# STRUCTURE-FUNCTION ANALYSIS OF PHOTOTROPIN RECEPTOR KINASES

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## Abstract

The ability of plants to convert energy provided by the sun into a form accessible by heterotrophic organisms ensures that they form a basal part of most ecosystems. However, in addition to being a vital energy source light can also serve as an environmental indicator. In order to maximise light perception, plants have evolved a suite of photosensors with differing sensitivities, which in combination provide detailed information regarding light availability and quality. The major photoreceptor families identified in plants include the phytochromes, which are most sensitive to red and far-red light, and the cryptochromes and phototropins, which are UV-A and blue light receptors. Additional photosensors, including the recently identified ZEITLUPE/ADAGIO family, have roles in modulating the action of these main components. Plants can also respond to UV-B and green light, although the photoreceptors responsible for their detection remain elusive.

Phototropins are blue light sensors that are responsible for a range of responses (including phototropism, chloroplast movement and stomatal opening) that combine to increase the photosynthetic efficiency of plants. Initially identified in the model plant *Arabidopsis thaliana*, phototropins have since been characterised in the unicellular alga *Chalmydomonas reinhardtii*, pteridophytes and angiosperms. The light sensitivity of phototropins is derived from the action of two highly conserved regions known as LOV domains which subsequently induce activity of an integral serine/threonine kinase domain via movement of a conserved α-helix (Jα-helix).

Although little is known regarding phototropin signal transduction an obvious biochemical consequence of phototropin light stimulation is autophosphorylation. Phototropin autophosphorylation has previously been studied using a baculovirus/insect cell expression system, with the consequences of phototropin mutation on phototropin kinase activity within this heterologous system having comparable effects to those when identically-mutated phototropins were introduced into transgenic *Arabidopsis*. Whilst the ability of phototropins to act as light-regulated kinases is well established, the mechanism by which this occurs remains unclear. In this study a baculovirus/insect cell expression system is used to further characterise the mode of phototropin autophosphorylation and the functionality of a mutated

phototropin 1 which demonstrates increased autophosphorylation activity in this system is assessed *in planta* using transgenic *Arabidopsis*.

It was initially of interest to further evaluate the mode of phototropin autophosphorylation. For example, it is unclear whether phototropin autophosphorylation occurs via an intramolecular mechanism or whether this process involves cross-phosphorylation between phototropin molecules. In Chapter 3 the mode of phototropin autophosphorylation activity was further examined using the baculovirus/insect cell expression system to assess the effect of protein truncations and specific point mutations on phototropin kinase activity. Such mutational analysis reveals that phototropin 1 is capable of intermolecular phosphorylation *in vitro* and also suggests that further phototropin autophosphorylation sites exist in addition to those previously mapped. Additionally, the importance of LOV2 and the Jα-helix as components of the phototropin receptor activation are confirmed. The implications of such findings for our understanding of phototropin autophosphorylation are discussed.

LOV domains bind flavin mononucleotide as a chromophore. The light-induced formation of a covalent adduct between the LOV domain and the associated chromophore is thought to induce conformational changes culminating in phototropin kinase activation. In Chapter 4 the two leading hypotheses proposed to permit signal transmission between the light-sensitive LOV domains and the integral kinase domain are examined. Previous work has shown that photosensitivity within one of the two LOV domains (LOV2) is sufficient for light-regulated kinase activity, suggesting that light-induced kinase activation is primarily induced via LOV2. This allowed the effect of point mutations within LOV2 on the light-regulated kinase activation of the full-length phototropin protein to be assessed using the baculovirus/insect cell expression system. Work presented here suggests that a single point mutation within LOV2 ($Gln^{575}$→Leu; Q575L) is sufficient to reduce signal transmission between LOV2 and the phototropin kinase domain whilst not altering the photosensitivity of the LOV2 domain. In contrast, mutation of a highly conserved salt bridge at the surface of LOV2 does not alter LOV2 domain signal transmission. Interestingly, the reduced light-induced kinase activity of the phot1 Q575L mutant could be counteracted by an additional mutation within the Jα-helix ($Ile^{608}$→Glu; I608E) which has previously been shown to increase the autophosphorylation of mock-treated phot1 *in vitro* in

comparison with a wild-type control. Such findings suggest that conformational changes may occur in sequence to induce phototropin kinase activation.

The final component of this work involved transgenic *Arabidopsis* to examine the *in planta* functionality of phot1 I608E, which has been shown to have increased autophosphorylation *in vitro* when expressed using the baculovirus/insect cell system. However, rather than demonstrating phenotypes consistent with a constitutively-active form of phototropin, transgenic *Arabidopsis* expressing phot1 I608E appeared to have only partial functionality *in planta* and indeed appeared to inhibit phot-mediated phenotypes when expressed in a wild-type background. Possible explanations of the observed phenotypes are discussed.

The studies presented here further advance our knowledge of the light-induced mechanism which results in activation of the phototropin photoreceptor and also provide insight into the potential differing roles of phototropin autophosphorylation *in planta*.

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## Publications

Components of this thesis have been published in the following journal articles;

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Sullivan JS, Thomson CE, Lamont DJ, **Jones, MA** and Christie JM (2008) *In vivo* phosphorylation site mapping and functional characterisation of *Arabidopsis* phototropin 1. Mol Plant 1: 178-194

# Contents

## CHAPTER 3 - ANALYSIS OF PHOTOTROPIN AUTOPHOSPHORYLATION ACTIVITY

# List of Figures and Tables

# Abbreviations

The following abbreviations are used during this thesis;

| | |
|---|---|
| ADO | ADAGIO |
| $^{32}$P-ATP | Adenosine 5'-[γ-$^{32}$P] triphosphate |
| 35S | Cauliflower mosaic virus *35S* promoter |
| AGC | cAMP-dependent protein kinase A, cGMP-dependent protein kinase G, phospholipid-dependent kinase C |
| AKAP | A-kinase anchoring protein |
| ARF | ADP-ribosylation factor |
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| BTB/POZ | Broad complex, tramtrack, bric-a-brac/ pox virus and zinc finger |
| CAB | Chlorophyll a/b-binding protein |
| cAMP | Cyclic adenosine monophosphate |
| CCT | Cry C-terminal |
| cDNA | Complementary DNA |
| CHS | CHALCONE SYNTHASE |
| CHUP | CHLOROPLAST UNUSUAL POSITIONING |
| CO | CONSTANS |
| $CO_2$ | Carbon dioxide |
| COP | Constitutively photomorphogenic |
| CPT | COLEOPTILE PHOTOTROPISM |
| cry | Cryptochrome |
| CUL | CULLIN |
| DASH | *Drosophila*, *Arabidopsis*, *Synechocystis*, Human homology domain |
| $dH_2O$ | Distilled water |
| dNTPs | Deoxyribonucleotide triphosphates |
| DNA | Deoxyribonucleic acid |
| FAD | Flavin adenine dinucleotide |
| FBP | F-Box protein |

| | |
|---|---|
| FHL | FHY-LIKE |
| FHY | FAR RED ELONGATED HYPOCOTYL |
| FKF1 | FLAVIN BINDING, KELCH REPEAT, F-BOX |
| FMN | Flavin mononucleotide |
| FTIR | Fourier transform infra-red |
| GAF | cGMP-specific and -regulated cyclic nucleotide phosphodiesterase, Adenylyl cyclase and FhlA homology domain |
| GFP | Green fluorescent protein |
| GI | GIGANTEA |
| GST | Glutathione S-transferase |
| GUS | β-glucuronidase |
| HIR | High Irradiance Response |
| His-tag | Histidine-tag |
| IAA | Indole-3-acetic acid |
| IPTG | Isopropyl β-D-galactopyranoside |
| JAC | J-DOMAIN PROTEIN REQUIRED FOR CHLOROPLAST ACCUMULATION RESPONSE |
| kDa | Kilodaltons |
| L2K | LOV2 KINASE |
| LFR | Low fluence response |
| LKP2 | LOV KELCH PROTEIN |
| LOV | Light, Oxygen or Voltage sensitive |
| MG132 | Cbz-leu-leu-leucinal |
| mRNA | Messenger ribonucleic acid |
| MSG | MASSUGU |
| MTHF | Methenyltetrahydrofolate |
| neo | Neochrome |
| NLS | Nuclear localisation signal |
| nm | Nanometres |
| NMR | Nuclear magnetic resonance |
| NPH | NONPHOTOTROPIC HYPOCOTYL |
| NPH3-L | NPH3-LIKE |

| | |
|---|---|
| NRL | NPH3/RPT2-like |
| OD | Optical density |
| *p1p2* | *phot1-5 phot2-1* |
| PAPP5 | Phytochrome-associated protein phosphatase |
| PAS | Per/Arnt/Sim |
| PAT | Polar auxin transport |
| PCR | Polymerase chain reaction |
| PDK1 | 3-phosphoinositide-dependent protein kinase 1 |
| phot | Phototropin |
| PHR | Photolyase Homology Region |
| phy | Phytochrome |
| PID | PINOID |
| PIF | PHYTOCHROME INTERACTING FACTOR |
| PIN | PINFORMED |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKI | PROTEIN KINASE A INHIBITOR |
| PKS | PHYTOCHROME KINASE SUBSTRATE |
| PLP | PAS/LOV PROTEIN |
| PMI | PLASTID MOVEMENT IMPAIRED |
| PP1 | type 1 protein phosphatase |
| PP7 | PROTEIN PHOSPHATASE 7 |
| R | Regulatory |
| RPT | ROOT PHOTOTROPISM |
| SCF | SKP1–CUL1–FBP |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| Sf9 | *Spodoptera frugiperda* cell line |
| SKP | S-PHASE KINASE-ASSOCIATED PROTEIN |
| STAS | Sulphate transport/ anti-sigma factor |
| SUB | SHORT UNDER BLUE LIGHT |
| T-DNA | Transfer-DNA |

| | |
|---|---|
| TOC | TIMING OF CAB1 |
| U | Units |
| UGPase | UDP-glucose pyrophosphorylase |
| UV | Ultra-violet |
| UVR8 | UV-RESISTANCE LOCUS 8 |
| v/v | volume/volume |
| VfPIP | *Vicia faba* PHOTOTROPIN1A INTERACTING PROTEIN |
| VLFR | Very Low Fluence Response |
| w/v | weight/volume |
| ZTL | ZEITLUPE |

# Chapter 1-Light Perception and Signalling in *Arabidopsis*

## 1.1 The necessity of light for plant growth

Plants form a fundamental, basal part of most ecosystems by converting energy provided by the sun into a form accessible to heterotrophic organisms. In addition to being an energy source, light is a predictive environmental indicator. Shortening days imply the onset of winter and subsequent reductions in temperature whilst the spectrum of light provided by the sun is enriched in the blue portion of the spectrum at dawn and dusk relative to midday (Whitelam and Halliday, 2007). Given these properties it is vital that plants possess suitable mechanisms to determine light availability and quality. Plants are capable of detecting differences in light direction, duration, quantity and wavelength (Whitelam and Halliday, 2007). Perhaps more importantly, they are able to respond appropriately to each of these stimuli in order to maximise their survival. This is achieved through the optimisation of available resources and by utilising the plasticity inherent to plant development.

The process by which developmental alterations occur in response to the changing light environment is referred to as photomorphogenesis (Whitelam and Halliday, 2007). Levels of light are monitored throughout the lifecycle of the plant, and subtle alterations to the developmental pathway are made at each point, optimising survival. In order to fully assimilate all of the available light-derived information plants have evolved a suite of photoreceptors, each of which provide sensitivity to different portions of the light spectrum by binding a light absorbing co-factor (referred to as a chromophore; Briggs and Spudich, 2005). Red and far-red light (600-750 nm) is primarily detected by the phytochrome family (Rockwell *et al.*, 2006) while blue and UV-A light (320-500 nm) is sensed by cryptochromes, phototropins and members of the recently characterised ZEITLUPE/ADAGIO family (Christie, 2007, Li and Yang, 2007, Briggs, 2007, Briggs and Spudich, 2005). In addition to these characterised photosensors, plants are also able to respond to 'green' (500-600 nm) and UV-B (290-320 nm) light, although the photoreceptors responsible for these responses have not been elucidated (Mackerness, 2000, Folta and Maruhnich, 2007). The structure and primary signalling mechanisms of plant photoreceptors will be

discussed below in relation to the ultimate physiological responses controlled by photoactivation.

## 1.2 The role of phytochromes in red light-sensing

### *1.2.1 Phytochrome structure and physiological function*

Phytochromes were initially identified in 1959 as the photoreceptor that mediates plant photomorphogenesis in response to long-wavelength visible light (Butler *et al.*, 1959). The phytochrome family has since been found to be ubiquitous amongst seed plants and cryptophytes, with examples also being found in cyanobacteria, non-photosynthetic bacteria and fungi (Rockwell *et al.*, 2006). Phytochromes have a conserved N-terminal photosensory domain which is coupled to a variety of different effector domains in different species. In higher plants, this C-terminal region consists of two Per/Arnt/Sim (PAS) domains and a histidine kinase (Figure 1.1, Rockwell *et al.*, 2006). Although multiple phytochrome effector domains have been identified this review will be restricted to plant phytochromes and their role in light detection and signal transduction.

Phytochromes (phy) are sensitive to irradiation by both red and far-red light, and uniquely function by measuring the relative amount of each of these wavelengths. The phytochrome basal state (designated $P_r$) is sensitive to red light and upon irradiation is converted to a far-red sensitive state ($P_{fr}$). Reversion to the $P_r$ form occurs either after far-red light exposure or as a consequence of dark incubation (Figure 1.2). The relative amounts of each of these forms determine downstream signalling events, with the $P_{fr}$ form considered to be the active signalling state (Huq and Quail, 2005). Increased ratios of far-red:red light are indicative of shading by overlying vegetation as plants absorb relatively little far-red light compared to other portions of the light spectrum (Franklin and Whitelam, 2007). Responses to such shading include elongation of stems and petioles at the expense of leaf and storage organ development, increased hyponasty and reduced branching (Franklin and Whitelam, 2007). These developmental changes (collectively termed shade avoidance) act to reposition the plant into an unshaded position (Franklin and Whitelam, 2005). In the persistence of shaded conditions phytochromes promote flowering to enhance the probability of reproductive success (Franklin and Whitelam,

2005). Phytochromes also play an important role in seed germination under a broad range of low fluences (Smith, 2000) and have a role in regulating the circadian clock as *phy* mutants and lines over-expressing phytochromes display altered circadian rhythms (Somers *et al.*, 1998).

Higher plant genomes encode a suite of phytochrome proteins each with slightly diverged light-sensitivity and function. *Arabidopsis thaliana* has five phytochrome proteins (designated phyA-E), each of which has slightly altered spectral characteristics (Eichenberg *et al.*, 2000, Clack *et al.*, 1994). In *Arabidopsis*, phyA accumulates to high levels in the dark whilst phyB has the highest level of expression at other times (Mathews and Sharrock, 1997). PhyC, phyD and phyE have comparatively minor roles and act to modulate phyB signalling (Franklin *et al.*, 2003). Angiosperm phytochromes can be placed into two broad groups based upon the stability of the red light irradiated $P_{fr}$ form. PhyA is rapidly degraded after illumination and is primarily involved in very low light responses (VLFR) or those involving high far red:red ratios (HIR). These two signalling modes are functionally different and appear to operate through at least partially distinct pathways (Casal *et al.*, 2000). The remaining phytochromes (phyB-E) remain stable after illumination allowing these phytochromes to control plant responses to intermediate and persistent illumination (low fluence response, LFR; Sharrock and Clack, 2002, Franklin *et al.*, 2005). LFR responses, mediated by phyB-E, are reversible and are determined by the ratio of red and far red light used to irradiate the plant (Schafer and Bowler, 2002). VLFR, HIR and LFR interact to facilitate light sensitivity under a broad range of light conditions. As phyA is light-labile, phyA is generally considered to be the primary photoreceptor in the dark and under low-light conditions with a role in seed germination and seedling de-etiolation, with phyB and related phytochromes having greater importance in mature tissue with regards shade avoidance (Schafer and Bowler, 2002).

Phytochromes are dimers of approximately 125 kDa subunits, each of which is bound to a phytochromobilin chromophore via a thioether bond to a conserved cysteine residue. The conserved PAS domains in the C-terminal region of the protein are responsible for this interaction (Figure 1.1, Kim *et al.*, 2006). The high levels of phyA expression in dark grown seedlings ensures that phyA forms mostly homodimers, although phyB-E have been shown to exist as heterodimer complexes (Sharrock and Clack, 2004, Jones and Quail, 1986, Lagarias and Mercurio, 1985).

Red light illumination results in phytochrome sub-cellular relocalisation, altered interactions with binding partners and the initiation of signalling cascades which may include phytochrome phosphorylation and the phosphorylation of target proteins (reviewed in Huq and Quail, 2005).

### *1.2.2 Phytochrome photosensory domain structure and photochemistry*

The N-terminal phytochrome photosensory region is highly conserved, consisting of PAS, GAF and phy domains in combination. GAF is an acronym of the founding members of this domain family; c**G**MP-specific and -regulated cyclic nucleotide phosphodiesterase, **A**denylyl cyclase, and **F**hlA. Phy domains are unique to the phytochrome family. The phytochrome PAS and GAF domains are required in combination for binding of the chromophore phytochromobilin, while mutation of the phy domain alters the spectral and photochemical properties of the domain (Rockwell *et al.*, 2006). Recent X-ray crystallography analyses have elucidated the interlinking structure of the PAS and GAF domains that are necessary for chromophore binding of a bacterial phytochrome (Wagner *et al.*, 2005, Wagner *et al.*, 2007). A high degree of sequence similarity within these domains between otherwise divergent phytochromes provides confidence that this structure is conserved between phytochromes and allows more informative modelling of primary phytochrome photochemistry.

Although the phytochrome photocycle still remains to be formalised structural insights from X-ray crystallography have been combined with other data to provide a tenable model of the interconversion between the $P_r$ and $P_{fr}$ forms (Rockwell *et al.*, 2006). Such a model proposes that red and far-red light-induced conformational changes within the chromophore are followed by subsequent light-independent reactions that result in formation of the appropriate signalling state (Figure 1.2). These conformational changes within the chromophore are suggested to modulate the surrounding protein structure to allow intra- and inter-molecular signalling (Rockwell *et al.*, 2006). Although these protein structural changes have not yet been elucidated a significant proportion of isolated loss-of-function and gain-of-function point mutations within the phytochrome photosensory motif are located within a group of GAF domain $\alpha$-helices, suggesting that movement within this region is important for signal transmission (Rockwell *et al.*, 2006). For example, mutation of a conserved tyrosine to histidine in both phyA and phyB causes constitutive phytochrome

signalling which is dependent on the presence of chromophore (Su and Lagarias, 2007). It has therefore been suggested that this mutation induces chromophore conformational changes that mimic those induced by red light irradiation. Additionally, expression of the phyA mutant (phyA Y242H) in a wild-type *Arabidopsis* background caused a dominant negative phenotype under HIR conditions (Su and Lagarias, 2007). The authors suggest that this is caused by heterodimer formation between wild-type phyA and phyA Y242H. Previous work has suggested that HIR requires phyA to converted to the $P_{fr}$ form and back again before it becomes functionally active (Shinomura *et al.*, 1996). PhyA Y242H is proposed to resist reconversion to the $P_r$ form, thereby inhibiting wild-type phyA function for this response (Su and Lagarias, 2007).

Several studies have reported conformational differences between the $P_r$ and $P_{fr}$ forms of phytochrome. While these studies are only of limited resolution (X-ray crystallography of the chromophore binding domains has thus far only been applied to the $P_r$ form of phytochrome; Wagner *et al.*, 2005, Wagner *et al.*, 2007), it appears that conversion to the $P_{fr}$ form does induce structural changes outwith the photosensory core. Limited proteolysis studies have shown that part of the phy domain becomes more susceptible to degradation while the ability of monoclonal antibodies to bind phytochrome differs between $P_r$ and $P_{fr}$ forms (Natori *et al.*, 2007, Noack *et al.*, 2007). These conformational changes ultimately result in phytochrome activation, which typically includes relocalisation to the nucleus from the cytoplasm and phytochrome phosphorylation (Kevei *et al.*, 2007, Kim *et al.*, 2005) although roles for activated phytochrome in the cytoplasm have also been identified (Rosler *et al.*, 2007).

The mechanism of phy nuclear translocation is still not fully understood but appears to act via a number of different mechanisms as the kinetics of phyA and phyB nuclear accumulation differ (Kevei *et al.*, 2007). Nuclear import appears to be necessary for most phytochrome functions, as mutants deficient in light-induced nuclear localisation, or phytochrome artificially retained in the cytoplasm, do not mediate certain downstream signalling events (Huq *et al.*, 2003, Matsushita *et al.*, 2003). Once localised to the nucleus phytochromes tagged with green fluorescent protein (GFP) form distinctive speckles that are thought to represent areas of transcriptional activity (Kircher *et al.*, 2002, Yamaguchi *et al.*, 1999). PhyA speckle formation is also associated with desensitisation as phyA speckles co-localise with

CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), an E3 ligase known to have a role in phyA degradation (Seo *et al.*, 2004). The nuclear relocalisation of phyA requires interaction with two plant specific proteins, FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and FHY1-LIKE (FHL; Hiltbrunner *et al.*, 2006, Hiltbrunner *et al.*, 2005). Interactions between phyA, FHY1 and FHL are enhanced by red light *in vitro*, suggesting that a conformational change subsequent to formation of phyA $P_{fr}$ promotes FHY1 and FHL binding and nuclear translocation. PhyB similarly undergoes nuclear transport after red light irradiation which requires a conformational change within the protein to expose one of the C-terminal PAS domains (Chen *et al.*, 2005). Whether this structural change reveals a non-canonical nuclear localisation signal (NLS) or facilitates binding of proteins containing an NLS is unclear at present (Kevei *et al.*, 2007).

A controversial consequence of phytochrome activation is phytochrome autophosphorylation. Phytochrome phosphorylation acts to modulate phytochrome interactions with partner signalling proteins and also has a role in receptor desensitisation (Kevei *et al.*, 2007, Kim *et al.*, 2005, Kim *et al.*, 2004). However, whether phytochromes are capable of autophosphorylation has yet to be formally determined. Phytochromes are likely descended from prokaryotic phytochromes which display light-regulated histidine kinase activity (Montgomery and Lagarias, 2002) and phytochrome phosphorylation has been observed in oat phytochromes heterologously expressed in yeast (Yeh and Lagarias, 1998). However, it has been shown that the C-terminal region of phytochrome containing the proposed kinase domain is not required for phytochrome function *in planta* (Matsushita *et al.*, 2003). Phytochrome autophosphorylation may therefore act to modulate phytochrome signalling rather than as a primary signalling mechanism (Huq and Quail, 2005), although phytochrome does appear capable of phosphorylating some phytochrome-interacting partners such as PHYTOCHROME KINASE SUBSTRATE 1 (PKS1; Fankhauser *et al.*, 1999). Phosphorylation of phyA increases in the $P_{fr}$ form and phosphorylation at the phytochrome N-terminus is thought to promote protein degradation (Kim *et al.*, 2005) while an additional phosphorylation at a specific serine residue within phyA ($Ser^{598}$) prevents the interaction between phyA and interacting partners such as PHYTOCHROME INTERACTING FACTOR 3 (PIF3; Kim *et al.*, 2004, Lapko *et al.*, 1997, Lapko *et al.*, 1999). Phytochrome phosphorylation may be reversed by the action of the phosphatases such as PHYTOCHROME-ASSOCIATED

PROTEIN PHOSPHATASE 5 (PAPP5) which preferentially dephosphorylates both phyA and phyB in the $P_{fr}$ form (Ryu *et al.*, 2005). It therefore appears that a complex interplay between $P_{fr}$-dependent phosphorylation and dephosphorylation modulates phytochrome biological activity and its degradation.

### *1.2.3 Signalling cascades induced by phytochrome activation*

The majority of phytochrome-mediated responses occur subsequent to phytochrome nuclear translocation and it has been estimated that approximately 10% of the *Arabidopsis* genome is altered after prolonged irradiation of etiolated seedlings (Quail, 2007b). Regulation of these diverse responses occurs in part through the action of phytochrome interacting factors (PIFs), of which 15 have now been identified (Quail, 2007a). These PIFs include a number of DNA-binding proteins which are thought to have a direct role in gene expression regulation. PIF single mutants often display clear mutant phenotypes and as such appear to have limited functional redundancy, implying that multiple signalling cascades are initiated by each phytochrome (Castillon *et al.*, 2007). The best characterised gene expression signalling cascade is that of PIF3, the first PIF to be identified (Ni *et al.*, 1998).

Etiolated *pif3* seedlings have shorter hypocotyls and expanded cotyledons compared to wild-type and so PIF3 is thought to act as a negative regulator of photomorphogenesis in etiolated seedlings (Kim *et al.*, 2003). PIF3 interacts with both phyA and phyB in the $P_{fr}$ form (Shimizu-Sato *et al.*, 2002, Ni *et al.*, 1999, Zhu *et al.*, 2000) and is phosphorylated and rapidly degraded in a phytochrome-dependent manner (Al-Sady *et al.*, 2006). It has been proposed that this degradation releases PIF3-mediated inhibition of gene expression (Castillon *et al.*, 2007). The acceptance of such a model is complicated by the lack of example gene promoters which are regulated in this fashion and additionally it remains unclear whether phytochrome itself phosphorylates PIF3 as N-terminal fragments of phyB artificially targeted to the nucleus are able to initiate phyB-dependent signalling. This suggests that phytochrome kinase function is partially dispensable (Krall and Reed, 2000, Matsushita *et al.*, 2003). Definitive identification of the PIF3 kinase and the use of chromatin immunoprecipitation assays to identify promoters which interact with PIF3 will greatly enhance understanding of this signalling cascade.

Although the majority of phytochrome signalling is dependent upon

phytochrome translocation to the nucleus (Quail, 2007b), an increasing body of evidence suggests that phytochrome is functionally active whilst localised to the cytoplasm. Analysis of phyA function in mutant *Arabidopsis* plants lacking both FHY1 and FHL, which are proteins thought necessary for phyA nuclear translocation (Hiltbrunner *et al.*, 2006, Hiltbrunner *et al.*, 2005), has allowed elucidation of physiological responses induced by the activity of cytoplasmically localised phyA (Rosler *et al.*, 2007). Whilst the *fhy1 fhl* double mutant phenotype strongly resembles that observed in *phyA* mutants these plants retained a subset of phyA-dependent physiological responses including an enhanced phototropic response to blue light after red light pre-treatment (Rosler *et al.*, 2007). Although phototropism is primarily mediated by the phototropin blue-light photoreceptors (discussed in further detail in Section 1.12.1), phyA modulates this response by inhibiting the gravitropic signalling pathway which would otherwise conflict with a lateral phototropic response (Lariguet and Fankhauser, 2004).

An example of a phytochrome-interacting factor functioning outwith the nucleus is PKS1. PKS1 is localised to the cytoplasm and plasma membrane and is phosphorylated by phyA in a light-dependent fashion *in vitro* (Fankhauser *et al.*, 1999, Lariguet *et al.*, 2006) whilst phosphorylated PKS1 is only detected *in vivo* after light irradation, suggesting that phytochrome acts as a light-activated kinase (Fankhauser *et al.*, 1999). PKS1 and its closest homologue (PKS2) act antagonistically to regulate the VLFR branch of phyA signalling (Lariguet *et al.*, 2003). Data presented suggests that whilst both PKS1 and PKS2 positively regulate the phyA VLFR response they also inhibit each others function, possibly via the formation of inactive heterodimers in contrast to active homodimers (Lariguet *et al.*, 2003). It has also been suggested that the proportions of a phyA signalling pool involved in the HIR response are reduced in the *pks1 pks2* double mutant, although it is unclear whether this is a direct consequence of PKS inactivity or a mechanism to compensate for the altered phyA signalling caused by PKS mutation (Sineshchekov and Fankhauser, 2004).

Interestingly, PKS1 has recently been shown to interact with a blue light photoreceptor (phototropin 1) in addition to phytochrome (Lariguet *et al.*, 2006) which suggests that PKS1 has a role in interlinking phytochrome- and phototropin-mediated signalling. *Arabidopsis pks1* mutants demonstrate defective phototropism (Lariguet *et al.*, 2006) and PKS1 may therefore facilitate the previously observed

phytochrome-dependent modulation of the primarily blue light-mediated phototropism response (Hangarter, 1997, Lariguet *et al.*, 2006). In agreement with this proposal, phototropism in response to a lateral blue light stimulus in the *fhy1 fhl* double mutant was unaltered (Rosler *et al.*, 2007), suggesting that phyA localised to the cytoplasm is necessary for this response. Phytochromes therefore not only initiate multiple signalling cascades in both the nucleus and cytoplasm but also interact with other photoreceptors and their signalling networks. Such a combination of responses increases the ability of plants to respond appropriately to their environments.

