-mCitrine from our collaborators showed the same complementation of the *phot1-5 phot2-1* double mutant (Chapter 5). Although the kinetics and fluence response of phototropism was measured, first positive phototropism was not. This response occurs proportionally to the light intensities (Sakai and Haga, 2012). Taking in to consideration the slightly reduced phototropic curvature in the farnesyl-tagged phot1-GFP lines (section 5.2.4) there may be a change in first positive phototropic curvature as it occurs before the phototropism measured in this study. Alongside this the refractory period after saturating light conditions would be interesting to measure as these are both responses that are much more fine tuned than the whole plant response measured here. It would not be surprising to discover that internalisation from the membrane is required for more fine control of plant responses or desensitisation of the receptor since the relocalisation occurs within a small period of time following blue light illumination.

There are other responses that are controlled by phot1 that were not tested such as rapid hypocotyl growth inhibition, although it seems unlikely that a growth response would be affected given the complementation of the *phot1-5 phot2-1* double mutant for other phot-mediated responses. The phosphorylation of *BLUS1* by phot1 is likely to occur in the cytoplasm as *BLUS1* was found to localise to this subcellular compartment (Takemiya et al., 2013). Perhaps cytosolic phot1 is required for this phosphorylation and subsequent stomatal opening. This is another response that could also be tested in the lipid modified phot1 lines as stomatal opening is also required for increasing photosynthetic efficiency. Another response that may however be affected is the mRNA stability

controlled by phot1. Under high fluence rates of blue light, equivalent to one minute of sunlight, phot1 destabilises specific light-harvesting, chlorophyll-binding (*Lchb*) transcripts resulting in degradation (Folta and Kaufman, 2003). In the *phot1-5* mutant however, the transcript levels accumulate. The cytoplasmic strands visualised after blue light treatment may be required for interaction with the mRNA of *Lchb* and perhaps other transcripts involved in photosynthesis. The transcript levels of *Lchb* could be easily measured in the farnesyl-tagged phot1-GFP lines using qPCR and perhaps shed some light on why phototropins relocalise in response to illumination.

The orthogonal methods used to constitutively localise phot1 to the plasma membrane illustrate that trafficking of phot1 to the plasma membrane is not affected by addition of a lipid modification sequence. The reasoning for constitutively localising phot1 to the plasma membrane is the findings that a red-light pretreatment enhances the phototropic response, probably due to phyA dependent retention of phot1 at the plasma membrane (Han et al., 2008). Since this response is also seen in the lipid modified versions of phot1 it would suggest this is not the case (Preuten et al., 2015). A complimentary experiment would be to tag phot2-GFP with farnesylation sequence and assess if the localisation to the Golgi following illumination was prevented. Since phot2 alone is responsible for chloroplast avoidance and has been found to localise to the chloroplast outer membrane, it would be of interest to see if constitutive localisation would change the function of phot2 (Kong et al., 2013). This would require microbeam analysis for the visualisation of fine movement of the chloroplasts.

6.2.5 Ostreococcus phototropin does not fully complement phot-mediated responses in the phot1-5 phot2-1 mutant

Phototropins have been identified in lower plants including the unicellular green algae *Chlamydomonas* and this protein has been shown to complement phot-mediated processes when expressed in the *phot1-5 phot2-1* mutant of *Arabidopsis* (Huang et al., 2002; Onodera et al., 2005). Recently the alga *Ostreococcus tauri* was sequenced and revealed to also contain a phototropin. Expression of the protein in insect cells and *N. benthamiana* revealed the protein to be membrane localised (Fig. 3.2 and Fig. 3.8) and similar to *Arabidopsis* phot1, Otphot can be solubilised from insect cell membranes using

the non-ionic detergents DDM and Triton X-100 suggesting the mechanism of membrane attachment is the same in lower plants. We therefore investigated the protein further, discovering that it also contained a LRM, however mutation of the LRM in Otphot does not result in soluble protein (Fig. 3.8). This indicates that the properties of *Arabidopsis* phot1 and Otphot are very similar and since *Chlamydomonas* phototropin was able to function in the *phot1-5 phot2-1* double mutant we investigated the function of Otphot in *Arabidopsis*.