## 1.3 The role of cryptochromes in blue light-sensing

### *1.3.1 Arabidopsis cryptochromes*

Plant cryptochromes are one of five subfamilies identified in the photolyase/cryptochrome family based on molecular phylogenetic analyses and functional similarity (Daiyasu *et al.*, 2004). Plant cryptochromes are blue light photoreceptors which have been identified in the model plant *Arabidopsis thaliana*, the closely related *Brassica napus*, and in a number of other model plant systems including *Pisum sativum* and *Oryza sativa* (Li and Yang, 2007). Although closely related to photolyases, plant cryptochromes are not able to repair DNA *in vitro* nor is cryptochrome expression sufficient to rescue photolyase activity in deficient *E. coli* (Malhotra *et al.*, 1995). The *Arabidopsis* genome encodes for two canonical plant cryptochrome proteins (cry1 and cry2) and one member of the Cry-DASH subfamily, which has recently been designated cry3 (Lin *et al.*, 1996, Kleine *et al.*, 2003, Ahmad and Cashmore, 1993). Despite its designation, cry3 is a member of a separate subfamily to that which includes the plant cryptochromes (Brudler *et al.*, 2003, Daiyasu *et al.*, 2004). Cry3 is targeted to mitochondria and chloroplasts and is capable of repairing single-stranded DNA (Kleine *et al.*, 2003, Selby and Sancar, 2006). However, a role for cry3 mediating blue light signal transduction beyond these photolyase abilities has not been elucidated (Pokorny *et al.*, 2005, Song *et al.*, 2006) and as such will not be considered further.

### *1.3.2 Cryptochrome domain structure and primary light-induced signalling events*

As with phytochromes, cryptochrome protein structure can be separated into two separate components; a light-sensing region and an associated effector. The N-terminal region of cryptochrome (or PHR for photolyase homology region) is required for light-sensing while the cry C-terminal (CCT) motif contains a unique motif with no known homology to previously characterised protein domains (Figure 1.1, Cashmore *et al.*, 1999, Lin, 2002). Expression of either the PHR or CCT region *in planta* has allowed individual roles to be assigned to each part of the photoreceptor. Cryptochrome activity requires dimerisation which is mediated by the PHR region although downstream signalling requires the additional presence of the CCT (Sang *et al.*, 2005).

The structure of the *Arabidopsis* cry1 PHR has been examined by X-ray crystallography and has been shown to be similar to that of other cryptochrome PHRs (Lin and Todo, 2005, Brautigam *et al.*, 2004). The PHR folds into two domains, consisting of an $\alpha/\beta$ domain and a helical motif which are connected by a loop wrapped around the $\alpha/\beta$ domain (Brautigam *et al.*, 2004). Such a conformation allows the non-convalent binding of two chromophores; flavin adenine dinucleotide (FAD) and methenyltetrahydrofolate (MTHF; Malhotra *et al.*, 1995, Lin *et al.*, 1995, Hoang *et al.*, 2007, Brautigam *et al.*, 2004). FAD is necessary for activity *in planta* whilst MTHF acts as a light-harvesting 'antenna' molecule to increase light sensitivity (Partch and Sancar, 2005, Hoang *et al.*, 2007).

Characterisation of the cry photocycle has been restricted due to difficulties in expressing sufficient levels of functional protein, although the high degree of structural similarity between the cry PHR region and ancestral photolyases allows the latter to be used as a model system (Partch and Sancar, 2005). In photolyases, MTHF photoexcitation allows inter-chromophore energy transfer to FAD which causes FAD reduction and induces DNA repair (Partch and Sancar, 2005). Such inter-chromophore excitation has also been observed in *Vibrio cholerae* cry1 *in vitro* although the role of such excitation is unclear (Saxena *et al.*, 2005, Worthington *et al.*, 2003). Catalytically active photolyases contain reduced FAD and it is possible that light-induced FAD reduction also stimulates cry signalling (Partch and Sancar, 2005). Such FAD reduction may occur via conserved trypophan residues with the PHR as mutation of these amino acids impairs FAD photoreduction (Zeugner *et al.*, 2005).

Consistent with this, recent data suggests that *Arabidopsis* cry1 and cry2 undergo a photocycle involving the accumulation of semi-reduced FAD upon blue light exposure (Banerjee *et al.*, 2007, Bouly *et al.*, 2007). It therefore appears that blue light irradiation of cry proteins results in reduction of the FAD chromophore, creating an active signalling state. Such chromophore modification appears to induce minimal conformational changes within the CCT domain as limited proteolysis experiments have indicated that this region is more sensitive to degradation after exposure to light (Partch *et al.*, 2005). This finding is supported by fourier transform infra-red (FTIR) spectroscopy which detected only slight differences between dark and irradiated structures (Kottke *et al.*, 2006a).

The primary effect of the above-mentioned changes is to allow CCT signalling. Expression of a truncated cryptochrome containing only cry1 PHR in a wild-type background inhibited cry1 function, suggesting that the PHR may act to inhibit CCT activity in the dark (Sang *et al.*, 2005). Cry1 dimerises via the PHR in a light-independent fashion, and it has been proposed that PHR photo-activation results in conformational changes within the cryptochrome dimer to re-orientate the CCT into an active conformation (Sang *et al.*, 2005). Cryptochrome dimerisation is critical for CCT-derived signalling as artificial cry1 CCT (CCT1) and cry2 CCT (CCT2) multimerisation using a GUS-tag is sufficient to induce constitutive cryptochrome functionality *in planta* (Yang *et al.*, 2000). Such an effect was not observed when either CCT was expressed without a GUS-tag, suggesting that GUS-induced multimerisation mimicked the light-induced conformational changes of the native protein (Sang *et al.*, 2005, Yu *et al.*, 2007). Further protein truncation analysis of cry2 has shown that the final 49 C-terminal residues of the protein are also dispensable for cry2 signalling, as a shortened version of CCT2 lacking these terminal residues retains constitutive signalling activity (Yu *et al.*, 2007).

Cry photoactivation results in light-dependent cry phosphorylation within the CCT domain (Bouly *et al.*, 2003, Shalitin *et al.*, 2002, Shalitin *et al.*, 2003, Ahmad *et al.*, 1998b, Yu *et al.*, 2007). Although cry proteins do not contain a canonical kinase domain, blue light-dependent phosphorylation of purified cry1 suggests that this protein is capable of autophosphorylation (Shalitin *et al.*, 2003). It is also interesting to note that inactive cry proteins expressed by certain *cry* alleles are incapable of cry phosphorylation (Shalitin *et al.*, 2003). Such a correlation suggests an important role for phosphorylation in cry-mediated signalling. Although the role of phosphorylation

in cry1 signalling is unclear, cry2 blue-light-dependent phosphorylation precedes protein degradation, suggesting that this post-translational modification may target the protein for degradation in addition to acting as a signalling state to downstream components (Shalitin *et al.*, 2002). Such degradation may explain why cry2 function is most discernable under low light intensities, as cry1 abundance is unaltered by light irradiation (Lin *et al.*, 1998).

### *1.3.3 Physiological responses mediated by Arabidopsis cryptochromes*

Cry1 and cry2 have partially redundant roles in a number of blue light-mediated plant responses including photomorphogenesis, stomatal opening and floral initiation (Li and Yang, 2007). In contrast to the phytochromes, cryptochromes are primarily localised to the nucleus and function mainly through the modulation of gene expression, although cytoplasmically localised cry1 retains a function in cotyledon expansion and the control of primary root growth (Lin and Todo, 2005, Wu and Spalding, 2007). *Arabidopsis* cry1 and cry2 are together thought to regulate approximately 10% of the *Arabidopsis* genome in response to blue light irradiation (Ma *et al.*, 2001). Constitutive cryptochrome activity using GUS-CCT fusion proteins causes a constitutive photomorphogenesis (COP) phenotype as seen in *Arabidopsis* mutants deficient in COP1 or COP9 signalosome complexes (Yang *et al.*, 2000, Wei *et al.*, 1994, Deng *et al.*, 1992). Importantly, the activity of GUS-CCT proteins are negated by the same point mutations which prevent full-length cryptochrome signalling (Yang *et al.*, 2000), suggesting that the activity of these truncated proteins is physiologically significant. Both cry1 and cry2 and their respective CCTs are capable of binding COP1 *in vitro* (Yang *et al.*, 2001, Wang *et al.*, 2001), while epistatic analysis has shown that COP1 acts downstream of cryptochromes in this response (Ang and Deng, 1994). These results suggest that cryptochromes control photomorphogenesis through negative regulation of COP1 action via a direct cry-COP1 interaction (Li and Yang, 2007).

One of the most intensely studied physiological responses induced by the onset of photomorphogenesis is the inhibition of hypocotyl elongation (Parks *et al.*, 2001). This response has been split into different temporal phases, each of which is controlled by different blue-light photoreceptors (Parks *et al.*, 2001). An initial transient phase of hypocotyl elongation occurring within 30 s of irradiation is

regulated by cry2 and the unrelated blue light receptor phototropin 1 (Folta and Spalding, 2001) whilst prolonged hypocotyl elongation inhibition is dependent on cry1 and cry2 activity and involves the activation of anion channels to induce plasma membrane depolarisation within hypocotyl cells (Parks *et al.*, 1998). Microarray analysis has identified a large number of transcripts that are up-regulated in a cry1-dependent manner within this timeframe (Folta *et al.*, 2003b). Over a period of several days a final phase of hypocotyl growth inhibition occurs by a mechanism that requires cry1 but which is independent of anion channel activation or cry2 (Parks *et al.*, 1998).

Although the mechanism by which cryptochrome-mediated photomorphogenic responses are controlled is unclear it is apparent that the cryptochrome-induced signal is modulated by both positive and negative regulatory factors. Identified signalling intermediates which act to positively regulate cryptochrome-mediated photomorphogenesis include PROTEIN PHOSPHATASE 7 (AtPP7; Moller *et al.*, 2003). Repression of *PP7* translation using *PP7* antisense RNA phenocopies a portion of *cry1* mutant phenotypes including a loss of hypocotyl elongation inhibition and reduced cotyledon expansion when grown under blue light (Moller *et al.*, 2003). However data suggesting that cryptochrome-mediated gene expression of selected genes was unaltered in these lines indicates that PP7 is not involved in the primary blue-light sensing response (Moller *et al.*, 2003). Conversely, signalling components have been identified which negatively regulate cryptochrome-mediated photomorphogenesis. The first isolated example of such a component is SHORT UNDER BLUE LIGHT (SUB1; Guo *et al.*, 2001). This protein was isolated as part of a mutant screen to identify proteins that displayed altered hypocotyl elongation specifically when grown under low fluences of blue light (3 μmol $m^{-2}$ $s^{-1}$), although *sub1* mutants also demonstrate reduced hypocotyl growth when grown under far-red light, implying an additional role in phyA signalling (Guo *et al.*, 2001). The shortened hypocotyl phenotype observed in *sub1* seedlings suggests that SUB1 is a negative regulator of photomorphogenesis. Epistatic analysis suggests that SUB1 functions downstream of both cry1 and cry2 primarily at low blue light intensities (Guo *et al.*, 2001). Although the physiological role of SUB1 is unknown, SUB1 is localised primarily to the nuclear envelope and cytoplasm and has been shown capable of binding $Ca^{2+}$ (Guo *et al.*, 2001). Such data is reminiscent of results suggesting a role for $Ca^{2+}$ signalling in cryptochrome signalling (Long and Jenkins, 1998). Further work is required to characterise this signalling pathway.

Although stomatal opening is predominantly controlled by the blue light-sensitive phototropins (discussed in further detail in Section 1.12.3) it is apparent that cryptochromes also modulate stomatal opening in response to blue light. Cry1 over-expression causes hypersensitivity with regards stomatal opening (Mao *et al.*, 2005) and only the stomata of a *cry1 cry2 phot1 phot2* quadruple mutant plants were unresponsive to irradiation with blue light (Mao *et al.*, 2005). The *cry1 cry2* double mutant shows enhanced drought tolerance which suggests that the role of these proteins is physiologically significant. The cryptochrome-mediated regulation of stomatal movement appears to be mediated by a COP1-dependent signal as the stomata of *cop1* mutant plants are constitutively open while epistatic analysis indicates that COP1 acts downstream of cryptochromes in this response as for photomorphogenesis (Mao *et al.*, 2005). It therefore appears that cryptochromes are an important modulator of stomatal opening in *Arabidopsis*.

Cryptochromes also have a role in the promotion of flowering and the entrainment of the circadian clock. The late flowering mutant *fha* is allelic to *cry2*, and cry1 and cry2 have been shown to act redundantly to promote flowering under monochromatic blue light (Mockler *et al.*, 1999, Koornneef *et al.*, 1991). The cryptochromes similarly act redundantly to reset the circadian clock in response to blue light (Devlin and Kay, 2000). These phenotypes may also be mediated via modulation of COP1 activity, although several additional layers of regulation (such as the interlinking role of the phytochrome photoreceptors) are also involved in these responses (Li and Yang, 2007).

## 1.4 Blue light-sensing using the LOV domain motif

Other than the cryptochromes, plants contain at least four other blue light photoreceptors. These include the two members of the phototropin (phot) family that are responsible for a range of blue light-induced plant 'photomovement' responses (Christie, 2007) and two of the three members of the ZEITLUPE/ADAGIO family (Sawa *et al.*, 2007, Kim *et al.*, 2007). All of these proteins contain a modular light sensitive motif referred to as the Light-, Oxygen- or Voltage-sensitive (LOV) domain (Crosson *et al.*, 2003, Huala *et al.*, 1997). The remaining member of the ZEITLUPE/ADAGIO family and another uncharacterised *Arabidopsis* LOV domain-containing protein (PAS/LOV protein, PLP) may also have a role in light detection

although such activities are as yet undefined (Ogura *et al.*, 2007, Crosson *et al.*, 2003).

LOV domains were originally identified on a broad structural level as a subfamily of the PAS domain super-family and have now been identified by sequence homology in a variety of prokaryotes, fungi and both lower and higher plants (Gu *et al.*, 2000, Briggs, 2007, Crosson *et al.*, 2003, Losi, 2004). Canonical PAS domains have been co-opted by evolution as either environmental sensors or as sites of protein dimerisation and are classified in broad groups based on their roles in these processes, although these roles are not mutually exclusive (Gu *et al.*, 2000). Environmental sensing requires co-factor binding and PAS domains bind a variety of co-factors including flavin mononucleotide (FMN), heme, and p-coumaric acid to facilitate this (Briggs *et al.*, 2001b, Crosson *et al.*, 2003, Gu *et al.*, 2000, Hoff *et al.*, 1994). LOV domains in particular have been shown to associate with either FMN or FAD when expressed in *E. coli* (Christie *et al.*, 1999, Imaizumi *et al.*, 2003, Schwerdtfeger and Linden, 2003, He *et al.*, 2002) and recent work has demonstrated that phototropin 1 associates with FMN *in planta* (Sullivan *et al.*, 2008). This and other recent work has allowed refinement of LOV domain classification to specifically describe motifs which are highly homologous to the domains found within the phototropins and which are predicted to form a photoproduct with the associated flavin chromophore upon irradiation (Briggs, 2007, Crosson *et al.*, 2003, Losi, 2004). This refined definition will be used throughout this review. FMN and FAD absorb light maximally in the UV-A and blue portions of the electromagnetic spectrum and as a consequence LOV domains are sensitive to both these qualities of light. Given the broad range of effector domains that are associated with LOV domains in a variety of species, it appears that LOV domains act as a light-sensing module that may be used to confer blue light specificity to a variety of protein functions (Briggs, 2007). The photochemistry of the LOV domains will be discussed in further detail in Section 1.8.

### 1.5 Blue light-sensing by ZEITLUPE/ADAGIO proteins

The ZEITLUPE/ADAGIO (ZTL/ADO) family consists of three members; ZEITLUPE (ZTL), FLAVIN BINDING, KELCH REPEAT, F-BOX 1 (FKF1) and LOV KELCH PROTEIN 2 (LKP2; Somers *et al.*, 2000, Nelson *et al.*, 2000, Schultz *et al.*, 2001). Each of these proteins have a conserved structure consisting of an N-terminal LOV

domain, an F-box domain which allows binding to a SKP1–CUL1–FBP (SCF) ubiquitin ligase and a region of kelch repeats which are also thought to allow protein-protein interactions (Somers, 2001). The existence of a light sensitive LOV domain coupled with an F-box suggested that these proteins may be involved in the light-dependent regulation of protein stability. Indeed, recent work has shown a role for ZTL and FKF1 in the circadian clock where their light-dependent function allows modulation of internal timing signals (Sawa *et al.*, 2007, Kim *et al.*, 2007).

ZTL was first identified from genetic screens to isolate *Arabidopsis* mutants with an altered circadian clock (Somers *et al.*, 2000, Kiyosue and Wada, 2000). *Ztl* mutants have a longer circadian period which is more obvious under low light intensities (Somers *et al.*, 2000), while ZTL over-expression disrupts circadian clock oscillation (Somers *et al.*, 2004). Although ZTL has recently been shown to have light-dependent activity (Kim *et al.*, 2007), ZTL also has a light-independent role in the circadian clock as *ztl* mutants continue to display aberrant circadian rhythms when grown under constant darkness (Somers *et al.*, 2004). ZTL controls the levels of at least two central circadian clock components (TIMING OF CAB1; TOC1 and PSEUDO-RESPONSE REGULATOR 5; PRR5) by regulating the proteosome-dependent degradation of these proteins in a light-dependent manner (Kiba *et al.*, 2007, Mas *et al.*, 2003). The stability of ZTL protein itself is increased by blue light-dependent binding to GIGANTEA (GI), a protein under circadian control with previously uncharacterised biochemical function (Park *et al.*, 1999, Kim *et al.*, 2007). Light-dependent ZTL activity and protein stability combine to provide increased circadian robustness and to allow day-length measurement.

In comparison to ZTL, FKF1 acts primarily as a modulator of circadian output signals rather than altering the clock itself (Imaizumi *et al.*, 2003). Specificially, FKF1 acts to induce flowering in long day conditions by promoting the expression of a key molecular flowering regulator, CONSTANS (CO; Nelson *et al.*, 2000, Imaizumi *et al.*, 2003). Recent work has shown that FKF1 also forms a heterodimer with GI under blue light (Sawa *et al.*, 2007), which subsequently mediates the degradation of a repressor of *CO* expression (Imaizumi *et al.*, 2005, Sawa *et al.*, 2007). These interactions allow plants to induce flowering at favourable times of year by responding to seasonal changes in day length through light-dependent modulation of circadian clock signals.

Compared with ZTL and FKF1, comparatively little is known about the role of LKP2. Over-expression of LKP2 delays flowering (Schultz *et al.*, 2001, Yasuhara *et al.*, 2004), and this protein has also been shown to interact *in vitro* with TOC1, CO and GI (Kim *et al.*, 2007, Fukamatsu *et al.*, 2005, Yasuhara *et al.*, 2004). Intriguingly LKP2 was localised to the nucleus when co-expressed with CO *in vivo*, suggesting that LKP2 may have a similar role to FKF1 in the regulation of CO activity (Fukamatsu *et al.*, 2005). Further work is required to assess the specific role of LKP2 and whether its activity is also modulated by light.

### 1.6 Phototropins- a second major class of plant blue light receptor

A second family of plant proteins utilising the blue light-sensitive LOV domain motif are the phototropins. Phots are plasma membrane-localised protein kinases which were initially characterised in *Pisum sativum* membrane extracts due to their blue-light dependent phosphorylation (Gallagher *et al.*, 1988). Since the identification of the *PHOT1* locus in *Arabidopsis* (Huala *et al.*, 1997), phototropins have been characterised in numerous other dicots and monocots, as well as in lower plants such as the fern *Adiantum capillis-veneris* (*Adiantum*; Briggs *et al.*, 2001a). Studies have identified two primary members of the phototropin family, phototropin 1 and 2 (Huala *et al.*, 1997, Briggs *et al.*, 2001b, Kagawa *et al.*, 2001), both of which are found in *Arabidopsis*.

Phot1 and phot2 appear to have evolved from a single gene duplication event after the evolution of seed plants (Huala *et al.*, 1997, Kagawa *et al.*, 2001, Lariguet and Dunand, 2005). Single copies of *PHOT* are found in pteridophytes and in the single-celled algae *Chlamydomonas reinhardtii* (Huang *et al.*, 2002, Nozue *et al.*, 1998) and are likely derived from the ancestral *PHOT* gene (Lariguet and Dunand, 2005). In addition to these sequences, a chimeric photoreceptor (neochrome 1, neo1) has been identified in *Adiantum* and the alga *Mougeotia scalaris* which contains the red light-sensing N-terminal region of a phytochrome fused with a complete phototropin protein (Suetsugu *et al.*, 2005b). This fusion event allows both red and blue light to be used to induce what are primarily thought to be blue light-mediated phot-dependent responses in higher plants. This is thought to be advantageous in the shaded, low light environments in which these plants are commonly found (Kawai *et*

*al.*, 2003). Indeed, neochrome is thought to have arisen on two independent occasions in cryptophytes (Suetsugu *et al.*, 2005b).

The phots have partially redundant roles in many responses in *Arabidopsis*, but have some diverged functions; in general phot1 is sensitive to lower fluences of light while phot2 acts in response to higher light intensities (Briggs and Christie, 2002). Like phytochromes and cryptochromes, phots are capable of eliciting changes in gene expression in response to blue light stimulation, although compared to the modulation of gene expression induced by cryptochrome activity this role is minor (Ohgishi *et al.*, 2004). Instead, phots are thought to act primarily at a post-transcriptional level to mediate responses to blue light. Phototropins have been shown to be the primary light receptors for a range of blue light-specific responses including phototropism (after which they were named), chloroplast accumulation, leaf positioning and expansion and also stomatal opening (Christie, 2007). In addition, phot1 has a role in mediating the blue light-induced stabilisation of specific mRNAs (Folta and Kaufman, 2003) while phot2 induces chloroplast avoidance movements under high light irradiation (Christie, 2007). These responses will be discussed in further detail in Section 1.11.

## 1.7 Phototropin protein structure

As with the phytochromes and cryptochromes the phototropin molecule can be thought of as two distinct components; a photosensory region and an accompanying effector domain (Figure 1.1). Uniquely amongst LOV-containing proteins, the phototropins contain two photochemically active LOV domains, designated LOV1 and LOV2 (Crosson *et al.*, 2003, Huala *et al.*, 1997). Both of these domains bind FMN in a non-covalent fashion in the dark (Christie *et al.*, 1999). These N-terminal LOV domains are coupled to a serine/threonine kinase domain (Huala *et al.*, 1997, Christie, 2007). The combination of these domains allow blue light-dependent phototropin autophosphorylation *in vivo* and *in vitro* (Briggs *et al.*, 2001b, Christie *et al.*, 1998, Christie *et al.*, 2002, Sakai *et al.*, 2001).

Phototropin kinase domains are highly homologous to members of the AGC family (named after the founding members cAMP-dependent protein kinase A, cGMP-dependent protein kinase G, phospholipid-dependent kinase C) and have been assigned to the plant-specific AGC-VIIIb subfamily (Bogre *et al.*, 2003). Interestingly, the highly conserved DFG residue triplet responsible for $Mg^{2+}$

coordination (which is necessary for catalytic activity) is altered to DFD in the AGC-VIII family (Bogre *et al.*, 2003). The consequences of this substitution are unclear, but mutation of the lead aspartate ($Asp^{806}$ in *Arabidopsis* phot1) causes a loss of kinase activity as observed in cAMP-dependent protein kinase A (PKA; Christie *et al.*, 2002), suggesting that the altered motif is required for phot kinase function. An additional difference between phots and other AGC-VIII kinases is the insertion of 32 residues into the activation-loop (T-loop) that has been structurally modelled to form an additional α-helix (Bogre *et al.*, 2003, Christie, 2007, Tokutomi *et al.*, 2008). Such an insertion within an otherwise highly conserved region may imply a modified function for this loop.

### 1.8 LOV domain structure and photochemistry

LOV domains have a highly conserved tertiary structure which facilitates the non-covalent binding of FMN or FAD (Crosson *et al.*, 2003). Phototropin and other plant LOV domains incorporate FMN whilst fungal LOV domains contain an 11 amino acid insertion to accommodate the larger chromophore FAD (Christie *et al.*, 1999, He *et al.*, 2002, Schwerdtfeger and Linden, 2003, Crosson *et al.*, 2003). The structure of LOV1 has been crystallised from the *Chlamydomonas reinhardtii* phototropin (Fedorov *et al.*, 2003), whilst the structure of LOV2 domains from *Adiantum* neochrome and *Avena* phot1 have also been elucidated (Crosson and Moffat, 2001, Halavaty and Moffat, 2007). The structures of LOV1 and LOV2 are almost identical and are likely to be good models of LOV domain structure given the highly conserved primary structure of the motif (Crosson *et al.*, 2003). LOV domains share a common structure consisting of three helical segments and a 5-stranded anti-parallel β-barrel (Crosson and Moffat, 2001, Fedorov *et al.*, 2003), which form a hydrophobic pocket in which the flavin chromophore resides. The predicted structure of *Arabidopsis* phot1 LOV2 with bound FMN is illustrated in Figure 1.3A.

The published crystal structures of LOV domains reveal that the FMN chromophore interacts with 11 separate amino acids via Van Der Waals forces in the dark (Crosson and Moffat, 2001). The non-covalent interaction between flavin and the LOV domain is altered after exposure to blue or UV-A light via a unique, self-contained photocycle which forms an adduct between the FMN chromophore and a conserved cysteine within the domain ($Cys^{512}$ in *Arabidopsis* phot1 LOV2, Kennis *et*

*al.*, 2003, Salomon *et al.*, 2000). Such a photocycle contrasts to that of the phytochromes and cryptochromes which involves light-dependent chromophore cis/trans isomerisation or chromophore reduction respectively (Hellingwerf, 2000, Bouly *et al.*, 2007, Williams and Braslavsky, 2001). Covalent adduct formation is thought to be the stimulus for the initiation of a signalling cascade in response to blue light as slight conformational changes are detected in the domain after light irradiation (Crosson and Moffat, 2002, Crosson *et al.*, 2003, Iwata *et al.*, 2003, Nozaki *et al.*, 2004, Swartz *et al.*, 2002).

In contrast to other plant photoreceptors, the compact nature of the LOV domain has allowed crystal structure to be elucidated for both dark and irradiated samples. This has facilitated LOV domain photocycle characterisation and the major photochemical products have now been identified, although the precise mechanism by which these states are interconverted remains debatable (Kottke *et al.*, 2003b, Neiss and Saalfrank, 2003, Kennis *et al.*, 2003). This photocycle is summarised in Figure 1.4. The LOV domain exists in three main forms that can be distinguished by their different spectral characteristics (Kottke *et al.*, 2006b). Phototropin LOV domains incubated in the dark spontaneously revert to the basal resting state ($LOV_{448}$) which has a peak of absorption at 448 nm. Absorption of a blue or UV-A photon by the FMN chromophore causes excitation to a higher energy state that is converted into a short-lived triplet state ($LOV_{660}$) with a maximal absorption at 660 nm (Kennis *et al.*, 2003). Stabilisation of this triplet state by the photoactive cysteine reduces the energy required for covalent bond formation and so permits adduct creation, resulting in the generally accepted signalling state of the LOV domain ($LOV_{390}$) that has a peak of absorption at 390 nm. $LOV_{390}$ has a variable half-life that ranges from tens of seconds for phototropin LOV2 to several hours in the case of ZTL/ADO LOV domains (Kasahara *et al.*, 2002b, Salomon *et al.*, 2000, Zikihara *et al.*, 2006). An explanation of these discrepancies is elusive at present, although it appears that single amino acid substitutions can have a significant effect on the dynamics of LOV domain dark-state reversion. The half-life of *Adiantum* neo1 $LOV2_{390}$ can be significantly extended by mutation of a single residue (Iwata *et al.*, 2005), while conversely the half-life of *Avena* phot1 $LOV2_{390}$ was significantly reduced by mutation of an alternate residue (Christie *et al.*, 2007). This suggests that even slight alterations to LOV domain structure have a significant effect on LOV domain photochemistry.

LOV domain photochemistry can be prevented by mutation of the photoactive $Cys^{512}$ to alanine or serine, which inhibits formation of the signalling cysteinyl-flavin adduct (Salomon *et al.*, 2000). Intriguingly, replacement of $Cys^{512}$ with methionine has been shown capable of creating a photochemically sensitive domain which forms a methionyl-flavin adduct upon light irradiation (Kottke *et al.*, 2003a). This alternate photoproduct is stable compared with the wild-type cysteinyl-flavin adduct as it does not revert to the basal state after dark incubation (Kottke *et al.*, 2003a). Whether this mutant photoproduct is capable of inducing light-dependent signalling is unclear at present. Several other mutations within the LOV domain have been identified which abolish photochemistry by preventing chromophore incorporation (Cheng *et al.*, 2003, Salomon *et al.*, 2000). The residues in the immediate vicinity of $Cys^{512}$ are highly conserved and particularly sensitive to substitution, suggesting that these residues have an important role in creating an appropriate environment for cysteinyl-flavin adduct formation (Cheng *et al.*, 2003, Salomon *et al.*, 2000, Crosson *et al.*, 2003, Christie, 2007).

### *1.8.1 Conformational changes associated with LOV domain photoactivation*

Crystallisation of both dark and illuminated LOV domain structures has greatly facilitated the examination of light-induced conformational changes (Zoltowski *et al.*, 2007, Crosson and Moffat, 2001, Fedorov *et al.*, 2003, Crosson and Moffat, 2002). These crystal structures have been used in combination with other independent techniques to identify modest conformational changes within the domain upon light irradiation including the slight movement of α helices and β-sheets (Iwata *et al.*, 2003, Nozaki *et al.*, 2004, Swartz *et al.*, 2002). Initial analysis identified a highly conserved chain of residues running from the chromophore to a salt bridge at the domain surface (Crosson *et al.*, 2003). This suggested a signalling mechanism whereby minimal chromophore movements could be transmitted to the surface of the domain and thereby alter interdomain binding affinities. Subsequent analysis revealed that β-sheet movement within the LOV2 domain could be abrogated by substitution of an FMN-interacting residue present within one of the β-sheets (Zoltowski *et al.*, 2007, Nozaki *et al.*, 2004). This conserved residue ($Gln^{575}$ in *Arabidopsis* phot1 LOV2) undergoes side chain rotation upon cysteinyl-flavin adduct formation (Crosson and Moffat, 2002), and its substitution with leucine limits β-sheet movement upon light

stimulation in isolated LOV domains from *Adiantum* neochrome and *Neurospora crassa* VIVID (Nozaki *et al.*, 2004, Zoltowski *et al.*, 2007). This mutation also appears to mitigate LOV domain function as recent work has shown that incorporation of this mutation prevents the light-dependent interaction *in vitro* between the FKF1 LOV domain and GIGANTEA protein (Sawa *et al.*, 2007). In support of the notion that LOV domain signalling occurs via changes in β-sheet conformation substitution of residues in alternate β-sheets within bacterial LOV domains enhance structural rearrangement within the domain upon light stimulation (Losi *et al.*, 2005).

Whilst light-induced structural differences within the LOV domain are relatively minor, examination of peptide fragments containing residues in addition to the canonical LOV domain show greater conformational changes. An amphipathic α-helix (designated the Jα-helix) encoded after the C-terminal of *Avena* phot1 LOV2 has been shown to become disorganised upon light irradiation (Harper *et al.*, 2003). Photochemical activity is necessary to induce this disorganisation as abolition of LOV2 photochemistry (through substitution of $Cys^{512}$→Ala) inhibited this light-dependent effect (Harper *et al.*, 2003). Studies using extended LOV2 constructs have since verified this movement (Iwata *et al.*, 2005, Corchnoy *et al.*, 2003, Eitoku *et al.*, 2005). Interestingly, disorganisation of the Jα-helix appears sufficient to induce kinase activity in full-length phot1 as substitutions within the Jα-helix induce increased phot1 autophosphorylation activity *in vitro* (Harper *et al.*, 2004a). Disorganisation of α-helices associated with LOV domains may be conserved as a mode of signal transmission amongst LOV domains as the crystal structure of the fungal photoreceptor VIVID also reveals an α-helix physically associated against the LOV domain (Zoltowski *et al.*, 2007), although in this latter case the α-helix is composed of residues N-terminal of the LOV domain. Whilst the VIVID α-helix does not become completely disorganised upon illumination its conformation is significantly altered (Zoltowski *et al.*, 2007). Additionally a mutated VIVID protein that retains photochemistry but lacks N-terminal conformational changes is non-functional *in vivo* (Zoltowski *et al.*, 2007). This further suggests that LOV domain conformational changes are necessary for biological activity.

## 1.9 LOV domains have different roles in the regulation of phototropin autophosphorylation activity

Given the ability of individual LOV domains to detect light *in vitro* (Salomon *et al.*, 2000), and the ability of proteins containing single LOV domains to act as photosensors (Sawa *et al.*, 2007, Kim *et al.*, 2007) the requirement of two LOV domains within the phot protein is not immediately apparent. By preventing the photochemistry of either LOV1 or LOV2 the individual contribution of each phot LOV domain to phot light-dependent autophosphorylation *in vitro* has been assessed (Cho *et al.*, 2007, Christie *et al.*, 2002). This work has shown that light-induced autophosphorylation requires only the photoreactivity of the LOV2 domain, with LOV1 photosensitivity apparently dispensable for the induction of autophosphorylation. The conclusion from this data that LOV2 is the primary phot photoreceptor is supported by analysis of these mutated phot proteins *in vivo*. These latter studies show that only LOV2 photosensitivity is necessary to complement phot-mediated physiological phenotypes (Cho *et al.*, 2007, Christie *et al.*, 2002). Phylogenetic analysis of LOV domains suggests that phot LOV1 domains are more similar to those found in other LOV proteins, with LOV2 domains forming a divergent group (Figure 1.3B). Photochemical studies have shown that LOV2 is a more efficient photosensor than LOV1 (Salomon *et al.*, 2000, Kasahara *et al.*, 2002b) and LOV2 domains may therefore represent specialised photosensors required for phot kinase activation. Insight into the relationship between LOV2 and the phot kinase domain may be gained through comparison with related kinases such as PKA.

Canonical PKAs have not been successfully identified in plants (Newton *et al.*, 1999, Trewavas *et al.*, 2002), but this family has been intensively studied in other model systems. PKA typically acts as a heterotetramer containing two regulatory (R) subunits and two catalytic subunits in the inactive state (Taylor *et al.*, 2004). It has been speculated that LOV2 may act as the 'R' regulatory subunit in phots as residues important for the interaction between PKA and a regulatory subunit are conserved in LOV2 but not LOV1. Light stimulation is proposed to inhibit this LOV2-mediated repression (Figure 1.5; Matsuoka and Tokutomi, 2005, Christie, 2007, Tokutomi *et al.*, 2008). Similar mechanisms have been suggested for other PAS-containing kinases such as the bacterial protein fixL and the human protein PAS kinase (Rutter *et al.*, 2001, Gong *et al.*, 1998, Amezcua *et al.*, 2002). PAS kinase also contains two PAS

domains (PAS-A and PAS-B) in series with a serine/threonine kinase domain. In this case, PAS-A acts as the regulatory domain and is proposed to disassociate from the integral kinase when PAS-A binds an aromatic compound (Amezcua *et al.*, 2002).

Support for this model is provided by the analysis of mutant and truncated phot proteins both *in vitro* and *in vivo*. The isolated phot2 kinase domain is constitutively active *in vitro* and its expression *in vivo* induces constitutive phot2-mediated phenotypes *in planta*, implying that the N-terminal photosensory region acts to repress phot2 kinase activity (Kong *et al.*, 2007, Matsuoka and Tokutomi, 2005). Individual phot2 LOV2 domains expressed in *E. coli* inhibit phot2 kinase activity *in vitro*, although this inhibition of phot2 kinase activity occurred only in the absence of light (Matsuoka and Tokutomi, 2005). Phot2 LOV1 fragments did not alter kinase activity (Matsuoka and Tokutomi, 2005). These data suggest that LOV2 acts as a light-controlled repressor of phot2 kinase activity, and are consistent with the requirement of LOV2 photochemistry for phot function *in vivo* (Cho *et al.*, 2007, Christie *et al.*, 2002).

Such a model does not exclude the possibility that phots act as a dimer (Christie, 2007) and evidence that this is the case arises from several sources. Irradiated maize phototropins can phosphorylate unirradiated pea phototropin *in vitro* (Reymond *et al.*, 1992), while a protein complex with a molecular weight of 335KDa (more than twice the calculated weight of the expressed protein) is identified alongside the monomer using a polyclonal anti-phot1 antibody (Briggs *et al.*, 2001b). Additionally, phot2 has been shown capable of phosphorylating phot1 *in vitro* (Cho *et al.*, 2007).

It has been speculated that phot dimerisation is mediated via LOV1 (Salomon *et al.*, 2004). *Avena* phot1 LOV1 fragments have been observed as dimers *in vitro*, while similar phot1 LOV2 remained monomeric (Salomon *et al.*, 2004). *Arabidopsis* phot1 LOV1 and FKF1 LOV domains have also been shown to dimerise (Nakasako *et al.*, 2004, Nakasako *et al.*, 2005), as have the LOV domains of bacterial LOV-containing proteins (Moglich and Moffat, 2007). This dimerisation hypothesis is given further credence by the fact that both ZTL and FKF1 demonstrate biologically-significant blue light-dependent interactions (Kim *et al.*, 2007, Sawa *et al.*, 2007). The recently characterised PAS/LOV protein (PLP) is also reported to have blue light-specific binding affinities in a yeast two-hybrid screen (Ogura *et al.*, 2007). Such binding may confer additional specificity over canonical PAS domain-mediated interactions

(Ballario *et al.*, 1998). While PAS-mediated dimerisation has been shown between PAS domains from phylogenetically distant species (eg. mammalian and *Drosophila*) in both *in vitro* and *in vivo* experiments (Huang *et al.*, 1993, Lindebro *et al.*, 1995, Sogawa, 1995) the LOV domain of the fungal protein WHITE COLLAR 1 does not interact with the PAS domain also present in this protein (Ballario *et al.*, 1998). This suggests a specialisation of the binding affinities of LOV domains within this protein, although at present the mechanism through which this is achieved remains obscure.

An alternative role for phot1 LOV1 is suggested by photochemical analysis of phot N-terminal regions containing both LOV1 and LOV2 domains (Song *et al.*, 2005, Kasahara *et al.*, 2002b). Such analysis of this phot fragment revealed that the presence of LOV1 extended the half-life of the LOV2 signalling state, although LOV1 photosensitivity was not required for this activity (Kasahara *et al.*, 2002b). This may suggest a role for LOV1 in extending the active signalling state of the full-length phot protein (Kagawa *et al.*, 2004, Christie, 2007, Christie *et al.*, 2002).

## 1.10 Phototropin autophosphorylation is likely to have multiple consequences for protein function

Light stimulation induces phot autophosphorylation primarily at the N-terminal of the protein and in the linker region between LOV1 and LOV2 (referred to as the LOV linker; Salomon *et al.*, 2003, Sullivan *et al.*, 2008). Light stimulation of LOV domains may induce conformational changes in both the immediate N- and C-terminal residues (Zoltowski *et al.*, 2007, Harper *et al.*, 2003, Halavaty and Moffat, 2007) and such changes may facilitate this phosphorylation. Interestingly, data has been presented which suggests that phosphorylation at different positions within the protein depends upon the intensity of the light source used (Salomon *et al.*, 2003). This would allow the initial physiological response to be followed by desensitisation (Salomon *et al.*, 2003). Low levels of blue light stimulate phosphorylation of serine residues in the region surrounding LOV1 while higher fluences of light allows phosphorylation of additional serine residues within the LOV linker sequence (Salomon *et al.*, 2003). Phot phosphorylation can most likely be reversed through the action of an unidentified phosphatase. Several studies have shown that light-irradiated phot1 returns to the non-phosphorylated state when incubated in the dark via intermediate phosphopeptides consistent with the dephosphorylation of phot1, with residues being

dephosphorylated contrary to the order of phosphorylation (Salomon *et al.*, 2003, Kinoshita *et al.*, 2003, Short *et al.*, 1992). If phot phosphorylation has alternate consequences dependent upon the sites phosphorylated such a process would provide additional regulation of phot signalling in response to variable light environments.

Phot phosphorylation sites in addition to those mapped are also likely to exist. A common characteristic of AGC kinase activation is phosphorylation within the activation- or T-loop (Hauge *et al.*, 2007). This phosphorylation has a role in kinase activation and is capable of inducing substantial changes in T-loop structure (Johnson *et al.*, 1996). Whilst the precise conformational changes induced by T-loop phosphorylation vary between kinases phosphorylation generally stabilises the enzyme in an extended conformation that allows substrate binding (Huse and Kuriyan, 2002). For example, the activity of the related *Arabidopsis* kinase PINOID is enhanced by phosphorylation within the T-loop by an upstream regulatory kinase (3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1; Zegzouti *et al.*, 2006a). Phots also have several conserved serine residues within the T-loop which may represent sites of phosphorylation. Additionally, the T-loops of the phots and PINOID differ from the canonical PKA motif, suggesting a divergence of T-loop function and kinase regulation. PINOID is also localised to the plasma membrane and analysis of this protein has indicated that the novel insertion within the T-loop is necessary and sufficient for plasma membrane localisation (Zegzouti *et al.*, 2006b). The mechanism by which phots are localised to the plasma membrane has yet to be determined and it will be interesting to confirm whether a similar mechanism allows phot plasma-membrane association.

An immediate consequence of phot irradiation is the partial relocalisation of phot from the plasma membrane. GFP-tagged versions of phot1 and phot2 both display altered localisation after blue light irradiation, with phot1 moving to the cytosol and phot2 becoming associated with Golgi bodies (Kong *et al.*, 2006, Sakamoto and Briggs, 2002, Wan *et al.*, 2007). Analysis using truncated versions of phot2 has allowed the role of individual domains to be elucidated. The plasma membrane localisation of full-length phot2 is apparently mediated by the kinase domain as a truncated phot2 containing only the N-terminal region is localised to the cytoplasm whilst light-induced phot2 movement is dependent upon phot2 kinase activity (Kong *et al.*, 2006). This suggests that phot2 autophosphorylation regulates phot2 redistribution from the plasma membrane (Kong *et al.*, 2006). However, a

truncated version of phot2 containing only the kinase domain was localised to both the plasma membrane and the Golgi when expressed *in planta*, an effect which persisted when the catalytic activity of the isolated kinase domain was abolished (Kong *et al.*, 2006). This suggests that the N-terminal region of phot2 acts to inhibit kinase domain-mediated Golgi association of the full-length protein in the absence of light (Kong *et al.*, 2006) and indicates that an interaction between the photosensory N-terminal and kinase domain is required for correct full-length protein localisation. The role of phot redistribution in mediating phot signalling has yet to be elucidated. A possibility is that the removal of phosphorylated phot from the membrane allows retention of light sensitivity, although the redistribution of phot2 to the Golgi implies that phot movement may also have a signalling role (Kong *et al.*, 2006).

An additional consequence of phot phosphorylation may be a modulation of protein dimerisation. If the LOV1 domain has a role in mediating interactions with other proteins (Salomon *et al.*, 2004) it is possible that phosphorylation of the sequence surrounding the domain may alter its binding affinities. Phosphorylation of the LOV linker sequence has previously been shown to be necessary for interactions between phot1 and 14-3-3 proteins in *Vicia faba*, with a specific phosphorylated residue necessary for this interaction (Kinoshita *et al.*, 2003). Although this residue is not conserved between *Vicia* phot and *Arabidopsis* phot1 this data indicates that phot phosphorylation can enhance interactions with downstream signalling factors. It will now be of interest to determine the specific role of each of the phot1 autophosphorylation sites mapped *in vivo*, and also to determine whether additional autophosphorylation sites are present within the kinase domain as for other AGC kinases (Sullivan *et al.*, 2008).

### 1.11 Plant physiological responses induced by green and UV-B light

Whilst the photoreceptors involved in plant responses to blue, UV-A, red and far-red light have been extensively characterised plant responses to the remainder of the visual spectrum is poorly understood. Although subtle compared to blue- and red-mediated responses it is thought that green and UV-B qualities of light are capable of eliciting specific photomorphogenic responses (Folta and Maruhnich, 2007, Ulm and Nagy, 2005). Such responses are thought to be important under specific environmental conditions such as growth under shaded canopies where the absorption

of red and blue portions of the visual spectrum enhances the relative amount of green present (Folta and Maruhnich, 2007).

Various studies have suggested that green light inhibits growth (reviewed in Folta and Maruhnich, 2007). Green light also induces phototropism (Liscum and Briggs, 1996) and inhibits stomatal opening (Frechilla *et al.*, 2000). Despite this, the existence of a novel green light photosensor is controversial as phytochromes and cryptochromes each have photochemical states that absorb in the green portion of the spectrum. Although phytochromes are very inefficient green light sensors the abundant quantity of phytochromes in etiolated seedlings is sufficient to stimulate germination in a phytochrome-dependent manner (Shinomura *et al.*, 1996). Similarly, phototropins are required for the green light-mediated phototropic response, suggesting that they are also capable of responding to green light (Liscum and Briggs, 1996). More critically, the photo-stimulated form of cryptochrome (Section 1.3.2) has enhanced absorption in the green portion of the spectrum and it has recently been shown that green light exposure reverses blue light-induced crypochrome photoproduct formation (Bouly *et al.*, 2007, Banerjee *et al.*, 2007). Such green light reversal of cryptochrome activation may account for the majority of the observed antagonistic roles of green light (Folta, 2004, Folta and Maruhnich, 2007). Despite this, certain green light-induced responses still defy explanation without the existence of a novel photoreceptor or a previously uncharacterised interaction between known receptors. An example of such a response is green light-mediated hypocotyl elongation, as this response is observed in both wild-type *Arabidopsis* and plants lacking cryptochromes (Folta, 2004). Although identification of the novel photoreceptor(s) necessary for such cryptochrome-independent green light-mediated responses is complicated by the roles of cryptochrome and phytochrome in green light sensing it is hoped that the use of double and triple photoreceptor mutants will facilitate characterisation (Folta and Maruhnich, 2007). As many of the identified green-light specific responses identified are the converse of known photomorphogenic responses to red and blue light it has been hypothesised that mutants deficient in the hypothetical green photoreceptor would display light-dependent hyper-photomorphogenic phenotypes (Folta and Maruhnich, 2007). Further characterisation of such mutants will be awaited with interest.

Plants can also respond specifically to the UV-B portion of the spectrum (280-320nm; Mackerness, 2000). Such responses can be divided into those caused by UV-

B damage inflicted by high intensities and a smaller subset which are induced under non-damaging levels of irradiation (Frohnmeyer and Staiger, 2003). These latter responses include photomorphogenesis, stomatal opening and the increased expression of proteins with a role in photo-protection such as CHALCONE SYNTHASE (CHS; Whitelam and Halliday, 2007). Although a DNA-binding protein with a specific role in mediating the up-regulation of *CHS* in response to UV-B has been characterised (UV-RESISTANCE LOCUS 8; Brown *et al.*, 2005), the photoreceptor involved in this response has yet to be identified.

## 1.12 Phototropin-mediated physiological responses and the signalling mechanisms involved

Phots are involved in a range of blue light-dependent responses including phototropism, petiole positioning, leaf expansion, stomatal opening and chloroplast movement which cumulatively increase the fitness of seedlings in low light conditions (Takemiya *et al.*, 2005a). Such activity requires phot kinase activity although phot autophosphorylation within the N-terminal region of the protein is not required for many of these responses (Christie *et al.*, 2002, Kagawa *et al.*, 2004, Cho *et al.*, 2007, Kong *et al.*, 2007, Sullivan *et al.*, 2008). Truncated forms of phot1 and phot2 lacking LOV1 and the interlinking sequence between LOV1 and LOV2 (referred to as phot1 LOV2-kinase and phot2 LOV2-kinase respectively) are capable of restoring a subset of phot-dependent phenotypes *in planta* (Sullivan *et al.,* Kagawa *et al.*, 2004), while the expression of the isolated phot2 kinase domain invokes constitutive responses (Kong *et al.*, 2007). In addition, *Chlamydomonas* phot (which lacks sequence N-terminal of LOV1 and approximately 70 residues from the LOV linker region) is capable of mediating a subset of phot-mediated responses when expressed in transgenic *Arabidopsis* lacking both phot1 and phot2 (Onodera *et al.*, 2005). The role of phots in initiating this variety of responses will be discussed below.

### *1.12.1 Phototropism*

Etiolated dicotyledon hypocotyls and monocot coleoptiles grow towards a directional blue light source (Liscum and Stowe-Evans, 2000). This directed growth response is termed phototropism. Roots also have a phototropic response, although these organs

display negative phototropism and as such grow away from the light source. The phototropic response (whether positive or negative) is regulated in combination with gravitropism to allow both seedlings and mature tissue to orientate themselves within their environment. Although phototropism is modulated by gravitropism (Iino, 2006) such interactions will not be considered for the purposes of this review as gravity is a constant vector in this study. The phototropic response may be separated into two distinct stages. First positive phototropic curvature occurs under very low fluences and obeys the Bunsen-Roscoe laws of reciprocity; that is the same degree of curvature is obtained for a given quantity of light, regardless of the fluence/time combinations used to supply the light (Iino, 2006). This initial response differs from second positive curvature that occurs in a time-dependent manner in response to long-term exposure to light and results in larger organ curvatures which are apparent to the naked eye (Iino, 2006).

The *PHOT1* locus was initially identified in a screen for mutant *Arabidopsis* seedlings defective for hypocotyl phototropism (Liscum and Briggs, 1995) and as such this physiological response provided inspiration when phot nomenclature was rationalised (Briggs *et al.*, 2001a). In *Arabidopsis*, both phot1 and phot2 have a role in mediating this response, with phot1 conferring sensitivity at lower light intensities and phot2 being functional at higher fluences (Sakai *et al.*, 2001). Such differing roles are partially explained by differential gene expression; only phot1 is expressed in etiolated seedlings (Sakamoto and Briggs, 2002) whilst *PHOT2* transcription increases upon exposure to light (Kagawa *et al.*, 2001). The shorter half-life of phot2 photocycle reversion may also allow phot2 to retain sensitivity at higher fluences (Christie *et al.*, 2002).

Although phototropins are necessary for directional phototropic responses the cryptochromes and phytochromes act to modulate the phot-derived signal. Increased *PHOT2* transcription in response to light exposure requires phyA (Tepperman *et al.*, 2001) while both phyA and phyB are necessary for the observed red light enhancement of first positive phototropic curvature (Janoudi *et al.*, 1997, Janoudi and Poff, 1992). Similarly, *Arabidopsis* mutants lacking both cry1 and cry2 have reduced first positive curvature (Ahmad *et al.*, 1998a). Phytochrome modulation of phototropism may occur through a repression of gravitropism which would otherwise contradict the light-derived stimulus (Stowe-Evans *et al.*, 2001, Hangarter, 1997, Lariguet and Fankhauser, 2004). A prime candidate to facilitate this crosstalk between

phytochrome and phototropin signalling is PKS1 (Lariguet *et al.*, 2006). As previously mentioned (Section 1.2.3), PKS1 is phosphorylated by phyA and yet PKS1 and phot1 interact *in vivo* (Fankhauser *et al.*, 1999, Lariguet *et al.*, 2006). PKS1 is expressed primarily in the elongation region of both the hypocotyl and the root in etiolated seedlings and its expression is up-regulated by blue light stimulation in a phyA-dependent fashion (Lariguet *et al.*, 2003, Lariguet *et al.*, 2006). Recent work has demonstrated roles for PKS1 in both the hypocotyl and root phototropic responses (Lariguet *et al.*, 2006, Boccalandro *et al.*, 2007) and it therefore appears that PKS1 has a central role in modulating the phototropic response in each of these organs.

Unilateral irradiation of oat coleoptiles induces a gradient of phot activation across the organ, with phot activity being greatest on the illuminated side (Salomon *et al.*, 1996, Salomon *et al.*, 1997). The over-expression of the constitutively active phot2 kinase domain in a wild-type background inhibits phototropic curvature, supporting the notion that a gradient of phot activity is required for phototropism (Kong *et al.*, 2007, Salomon *et al.*, 1997). Despite this, the mechanism by which downstream signalling initiates phototropism remains unclear. Second positive curvature occurs following a lateral redistribution of the phytohormone indole-3-acetic acid (IAA) across the photosensitive organ to the shaded side (Lino, 2001). Originally proposed 80 years ago, the Cholodny-Went hypothesis suggested that an unequal distribution of auxin between the shaded and illuminated sides of plant organs allowed differential growth, thereby facilitating directional growth (Cholodny, 1927, Went and Thimann, 1937). Such asymmetric auxin distributions have been observed in pea, *Brassica oleracea* and rice after organ illumination with unidirectional blue light (Haga *et al.*, 2005, Esmon *et al.*, 2006, Haga and Iino, 2006). Additionally, the use of transgenic *Arabidopsis* transformed with auxin-responsive reporter constructs has allowed indirect measurements of auxin redistribution to be made which correlate with the direct measurements made in other species (Friml *et al.*, 2002). Recent advances have begun to link phot autophosphorylation and auxin redistribution together and have suggested downstream signalling factors.

Phot activation has been biochemically linked to auxin redistribution via a phot1 interacting partner, NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3). NPH3 is necessary for phototropism in *Arabidopsis* and interacts with the photosensory N-terminal of phot1 (Motchoulski and Liscum, 1999, Lariguet *et al.*, 2006). NPH3 interacts with phot1 *in vitro* and undergoes dephosphorylation upon blue light

irradiation, a post-translational modification that is correlated with phototropism and which requires phot1 (Motchoulski and Liscum, 1999, Pedmale and Liscum, 2007). The rice NPH3 ortholog COLEOPTILE PHOTOTROPISM 1 (CPT1) is required for phototropic curvature and has additionally been shown necessary for unequal auxin distribution in rice coleoptiles (Haga *et al.*, 2005). It has therefore been proposed that phot1 autophosphorylation initiates NPH3 dephosphorylation which subsequently leads to auxin redistribution (Pedmale and Liscum, 2007). A related protein, ROOT PHOTOTROPISM 2 (RPT2), also interacts with phot1 and has a role in phot1-mediated phototropism and stomatal opening (Sakai *et al.*, 2000, Inada *et al.*, 2004 Okada & Shimura, 1992). Analysis of the *Arabidopsis* genome has identified 32 NPH3/RPT2-LIKE (NRL) proteins, each having a conserved structure consisting of a coiled coil and a BTB/POZ (**B**road complex, **T**ramtrack, **B**ric-a-brac/ **PO**x virus and **Z**inc finger) domain which are known protein dimerisation motifs (Celaya and Liscum, 2005). Similar investigations in rice have identified 24 NRL protein family members (Kimura and Kagawa, 2006). The presence of these motifs has lead to speculation that NRL proteins act as scaffolds to enhance interactions between phots and downstream signalling factors (Celaya and Liscum, 2005).

The precise mechanism by which phots modulate auxin distribution remains elusive. Auxin is transported between cells by a combination of diffusion along a chemiosmotic gradient and actively-mediated directional transport. Auxin is able to diffuse through the plasma membrane from the acidic extracellular cell wall but upon entry into the cytoplasm becomes ionic and requires active transport to exit the cell (Vieten *et al.*, 2007). Auxin import is also actively-mediated through the action of auxin influx carriers. Auxin influx and efflux transporters are often asymmetrically localised and therefore generate directional auxin transport and this polar auxin transport (PAT) has been shown necessary for phototropism (Vieten *et al.*, 2007, Friml *et al.*, 2002). A possible mode of action of phot activity therefore involves modulation of auxin influx and efflux transporters. Recent work has suggested a role for the putative auxin importer AUX1 in hypocotyl phototropism (Stone *et al.*, 2007).

The best-characterised auxin efflux transporters are the PIN-FORMED (PIN) proteins (Zažímalová *et al.*, 2007). Eight members of this family have been identified in *Arabidopsis* which are thought to be derived from a single ancestral gene (Paponov *et al.*, 2005). Although there is a great deal of functional redundancy between members of this family specific functions have been assigned to certain members

(Vieten *et al.*, 2007). Both PIN1 and PIN3 have been shown to have a role in phototropism (Blakeslee *et al.*, 2004, Friml *et al.*, 2002). PIN1 is localised to the basal cellular membrane and enhances auxin transport away from the shoot apical meristem (Gaelweiler *et al.*, 1998) whilst PIN3 is required for lateral PAT across the hypocotyl (Friml *et al.*, 2002). Upon blue light exposure PIN1 localisation becomes disorganised, a process that requires phot1 (Blakeslee *et al.*, 2004). Whilst the role of phot1 in this process is not well understood, the related kinase PINOID acts to relocalise PIN proteins via direct phosphorylation (Friml, 2004) and it is tempting to speculate that phot1 may modulate PIN1 localisation in a similar fashion.