The Otphot construct was transformed into *phot1-5 phot2-1* double mutant under the control of the *CaMV-35S* promoter. Whilst this promoter was also used to drive expression of *Chlamydomonas* phototropin it would be better to express all phototropin constructs under the control of the native *Arabidopsis PHOT1* promoter to ensure the correct spatial expression of the protein being analysed. This may also help ensure that T3 constructs are obtained as we experienced difficulties with producing homozygous lines. The T2 plants still allowed function to be assessed. Interestingly, Otphot was unable to complement the phototropism under a wide range of fluence rates tested (Fig. 5.16) whilst petiole positioning and leaf expansion were both complemented. The latter two responses are measured under relatively low fluence rates of light (70 μmol m^{-2} s^{-1} white light and would contain low levels of blue light) indicting that the protein is functional and must be light responsive. The photochemistry of Otphot has been examined in preliminary experiments from our group. The LOV1 and LOV2 domains from Otphot were expressed alone, as the LOV domains expressed alone are known to be a model for the photochemistry of the full-length photoreceptor (Kasahara, Swartz, et al., 2002). Indeed the initial results from our group indicate that the Otphot photocycle is slower than *Arabidopsis* phot1 and therefore requires more light for the photochemistry reaction. The effect of mutating the conserved cysteine residues within the LOV domains could also be measured to ascertain if the LOV2 domain is the main light sensor for Otphot as it is for the other phototropins assessed. Transformation of these mutants into the *phot1-5 phot2-1* double mutant and analysis of petiole positioning and leaf expansion would also be of interest to assess the impact of the LOV domains on these responses that are complemented by the full-length protein (Christie et al., 2002, Cho et al., 2007, Suetsugu et al., 2013).

The inability of Otphot to complement phototropism or chloroplast accumulation is of great interest as it is a function that appears to be complemented by all other phototropins assessed and is interesting as the other alga, *Chlamydomonas,* investigated in *Arabidopsis* was able to complement the double mutant. Remarkably however, Crphot is unable to complement phototropism in the low and medium expressing lines (Onodera et al., 2005), therefore perhaps expressing Otphot at a similar level to Crphot in *Arabidopsis* would result in complementation of phototropism.

It is intriguing that an evolutionary distant phototropin is unable to complement phototropism and chloroplast avoidance. Otphot is found at the base of the green lineage (Finet *et al.*, 2010, Figure A.2) and will not have the same requirements for phototropism as higher plants such as *Arabidopsis*. In *Chlamydomonas,* the phototropin is responsible for eyespot development, a part of the flagella important for phototaxis (Trippens et al., 2012). Phototactic movement is a movement of the organism in response to light in a similar way that the hypocotyls of *Arabidopsis* curve in response to light. Interestingly flagella are absent from *Ostreococcus* indicating that the organism does not use an eyespot for movement in a similar way to the eyespot of *Chlamydomonas* (Peers and Niyogi, 2008). This could help explain the lack of phototropism and chloroplast movement in the transgenic *Arabidopsis* lines expressing Otphot as the organism does not require the response.

There are another three *Ostreococcus* genomes that have been sequenced and it would be informative to assess if these contain phototropin homologs and if they are able to complement the phototropic response in *Arabidopsis*. As a control the *Chlamydomonas* phototropin could also be expressed this time under the control of the native *PHOT1* promoter. Alongside this, autophosphorylation activity in the insect cell system could be analysed. It would be of interest to know if the algal phototropins are active at the membrane in a similar way to *Arabidopsis* phot1. It would seem likely since Otphot is solubilised from insect cell membranes using non-ionic detergents. It would also be useful to analyse the sequenced *Ostreococcus* genomes to identify if there are homologs of the known phot1 interactors such as NPH3, required for phototropism, RPT2, PKS4 or the 14-3-3 proteins. Comparison of this with the *Chlamydomonas* genome would

provide an interesting insight into the phototropins, specifically what is different about Crphot compared to Otphot that means it is able to function in phototropism and chloroplast positioning. Perhaps the Otphot is unable to phosphorylate the downstream targets that *Arabidopsis* phot1 and phot2 interact with and is therefore unable to complement this function.

6.3 Conclusion

The aims of this project were to try and elucidate the mechanism of phot1 attachment to the plasma membrane. Solubilisation analysis of phot1 from insect cell membranes as well as *Arabidopsis* microsomal membranes indicated that the mechanism of attachment was likely through non-ionic interactions with an as yet unidentified lipid(s). Identification of a LRM within the kinase domain suggested that perhaps this positively charged region might be responsible for directing phot1 to the plasma membrane. Mutational analysis in *Arabidopsis* showed that this was not the case and the protein was fully functional with respect to the physiological responses tested. Following this, truncation analysis of the kinase domain of phot1 in insect cells revealed a possible small region of membrane interaction within the N-terminus of the kinase. This region was unable to target GFP to the plasma membrane in *N. benthamiana* indicating the role of other regions of the kinase domain are likely to be involved in the membrane association. Finally the role of internalisation of phot1 from the plasma membrane to cytosolic strands was investigated by the use of a farnesylation sequence to constitutively target phot1 to the plasma membrane. This revealed that the internalisation response is not essential for phototropism or responses that serve to optimise photosynthetic efficiency of the plant. The transgenic plants produced in this study will provide useful tools to analyse the small scale responses controlled by phot1 such as mRNA stability and the refractory period of phototropism. Overall the aims set out at the beginning of this study have largely been achieved.