Such an alteration in auxin distribution induces changes in gene expression. Recent microarray analysis using a *Arabidopsis thaliana* relative (*Brassica oleracea*) has shown that a subset of genes are differentially regulated on the shaded side of the hypocotyl compared to the illuminated side (Esmon *et al.*, 2006). This differential expression correlated with auxin levels and could be negated by auxin transport inhibitors (Esmon *et al.*, 2006), suggesting that this transcription regulation was mediated by auxin. In support of this a suite of auxin-regulated transcription factors including NON-PHOTOTROPIC HYPOCOTYL 4 (NPH4) and MASSUGU 2 (MSG2) are required for normal phototropic responses (Tatematsu *et al.*, 2004, Harper *et al.*, 2000, Stowe-Evans *et al.*, 1998). Tantalisingly, genes differentially regulated include members of the expansin protein family which are involved in cell wall expansion (Esmon *et al.*, 2006). This would provide a mechanistic explanation to account for the differential growth required for phototropism.

### *1.12.2 Blue light-mediated petiole positioning and leaf expansion*

The initial positioning of the petiole and subsequent leaf expansion allow the plant to further optimise its ability to photosynthesise under limiting light conditions. These two responses are strongly controlled by the phytochromes and cryptochromes as part of the shade avoidance response (Kozuka *et al.*, 2005) but phototropins have an important role in modulating these responses in the presence of blue light (Sullivan *et al.,* Takemiya *et al.*, 2005b, Inoue *et al.*, 2005, Inoue *et al.*, 2007). When grown under red light alone, *Arabidopsis* petioles are positioned approximately 30° from the horizontal. The addition of blue light causes the petioles to be angled at approximately 60°, a response which is lacking in the *phot1 phot2* mutant (Inoue *et al.*, 2007).

Similarly, plants grown under red light alone display an obvious leaf curling phenotype which requires either phot1 or phot2 activity in the presence of blue light to be complemented (Takemiya *et al.*, 2005b). In common with other phot-mediated signals both phot1 and phot2 are capable of inducing this phenotype although phot1 retains sensitivity at lower fluence levels than phot2 (Inoue *et al.*, 2007).

As these are recently identified phot-mediated responses, little is known about the downstream signalling necessary for these responses. Despite this, it appears that NPH3 acts in a phot1-specific manner. Under low light conditions (0.1 μmol $m^{-2}$ $s^{-1}$ blue light) both phot1 and NPH3 are required for wild-type petiole movement and leaf expansion responses. However under higher irradiation (5 μmol $m^{-2}$ $s^{-1}$ blue light) phot2 appears sufficient to complement both these responses in both *phot1* and *nph3* mutants (Inoue *et al.*, 2007).

### *1.12.3 Blue light-mediated stomatal opening*

The opening and closing of stomatal pores is controlled by the movement of a pair of guard cells which surround each pore. The guard cells actively transport $K^+$ and sugars to alter their osmotic potential, allowing turgor dependent movements (Shimazaki *et al.*, 2007). Because of the vital role of the stomata in regulating gaseous exchange between leaves and the environment stomatal opening is tightly regulated by several internal and external mechanisms including circadian cues, intracellular $CO_2$ levels, drought and both red and blue light (Shimazaki *et al.*, 2007). Blue light-dependent stomatal opening is controlled by both cryptochromes (Section 1.3.3) and phototropins. Data suggests that phot-mediated stomatal opening occurs at higher light intensities than those required for cryptochrome activity (Mao *et al.*, 2005). Blue light has been shown to rapidly induce stomatal opening in a phot-dependent fashion as the *Arabidopsis phot1 phot2* double mutant demonstrates an impaired response (Kinoshita *et al.*, 2001) while blue light-dependent stomatal opening is complemented by expression of a *PHOT1* transgene in this mutant background (Doi *et al.*, 2004). Compared to photosynthesis, stomatal opening occurs over a longer timescale and it is thought blue light-mediated opening may act to enhance stomatal opening at dawn to optimise $CO_2$ fixation (Shimazaki *et al.*, 2007).

Despite the known role of the phots in initiating this blue light-dependent opening it is clear that several intermediate steps exist before stomatal opening

occurs. Phot-mediated signalling includes blue-light induced phosphorylation and subsequent activation of a plasma membrane $H^+$-ATPase (Kinoshita *et al.*, 2001). This phosphorylation increases binding affinities of the $H^+$-ATPase with a 14-3-3 protein, and such binding is necessary for $H^+$-ATPase activation (Kinoshita and Shimazaki, 2002). Although phot1 also binds 14-3-3 proteins in a light-dependent fashion (Section 1.10; Kinoshita *et al.*, 2003), phots are not thought to be the kinases directly responsible for $H^+$-ATPase phosphorylation as the fungal toxin fusicoccin induces $H^+$-ATPase phosphorylation in the absence of either phot1 or phot2 (Kinoshita and Shimazaki, 2001, Ueno *et al.*, 2005). Whilst it has been speculated that 14-3-3 proteins may have a role in the facilitation of phototropin kinase interaction with downstream proteins, as in alternate model systems (Kinoshita *et al.*, 2003, Fantl *et al.*, 1994), recent work has suggested that a type 1 protein phosphatase (PP1) acts as an intermediary between phots and $H^+$-ATPases (Takemiya *et al.*, 2006). Pulvini-mediated leaf movement in *Phaseolus vulgaris* (which also requires changes in turgor pressure) similarly acts via a PP1 to alter $H^+$-ATPase activity (Inoue *et al.*, 2005). Work to identify additional intermediate factors has revealed that phot1 and phot2 signal partly through different intermediaries (Shimazaki *et al.*, 2007). Phot1 stomatal opening has been genetically linked to the NRL protein RPT2 (see Section 1.12.1). In the *rpt2* mutant, stomatal opening in response to blue light is significantly reduced, with no additive effect being observed in the *rpt2 phot1* double mutant (Inada *et al.*, 2004). Conversely, stomatal opening in response to blue light was abolished in the *rpt2 phot2* double mutant (Inada *et al.*, 2004).

Investigation of stomatal opening in *Vicia faba* has shown that a phot1 variant within this species interacts with a protein with high similarity to dynein light chain (Emi *et al.*, 2005). This protein (*Vicia faba* PHOTOTROPIN1A-INTERACTING PROTEIN, VfPIP) interacts with the LOV linker sequence in yeast two-hybrid screens but does not interact with the other known phot in this species *in vitro* (Emi *et al.*, 2005). A VfPIP-GFP fusion protein was localised to cortical microtubules and artificial disruption of microtubule structure partially inhibited blue light-mediated stomatal opening (Emi *et al.*, 2005). Interestingly microtubule organisation is blue light sensitive in *Arabidopsis* guard cells (Lahav *et al.*, 2004). In combination these data suggest that VfPIP may mediate microtubule rearrangement to facilitate stomatal opening in *Vicia faba*. It will be of interest to determine whether *Arabidopsis*

orthologs of VfPIP demonstrate altered microtubule organisation and stomatal opening.

### *1.12.4 Blue light-controlled chloroplast movement*

Chloroplast movement allows plants to further refine photosynthesis potential. Chloroplast arrangement within the photosynthetically important palisade mesophyll cell layer is typically described as having three configurations which are alternated dependent on ambient light conditions (Wada *et al.*, 2003). In the dark chloroplasts are minimally orientated within the palisade mesophyll cells, while illumination from above provides a directional stimulus and induces an 'accumulation' response at the dorsal surface. Higher light intensities stimulate further chloroplast movement whereby chloroplasts are relocated to the anticlinal cell walls, referred to as the 'avoidance' response (Wada *et al.*, 2003). Comparative studies have shown that these described chloroplast movements are more pronounced in plant species which occupy ecological niches with variable light exposure (Wada *et al.*, 2003). Chloroplast accumulation presumably acts to optimise photosynthesis under low light conditions, whilst it has been shown that the avoidance response prevents chloroplast damage under intense light irradiation (Kagawa, 2003, Suetsugu and Wada, 2007, Kasahara *et al.*, 2002a).

Chloroplast movement is primarily regulated by blue light, and phototropins are necessary for this response (Suetsugu and Wada, 2007). Both phot1 and phot2 are capable of mediating the accumulation response in *Arabidopsis* although only phot2 has a role in the higher-fluence avoidance movement (Kagawa *et al.*, 2001, Sakai *et al.*, 2001). Phot-interacting partners necessary for chloroplast movement have not yet been characterised although progress is being made to identify components of this signalling pathway (Suetsugu and Wada, 2007). Two proteins have been identified which are required for both chloroplast accumulation and avoidance, CHLOROPLAST UNUSUAL POSITIONING 1 (CHUP1) and PLASTID MOVEMENT IMPAIRED 1 (PMI1) (Oikawa *et al.*, 2003, DeBlasio *et al.*, 2005). CHUP1 is an F-actin binding protein which is consistent with the known requirement of cytoskeletal rearrangement for chloroplast movement (Wada *et al.*, 2003, Oikawa *et al.*, 2003) and the N-terminal region of CHUP1 is sufficient to target GFP to the chloroplast envelope, suggesting that CHUP1 may mediate interactions between the

chloroplasts and the cytoskeleton (Oikawa *et al.*, 2003). PMI1 mutants also display defects in both chloroplast accumulation and avoidance movements despite the cytoskeletal structure of *pmi1* mutants appearing to be intact (DeBlasio *et al.*, 2005). PMI1 is a protein with unknown function, although it has homology to several signalling peptides (DeBlasio *et al.*, 2005). Further work is required to identify the biochemical function of this protein.

Mutant screens have identified several proteins required specifically for either chloroplast accumulation or avoidance. Proteins necessary for the accumulation response include J-DOMAIN PROTEIN REQUIRED FOR CHLOROPLAST ACCUMULATION RESPONSE 1 (JAC1; Suetsugu *et al.*, 2005a). JAC1 is present in cytoplasmic fractions and its C-terminus has homology to auxilin. Auxilin has a key role in clathrin-mediated endocytosis in animal cells although this function has not yet been assigned to JAC1 (Suetsugu *et al.*, 2005a). Proteins required specifically for chloroplast avoidance include PMI2 and PMI5 (Luesse *et al.*, 2006). Again, the biochemical function of these proteins remains elusive although they contain conserved coiled-coil protein interaction domains (Luesse *et al.*, 2006). Known phot interactors (such as NPH3 and RPT2, see Section 1.12.1) also contain coiled coil motifs and it will be interesting to determine whether PMI2 and PMI5 are also capable of directly interacting with phot2. Such interactions may involve the C-terminal region of the phot2 protein as a truncated phot2 protein lacking the terminal 40 amino acids (but retaining an intact kinase domain) was unable to complement chloroplast avoidance when transiently expressed in *Adiantum* (Kagawa *et al.*, 2004). This extreme C-terminal region contains two highly conserved proline residues which have been speculated to have a functional role (Kagawa *et al.*, 2004), although further work is required to determine whether these are necessary for phot2 complementation of chloroplast avoidance.

### *1.12.5 $Ca^{2+}$ as a potential intermediary of phot signalling cascades*

As phots mediate a broad array of different physiological responses without having a significant effect on transcription (Christie, 2007, Ohgishi *et al.*, 2004) considerable efforts have been made to isolate intermediate signalling components. Whilst NPH3 and RPT2 have been identified as phot-interacting partners involved in multiple phot-mediated physiological responses (Motchoulski and Liscum, 1999, Inada *et al.*, 2004,

Sakai *et al.*, 2000, Inoue *et al.*, 2007) it is becoming increasingly apparent that calcium ions ($Ca^{2+}$) act as an additional mediator of phot signalling (Harada and Shimazaki, 2007). $Ca^{2+}$ has previously been shown to be a versatile intracellular messenger and changes in intracellular calcium levels are observed in stomatal opening, chloroplast movement and the rapid inhibition of hypocotyl elongation which are all blue light responses initiated by phots (Folta *et al.*, 2003a, Wada *et al.*, 2003, Dietrich *et al.*, 2001). $Ca^{2+}$ release from different intracellular stores is thought to create varied '$Ca^{2+}$ signatures' which are interpreted by appropriate downstream factors (Harada and Shimazaki, 2007). Both phot1 and phot2 are capable of increasing cytoplasmic levels of $Ca^{2+}$ although the $Ca^{2+}$ source used varies (Babourina *et al.*, 2002, Baum *et al.*, 1999, Harada *et al.*, 2003). The correlation between intracellular calcium and phot-mediated signalling extends to light-dependency as both phot2-mediated $Ca^{2+}$ release and phot2-mediated physiological responses require higher blue light fluence rates than those dependent on phot1 (Harada *et al.*, 2003, Sakai *et al.*, 2001, Kagawa *et al.*, 2001). Interestingly, it has been speculated that phot1 may suppress phot2-mediated $Ca^{2+}$ import in mature leaves (Harada and Shimazaki, 2007). Although a promising avenue of research, progress in this field has been inhibited by a lack of appropriate reagents (Harada and Shimazaki, 2007). Future developments will be monitored with interest.

## 1.13 Project aims

Although the characterised plant photoreceptors are divergent in their structure, certain parallels can be drawn from their activity. Phytochromes, cryptochromes and phototropins each contain a photosensitive regulatory domain that controls the activity of an integral signalling region following light-induced conformational change (Christie, 2007, Li and Yang, 2007, Rockwell *et al.*, 2006). In addition, each is phosphorylated in a light-dependent fashion (Shalitin *et al.*, 2003, Kim *et al.*, 2005, Christie *et al.*, 1998), although phots are the only *Arabidopsis* photoreceptors with a canonical serine/threonine kinase domain. Whilst phot phosphorylation has been characterised for almost 20 years (Gallagher *et al.*, 1988) remarkably little is known regarding the mechanism regulating this process. For example, although phots are known to be autophosphorylated it is unclear whether this autophosphorylation occurs via an intramolecular mechanism or whether phots act as multimers and cross-

phosphorylate associated phot molecules. Additionally, whilst sites of phot1 autophosphorylation have been mapped to the N-terminal portion of the protein a truncated version of phot1 lacking these sites retains activity *in planta* (Sullivan *et al.*, 2008). The first goal of this project was to further refine our understanding of phot kinase activity by assessing the ability of phot1 to complete intermolecular phosphorylation and to evaluate the effect of protein truncation on phot autophosphorylation. This work was completed *in vitro* using phot protein samples heterologously expressed using a baculovirus/insect cell expression system, an approach which has previously been used to evaluate phot autophosphorylation (Christie *et al.*, 1998).

An atypical characteristic of phot autophosphorylation compared to related protein kinases is that phot kinase activity appears to be autoregulated in response to light, rather than being modulated by an upstream kinase. Whilst the photochemical mechanism of light detection by the integral LOV domains is well understood (Kottke *et al.*, 2006b) how this photochemical activity regulates phot kinase is less clear, with at least two mechanisms of LOV2 signal transmission proposed (as outlined in Section 1.8.1). In an attempt to distinguish between these two proposals the baculovirus/insect cell system was used to evaluate the effect of selected point mutations on the autophosphorylative ability of phot1. Such an approach had previously identified a point mutation within phot1 which increases the basal level of phot1 autophosphorylation in mock-treated samples *in vitro* (Harper *et al.*, 2004a). The final component of this research set out to characterise the physiological consequences of expression of this constitutively-phosphorylated version of phot1 *in planta*. Known phot1-mediated physiological responses were assessed using transgenic *Arabidopsis* expressing the mutated phot1 protein in either a *phot1 phot2* or wild-type *Arabidopsis* background.

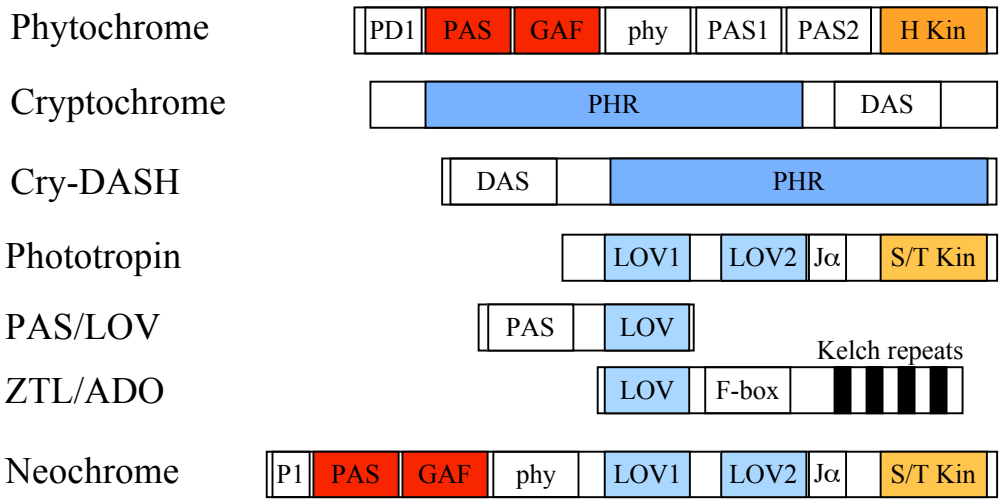



**Figure 1.1 Plant photoreceptor domain structure.** Schematic diagram illustrating major domain structure of plant photoreceptors. Domains necessary for red light detection are shown in red, whilst those for blue light detection are shown in blue. The N-terminal phytochrome PAS and GAF domains interlink to allow binding of a phytochromobilin chromophore whilst the cryptochrome PHR domain associates with FAD and MTHF chromophores. LOV domains bind a FMN chromophore. Kinase domains are highlighted in orange. Abbreviations (in alphabetical order) as follows; DAS- ***D****rosophila, **A**rabidopsis, **S**ynechocystis* cryptochrome domain; FAD- **F**lavin **A**denosine **D**inulceotide; FMN- **F**lavin **M**ono-**N**ucleotide; GAF- c**G**MP-specific and -regulated cyclic nucleotide phosphodiesterase, **A**denylyl cyclase, and **F**hlA; H Kin- **H**istidine **kin**ase; Jα- Jα-helix; LOV- **L**ight/**O**xygen/**V**oltage sensitive; MTHF- **M**ethenyl**t**etra**h**ydro**f**olate; PAS- **P**er/**A**rnt/**S**im; PD1- **P**hytochrome **D**omain 1; PHR- **P**hotolyase **H**omology **R**egion; phy-**Phy**tochrome domain 4; S/T Kin- **S**erine/**T**hreonine **kin**ase.

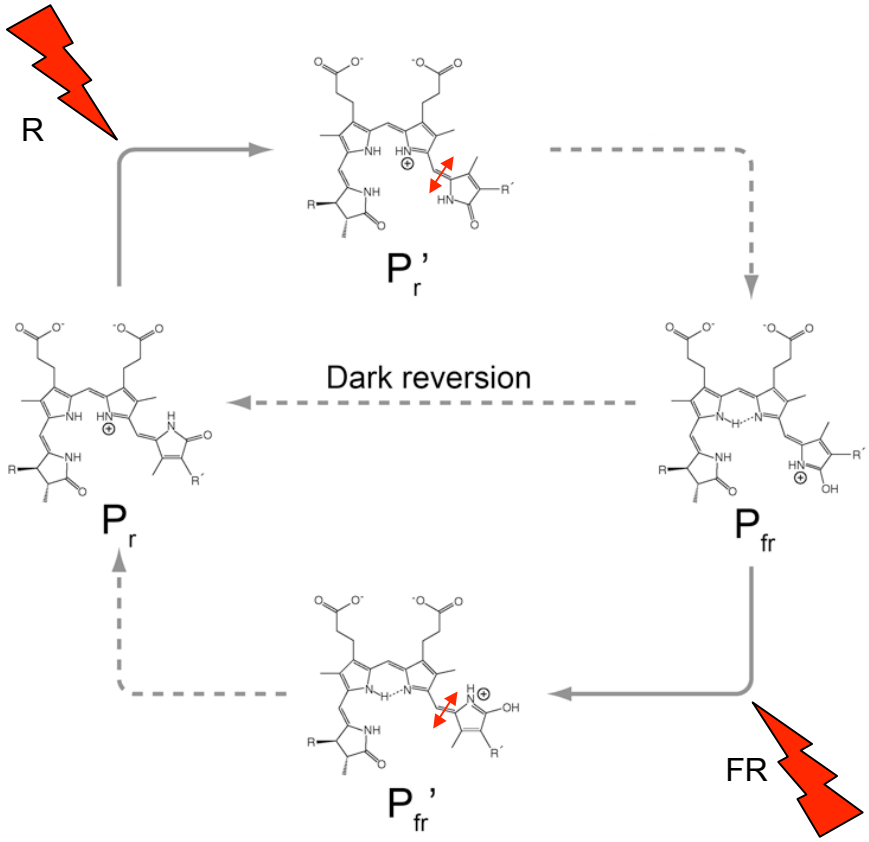



**Figure 1.2 Proposed photocycle of the phytochrome chromophore phytochromobilin.** The phytochrome chromophore (phytochromobilin) interchanges between two forms determined by the ambient light conditions. Irradiation with red light (R) induces photoisomerisation around a double bond to form $P_r$' (red arrow). This is subsequently converted to $P_{fr}$ via several light independent steps (dashed line). Illumination with far red light (FR) accelerates dark version by inducing the reverse photoisomerisation to produce $P_{fr}$' which then reverts to $P_r$ via several light independent steps (dashed line). Modified from Rockwell *et al.,* 2006.

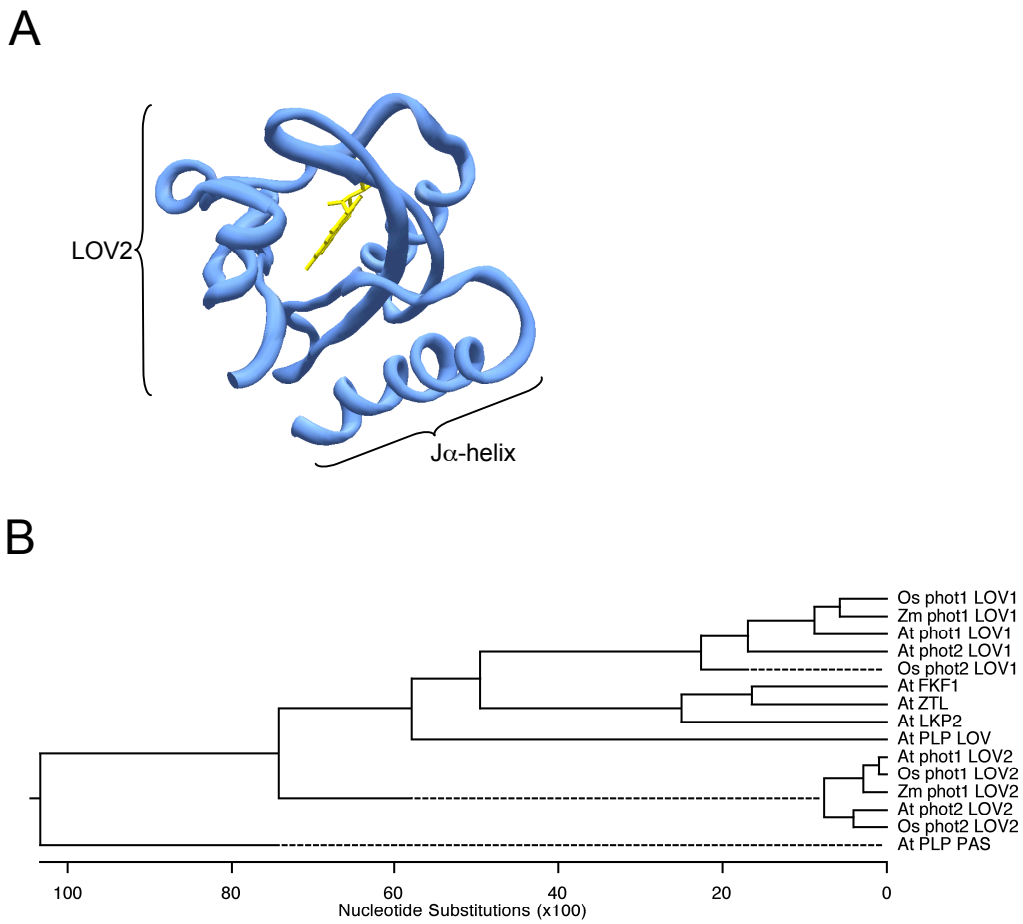



**Figure 1.3 Phototropin LOV domain tertiary structure and evolutionary context. (A)** Protein structure of the LOV2 domain and associated Jα-helix of *Arabidopsis* phot1. The structure of *Arabidopsis* phot1 LOV2 was modelled using the program Swiss Model (http://www.expasy.org; Christie 2007). The backbone structures of α-helices, turns and β-sheets are coloured in blue. The FMN chromophore is shown in yellow. **(B)** Phylogenetic tree of LOV domains from phototropins and angiosperm LOV domain-containing proteins. Amino acid alignments were completed using the clustalW algorithm provided by the program MegAlign (http://www.dnastar.com). The PLP PAS domain is used as an outgroup due to the lack of conserved flavin-interacting residues necessary for photochemistry compared to the PLP LOV domain. Abbreviations (in alphabetical order); At- ***A**rabidopsis **th**aliana*; FKF1- **F**LAVIN BINDING, **K**ELCH REPEAT, **F**-BOX 1; LKP2- **L**OV **K**ELCH **P**ROTEIN 2; LOV- **L**ight-, **O**xygen- or **V**oltage-sensitive; Os- ***O**ryza **s**ativa*; PAS- **P**er/**A**rnt/**S**im phot- **phot**otropin; PLP- **P**AS/**L**OV **P**ROTEIN; Zm- ***Z**ea **m**ays*; ZTL- **Z**EI**TL**UPE.

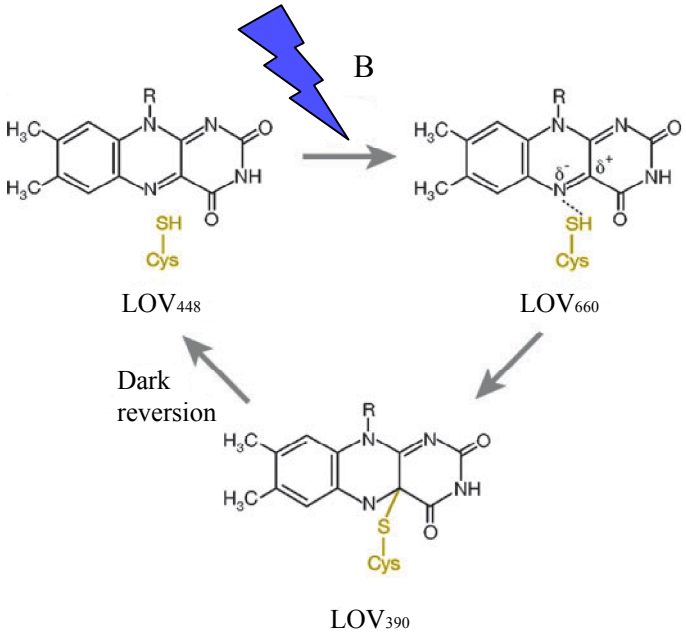



**Figure 1.4 Schematic representation of the LOV domain photocycle.** In the dark resting state the FMN chromophore is non-covalently bound within the LOV domain, forming a species that absorbs maximally at 448 nm ($LOV_{448}$). Blue light (B) drives the production of a highly reactive triplet-state flavin ($LOV_{660}$) that subsequently decays to allow formation of a covalent adduct between the C(4a) carbon of the FMN chromophore and a conserved cysteine residue (yellow) within the LOV domain. This photoproduct ($LOV_{390}$) has a peak of absorption at 390 nm. The photoreaction is fully reversible in darkness. Modified from Christie 2007.

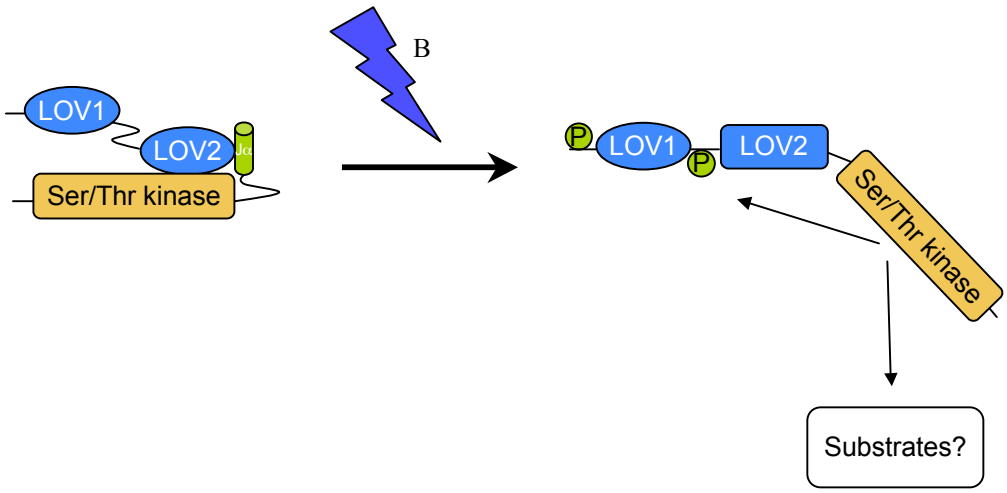



**Figure 1.5 Schematic overview of phototropin activation by light.** In the dark state, the phototropin receptor is unphosphorylated and inactive. Absorption of blue light (B) by the predominant light sensor LOV2 results in conformational change and the disordering of the associated Jα-helix. This leads to activation of the C-terminal kinase domain and autophosphorylation of both the photoreceptor and possibly unidentified protein substrates. LOV domains and the serine/threonine kinase are shown in blue and orange respectively. The approximate position of mapped phototropin phosphorylation sites are indicated in green (P). Modified from Christie 2007.

# Chapter 2- Materials and Methods

## 2.1 Materials and reagents

All chemicals were provided by Sigma-Alrich Ltd (Poole, UK), Fisher Scientific UK (Southampton, UK) or VWR International Ltd. (Poole, UK) unless otherwise stated. Agarose MP (cat# 11388991001) was supplied by Roche Applied Science (Mannheim, Germany). Restriction enzymes and T4 ligase were provided by either New England Biolabs Ltd. (Hitchin, UK) or Promega (Southampton, UK). Taq polymerase and dNTPs were provided by Promega (Southampton, UK). Pfu Turbo® (cat# 600250) was sourced from Stratagene (CA, USA). DNA molecular weight markers were provided by Invitrogen Ltd. (Paisley, UK) and New England Biolabs Ltd. (Hitchin, UK). QIA-quick® PCR purification kit (cat# 28104), QIA-quick® gel extraction kit (cat# 28704), QIA-prep® spin plasmid mini kit (cat# 12125) and DNeasy® DNA isolation kit (cat# 69104) were provided by Qiagen Ltd. (Crawley, UK). Luria-Bertani–broth (cat# 1102850500) and –agar (cat# 1102830500) were provided by Merck KGaA (Darmstadt, Germany).

BD BaculoGold™ baculovirus transfection kits (cat# 554740) supplied by BD Biosciences (CA, USA). Cell culture flasks (cat# 43072) provided by Corning (NY, USA). Insect cell media (SF900II, cat# 10902-088) sourced from Invitrogen Ltd. (Paisley, UK). Fetal bovine serum (cat# S1810) provided by Biosera (Ringmer, UK). 37.5:1 acrylamide:bis-acrylamide solution, Bradford reagent, nitrocellulose membrane (cat# 162-0115) and Poly-prep® chromatography columns (cat# 731-1550) were provided by Bio-Rad Laboratories (England) Ltd. (Bramley, UK). Protein molecular weight markers (cat# P7708S) sourced from New England Biolabs Ltd. (Hitchin, UK). Complete® protease inhibitor cocktail Tablets (EDTA-free, cat# 11836170001) supplied by Roche Applied Science (Mannheim, Germany). Ponceau S (cat# P3504) and BCIP/NBT solution (cat #B6404) sourced from Sigma-Aldrich. ECL+™ (cat# RPN2132) was provided by Amersham. Affinity® protein expression system (cat# 204302) from Stratagene (CA, USA), MagneGST™ protein purification system (cat# V8600) from Promega (Southampton, UK). Amicon Ultra-15 Centrifugal Filter Units (cat # UFC901008) provided by (Millipore, USA). Redivue

adenosine 5'-[γ-$^{32}$P] triphosphate (cat# AA0068) was sourced from Amersham Biosciences UK Ltd. (Bucks. UK). X-ray film (cat# 814 3059) was supplied by Kodak (USA).

## 2.2 DNA cloning and manipulation

### *2.2.1 Agarose gel electrophoresis of DNA*

The integrity and molecular weight of DNA samples was routinely checked using horizontal agarose gel electrophoresis. Samples were resuspended in 1 x Loading Buffer (6% (v/v) glycerol, 0.05% (v/v) bromophenol blue, 0.05% (v/v) xylene cyanol FF) and resolved by electrophoresis in 1 x TAE (40 mM Tris, 2 mM ethylenediaminetetraacetic acid (EDTA), 1.15% (v/v) glacial acetic acid) at 100 V. Agarose gels used contained 1% agarose MP (w/v) and 0.1 μg ml$^{-1}$ ethidium bromide. Ready-Load™ 1Kb Plus DNA ladder (Invitrogen, UK) was loaded alongside samples expected to be between 1 Kb and 12 Kb in length while 100 bp DNA ladder (NEB, UK) was used for samples expected to be ≤1 Kb. This allowed estimation of sample molecular weight. Ethidium bromide stained gels were visualised under ultra-violet light using a Bio-Rad Gel-Doc 2000 in combination with the program Quantity One (http://www.bio-rad.com).

### 2.2.2 *Isolation of plasmid DNA from Escherischia. coli*

XL-1 Blue Super-competent *Escherichia coli* (*E. coli*; Stratagene, USA) were used for cloning of plasmids mutated using site-directed mutagenesis (Section 2.2.5) and newly-ligated plasmids (Section 2.2.6). Chemically competent XL-1 Blue *E.coli* were gently thawed before being incubated with 0.024 M β-Mercaptoethanol (Stratagene, USA) on ice for 10 min. Plasmid DNA was then added and cells incubated for a further 30 min on ice before cells were heat-shocked (42°C, 45 s). Treated cells were returned to ice for 5 min. Luria-Bertani (LB) broth was then added before *E. coli* were incubated at 37°C for one hour. Transformed *E. coli* were selected using LB-agar plates containing appropriate antibiotic.

Transformed *E. coli* were grown overnight at 37°C with shaking (200 rpm) in a 15 ml culture. Plasmid DNA was isolated using the QIA-prep spin plasmid mini kit

(Qiagen, UK) according to the manufacturer's protocol. The quantity and purity of isolated DNA was assessed using a spectrometer (Bio-rad SmartSpec 3000) against a $dH_2O$ blank (Sambrook *et al.*, 1989). 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 (Section 2.2.1).

### *2.2.3 Polymerase chain reaction techniques*

Polymerase chain reactions (PCR) were completed using a MJ Research DNA Engine PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation, Essex, UK). DNA oligonucleotides (primers) were designed *de novo* or with the program PrimerSelect (http://www.dnastar.com). Primers used in this study are listed in Appendix I (Table 7.1).

PCR reactions were completed in a final volume of 25 μl. Template DNA (0.2 μg) was added to 1 x GoTaq® reaction buffer (Promega, UK) in combination with 1 μM of each primer, 200 μM dNTPs and 1.25 U GoTaq® DNA polymerase. Amplification was performed using a suitable number of cycles following an initial denaturation step of 2 min at 94°C. A typical cycle consisted of an additional denaturation at 95°C for 30 s, an annealing step at 58°C for 1 min and an extension step at 72°C for 1 min per Kb template. Modifications were made to this basic program when required by specific template DNA or primers.

PCR of cDNA to be used for protein expression or *Arabidopsis thaliana* transformation was completed using Pfu Turbo® DNA polymerase (Stratagene, USA). This was used according to the manufacturer's protocol. Amplification was performed using a suitable number of cycles following an initial denaturation step of 30 s at 95°C. A typical cycle consisted of an additional denaturation at 95°C for 30 s, an annealing step at 55°C for 1 min and an extension step at 68°C for 2 min per Kb template.

### *2.2.4 Colony PCR*

Transformed *E. coli* were transferred from a selective LB-agar plate to a thin-walled 200 μl PCR tube containing 5 μl $dH_2O$ using a sterile pipette tip. A portion of each transferred colony was re-streaked onto a new selective LB-agar plate. PCR was

completed as described (Section 2.2.3). PCR samples were examined by agarose gel electrophoresis (Section 2.2.1) and colonies containing the desired plasmid identified. Plasmid DNA was isolated as described (Section 2.2.2).

### *2.2.5 Site-directed mutagenesis of plasmid DNA*

Site-directed mutagenesis was completed using the QuikChange® Site-directed Mutagenesis kit (Stratagene, USA). High-fidelity PCR (Section 2.2.3) was completed and used on the appropriate template DNA using specifically-designed primers and the DNA polymerase Pfu Turbo® (Stratagene, USA). Primers were designed to contain ~10-15 bases either side of the mutated codon, with care taken that the GC content was a minimum of 40%. The melting temperature ($T_m$) of each primer was ≥78°C according to the following formula;

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$$

where %GC and %mismatch are expressed as whole numbers and N is the primer length (in bases). Primers used for site-directed mutagenesis reactions are listed in Appendix I (Table 7.2).

After completion of PCR the template DNA was removed by digestion with Dpn1 for one hour at 37°C. The remaining amplified PCR product was purified using the QIA-quick PCR purification kit (Qiagen, UK). XL-1 Blue Super-competent *E. coli* were transformed with the mutated plasmid as described (Section 2.2.2). DNA sequencing was completed to confirm insertion of the desired mutation ('The Sequencing Service', University of Dundee, UK). Further rounds of mutagenesis were completed to insert additional mutations when desired (Sakai *et al.*, 2001).

### *2.2.6 DNA plasmid ligation*

DNA to be digested (0.5 μg plasmid DNA or 2.5 μg PCR product) was prepared in 30 μl total volume containing 1 x suggested buffer and 2.5 U of the restriction enzyme. Reactions were incubated in a water bath at 37°C for one hour before being purified using the QIA-quick PCR purification kit (Qiagen, UK) in accordance with the manufacturer's instructions. Alternately, digested DNA was run on a 1% agarose gel

and visualised using ethidium bromide staining (Section 2.2.1) before the appropriately sized band was excised and the DNA contained therein extracted using the QIA-quick gel extraction kit (Qiagen, UK). Recovery of digested DNA from the column via either method was assessed by examination of an aliquot of the reaction using agarose gel electrophoresis (Section 2.2.1).

DNA ligation was completed using T4 ligase. The insert and vector were combined in a 3:1 ratio in the presence of T4 ligase (400 U) and 1 x T4 ligase buffer. The reaction was allowed to proceed at room temperature (~22°C) for one hour before being transferred to 4°C overnight. Ligated DNA was purified using the QIA-quick PCR purification kit (Qiagen, UK) in accordance with the manufacturer's instructions before being transformed into *E. coli* (Section 2.2.2). Expression vectors created for this study are listed in Table 2.1.

## 2.3 Protein gel electrophoresis

### *2.3.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

Protein samples were denatured by boiling at 100°C after resuspension in 1 x SDS Loading Buffer (62.5 mM Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.004% (w/v) bromophenol blue, pH 6.8). Denatured samples were were electrophoresed at 200 V according to the method initially outlined by Laemmli (1970) using SDS-PAGE gels containing either a 7.5% or 12.5% polyacrylamide separating gel and a 5% polyacrylamide stacking gel. Protein molecular weights were estimated by loading 'Broad Range Pre-stained Molecular Weight' marker (New England Biolabs, UK) alongside the samples.

### *2.3.2 Western blotting*

Primary antibodies were sourced as indicated in Table 2.2. Blots using horseradish peroxidase (HRP)-conjugated secondary antibodies (Promega, UK, cat# W4011) were developed using the ECL+™ system (Amersham, UK) and exposed onto X-ray film. Alkaline phosphatase (AP)-conjugated secondary antibodies (Promega, UK, cat# S3731) were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT) solution (Sigma-Alrich, UK, cat# B6404).

Proteins were electro-transferred at 100 V for one hour onto nitrocellulose membrane in Transfer Buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol). Transferred membranes were stained in Ponceau Solution (0.1% (w/v) Ponceau S, 1% (v/v) acetic acid) to confirm equal protein loading by visualisation of the transferred bands. After removing the stain the membrane was blocked for at least one hour in 8% (w/v) milk powder dissolved in 1 x TBST (25 mM Tris-HCl, 137 mM sodium chloride, 2.7 mM potassium chloride, 0.1% (v/v) Triton X-100). After blocking, primary antibodies were incubated with the membane at the dilutions indicated (Table 2.2) for one hour or overnight at 4°C. The membrane was then washed three times in 1 x TBST before the secondary antibodies were added at the dilutions indicated (Table 2.2) for one hour at room temperature. The membrane was washed a further five times in 1 x TBSTT (25 mM Tris-HCl, 137 mM sodium chloride, 2.7 mM potassium chloride, 0.2% (v/v) Triton X-100, 0.05% (v/v) Tween-20) before a final wash in 1 x TBS (25 mM Tris-HCl, 137 mM sodium chloride, 2.7 mM potassium chloride).

### *2.3.3 Staining and drying of SDS-PAGE gels and nitrocellulose membranes*

Coomassie staining of SDS-PAGE gels was completed after removal of the stacking gel. The resolved gel was submerged in Coomassie Stain Solution (0.1% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid) for a minimum of 30 min. The stained gel was then destained in several changes of Destain Solution (50% (v/v) methanol, 10% (v/v) acetic acid). Destained gels were stored in Rehydration Buffer (10% (v/v) ethanol, 5% (v/v) acetic acid).

Silver staining of SDS-PAGE gels was completed after removal of the stacking gel. The resolved gel was soaked overnight in 50% (v/v) methanol before being transferred into Silver Stain Solution (0.21 M ammonium hydroxide, 20 mM sodium hydroxide, 50 mM silver nitrate) for 15 min. The gel was then washed several times in $dH_2O$ before being transferred to Silver Stain Developer (0.26 μM citric acid, 0.02% (w/v) formaldhyde) until bands became visible. Developed gels were washed with 10% (v/v) methanol before drying.

SDS-PAGE gels and nitrocellulose membranes were dried onto Whatmann 3 mm chromatography paper using a Scie Plas Gel Drier (model GD4535) connected to a Vacuubrand MZ C2 vacuum pump.

### *2.4* Protein expression and purification using *Escherichia coli*

The Affinity™ expression system (Stratagene, USA) was used to express fusion proteins with an N-terminal calmodulin-binding protein tag. Protein concentrations were determined at each appropriate step using the Bradford protein assay (Bio-Rad, USA) using BSA (1 μg $ml^{-1}$) as standard.

DNA fragments were cloned into the pCAL-n-EK vector and transformed into BL21 (DE3) pLysS *E. coli* (Merck Chemicals Ltd., Germany) for expression studies. Chemically competent BL21 (DE3) pLysS *Escherichia coli* (*E.coli*) were gently thawed before being incubated with plasmid DNA for 30 min on ice prior to heat-shock (42°C, 45 s). Treated cells were returned to ice for five minutes. 200 μl LB was then added before *E. coli* were incubated at 37°C for one hour. Transformed *E. coli* were incubated at 37°C overnight on LB-agar plates containing 100 μg $ml^{-1}$ ampicilin.

Antibiotic resistant *E. coli* were transferred to a 15 ml culture containing LB and 100 μg $ml^{-1}$ ampicilin using a sterile pipette tip. This culture was grown overnight at 37°C with shaking (200 rpm). In order to express protein, 5 ml of the initial culture was added to 500 ml LB supplemented with 100 μg $ml^{-1}$ ampicilin. This expression culture was incubated at 37°C for approximately two hours until it reached $OD_{600}$ 0.4. Protein expression was induced by addition of isopropyl β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. The expression culture was then grown in the dark for three hours at 28°C with shaking (200 rpm). Cells were pelleted by centrifugation at 5 000 *g*, 4°C for 10 min before being resuspended in 5 ml Calcium Binding Buffer (50 mM Tris-HCl, 150 mM sodium chloride, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM calcium chloride, pH 8, containing Complete® EDTA-free protease inhibitor cocktail). Cells were then lysed using a French® pressure cell press (Thermo Life Sciences, UK) followed by brief sonication (using a MSE Soniprep). Cell debris was removed by centrifugation at 16 000 *g,* 4°C for 20 min. The supernatant containing the soluble protein fraction was removed to a fresh tube and either used immediately or stored overnight on ice.

Bacterially-expressed proteins were purified using Calmodulin Affinity Resin (Stratagene, USA). This resin equilibrated by washing twice in five volumes Calcium Binding Buffer before adding to a 5 ml Poly-prep® chromatography column (Bio-Rad, UK). The resin was allowed to settle before an additional five resin volumes

Calcium Binding Buffer were passed down the column. Crude soluble protein extract was subsequently passed over the column and the column washed in five resin volumes Calcium Binding Buffer. Bound protein was then eluted in Elution Buffer (50 mM Tris-HCl, 10 mM β-mercaptoethanol, 2 mM EGTA, 150 mM sodium chloride, pH 8) in 500 µl fractions. Purified protein was checked for purity by SDS-PAGE (Section 2.3.1). If necessary, proteins were concentrated in Amicon® Ultra centrifugation filter devices (Millipore, USA).

## 2.5 Absorbance Spectroscopy

Absorption spectra were measured over a 100 ms time period using a Shimadzu MultiSpec-1501 diode array spectrometer at room temperature (~22°C) using the program Shimadzu Hyper UV (http://www.shimadzu.co.uk/uv-vis/software.html). The optical path length was 5 mm. Before analysis, the protein concentration of each sample was equalised using the Bradford protein assay using BSA (1 µg $ml^{-1}$) as standard unless stated otherwise. Exponential fitting was completed using the program SigmaPlot (http://www.systat.com) when required.

Photoproduct formation kinetics were determined by monitoring light-induced absorbance changes at the peak level of flavin absorption (~448 nm) whilst the sample was exposed to either 300 or 15 000 µmol $m^{-2}$ $s^{-1}$ blue light provided by a Zeiss KL1500 LCD light source (Carl Zeiss Ltd, USA). Spectra were recorded every 100 ms. Dark-regeneration kinetics were measured by monitoring the recovery of absorption at 448 nm following a pulse of white light (provided by a Cobra 440 AF camera strobe flash) as described (Christie *et al.*, 2002). Spectra were recorded every 10 s for wild-type LOV2 and every 150 s for LOV2 Q575L, with the light source shutter closed between readings.

To compare the relative bleaching of purified samples over a range of light intensities absorbance changes were monitored at 448 nm. Relative bleaching was achieved by reducing the intensity of a Cobra 440 AF camera strobe flash using neutral density filters (OC Oerlikon Balzers Ltd, Liechtenstein) as previously described (Christie *et al.*, 2002). Protein concentrations were corrected using Beer's law so that the $OD_{448}$ of each sample was 0.2. The entire sample was irradiated for each spectral measurement, with the same equipment geometry used in each case.

## 2.6 Fluorescence emission and excitation spectroscopy of isolated LOV domains

Fluorescence emission and excitation spectra of protein samples were recorded using a PerkinElmer LS-55 luminescence spectrometer (PerkinElmer, USA) using the program FL Winlab (http://las.perkinelmer.com). Protein concentrations were determined by the Bradford protein assay. Fluorescence excitation spectra were recorded by monitoring the emission at 520 nm. Fluorescence emission spectra were obtained by using an excitation wavelength of 380 nm. In each case the excitation slit was set to 5 nm and the emission slit set to 7 nm.

### *2.6.1 Assessment of bound flavin in isolated LOV domains*

All manipulations were carried out under a dim red light (60 W bulb filtered by a Kodak safe light filter, Cat# 1521509). Individual LOV2 domains expressed in *E. coli* were purified as described (Section 2.4) and then incubated overnight in darkness at 4°C to ensure photorecovery of the LOV domain back to its ground state before flavin release. Flavin was released by boiling at 100°C in the presence of Elution Buffer (Section 2.4). Boiled samples were chilled on ice for 5 min and centrifuged (13 000 *g*, 5 min). The supernatant obtained (containing released flavin) was cleared using a Qia-quick PCR purification column (Qiagen, UK) to remove further protein contaminants. Flavin fluorescence emission and excitation of the purified supernatant were then measured as described (Section 2.6).

## 2.7 Protein expression and purification using *Spodoptera frugiperda* cells

### *2.7.1 Protein expression and purification*

*Spodoptera frugiperda* cells (Sf9, from Invitrogen, UK) were used for expression of full-length and truncated versions of *Arabidopsis thaliana* phototropin 1 and 2 (Cho *et al.*, 2007, Christie *et al.*, 1998, Sakai *et al.*, 2001). Recombinant baculovirus encoding *Arabidopsis thaliana PHOT1*, *PHOT2* or truncated versions of either were generated using the BaculoGold™ Transfection kit (BD Biosciences, USA) in accordance with the manufacturer's instructions. Recombinant baculovirus was titred by end point dilution and used to infect additional Sf9 cells. Viral co-transfection was completed

using a 1:1 ratio of baculovirus when expressing two His-tagged protein or a 10:1 ratio (His:GST) when expressing a combination of His- and GST-tagged protein. Protein concentrations were determined at each appropriate step using the Bradford protein assay (Bio-Rad, USA) using BSA (1 μg $ml^{-1}$) as standard.

Infected cells were grown in serum free medium (SF900II, Invitrogen, UK) supplemented with 10% (v/v) fetal bovine serum (Biosera, UK) at 27°C for three days in cell culture flasks (Corning, UK) wrapped in aluminum foil. All subsequent manipulations were completed under dim red light (60 watt bulb filtered by a Kodak safe light filter, Cat# 1521509). Cells were harvested through gentle washing of the flask floor. Harvested cells were centrifuged (1 000 *g*, 1 min) and the supernatant removed before pelleted cells were resuspended in 100 μl 1 x Phosphorylation Buffer (37.5 mM Tris-HCl, 5.3 mM magnesium sulphate, 150 mM sodium chloride, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol, pH 7.5 containing Complete® EDTA-free protease inhibitor cocktail). Cells were lysed by sonication (using a MSE Soniprep) and cell debris removed by centrifugation (16 000 *g*) for 3 min. The crude soluble fraction was removed to a fresh tube and stored on ice before use. When necessary microsomal membranes were isolated by ultra-centrifugation (100 000 *g*, 4°C) of the crude soluble fraction for 1 hour. The resultant supernatant was removed to a fresh tube and the membrane pellet resuspended in 30 μl 1 x Phosphorylation Buffer before brief sonication.

GST-tagged proteins were purified using the MagneGST™ purification system (Promega, UK) according to the manufacturer's instructions. GST-tagged proteins were expressed in Sf9 cells using 75 $cm^2$ cell expression flasks and harvested as described above. Harvested cells were centrifuged (1 000 *g*, 1 min) and the supernatant removed before pelleted cells were resuspended in 185 μl 1 x Phosphorylation Buffer and lysed by sonication. Cell lysate was added to equilibrated MagneGST™ beads in Bind/Wash Buffer (4.2 mM di-sodium hydrogen orthophosphate, 2 mM potassium di-hydrogen orthophosphate, 140 mM sodium chloride, 10 mM potassium chloride, 0.5% (v/v) NP40) and incubated for 30 min with agitation. MagneGST™ beads were then washed in three times 300 μl Bind/Wash Buffer before elution in 1 x Glutathione Elution Buffer (50 mM glutathione, 50 mM Tris-HCl, pH 8).

### *2.7.2 In vivo light irradiation of Sf9 cells*

Sf9 cells were infected and grown as described (Section 2.7.1). All manipulations were completed under dim red light. Cells were harvested through gentle washing of the flask floor and split into two aliquots. Samples were given a mock (dark) treatment or irradiated with 30 000 μmol $m^{-2}$ $s^{-1}$ white light for 10 s before incubation at room temperature for 2 min. Crude soluble protein extract was then prepared as described (Section 2.7.1).

### *2.7.3 In vitro kinase assays using protein extracts from Sf9 cells*

Phototropin kinase activity *in vitro* was monitored as previously described (Cho *et al.*, 2007, Christie *et al.*, 1998, Sakai *et al.*, 2001). All manipulations were completed under dim red light. 20 μg protein prepared from Sf9 cells (Section 2.7.1) was suspended in 1 x Phosphorylation Buffer. Redivue adenosine 5'-[γ-$^{32}$P] triphosphate ($^{32}$P-ATP; Amersham, UK) was diluted 1:5 with 10 μM unlabelled ATP and 1 μl of diluted $^{32}$P-ATP added to each reaction. Samples were either given a mock (dark) treatment or exposed to 30 000 μmol $m^{-2}$ $s^{-1}$ white light for 10 s before being incubated at room temperature for a total of 2 min. Reactions were terminated through the addition of an equal volume 2 x SDS Loading Dye (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.008% (w/v) bromophenol blue, pH 6.8). Samples were separated by 7.5% SDS-PAGE, Coomassie-stained and dried as described (Section 2.3). Incorporation of $^{32}$P-ATP was assessed using autoradiography and phosphorimaging.

Dried radioactive gels and blots were exposed onto X-ray film for at least three hours at room temperature. When necessary due to weak signal dried gels were exposed onto X-ray film between two intensifying screens at -70°C overnight. X-ray film was developed using a Kodak X-OMAT processor (model ME-3). For quantification of phosphorylation assays dried radioactive gels were phosphoimaged. Dried gels were exposed to a pre-blanked imaging plate for 30 min in a cassette at room temperature. Exposed plates were imaged using a Fuji FLA-5000 phosphorimager (Fuji Photo Film Co. Ltd, Japan) in combination with the programs 'Image Reader' and 'Image Gauge' (Fuji Photo Film Co. Ltd, Japan).

### *2.7.4 Measurement of flavin incorporation in crude Sf9 protein extracts*

To measure flavin incorporation in crude Sf9 insect cell extract, dark-grown Sf9 cultures (Section 2.7.1) were harvested by centrifugation (1 000 *g*, 1 min) and were washed twice in 1 x PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM di-sodium hydrogen orthophosphate, 2 mM potassium di-hydrogen orthophosphate). Protein concentrations were determined using the Bradford protein assay. The FMN chromophore was released by boiling samples in the presence of 1 x Phosphorylation Buffer for 5 min. Boiled samples were chilled on ice for 5 min and centrifuged (13 000 *g*, 5 min). The supernatant obtained (containing released flavin) was cleared using a Qia-quick PCR purification column (Qiagen, UK) to remove further protein contaminants. Flavin fluorescence present in the purified supernatant was measured as described (Section 2.6.1).

## 2.8 *Arabidopsis thaliana* material and growth conditions

### 2.8.1 *Previously reported Arabidopsis thaliana material*

*Arabidopsis thaliana* (cv. Columbia, *gl1* background) was used as wild-type for this study. Mutant plants deficient for either phototropin 1 (*phot1-5*) or phototropin 2 (*phot2-1*) expression in this background have been characterised previously, as has the double mutant deficient in the expression of both phot1 and phot2 (*phot1-5 phot2-1*; (Jarillo *et al.*, 2001, Kagawa *et al.*, 2001, Liscum and Briggs, 1995, Sakai *et al.*, 2001)).

*Phot1-5 phot2-1* double mutants have subsequently been transformed with a variety of phot-containing constructs to confirm function. Previously characterised lines created in this manner which are used in this study include PHOT1::PHOT1-GFP (Sakamoto and Briggs, 2002), 35S::PHOT1 (Cho *et al.*, 2007) and 35S::PHOT1 LOV2 KINASE, a line containing a truncated version of phot1 (Sullivan *et al.*, 2008).

### *2.8.2 Transformation of Arabidopsis thaliana to create stable transgenic lines*

*Agrobacterium tumefaciens* (strain GV3101 (pMP90)) were transformed with a binary vector containing a cDNA of interest (Table 2.1) and a kanamycin resistance marker gene. Transformed *Agrobacterium* were used to infiltrate *Arabidopsis thaliana* plants

by the floral dipping method (Clough and Bent, 1998). Kanamycin-resistant plants containing the expression vector were selected by plating sterilised seed (Section 2.9) onto agar plates containing 0.5 x Murashige & Skoog (MS) media, 0.8% agarose and 75 μg $ml^{-1}$ kanamycin (pH 6.8). Based on the segregation of kanamycin resistance, T2 lines containing a single transgene locus were identified and homozygous T3 seed obtained. Homozygous T3 transgenic plants were used for physiological studies unless otherwise stated.

### 2.9 *Arabidopsis thaliana* seed sterilisation

Seed was surface sterilised using a bleach solution (50% (v/v) sodium hypochlorite, 0.2% (v/v) Tween-20). Seed was shaken for five minutes in 1 ml of this solution and left to settle before the bleach was removed. Seed was washed four times in 1 ml sterile $dH_2O$ before resuspension in an appropriate volume of sterile $dH_2O$.