Appendix

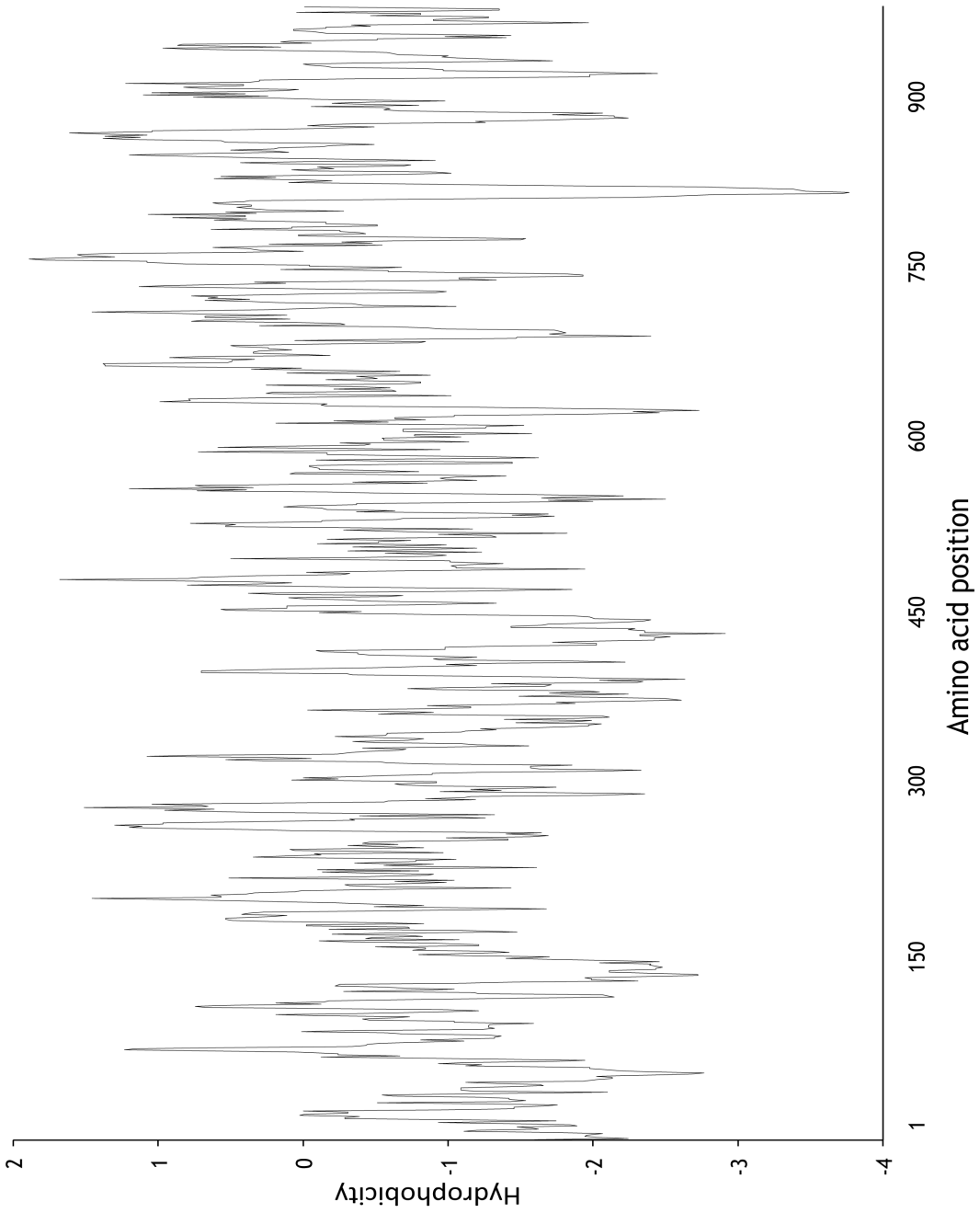


Figure A-1: Hydrophobicity plot of *Arabidopsis* phot1

Hydropathy plot corresponding to sequences spanning the entire phot1 protein. The hydropathy plot (mean values for a window of 19 amino acids) was constructed using the Kyte–Doolittle (KD) hydrophobicity index. Negative values represent highly hydrophilic regions.