### 2.10 Isolation of genomic DNA from *Arabidopsis thaliana*

Plant leaf material was collected and immediately frozen in liquid nitrogen. Genomic DNA was isolated using the DNeasy DNA isolation kit (Qiagen, UK) according to the manufacturer's protocol. The quantity, purity and integrity of isolated DNA was assessed by 1% (w/v) agarose gel electrophoresis as described (Section 2.2.1).

### 2.11 Examination of phot1-GFP localisation via confocal microscopy

Green fluorescent protein (GFP) fluorescence was visualised using 20 x or 40 x objective lenses by confocal laser scanning microscopy using a Zeiss LSM510 microscope. Samples were examined using an excitation wavelength of 488 nm and an emission range between 505 and 530 nm. Images were processed using the programs Zeiss LSM Image Browser (http://www.zeiss.com) and Adobe Photoshop (http://www.adobe.co.uk).

To examine the relocalisation of phot1-GFP from the plasma membrane following blue light irradiation three-day-old etiolated *Arabidopsis* seedlings were continuously irradiated with an excitation wavelength of 488 nm for 12 min (with the laser power was set to 5.1 A). During irradiation the seedling was imaged every 10 s.

## 2.12 Protein extraction from *Arabidopsis thaliana* transgenic plants

Plant tissue was extracted at 4°C under dim red light. Plant tissue was ground in 1 ml 1 x Homogenisation Buffer (25 mM 4-morpholine-prolinesulfonic acid (MOPS), 0.25 M sucrose, 0.1 mM magnesium chloride, Complete® EDTA-free protease inhibitor cocktail, pH 7.8) using a mortar and pestle and filtered through 70 μm mesh cloth before being clarified by centrifugation (10 000 *g*, 4°C, 10 min). The supernatant containing the Crude Soluble Protein fraction was removed to a fresh tube, and protein concentrations determined when appropriate using the Bradford protein assay. 30 μg Crude Soluble Protein was denatured by boiling for 5 min in the presence of 1 x SDS Loading Buffer before being separated by 7.5% SDS-PAGE (Section 2.3.1).

## 2.13 Examination of phototropin phosphorylation in crude plant microsomal extracts using *in vitro* kinase assays

Sterilised seed (Section 2.8.2) was plated onto petri dishes containing 0.5 x Murashige & Skoog (MS) media, 0.8% agarose (pH 6.8) (Murashige and Skoog, 1962). Seed was wrapped in aluminium foil and transferred to a cold room (4°C) for three days to allow stratification. Plates were then treated with 50 μmol $m^{-2}$ $s^{-1}$ white light for four hours (to induce uniform germination) before being re-wrapped in aluminum foil. Treated plants were transferred to a controlled environment room (Fititron, Weiss-Gallenkamp, Loughborough, UK) for three days.

The Crude Soluble Protein fraction was extracted as described (Section 2.12). To isolate crude microsomal membranes, the Crude Soluble Protein fraction was separated by ultra-centrifugation (100 000 *g*, 4°C) for 1 hour. The resultant supernatant was removed to a fresh tube and the remaining membrane pellet resuspended in 50 μl Resuspension Buffer (250 mM sucrose, 4 mM potassium nitrate, 5 mM di-potassium hydrogen orthophosphate, Complete® EDTA-free protease inhibitor cocktail) before brief sonication.

30 μg plant crude microsomal membranes were suspended in 1 x Phosphorylation Buffer + Triton (37.5 mM Tris-HCl, 5.3 mM magnesium sulphate, 150 mM sodium chloride, 1 mM EGTA, 1 mM dithiothreitol, 1% (v/v) Triton X-100, pH 7.5 containing EDTA-free Complete protease inhibitor cocktail). $^{32}$P-ATP was diluted 1:5 with 10 μM unlabelled ATP and 1 μl was added to each reaction. Samples

were either given a mock (dark) treatment or exposed to 30 000 μmol $m^{-2}$ $s^{-1}$ white light for 10 s before being incubated at room temperature for a total of 2 min. Reactions were terminated through the addition of an equal volume 2 x SDS Loading Dye. Samples were separated by 7.5% SDS-PAGE, Coomassie-stained and dried. Incorporation of $^{32}$P-ATP was assessed using autoradiography and phosphorimaging as per Section 2.7.3.

## 2.14 Assessment of NPH3 electrophoretic mobility in light-treated etiolated seedlings

Blue light-induced alterations in NPH3 electrophoretic mobility was assessed as previously described (Pedmale and Liscum, 2007). Three-day-old etiolated seedlings were treated with 10 μmol $m^{-2}$ $s^{-1}$ blue light supplied by a 18 W white fluorescent lamp (cat# 26-835, Osram, Germany) filtered through blue plexiglass for four hours or were given a mock dark treatment before Crude Soluble Protein extracts were isolated in 1 x Homogenisation Buffer as described (Section 2.12). 30 μg of Crude Soluble Protein extract was denatured by boiling at 100°C for 5 min in the presence of 1 x SDS Loading Buffer. Samples were separated by 7.5% SDS-PAGE gel (Section 2.3.1) and western blotting completed using anti-NPH3 antibody (Section 2.3.2; Table 2.2).

## 2.15 Physiological characterisation of *Arabidopsis thaliana* transgenic plants

### *2.15.1 Characterisation of phototropic curvature in response to unilateral blue light exposure*

Second positive hypocotyl curvature was measured as described (Lasceve *et al.*, 1999). Surface sterilised seed (Section 2.8.2) were planted individually in horizontal lines onto square petri dishes containing 0.5 x Murashige & Skoog (MS) media, 0.8% agarose (pH 6.8) (Murashige and Skoog, 1962). Seed was wrapped in aluminium foil and transferred to a cold room (4°C) for three days to allow stratification. Plates were then treated with 50 μmol $m^{-2}$ $s^{-1}$ white light for four hours (to induce uniform germination) before being re-wrapped in aluminum foil. Treated plates were transferred to a controlled environment room (Fititron, Weiss-Gallenkamp,

Loughborough, UK) and stored vertically for three days so that the seedlings grew across the agar surface. These three-day-old etiolated seedlings were exposed to unilateral blue light to induce phototropism. Blue light ≤10 μmol $m^{-2}$ $s^{-1}$ was provided by a white fluorescent lamp (18 W, cat# 26-835, Osram, Germany) filtered through blue plexiglass. Higher fluence rates of blue light were provided by a slide projector (Toshiba TLP-T50) in combination with a water filter and blue plexiglass. Images were digitally captured using an UMax PowerLook scanner and hypocotyl curvature quantified using the program ImageJ (http://rsb.info.nih.gov/ij).

### *2.15.2 Characterisation of petiole positioning in Arabidopsis seedlings*

Seed was sown on soil and stratified for three days at 4°C before being transferred to a controlled environment room. Plants were grown under 50 μmol $m^{-2}$ $s^{-1}$ white light in Long Day conditions (16/8 hours light/dark) until the first leaves began to develop (at approximately seven days). Seedlings were then transferred to 50 μmol $m^{-2}$ $s^{-1}$ constant white light for an additional five days before representative seedlings were photographed. The petiole angle of the first true leaves was measured using the program ImageJ (http://rsb.info.nih.gov/ij) as described (Inoue *et al.*, 2007).

### *2.15.3 Characterisation of leaf expansion in Arabidopsis*

Seed was sown on soil and stratified as described (Section 2.15.2) before being grown for 21 days under 70 μmol $m^{-2}$ $s^{-1}$ white light in Long Day conditions. The fifth rosette leaves of representative plants were detached and scanned using a UMax PowerLook scanner before being artificially flattened and rescanned. Leaf area before and after flattening was measured using the program ImageJ (http://rsb.info.nih.gov/ij) as outlined (Takemiya *et al.*, 2005). The leaf expansion index is defined as the ratio of the unflattened leaf area to the area of the artificially flattened leaf.

### *2.15.4 Assessment of chloroplast positioning in mature Arabidopsis tissue*

*Arabidopsis* seedlings were sown on soil and grown as described for 28 days under 70 μmol $m^{-2}$ $s^{-1}$ white light in Long Day conditions. Leaves were detached and

transplanted to 0.5 x MS plates before being exposed to either darkness, low blue (1 μmol $m^{-2}$ $s^{-1}$) or medium blue (10 μmol $m^{-2}$ $s^{-1}$) for three hours as described (Onodera *et al.*, 2005). Blue light was provided fluorescent white lamps (18 W, cat# 26-835, Osram, Germany) in combination with blue plexiglass filters. Chloroplast positioning in the palisade mesophyll layer of treated leaves was determined by confocal microscopy of chlorophyll autofluorescence. Samples were imaged using a 20 x objective lense by confocal laser scanning microscopy (Zeiss LSM510) using an excitation wavelength of 488 nm and an emission range ≥560 nm. Images were processed using the programs Zeiss LSM Image Browser (http://www.zeiss.com) and Adobe Photoshop (http://www.adobe.co.uk).

| Expression system | Name | Tag | Protein encoding | Source/Primers used |
|---|---|---|---|---|
| Baculovirus | pAcHLT-A phot1 | 6xHis (N-term) | phot1 (7-996) | (Christie *et al.*, 1998) |
| " | pAcHLT-A phot1 D806N | 6xHis (N-term) | phot1 D806N (7-996) | D806N mutagenesis primers |
| " | pAcHLT-A phot1 S849A | 6xHis (N-term) | phot1 S849A (7-996) | S849A mutagenesis primers |
| " | pAcHLT-A phot1 C512M | 6xHis (N-term) | phot1 C512M (7-996) | C512M mutagenesis primers |
| " | pAcHLT-A phot1 I608E | 6xHis (N-term) | phot1 I608E (7-996) | I608E mutagenesis primers |
| " | pAcHLT-A phot1 C512A | 6xHis (N-term) | phot1 C512A (7-996) | (Christie *et al.*, 2002) |
| " | pAcHLT-A phot1 E506K | 6xHis (N-term) | phot1 E506K (7-996) | E506K mutagenesis primers |
| " | pAcHLT-A phot1 Q575L | 6xHis (N-term) | phot1 Q575L (7-996) | Q575L mutagenesis primers |
| " | pAcHLT-A phot1 Q575H | 6xHis (N-term) | phot1 Q575H (7-996) | Q575H mutagenesis primers |
| " | pAcHLT-A phot1 Q575N | 6xHis (N-term) | phot1 Q575N (7-996) | Q575N mutagenesis primers |

| Expression system | Name | Tag | Protein encoding | Source/Primers used |
|---|---|---|---|---|
| Baculovirus | pAcHLT-A phot1 C512A I608E | 6xHis (N-term) | phot1 C512A I608E (7-996) | C512A+I608E mutagenesis primers |
| " | pAcHLT-A phot1 C512A Q575L | 6xHis (N-term) | phot1 C512A Q575L (7-996) | C512A+Q575L mutagenesis primers |
| " | pAcHLT-A phot1Q575L I608E | 6xHis (N-term) | phot1 Q575L I608E (7-996) | Q575L+I608E mutagenesis primers |
| " | pAcHLT-A phot1 LOV2 Kinase | 6xHis (N-term) | P1 L2K (448-966) | Sub-cloned from pEZR phot1 LOV2 kinase (EcoR1, Sma1) |
| " | pAcHLT-A phot1 LOV2 Kinase D806N | 6xHis (N-term) | P1 L2K D806N (448-966) | D806N mutagenesis primers |
| " | pAcG3X phot1 | GST (N-term) | phot1 (1-996) | BamH1-ATGGAACCAACAGAAAAACCATCGACC; EcoR1-TCAAAAAACATTTGTTTGCAG |
| " | pAcG3X phot1 D806N | GST (N-term) | phot1 D806N (1-996) | D806N mutagenesis primers |
| " | pAcHLT-A phot2 | 6xHis (N-term) | phot2 (1-916) | (Sakai *et al.*, 2001) |
| " | pAcHLT-A phot2 V522E | 6xHis (N-term) | phot2 V522E (1-916) | V522E mutagenesis primers |

| Expression system | Name | Tag | Protein encoding | Source/Primers used |
|---|---|---|---|---|
| Baculovirus | pAcHLT-A phot2 Q489L | 6xHis (N-term) | phot2 Q489L (1-916) | Q489L mutagenesis primers |
| " | pHLT-A phot2 LOV2 kinase | 6xHis (N-term) | p2 L2K (362-916) | Nco1-AGGGACAGTTGGGACC TATC EcoR1-GAAGAGGTCAATGTC CAA |
| " | pAcHLT-A phot2 LOV2 kinase D720N | 6xHis (N-term) | p2 L2K D720N (362-916) | D720N mutagenesis primers |
| *E.coli* | pCAL-n-EK LOV2 Jα | CBP (N-term) | P1 LOV2 (448-657) | EcoR1-GAGAGTGTGGATGA TAAAG; Sal1- TCACGGTTCACCACT TTCC |
| " | pCAL-n-EK LOV2 Jα E506K | CBP (N-term) | P1 LOV2 E506K (448-657) | E506K mutagenesis primers |
| " | pCAL-n-EK LOV2 Jα Q575L | CBP (N-term) | P1 LOV2 Q575L (448-657) | Q575L mutagenesis primers |
| " | pCAL-n-EK LOV2 Jα Q575H | CBP (N-term) | P1 LOV2 Q575H (448-657) | Q575H mutagenesis primers |
| " | pCAL-n-EK LOV2 Jα Q575N | CBP (N-term) | P1 LOV2 Q575N (448-657) | Q575N mutagenesis primers |
| *A. thaliana* | pEZR phot1 LOV2 Kinase | 6xHis (N-term) | P1 L2K (448-966) | (Sullivan *et al.*, 2008) |
| " | pEZR phot1 | 6xHis (N-term) | phot1 (7-996) | (Cho *et al.*, 2007) |

| Expression system | Name | Tag | Protein encoding | Source/Primers used |
|---|---|---|---|---|
| *A. thaliana* | pEZR phot1 I608E | 6xHis (N-term) | phot1 I608E (7-996) | I608E mutagenesis primers |
| " | pEZR-LC phot1 | GFP (C-term) | phot1 (7-996) | Sub-cloned from pEZR-LC phot1 (EcoR1, BamH1) |
| " | pEZR-LC phot1 I608E | GFP (C-term) | phot1 I608E (7-996) | I608E mutagenesis primers |

**Table 2.1 Expression vectors used in this study.** Vectors are listed by expression system (column 1). Initial source, or details of vector construction, are given in column 5.

| Primary Antibodies | | | |
|---|---|---|---|
| | **Host** | **Dilution** | **Reference/source** |
| Anti-phot1 | Rabbit | 1/5000 | (Christie *et al.*, 1998) |
| C-terminal anti-phot1 | Rabbit | 1/5000 | (Cho *et al.*, 2007) |
| Anti-NPH3-L | Rabbit | 1/5000 | Eurogentec, Belgium |
| Anti-NPH3 | Rabbit | 1/10 000 | (Pedmale and Liscum, 2007) |
| Anti-UGPase | Rabbit | 1/5000 | Agrisera, Vännäs, Sweden |
| Anti-phot2 | Rabbit | 1/5000 | (Cho *et al.*, 2007) |
| **Secondary Antibodies** | | | |
| | **Host** | **Dilution** | **Reference/source** |
| Anti-rabbit-AP | Goat | 1/5000 | Promega |
| Anti-rabbit-HRP | Goat | 1/5000 | Promega |

**Table 2.2 Antibodies used for western blot analysis in this study.** Primary and secondary antibodies are shown. Initial source is indicated in column 4.

# Chapter 3 - Analysis of Phototropin Autophosphorylation Activity

## 3.1 Introduction

The *Arabidopsis* genome encodes two closely related phototropin (phot) proteins, phot1 and phot2 (Christie, 2007). These proteins have a conserved structure, consisting of two N-terminal light-sensitive motifs (referred to as LOV domains) and a C-terminal serine/threonine kinase domain and display increased autophosphorylation after irradiation that can be conveniently monitored *in vitro* (Chapter 1; Christie *et al.*, 1998, Sakai *et al.*, 2001, Christie, 2007). However, such a model leaves several aspects of phot autophosphorylation unresolved. For example, it is unclear whether phot autophosphorylation occurs via an intramolecular mechanism or whether this process involves cross-phosphorylation between phot molecules. In this chapter the mode of phot autophosphorylation activity will be further examined using a heterologous expression system to assess the effect of protein truncations and specific point mutations on phot kinase activity.

## 3.2 Results

### *3.2.1 Phototropins expressed using a baculovirus/insect cell expression system exhibit light-induced kinase activity in vitro*

The activity of phot1 and phot2 can be conveniently monitored *in vitro* using protein extracted from a baculovirus/insect cell expression system (Christie *et al.*, 1998, Sakai *et al.*, 2001). The short timescale from recombinant virus creation to the expression of a suitable quantity of phot1 for autophosphorylation analysis (~2 weeks) provides a convenient means of assessing the effect of point mutations and protein truncation on phot activity. Previous studies have reported point mutations which attenuate phot light-induced autophosphorylation *in vitro* which have subsequently been shown to prevent physiological function when introduced into transgenic *Arabidopsis* (Cho *et al.*, 2007, Christie *et al.*, 2002). This suggests that the study of phot autophosphorylation activity *in vitro* provides insight into the mechanism of phosphorylation of phots *in planta*.

Previous work had been completed to introduce *PHOT1* and *PHOT2* cDNA into appropriate transfer vectors (Figure 3.1A) which were then co-transfected into *Spodoptera frugiperda* (Sf9) insect cells with linearised baculovirus DNA. Homologous recombination produced a viable baculovirus capable of expressing either His-phot1 (phot1) or His-phot2 (phot2). Sf9 cells infected with this recombinant virus express fusion proteins as shown by nitrocellulose blots probed with anti-His antibody (Figure 3.1B). Both phot1 and phot2 expressed in this fashion display increased autophosphorylation upon irradiation *in vitro* as previously described (Figure 3.1B; Christie *et al.*, 1998, Sakai *et al.*, 2001). These fusion proteins were used for subsequent analysis of phot autophosphorylation activity *in vitro*.

Both phot1 and phot2 are plasma membrane-localised proteins *in planta*, with a fraction of phot1 and phot2 being redistributed to the cytoplasm and Golgi apparatus respectively upon light irradiation (Short *et al.*, 1993, Briggs *et al.*, 2001, Sakamoto and Briggs, 2002, Kong *et al.*, 2006). Phots do not contain any clear membrane-spanning sequence and the mechanism by which phots are associated with the plasma membrane is unclear (Briggs *et al.*, 2001). Despite this, analysis of truncated versions of phot2 *in planta* have suggested that the phot2 kinase domain, or the sequence immediately C-terminal to it, is responsible for membrane localisation (Kong *et al.*, 2007). It has also been suggested that post-translational modification may be required for plasma membrane association (Christie, 2007). Given the functionality of phot1 derived from the baculovirus/insect cell expression system it was of interest to determine whether phot1 was also membrane-localised when expressed using this heterologous system. To assess this possibility Sf9 cells were infected with baculovirus encoding phot1 and the protein extracts obtained fractionated into soluble and microsomal fractions by ultra-centrifugation. Intriguingly, although phot1 was detected in both soluble and crude microsomal membrane fractions by western blot (Figure 3.1C) phot1 autophosphorylation activity was only detected in phot1 localised to the crude microsomal membrane fraction (Figure 3.1C). This suggests that only phot1 localised to the crude microsomal membrane fraction is capable of autophosphorylation *in vitro*.

### *3.2.2 In vivo irradiation of Sf9 cells infected with recombinant baculovirus does not affect in vitro phototropin autophosphorylation*

Phots are phosphorylated *in planta* in response to a blue light stimulation and this *in vivo* activity may be monitored by assessing the reduced incorporation of $^{32}$P-ATP in subsequent *in vitro* kinase assays (Sakai *et al.*, 2001, Huala *et al.*, 1997, Short and Briggs, 1990). Such attenuation of phot autophosphorylation *in vitro* is presumably caused by the reduced number of available phosphorylation sites after *in vivo* phosphorylation and as such *in vitro* kinase assays have been used to elegantly demonstrate phot phosphorylation and subsequent dephosphorylation *in vivo* (Salomon *et al.*, 1996, Short *et al.*, 1992). Given that insect-expressed phot1 and phot2 similarly display light-regulated autophosphorylation *in vitro* when expressed in the dark it was of interest to determine whether these heterologously-expressed phots were phosphorylated *in vivo* if provided with a light stimulus in a manner analogous to that observed *in planta*. The ability of phots expressed using the baculovirus/insect cell system to retain *in vivo* activity was assessed by illuminating infected insect cells with high-intensity white light prior to cell disruption (Figure 3.2). However, in contrast to phot kinase activity *in planta* sample irradiation with white light did not inhibit subsequent *in vitro* light-dependent autophosphorylation of either phot1 or phot2 expressed in insect cells (Figure 3.2A and B respectively). Western blot analysis shows that protein levels were similarly unaltered by *in vivo* illumination (Figure 3.2A and B). It therefore appears that irradiated phot samples expressed in insect cells retain the ability to be autophosphorylated *in vitro*.

### *3.2.3 Addition of a GST-tag does not affect insect-expressed phot1 activity in vitro*

As previously stated, phot autophosphorylation upon illumination is well characterised (Figure 3.1; Christie, 2007). Despite this, the precise mechanism by which this occurs is unknown although cross-phosphorylation between different phot proteins has previously been characterised (Reymond *et al.*, 1992a). The closely-related *Arabidopsis* protein kinase PINOID is also autophosphorylated, although this activity is regulated by an upstream regulatory kinase rather than light (Zegzouti *et al.*, 2006a, Benjamins *et al.*, 2003, Christensen *et al.*, 2000). Incubation of a kinase-inactive, GST-tagged version of PINOID with an active, His-tagged counterpart indicated that PINOID autophosphorylation is exclusively intramolecular, although

such autophosphorylation promoted intermolecular phosphorylation of an artificial substrate (Zegzouti *et al.*, 2006a). To assess whether phot1 autophosphorylation similarly occurred via intramolecular phosphorylation alone or involved intermolecular phosphorylation between phot1 molecules a GST-tagged phot1 was created. Full-length phot1 was introduced into an appropriate transfer vector which was then used to produce viable baculovirus capable of expressing the GST-phot1 fusion protein (Figure 3.3A).

Initially it was necessary to confirm that GST-phot1 present in the crude soluble extract isolated from infected insect cells was capable of light-induced autophosphorylation. As previously observed for His-tagged phot1 (Christie *et al.*, 1998), GST-phot1 expressed in Sf9 cells retained light-induced phosphorylation activity (Figure 3.3B). However, in order to assess intermolecular autophosphorylation a kinase-inactive version of GST-phot1 was also required. Phot1 autophosphorylation activity has previously been shown to be abolished by mutation of a conserved aspartic acid residue with asparagine within the kinase domain ($Asp^{806}$→Asn; Christie *et al.*, 2002). This aspartic acid residue is necessary for co-ordination of the catalytic $Mg^{2+}$ ion in related kinases (Hanks and Hunter, 1995) and the $Asp^{806}$→Asn mutation presumably acts by disrupting $Mg^{2+}$ co-ordination. Mutation of $Asp^{806}$ in GST-phot1 (indicated by a strikethrough) similarly abolished GST-~~phot1~~ autophosphorylation *in vitro* (Figure 3.3B), allowing the light-induced phosphorylation of GST-phot1 to be assigned to the activity of the integral phot1 kinase domain rather than a promiscuous Sf9 kinase. Given that His-tagged phot1 has previously been shown to have autophosphorylation activity only in the crude microsomal membrane fraction when expressed in Sf9 cells (Figure 3.1C) it was also of interest to determine whether a similar effect was observed with GST-phot1. In agreement with the data presented previously, only GST-phot1 localised to the crude microsomal membrane fraction displayed kinase activity (Figure 3.3C).

The incorporation of a GST-tag has the additional benefit of allowing purification of tagged proteins. Although a His-tag may be used for protein purification using affinity chromatography, attempts to purify active phot1 using this method resulted in a loss of light-induced phot1 kinase activity (data not shown). Protein purification was therefore attempted using the GST-phot1 fusion protein expressed in insect cells. Protein purification resulted in an enrichment of phot1

protein levels which could be visualised by silver staining (Figure 3.3D), and this purified phot1 retained its light-induced kinase activity (Figure 3.3D). Although contaminants remained after protein purification the use of increased salt concentrations to improve stringency resulted in GST-phot1 inactivation (data not shown). Equally, attempts to increase the yield of enriched GST-phot1 using increased concentrations of glutathione also caused a loss of kinase activity. It is possible that deviation from mild elution techniques resulted in chromophore loss and subsequent abolition of light-induced kinase activity. As the purification step yielded only low levels of active GST-phot1 crude protein extracts isolated from infected insect cells were used to assess whether phot1 is capable of intermolecular phosphorylation.

### *3.2.4 Phot1 in vitro autophosphorylation includes intermolecular phosphate incorporation*

To determine whether individual phot1 proteins were capable of intermolecular phosphorylation insect cells were infected with a mixed viral population encoding a combination of either phot1 and GST-phot1 or the appropriate kinase-inactive versions. Phosphorylation of the kinase-inactive phot1 or GST-phot1 (indicated by a strikethrough) by the active counterpart would be an indication of intermolecular phosphorylation (Figure 3.4A). As a negative control GST-~~phot1~~ and ~~phot1~~ were also used to co-infect insect cells. The lack of light-induced autophosphorylation in either protein confirms that any phosphorylation observed is caused by phot1 kinase activity (Figure 3.4A). *In vitro* kinase assays completed with protein extracts from cells infected with different combinations of both tagged versions indicate that ~~phot1~~ and GST-~~phot1~~ are phosphorylated by active partners in a light-dependent fashion (Figure 3.4A). A western blot to confirm protein levels is shown below (Figure 3.4B). These data suggest that phot1 is capable of intermolecular phosphorylation, although it is important to note that the presented data does not exclude intramolecular phosphorylation as an additional mechanism of phot1 autophosphorylation.

### *3.2.5 Phot1 retains function in planta after protein truncation to remove the major sites of autophosphorylation*

Phot1 kinase activity results in the phosphorylation of serine residues in the N-terminal region of phot1 before LOV2, both in the interlinking sequence between LOV1 and LOV2 and at the extreme N-terminal of the protein (Salomon *et al.*, 2003, Sullivan *et al.*, 2008). Previous studies using a truncated version of phot1 lacking both LOV1 and the major mapped sites of autophosphorylation have shown that this truncated protein (referred to as phot1 LOV2-kinase, phot1 L2K) is capable of complementing a subset of phot1-mediated phenotypes such as phototropism, leaf expansion and chloroplast movement *in planta* when expressed in *phot1 phot2* plants under the control of a *35S* promoter (Figure 3.5A; Sullivan *et al.*, 2008). As little is known regarding downstream phot1 signalling other than the requirement of phot1 kinase activity (Christie, 2007) there are several alternative mechanisms which may explain this complementation. Phot1 L2K may retain autophosphorylation at sites other than those which have been identified, or may remain unphosphorylated and phosphorylate unidentified substrates *in planta*. In order to determine whether the truncated phot1 L2K protein retained autophosphorylation *in vitro* kinase assays were completed using protein extracts from transgenic *Arabidopsis* expressing phot1 L2K.

Phot1 is associated with the plasma membrane *in planta* and therefore crude microsomal extracts are typically used for *in vitro* kinase assays using plant protein extracts (Sakai *et al.*, 2001, Cho *et al.*, 2007, Christie *et al.*, 2002, Briggs *et al.*, 2001, Sakamoto and Briggs, 2002, Liscum and Briggs, 1995). To ensure that protein truncation did not alter phot1 L2K localisation protein extracts from transgenic *Arabidopsis* were fractionated into soluble and microsomal fractions by ultra-centrifugation. These fractions were then assayed for phot1 L2K presence by western analysis using an antibody raised against a C-terminal portion of phot1 (Cho *et al.*, 2007). A UDP-GLUCOSE PYROPHOSPHORYLASE (UGPase) antibody was used as an indicator of soluble fraction purity. Such analysis confirmed the presence of phot1 L2K in the microsomal fraction (Figure 3.5B).

To establish whether phot1 L2K retained autophosphorylative activity *in vitro* kinase assays were completed using microsomal fractions extracted from transgenic *Arabidopsis* expressing phot1 L2K. Although a strong light-inducible phosphorylation response was observed in extracts from wild-type plants (representing native phot1

activity) a $^{32}P$ signal representing represent phot1 L2K phosphorylation could not be detected despite the presence of comparable levels of protein by western analysis (Figure 3.5C). This is perhaps unsurprising given the loss of the major sites of phosphorylation (Salomon *et al.*, 2003, Sullivan et al., 2008). However, these findings may be a reflection of the sensitivity of *in vitro* kinase assays rather than represent a true abolition of autophosphorylation by the phot1 L2K protein.

### *3.2.6 Truncated LOV2 kinase proteins retain light-induced autophosphorylation activity in vitro when expressed in insect cells*

In order to determine conclusively whether phot1 L2K retained light-dependent autophosphorylation the truncated phot1 L2K protein was expressed using the baculovirus/insect cell expression system. It was hoped that over-expression of phot1 L2K using this system would produce larger quantities of protein more amenable to sensitive kinase assay analysis. Phot1 L2K cDNA was introduced into an appropriate transfer vector (Figure 3.6A) which was then used to produce viable baculovirus capable of expressing His-tagged phot1 L2K protein when used to infect insect cells. A kinase-inactive form of phot1 L2K (~~phot1 L2K~~) was also produced by introducing the $Asp^{806}\rightarrow$Asn mutation that has previously been shown to negate phot1 autophosphorylation (Figure 3.3B; Christie *et al.*, 2002). *In vitro* kinase assays performed using these truncated proteins showed that phot1 L2K is capable of light-inducible autophosphorylation, while ~~phot1 L2K~~ did not demonstrate this activity (Figure 3.6B). This suggests that the observed phosphorylation activity observed is caused by phot1 L2K autophosphorylation rather than by a promiscuous insect protein kinase. As phot1 L2K lacks the mapped sites of autophosphorylation (Sullivan et al., 2008) it was hypothesised that the $^{32}P$ signal detected from the truncated protein would be less than that of full-length phot1. To assess this possibility *in vitro* kinase assays were completed using baculovirus encoding either full-length phot1 or phot1 L2K. Whilst protein levels of phot1 and phot1 L2K were broadly comparable (as detected by western blot) the $^{32}P$ signal from irradiated phot1 L2K was reduced compared to irradiated phot1 (Figure 3.6C). This implies that less $^{32}P$ incorporation occurs in phot1 L2K compared with the full-length control *in vitro*, in agreement with the suggested hypothesis.

To confirm whether autophosphorylation of the region encoded by phot1 L2K occurred in other phots, a baculovirus stock encoding a similarly truncated version of *Arabidopsis* phot2 (phot2 LOV2-kinase, phot2 L2K) was created (Figure 3.6D). A kinase-inactive version of phot2 L2K (~~phot2 L2K~~) was also produced by introducing an $Asp^{720}$→Asn point mutation into the kinase domain of the truncated protein. $Asp^{720}$ corresponds to the $Asp^{806}$ residue mutated to abolish kinase activity in the GST-~~phot1~~ and ~~phot1 L2K~~ proteins. As for phot1 L2K, a light-dependent increase in phosphorylation activity was observed for phot2 L2K which was absent in the ~~phot2 L2K~~ mutant, suggesting that phot2 L2K is also capable of light-regulated autophosphorylation (Figure 3.6E). The light-induced phosphorylation observed in truncated versions of both phot1 and phot2 suggests that sites of phosphorylation exist other than those previously identified by mass spectroscopy and 2-dimensional thin layer electrophoresis and chromatography (Salomon *et al.*, 2003, Sullivan *et al.* 2008).

### *3.2.7 Phot1 L2K retains the ability to phosphorylate full-length phot1 in vitro*

Although phot1 L2K is capable of autophosphorylation *in vitro* when expressed in insect cells, it is unclear whether this truncated protein would retain the ability to phosphorylate full-length phot1 by intermolecular phosphorylation. The function of the N-terminal region containing LOV1 in full-length phot1 is unknown at present, although it has been hypothesised that LOV1 may allow phot homodimerisation (Salomon *et al.*, 2004, Christie, 2007). If this were the case phot1 L2K would be expected to be unable to phosphorylate ~~phot1~~ as the sequence necessary for dimerisation would be absent. In order to assess the consequences of phot1 truncation on intermolecular phosphorylation Sf9 insect cells were infected with a mixed baculovirus population containing both full-length phot1 and the truncated phot1 L2K. The kinase activity of protein extracts containing both forms of the protein were then assayed, using either ~~phot1~~ or ~~phot1 L2K~~ as a substrate of the active counterpart (Figure 3.7). Protein levels of phot1 L2K and ~~phot1 L2K~~ were reduced compared to full-length phot1, which was most likely a consequence of differences in viral titre. Whilst these expression level differences limited data interpretation it is apparent that ~~phot1~~ is phosphorylated in a light-dependent fashion in the presence of phot1 L2K (Figure 3.7). Such an increase was not seen when ~~phot1~~ was co-expressed with ~~phot1 L2K~~ as a negative control. These findings indicate that phot1 L2K is capable of

phosphorylating full-length phot1 *in vitro* and suggests that protein truncation does not abolish intermolecular kinase activity. Although the reciprocal light-dependent phosphorylation of phot1 L2K by phot1 was considerably weaker the lower expression levels of phot1 L2K, combined with the absence of major phosphorylation sites in the truncated protein, may provide an explanation for the observed reduction in signal (Salomon *et al.*, 2003, Sullivan et al., 2008).

### *3.2.8 Mutation of the phot1 kinase T-loop reduces light-induced autophosphorylation*

In order to identify further potential sites of phosphorylation within the truncated L2K proteins the primary sequences of phots and related kinases were compared. Phot1 is a member of the AGC group of serine/threonine kinases which includes cAMP-dependent protein kinase (PKA; Bogre *et al.*, 2003). The activity of this group of kinases is often regulated by phosphorylation of subdomain VIII, which is commonly referred to as the activation- or T- loop (Adams, 2003). Potential sites responsible for the autophosphorylation activity observed in phot1 L2K therefore include conserved serine/threonine residues found within the T-loop. This normally conserved region contains a 30 residue insertion in phots (Tokutomi *et al.* 2008*,* Bogre *et al.*, 2003, Christie, 2007). Figure 3.8A shows an alignment of phot kinase domain T-loops from a number of different plant species compared with *Mus musculus* PKA. This alignment reveals five highly conserved serine/threonine residues within the phot T-loop. Three of the conserved residues are present in the T-loop extension ($Ser^{810}$, $Thr^{813}$ and $Ser^{814}$, numbered from *Arabidopsis* phot1) and are therefore only conserved in the phot protein sequences (Figure 3.3A) while the other two ($Ser^{849}$ and $Ser^{851}$) are highly conserved amongst AGC kinases (Adams, 2003).

In addition to the high degree of homology between PKA and the phot kinase domain, bovine PKA has previously been shown capable of phosphorylating phot1 (Salomon *et al.*, 2003). AGC kinases such as PKA only phosphorylate serine/threonine residues surrounded by specific motifs (Ubersax and Ferrell Jr, 2007), and such motifs therefore provide a reasonable prediction of the residues recognised and phosphorylated by the phot kinase domain. To further define potential phot1 phosphorylation sites the conserved serine/threonine residues within the phot T-loop extension were compared with motifs recognised by canonical PKA kinase

domains. One of the motifs recognised by PKA is RxS (Ubersax and Ferrell Jr, 2007) and the sequence surrounding $Ser^{849}$ matches this sequence. $Ser^{849}$ was therefore selected for initial analysis using site-directed mutagenesis in combination with the baculovirus/insect cell expression system. A $Ser^{849}$→Ala (S849A) point mutation was introduced into *PHOT1* cDNA using site-directed mutagenesis and recombinant baculovirus created incorporating this mutated cDNA. The autophosphorylation activity of the phot1 S849A variant was compared with wild-type phot1. Compared to wild-type phot1 (which shows a five-fold increase in autophosphorylation activity after light irradiation compared to dark controls) the phot1 S849A mutant exhibits a reduced (three-fold) light-induced increase in autophosphorylation (Figure 3.8C). It therefore appears that mutation of this residue reduces light-induced kinase activity of phot1, although further interpretation of this data is somewhat complicated as kinase domain mutation may have additional negative implications other than a reduction of the number of sites available for autophosphorylation.

### *3.2.9 Methionyl-flavin adduct formation within LOV2 is sufficient to induce phot1 kinase activation in vitro*

Although sharing homology with AGC kinases, phots are atypical of the family as they appear to regulate their own kinase activity in response to light rather than being activated by an upstream kinase as part of a signalling cascade (Bogre *et al.*, 2003, Christie, 2007). Current models of phot receptor activation state that LOV2 acts as a repressor of the C-terminal kinase domain in the dark with this repression thought to be alleviated by photoexcitation of the LOV2 domain (Tokutomi *et al.*, 2008, Matsuoka *et al.*, 2007, Christie, 2007).

The key residue required for LOV domain photochemistry is a cysteine residue highly conserved amongst LOV domains ($Cys^{512}$ in *Arabidopsis* phot1 LOV2; Crosson *et al.*, 2003, Briggs, 2007). LOV domain photoproduct formation occurs after a cysteinyl-flavin adduct is created between $Cys^{512}$ and the flavin mononucleotide (FMN) chromophore upon light stimulation (Salomon *et al.*, 2000, Crosson and Moffat, 2002, Fedorov *et al.*, 2003, Salomon *et al.*, 2001). Substitution of this cysteine with alanine within LOV2 ($Cys^{512}$→Ala, C512A) causes a loss of photoreactivity by preventing adduct formation and phot1 containing the C512A mutation also lacks light-induced kinase activity when expressed using the

baculovirus/insect cell expression system (Figure 3.9B; Christie *et al.*, 2002, Salomon *et al.*, 2000). Phot1 light-dependent autophosphorylation requires only the photochemical activity of LOV2 as phot1 L2K is functional *in planta* (Sullivan et al., 2008) and mutant phot1 deficient in LOV2 photochemistry is unable to complement phot1-mediated physiological responses (Cho *et al.*, 2007, Christie *et al.*, 1998).

Additional photochemical studies of isolated LOV domains have suggested that methionine can partially mimic the role of the photoreactive cysteine (Kottke *et al.*, 2003). When the Cys→Met mutation was introduced into *Chlamydomonas reinhardtii* phot LOV1 the isolated domain retained its photosensitivity via light-dependent formation of a methionyl-flavin covalent bond rather than the typical cysteinyl-flavin adduct (Kottke *et al.*, 2003). However, it is unclear whether this alternate photoproduct is capable of acting as an active signalling state. To confirm whether the formation of this alternate photoproduct was sufficient to upregulate phot1 autophosphorylation activity the corresponding LOV2 $Cys^{512}$→Met (C512M) mutation was introduced into full-length *Arabidopsis* phot1, which was then expressed using the baculovirus/insect cell expression system. The *in vitro* autophosphorylation activity of phot1 C512M was then compared to wild-type phot1 and phot1 C512A. Figure 3.9B shows that this mutant variant retained a degree of light-induced phot1 autophosphorylation when compared to the phot1 C512A mutant which lacks light-induced autophosphorylation activity (Christie *et al.*, 2002). However, the light-induced kinase activity of phot1 C512M is considerably reduced compared with wild-type phot1 (Figure 3.9C). Wild-type phot1 shows an approximate five-fold increase in autophosphorylation activity upon illumination whereas phot1 C512M autophosphorylation activity was only 1.5 times that of the dark basal level of kinase activity. These data suggest that the LOV2 $Cys^{512}$→Met mutation does not prevent light-induced signalling from LOV2 to the kinase domain.

### *3.2.10 Phot1 intermolecular autophosphorylation can be promoted by point mutation of a conserved α-helix associated with LOV2*

Although the LOV2 domain is sufficient to allow light-regulated kinase activity, it is only recently that the role of a conserved amphipathic α-helix located after the C-terminus of LOV2 in signal transmission has been elucidated. This α-helix (referred to as the Jα-helix) has been shown to 'dock' against the LOV2 domain in phot fragments

expressed in *E. coli* (Figure 3.10A; Harper *et al.*, 2003). Upon irradiation the Jα-helix becomes disordered suggesting a structural mechanism by which LOV2 photochemistry can be linked with phot1 kinase activation (Harper *et al.*, 2003). Jα-helix disorganisation can be constitutively induced by the substitution of uncharged amino acids along the hydrophobic surface of the Jα-helix with polar residues (e.g. $Ile^{608}$→Glu, I608E; Harper *et al.*, 2004) while the introduction of corresponding mutations into full-length phot1 expressed in insect cells causes increased basal levels of autophosphorylation (Figure 3.10B; Harper *et al.*, 2004). This suggests that Jα-helix disruption is sufficient to mimic LOV2 signal transmission after light irradiation.

Previous work in this chapter has shown that phot1 is capable of intermolecular autophosphorylation between phot1 molecules (Figure 3.4). Given that phot1 I608E displays higher levels of kinase activity (Figure 3.10B) it was of interest to establish whether the increased autophosphorylation caused by Jα-helix mutation also induced an increase in intermolecular phosphorylation. A mixed population of baculovirus encoding phot1 I608E and GST-~~phot1~~ was used to infect insect cells which were subsequently harvested for use in *in vitro* kinase assays. Consistent with previous data (Figures 3.4 and 3.10B) the phot1 I608E mutant displays increased intermolecular phosphorylation of GST-~~phot1~~ in the absence of light exposure compared to the wild-type phot1 control (Figure 3.10C). Phosphorylation of phot1 samples was not observed in the negative control containing two kinase-inactive phot1 proteins. These data suggest that intermolecular phosphorylation can be artificially promoted by mutation of the Jα-helix, further supporting the role of this mutation in phot1 kinase activation.

### *3.2.11 Incorporation of the Jα-helix mutation restores autophosphorylation activity when introduced in combination with light-insensitive phot1 LOV2*

If Jα-helix disorganisation is a consequence of LOV2 signal transmission constitutive disorganisation via the phot1 I608E point mutation should supplant the inhibition of kinase activity caused by a loss of LOV2 photosensitivity. To test this hypothesis a phot1 double mutant variant was created containing both C512A and I608E substitutions, and this mutant cDNA used to create competent baculovirus. *In vitro* kinase assays were used to study the combined effects of both these mutations on

phot1 autophosphorylation activity. As shown previously, phot1 C512A is incapable of mediating a light-induced increase in autophosphorylation (Christie *et al.*, 2002, Salomon *et al.*, 2000) while the I608E substitution increases basal levels of phot1 autophosphorylation activity (Figure 3.11). Activity of the phot1 C512A I608E double mutant was comparable to that of the phot1 I608E single mutant, suggesting that mutation of the Jα-helix is sufficient to induce autophosphorylation activity in a phot1 mutant lacking LOV2 photosensitivity.

### *3.2.12 Mutation of the phot2 Jα-helix also causes an increase in basal levels of autophosphorylation* in vitro

Given the dominance of the Jα-helix over LOV2 photosensitivity in phot1 it was of interest to discover whether the corresponding point mutation in phot2 had a similar effect, as this would suggest a conserved mode of activation between phots. Such conservation is plausible as a phot2 mutation to prevent LOV2 photochemistry inhibits both phot2 autophosphorylation activity *in vitro* and the function of phot2 *in planta* in a similar way to that observed for phot1 (Cho *et al.*, 2007, Christie *et al.*, 2002). Although the Jα-helix is not found in association with LOV1, this α-helix is highly conserved between phots in the sequence succeeding the LOV2 domain. The degree of autophosphorylation induced by phot1 Jα-helix mutation varies depending upon the precise residue mutated, with the substitution of $Ile^{608}$ causing the most substantial increase in autophosphorylation in the dark (Harper *et al.*, 2004). In an attempt to directly mimic the effects of this substitution in phot2 the Jα-helices of *Arabidopsis* phot1 and phot2 were aligned (Figure 3.12A). Based on this alignment phot2 $valine^{522}$ was selected for mutation due to its similar biochemical characteristics and its physical location within the Jα-helix compared to phot1 $Ile^{608}$ (Figure 3.12A). Phot2 $valine^{522}$ was substituted with glutamine ($Val^{522}$→Glu, V522E) and the mutant protein expressed using the baculovirus/insect cell expression system. When the autophosphorylation activity was assessed it was apparent that phot2 V522E had altered kinase activity compared to wild-type phot2 (Figure 3.12B and C). Wild-type phot2 shows an approximate five-fold increase in autophosphorylation activity upon irradiation, similar to that observed for phot1. Such an increase was not apparent in the phot2 V522E mutant (Figure 3.12C). Although phot2 V522E was unresponsive to light, the level of autophosphorylation in the phot2 V522E mutant in either the dark or

after illumination was consistently twice that observed in the mock-irradiated wild-type phot2 control (Figure 3.12C). Although not a facsimile of phot1 I608E autophosphorylation activity (Figure 3.10B) the ability of a point mutation outwith both the phot2 LOV2 motif and the kinase domain to alter phot2 light-induced autophosphorylation suggests a role for the Jα-helix in phot2 LOV2 signal transmission to the kinase domain.

## 3.3 Discussion

### *3.3.1 Light-dependent regulation of phot1 autophosphorylation*

Phots have been assigned to the AGC-VIIIb subfamily of protein kinases based on sequence homology (Bogre *et al.*, 2003, Hanks and Hunter, 1995). Of this unique plant subfamily only the phots have been characterised beyond initial identification within the *Arabidopsis* genome, although members of the related AGC-VIIIa subfamily such as PINOID have also been assigned specific functions (Bogre *et al.*, 2003, Christensen *et al.*, 2000). Both AGC-VIIIa and AGC-VIIIb subfamilies bear a close resemblance to cAMP-dependent protein kinase A (PKA; Bogre *et al.*, 2003), although there are notable differences between the plant AGC-VIII kinases and the canonical domain. For example, the highly conserved DFG residue triplet responsible for $Mg^{2+}$ coordination is altered to DFD in the AGC-VIII family (Figure 3.3A; Bogre *et al.*, 2003). The consequences of this substitution are unclear, but mutation of the lead aspartate ($Asp^{806}$ in *Arabidopsis* phot1) causes a loss of phot1 autophosphorylation activity as observed in PKA (Figure 3.3B; Christie *et al.*, 2002), suggesting that the altered DFD motif is still required for kinase function.

Canonical PKAs have not been successfully identified in plants (Newton *et al.*, 1999, Trewavas *et al.*, 2002), but this family has been intensively studied in other model systems. PKA typically acts as a heterotetramer containing two regulatory (R) subunits and two catalytic subunits in the inactive state (Taylor *et al.*, 2004). Upon cAMP binding the catalytic domains are released and subsequently facilitate protein phosphorylation. Plant homologues of the R subunit have not been identified based on primary structure comparisons, although it is hoped that improved tertiary structure predictive modelling will facilitate identification (Diller *et al.*, 2001). Phots are unique amongst plant AGC kinases in that their activity does not appear to be controlled by

an upstream kinase (Bogre *et al.*, 2003) and given the presence of integral light-sensitive LOV domains it has been speculated that LOV2 may act as the 'R' regulatory subunit in phots (Tokutomi *et al.* 2008). In support of this view, it has been shown that the ability of phot2 kinase to phosphorylate casein *in vitro* can be mitigated by the addition of isolated phot2 LOV2 fragments (Matsuoka and Tokutomi, 2005). Light irradiation released this LOV2-mediated repression.

A cysteinyl-flavin adduct formed between the LOV2 domain and the associated chromophore induces a conformational change within LOV2 (Corchnoy *et al.*, 2003, Eitoku *et al.*, 2005, Iwata *et al.*, 2003, Iwata *et al.*, 2002, Nakasako *et al.*, 2004, Nozaki *et al.*, 2004, Swartz *et al.*, 2002). These structural changes are necessary for phot kinase activation as mutation of LOV2 to inhibit cysteinyl-flavin adduct formation abolishes light-dependent kinase activity *in vitro* and prevents functional complementation when introduced *in planta* (Figure 3.9C; Cho *et al.*, 2007, Christie *et al.*, 2002). It is therefore feasible that light stimulation alters LOV2 conformation sufficiently to release kinase inhibition. Recently it has been shown that substitution of the photoreactive cysteine ($Cys^{512}$ in *Arabidopsis* phot1) with methionine does not abolish photochemical reactivity and allows conformational changes within the LOV2 domain to occur upon illumination (Kottke *et al.*, 2003). Data presented here suggests that formation of this alternate photoproduct within LOV2 is sufficient to allow light-dependent autophosphorylation of the phot1 C512M mutant, although the degree of autophosphorylation observed was greatly reduced (Figure 3.9B and C).

### *3.3.2 Phot1 is capable of intermolecular autophosphorylation in vitro*

Despite phot1 first being identified as a light-regulated autophosphorylating kinase almost ten years ago (Christie *et al.*, 1998) the precise mechanism of this phosphorylation remains unclear. Autophosphorylation of the related *Arabidopsis* kinase PINOID occurs only through intramolecular phosphorylation *in vitro* (Zegzouti *et al.*, 2006a). However, artificial mixing of plasma membrane preparations from different species allows intermolecular phosphorylation between phots (Reymond *et al.*, 1992b), while phot2 is capable of phosphorylating phot1 *in vitro* using protein expressed using the baculovirus/insect cell expression system (Cho *et al.*, 2007). Fragments of phot2 expressed in *E. coli* have also been shown capable of phosphorylating the artificial substrate casein *in vitro* (Cho *et al.*, 2007, Matsuoka and

Tokutomi, 2005). In contrast, preliminary experiments using phot1 expressed using the baculovirus/insect cell expression system did not induce phosphorylation of either phot2 or casein *in vitro* (data not shown). The inability of insect-expressed phot1 to phosphorylate casein may be reflective of the differing protein expression systems used as Matsuoka *et al*. (2005) used phot2 fragments purified from *E. coli* in contrast to the crude insect cell protein extract used to assess the activity of full-length phot1. Despite this, the baculovirus/insect cell expression system has previously been used to examine the activity of multiple recombinant phot proteins (Cho *et al.*, 2007) and this system was therefore used in combination with alternately-tagged versions of phot1 to clarify whether intermolecular phot1 autophosphorylation occurred *in vitro*.

GST-phot1 retains light-dependent phosphorylation and this activity is lost when the catalytic $Asp^{805}$ is mutated (Figure 3.3B). Kinase-inactive GST-phot1 (GST-~~phot1~~) is therefore a suitable potential substrate of phot1. The ability of phot1 to phosphorylate GST-~~phot1~~ and GST-phot1 to phosphorylate ~~phot1~~ indicates that phosphorylation occurs between phot1 molecules *in vitro*, although this data does not exclude intramolecular phosphorylation as a mechanism of phot1 autophosphorylation (Figure 3.4). Given the observed intermolecular phosphorylation it is tempting to speculate that phot1 may act as a dimer. However, it has not been possible to confirm phot1 multimerisation *in vitro* using pull-down assays and therefore it cannot be discounted that the interactions which result in intermolecular phosphorylation may be of a transient and sporadic nature. It will be of interest to discover whether similar intermolecular phosphorylation occurs *in planta* and this will be examined in Chapter 5.

It was also interesting to note that the N-terminal region of phot1 containing LOV1 and the linker region between LOV1 and LOV2 was not required for intermolecular phosphorylation as phot1 and phot1 L2K were each capable of phosphorylating each other *in vitro* (Figure 3.7). As LOV2 has previously been identified as the predominant photoreceptor that is required for phot autophosphorylation and physiological activity in planta (Christie *et al.*, 2002) it has previously been suggested that LOV1 has a role in protein dimerisation. In support of this hypothesis, LOV1 fragments expressed in *E. coli* dimerise whereas isolated LOV2 domains do not (Salomon *et al.*, 2004). However, the ability of phot1 L2K to phosphorylate full-length ~~phot1~~ *in vitro* in a light-dependent manner (Figure 3.7), suggests that homodimerisation between LOV1 domains is not necessary for

intermolecular phosphorylation, although interactions between LOV1 and other segments of the protein cannot be discounted based on this dataset.

An alternate LOV1 function other than as the primary light sensor or as a point of homodimerisation is suggested by photochemical analysis of the LOV domains (Christie *et al.*, 2002, Kagawa *et al.*, 2004). Constructs expressed in *E. coli* containing both LOV domains remain in the light-activated form for a longer period than fragments containing either domain alone. This suggests that the function of LOV1 is to delay LOV2 photorecovery and so facilitate increased signal transmission to the kinase domain, rather than LOV1 being the instigator of light-induced phot1 autophosphorylation *per se* (Christie, 2007, Matsuoka and Tokutomi, 2005). If this were the case, it may be predicted that phot1 L2K would remain in the light-induced 'kinase-active' conformation for a shorter period than full-length phot1, although preliminary experiments to assess the ability of phot1 L2K to remain in an active state after an initial light pulse *in vitro* using insect cell-expressed protein proved inconclusive (data not shown).

### *3.3.3 Phototropin autophosphorylation occurs at sites in addition to those previously identified*

Recent work by Sullivan *et al*. (2008) has identified three major sites of phosphorylation within the N-terminal region of *Arabidopsis* phot1 that are phosphorylated in a light-dependent fashion *in planta*. The location of these sites is in broad agreement with phosphorylation sites which were identified *in vitro* using bovine PKA to phosphorylate the N-terminal region of *Avena* phot1 heterologously expressed in *E. coli* (Salomon *et al.*, 2003). Protein truncation to remove this N-terminal portion of phot1 prevents detectable autophosphorylation *in planta* (Figure 3.5C), yet the phot1 L2K protein fragment retains the ability to complement a subset of phot-dependent responses when expressed in a *p1p2* mutant background (Sullivan et al., 2008). However, transgenic plants expressing phot1 L2K displayed an increased tolerance of drought which may imply that stomatal opening is impaired compared to wild-type (Sullivan et al., 2008). An interaction between phot1 and a 14-3-3 protein necessary for phot1-mediated stomatal opening requires phosphorylation of the interlinking region between LOV1 and LOV2 *in planta* (Kinoshita *et al.*, 2003) and this sequence is lacking in the phot1 L2K truncated protein. This suggests that the

multiple physiological responses mediated by phot1 are induced by phot1 autophosphorylation at specific sites, although phosphorylation of additional unidentified substrates remains a strong possibility.

Heterologous expression of phot1 L2K using the baculovirus/insect cell system demonstrated a retention of light-regulated autophosphorylation in the truncated protein (Figure 3.6B). Therefore additional sites of phot1 autophosphorylation are likely to exist other than those previously mapped in the N-terminal region of phot1. The light-induced autophosphorylation observed in phot1 L2K expressed in Sf9 cells was less pronounced than that typical of full-length phot1 (Figure 3.6C) which correlates with the lack of phot1 L2K phosphorylation activity observed in protein extracts from transgenic *Arabidopsis* expressing phot1 L2K (Figure 3.5C). Such data are consistent with the notion that phot1 L2K contains less phosphorylation sites than the full-length phot1 protein. Although the sites of phot2 autophosphorylation have not been identified a comparable truncated version of phot2 (phot2 L2K) has previously been shown to be sufficient for complementing phot2-dependent phenotypes (Kagawa *et al.*, 2004). This suggests that the N-terminal region of phot2 is similarly dispensable for phot2-mediated signalling. Data shown here demonstrates that phot2 L2K retains light-regulated autophosphorylation *in vitro* (Figure 3.6E), and suggests that sites of phot2 autophosphorylation are similarly located within the sequence of the truncated phot2 L2K protein.

The autophosphorylation observed in the phot1 L2K and phot2 L2K insect-derived protein may be caused by phosphorylation of the highly conserved residues found within the phot kinase activation- or T-loop, a region commonly phosphorylated in other AGC kinases. Such phosphorylation is capable of inducing substantial changes in T-loop structure which stabilises the enzyme in an extended conformation to allow substrate binding, although the precise conformational changes induced by T-loop phosphorylation vary between kinases (Huse and Kuriyan, 2002, Johnson *et al.*, 1996). This phosphorylation may either be mediated by a regulatory upstream kinase or by autophosphorylation (Johnson *et al.*, 1996). For example, the kinase activity of the closely related *Arabidopsis* kinase PINOID is enhanced by phosphorylation within the T-loop by 3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1 (PDK1; Zegzouti *et al.*, 2006a).