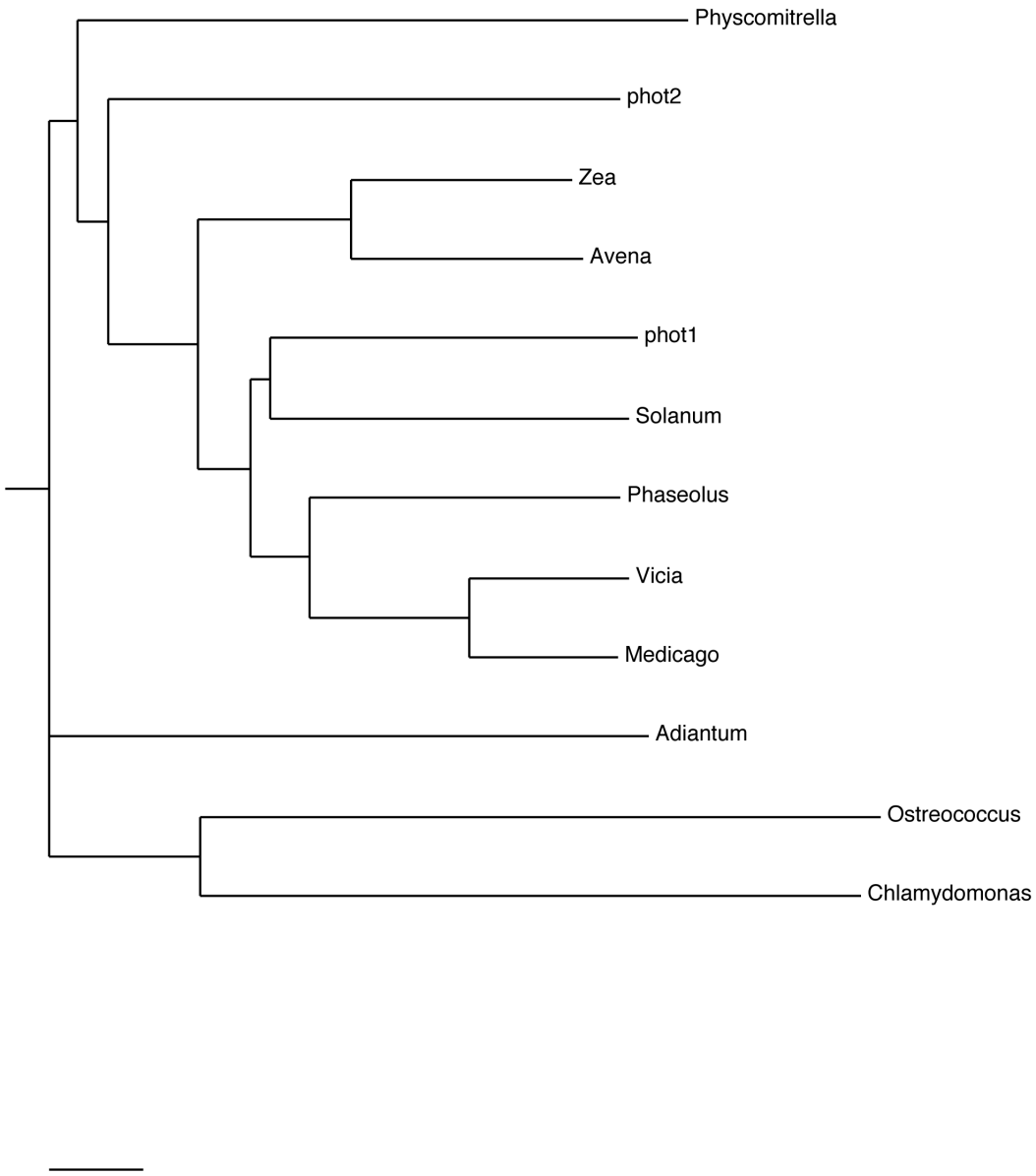


Figure A-2: Phylogenetic tree of phototropin proteins from lower and higher plants

Phylogenetic tree illustrating the position of *Arabidopsis* phot1 and phot2 relative to other phototropins. The tree was built using the Phylogeny program from ClustalW. Scale bar represents a genetic change of 0.1. Sequences are derived from *Physcomitrella patens* photB2, *Adiantum capillus-veneris* phot1, *Arabidopsis* phot2, *Zea mays* phot1, *Avena sativa* phot1, *Arabidopsis* phot1, *Solanum lycopersicum* phot1, *Phaseolus vulgaris* phot1a, *Vicia faba* phot1, *Medicago truncatula* phot2, *Ostreococcus tauri* phot, *Chlamydomonas reinhardtii* phot.

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