The T-loop of the phots and related kinases differs from the canonical PKA motif as they contain a large insertion within the T-loop which has been structurally

modelled to form an additional α-helix (Bogre *et al.*, 2003, Adams, 2003, Christie, 2007, Tokutomi *et al.* 2008). The extended phot T-loop contains five highly conserved potential sites of phosphorylation, two of which are conserved with canonical PKAs (Figure 3.8A). A recent review has implied that T-loop phosphorylation is required for phot1 function (Tokutomi *et al.*, 2008) and mutation of one of these residues ($Ser^{849}$) inhibited light-induced phot1 autophosphorylation activity (Figure 3.8), suggesting that this residue has a role in mediating T-loop function within the phot1 kinase domain as for other AGC kinases (Huse and Kuriyan, 2002). Although there was a clear effect of $Ser^{849}$ mutation on phot1 autophosphorylation activity (Figure 3.3B and C), this site was not identified from mass spectroscopy analysis of phot1 samples purified from *Arabidopsis* (Sullivan et al., 2008). Indeed, none of the potential phosphorylation sites suggested by activation loop alignment were identified by mass spectroscopy. This may be a consequence of the limited sensitivity of mass spectroscopy to detect site-specific phosphorylation, as detection of infrequently phosphorylated sites by this method can be problematic (Steen *et al.*, 2006). An alternative conclusion is that $Ser^{849}$ mutation may have deleterious effects on phot1 kinase function other than phosphorylation site removal. Despite these considerations, mutational analysis described here suggests that the phot1 T-loop has a role in kinase activation in agreement with more typical AGC kinases. Given the diminished autophosphorylation activity of the phot1 S849A mutant and this implied role of T-loop phosphorylation in phot1 autophosphorylation it is now necessary to determine whether $Ser^{849}$ is phosphorylated by the phot1 kinase domain. Although mass spectroscopy analysis has previously been unable to detect phosphorylation within the region encoded by phot1 L2K (Sullivan et al., 2008), examination of the truncated phospho-protein expressed either *in planta* or using insect cells may identify novel sites such as those located in the kinase T-loop.

### *3.3.4 Membrane localisation of phot1 occurs both in planta and when expressed in Sf9 cells*

The mechanism by which phots are associated with the plasma membrane is unclear but may involve post-translational modification of the protein (Christie, 2007). Although the role of the T-loop insertion within the phot kinase domain remains undefined (Tokutomi *et al.*, 2008, Christie, 2007) recent analysis has shown that the

sequence encoding the equivalent insertion within the related plasma membrane-localised kinase PINOID is sufficient to induce plasma membrane-localisation of green fluorescent protein (Zegzouti *et al.*, 2006b). The phot T-loop insertion may therefore be involved in associating phots with the plasma membrane. In support of this notion, removal of the N-terminal region of phot1 prior to LOV2 did not prevent the localisation of the truncated phot1 L2K protein to the crude microsomal fraction *in planta* (Figure 3.5B), suggesting that the protein motif required for microsomal localisation is present in the C-terminal region of phot1. However, phot plasma membrane-association may involve the action of multiple motifs, as analysis of N- and C-terminal truncations of phot2 revealed different sub-cellular localisations for each fragment compared to the full-length photoreceptor (Kong *et al.*, 2007). It was also of interest to note that phot1 expressed in the baculovirus/insect cell system was active only in the crude microsomal fraction (Figures 3.1C and 3.3C). The inactivity of phot1 present in the soluble fraction may represent incorrectly folded protein and is possibly a consequence of protein over-expression. Regardless, these results suggest that competent phot1 is solely associated with the plasma membrane. Whether membrane localisation confers kinase activity, or is merely a consequence of correct protein folding, is unclear from these data. Additionally, these findings suggest the possibility that phot membrane localisation occurs via a post-translational process conserved between plants and insects although further investigation is required to suggest candidate modifications.

### *3.3.5 Point mutations indicate the importance of the Jα-helix in regulating phot light-regulated autophosphorylation*

LOV2 photostimulation results in the disorganisation of an associated α-helix (referred to as the Jα-helix) which docks against the LOV2 domain in the dark (Harper *et al.*, 2003). This Jα-helix disorganisation provides a mechanism by which the relatively small conformational changes induced by cysteinyl-flavin adduct formation can be transduced into a light-dependent increase in phot1 autophosphorylation (Harper *et al.*, 2004, Harper *et al.*, 2003). Constitutive disorganisation of the phot1 Jα-helix via a single point mutation ($Ile^{608}$→Glu) increases the basal levels of kinase activity observed *in vitro* using insect cell extracts (Figure 3.10B; Harper *et al.*, 2004). Work presented here suggests that Jα-helix

mutation is sufficient to cause increased autophosphorylation activity in phot1 double mutants containing both C512A and I608E mutations (Figure 3.11). The ability of Jα-helix mutation to induce phot1 autophosphorylation in the absence of a phot1 LOV2 photochemical stimulus supports the view that Jα-helix disorganisation is a subsequent step in phot1 kinase activation.

One initially puzzling point regarding these data is that if phot1 I608E has constitutive autophosphorylative activity it should be unable to incorporate $^{32}P$ *in vitro* as a consequence of all available phosphorylation sites being filled *in vivo* prior to completion of the assay. However, the retention of autophosphorylative activity *in vitro* by phot1 I608E (Figure 3.10; Harper *et al*., 2004) correlates with data from Sf9 cells expressing phot which were irradiated *in vivo* prior to completion of *in vitro* kinase assays (Figure 3.2). In this latter case *in vivo* irradiation of phot1 or phot2 using the baculovirus/insect cell expression system did not alter subsequent autophosphorylation *in vitro* (Figure 3.2), in contrast to endogenous phot1 irradiated *in planta* which induces an alteration in both the electrophoretic mobility of phot1 and prevents incorporation of $^{32}P$ in subsequent *in vitro* kinase assays (Sakai *et al.*, 2001, Huala *et al.*, 1997, Short and Briggs, 1990). These findings suggest that either phot1 and phot2 expressed in Sf9 cells are not capable of autophosphorylation *in vivo*, or that a promiscuous insect phosphatase is able to strip any incorporated phosphate prior to completion of the *in vitro* kinase assays, although the data presented here does not allow these alternate hypotheses to be distinguished.

In order to further characterise the role of Jα-helix mutation in phot1 autophosphorylation the baculovirus/insect cell expression system was used to evaluate phot1 intermolecular phosphorylation in the presence of the $Ile^{608}\rightarrow$Glu mutation. In agreement with the previously observed increase in autophosphorylation (Figure 3.10B; Harper *et al.*, 2004), phosphorylation of GST-~~phot1~~ in the presence of phot1 I608E was greater in the dark compared to the kinase activity of wild-type phot1 (Figure 3.10C). This suggests that Jα-helix mutation enhances inter- as well as intramolecular phot1 autophosphorylation.

As the Jα-helix is a conserved structure found after phot LOV2 domains (but not after LOV1; Harper *et al.*, 2003) it is possible that Jα-helix mutation is a common feature of phot light-induced kinase activation. Point mutation of the phot2 Jα-helix also altered the autophosphorylation activity of this protein *in vitro* although the

increase in activity was not as striking as that for phot1 I608E (Figures 3.10B, 3.12B and C). The marginal increases in basal kinase levels are reminiscent of those observed for alternate phot1 Jα-helix mutants other than phot1 I608E (e.g. V601E, A605E, see Harper *et al.*, 2004), suggesting that alternate substitutions within the phot2 Jα-helix may induce more substantial effects. However, the reduction in kinase activity described here for phot2 V522E was not observed in the phot1 Jα-helix mutational series. This may indicate that Jα-helix mutation is insufficient to fully mimic light stimulation in phot2.

Previous work using the bacterial LOV protein YtvA has indicated that photoproduct formation within the LOV domain causes conformational changes within the STAS (sulphate transport/ anti-sigma factor) domain at the C-terminal of the protein (Bednarz *et al.*, 2004). The YtvA LOV domain is also associated with a C-terminal Jα-helix (Losi *et al.*, 2005), suggesting that LOV domain signalling in this protein also occurs through associated α-helix disruption. Whilst photochemical activity of LOV2 has been shown to be necessary for light-induced Jα-helix disorganisation (Harper *et al.*, 2003) the mechanism by which conformational changes within LOV2 are transferred to the surface of the domain have not yet been elucidated.

Although further work is required to determine whether Jα-helix mutation is sufficient to induce phot2 autophosphorylation, data provided here supports the notion that phot1 LOV2 signals the integral kinase domain via disorganisation of the phot1 Jα-helix. However, it is still unclear how these two known mechanisms are linked. In Chapter 4 I will discuss possible mechanisms which have been hypothesised to link LOV2 photostimulation with Jα-helix disorganisation within phot1 by using the baculovirus/insect cell system and site-directed mutagenesis to probe the effects of specific mutations on light-induced phot1 autophosphorylation.

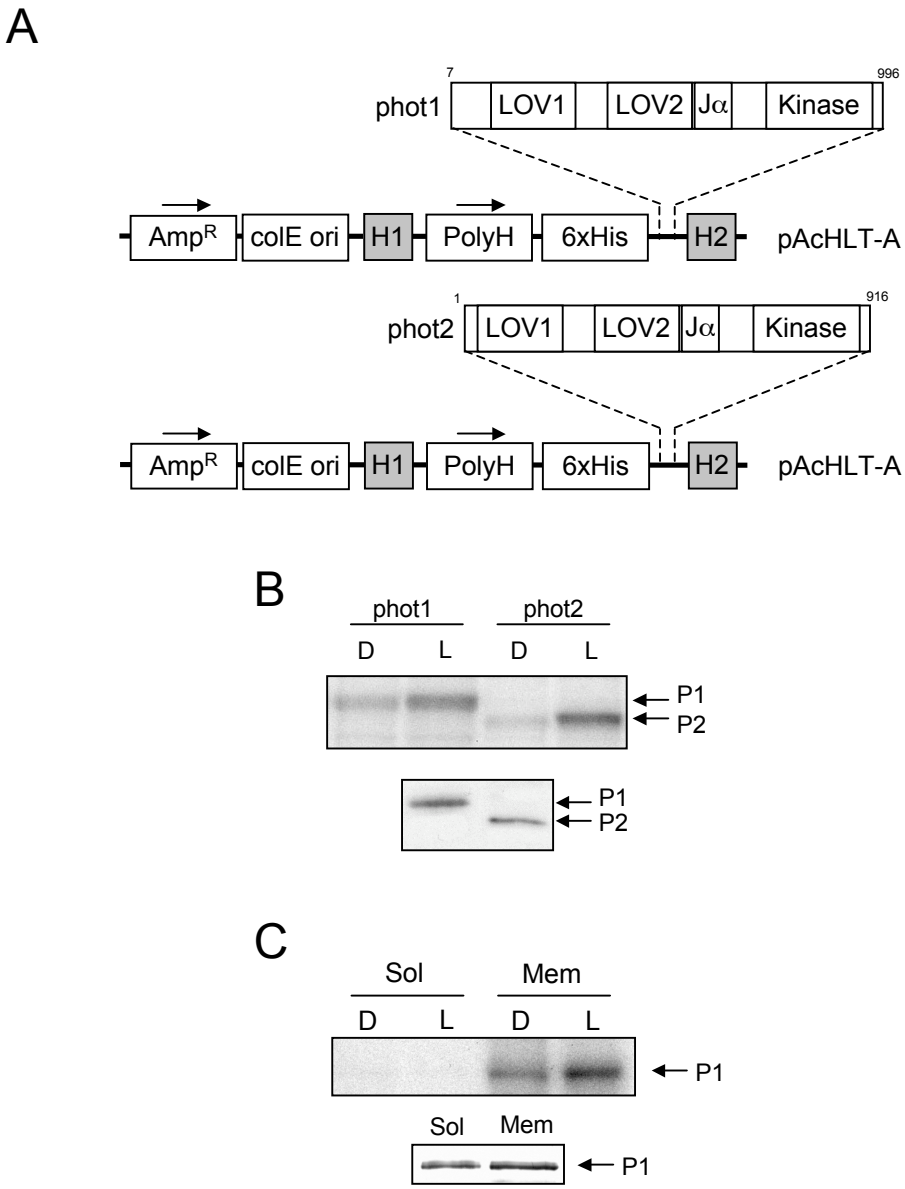



**Figure 3.1 Phototropin expression and autophosphorylation using a baculovirus/insect cell transfection system. (A)** Diagram illustrating creation of transfer vectors containing *PHOT1* and *PHOT2* cDNA. Vectors were used to create recombinant baculovirus containing either His-tagged phot1 (phot1) or His-tagged phot2 (phot2). Amino acid positions are shown. H1 and H2 are homologous regions that allow recombination with linearised baculovirus to produce recombinant viral stocks. **(B)** Autoradiograph showing light-induced autophosphorylation activity of phot1 prepared from insect cells. His-phot1 (P1) and His-phot2 (P2) are indicated. All manipulations were completed under dim red light. Samples were given a mock irradiation (D) or irradiated with white light (L) at a total photon fluence rate of 30,000 μmol $m^{-2}$ $s^{-1}$ prior to the addition of radiolabelled ATP. Western blot analysis of phot protein levels using an anti-His antibody is shown below. **(C)** Autoradiograph showing light-dependent phosphorylation of phot1 in soluble (sol) and crude microsomal membrane (mem) fractions isolated from Sf9 cells. P1 is indicated. Sol and mem fractions were separated by ultra-centrifugation before experimental procedures were performed as described in (B). Western blot analysis of P1 protein levels using a C-terminal anti-phot1 antibody is shown below.

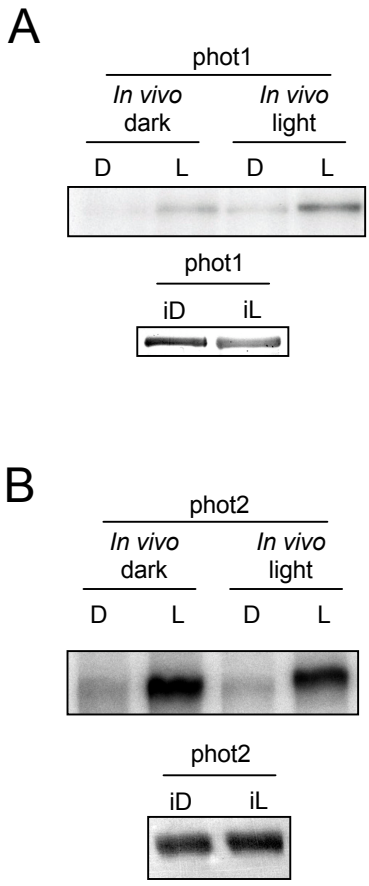
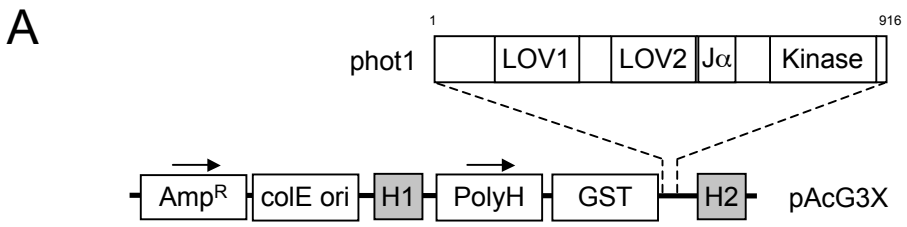



**Figure 3.2 Effect of *in vivo* light pulses on autophosphorylation activity of phot1 and phot2 (A)** Autoradiograph showing the effect of *in vivo* light pulses on the *in vitro* autophosphorylation activity of phot1. All manipulations were completed under dim red light. Infected insect cells were harvested and given a mock irradiation (*in vivo* dark) or irradiated with white light (*in vivo* light) at a total photon fluence rate of 30,000 μmol $m^{-2}$ $s^{-1}$ for 10 secs. *In vitro* kinase assays were then completed as described in Figure 3.1B. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. Aliquots were taken from phot1 samples that had either been mock irradiated (iD) or irradiated with 30,000 μmol $m^{-2}$ $s^{-1}$ (iL) *in vivo*. **(B)** Autoradiograph showing the effect of *in vivo* light pulses on the *in vitro* autophosphorylation activity of phot2. Sample preparation and light treatment as described in A. Western blot analysis of phot2 protein levels using a C-terminal anti-phot2 antibody is shown below. Aliquots were taken from phot2 samples that had either been mock irradiated (iD) or irradiated with 30,000 μmol $m^{-2}$ $s^{-1}$ (iL) *in vivo*.

**Figure 3.3 Phot1 expression and autophosphorylation using a baculovirus/insect cell transfection system. (A)** Schematic diagram illustrating creation of pG3X transfer vector containing *PHOT1* cDNA. This vector was used to create recombinant baculovirus encoding GST-tagged phot1 (GST-phot1). Amino acid positions are shown. H1 and H2 are homologous regions that allow recombination with linearised baculovirus to produce recombinant viral stocks. **(B)** Autoradiograph showing the effect of the single D806N point mutation on GST-phot1 autophosphorylation activity in soluble protein extracts prepared from insect cells. GST-phot1 containing the D806N mutation, which inactivates the phot1 kinase domain (Christie *et al.,* 2002), is indicated by a strike-though. All manipulations were completed under dim red light. Samples were given a mock irradiation (D) or irradiated with white light (L) at a total photon fluence rate of 30,000 μmol $m^{-2}$ $s^{-1}$ prior to the addition of radiolabelled ATP. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. **(C)** Autoradiograph showing light-dependent phosphorylation of GST-phot1 in soluble (sol) and crude microsomal membrane (mem) fractions isolated from Sf9 cells. Sample preparation and experimental procedures were performed as described in Figure 3.1C. Western blot analysis of P1 protein levels using a C-terminal anti-phot1 antibody is shown below. **(D)** Enrichment of GST-phot1 expressed in insect cells. Silver stain showing enrichment of GST-phot1. Protein from insect cells infected with baculovirus containing GST-phot1 (GST-P1) or GST-tag (GST) was prepared from insect cells and batch purified using the MagneGST™ system (Promega). Proteins were separated by 12.5% SDS-PAGE and silver stained (left). Molecular masses of marker proteins are indicated in kilodaltons (kDa) and expected sizes of GST-phot1 (GST-P1) and GST are shown. Autoradiograph showing GST-phot1 light-dependent autophosphorylation after purification is shown on the right. Experimental procedures are as described in (B).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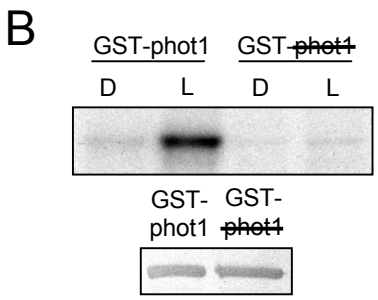
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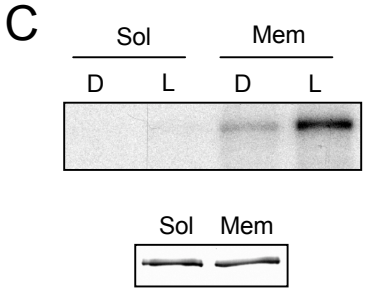
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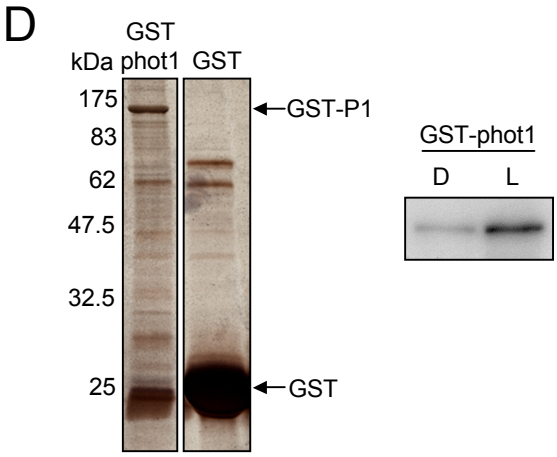
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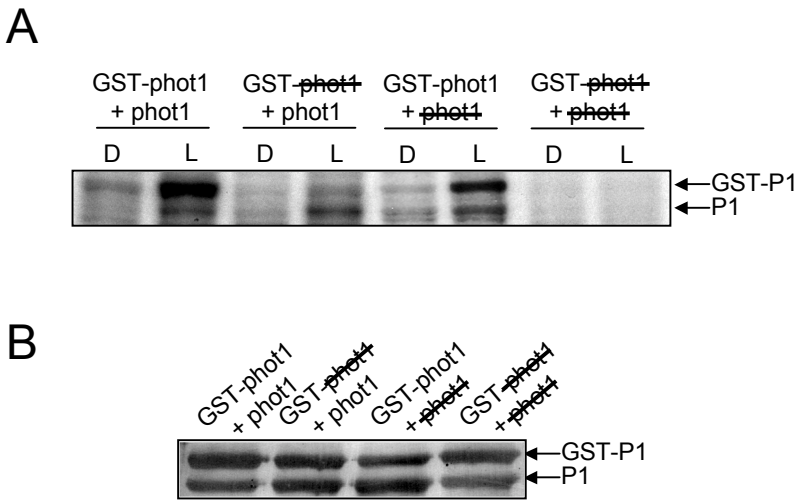



**Figure 3.4 Analysis of phot1 intermolecular autophosphorylation.** Autoradiograph showing light-dependent phosphorylation of inactive phot1 in the presence of active phot1. Insect cells were co-infected with active or inactive versions of phot1 and GST-phot1 as indicated. Kinase-inactive phot1 is represented by a strike-though. Sample preparation and experimental procedures were performed as described in Figure 3.1B. **(B)** Western blot analysis of phot1 protein levels as described in (A) using a C-terminal anti-phot1 antibody. Phot1 (P1) and GST-phot1 (GST-P1) are indicated.

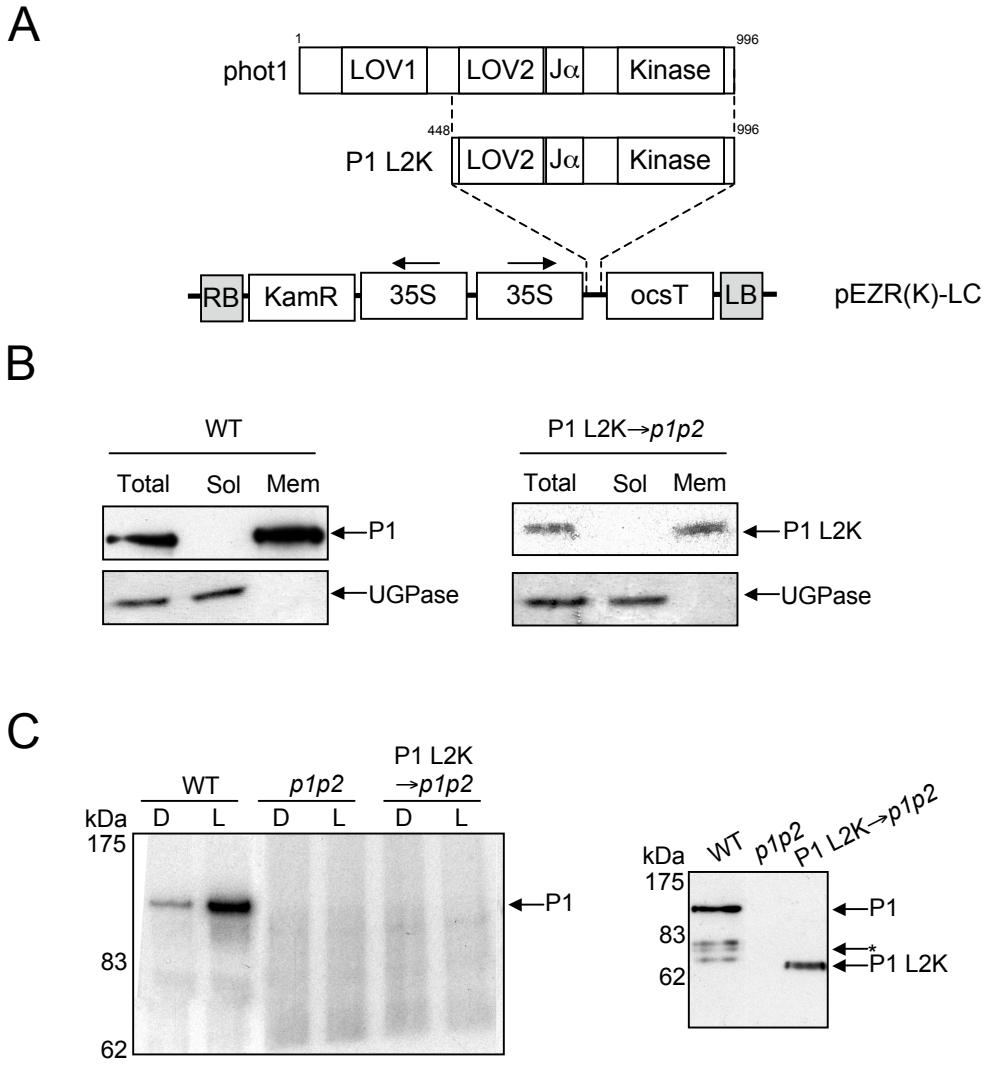
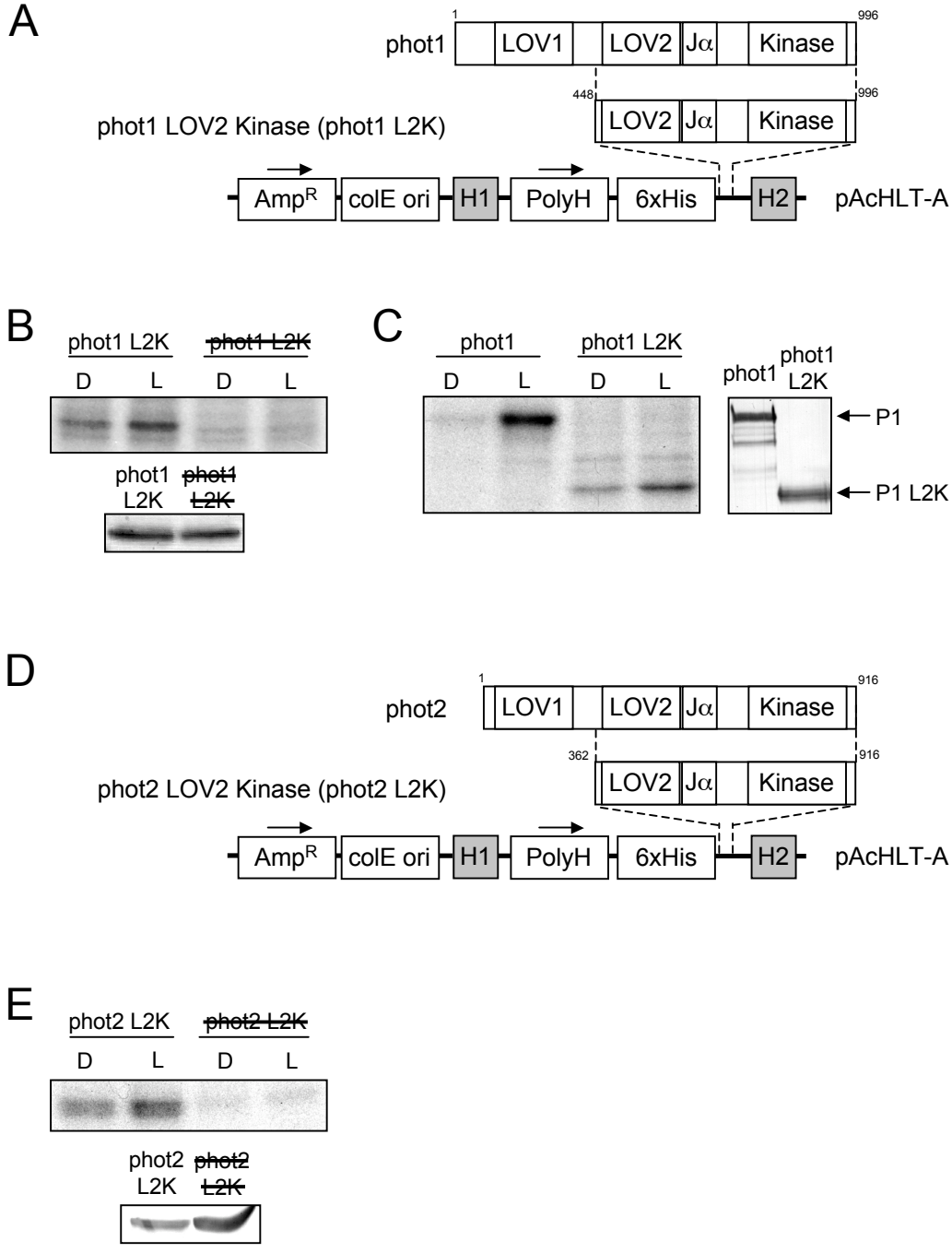



**Figure 3.5 Autophosphorylation analysis of phot1 LOV2-kinase (P1 L2K) expressed *in planta*. (A)** Diagram showing construction of expression vector used to transform *phot1-5 phot2-1* (p1p2) plants. Amino acid positions are indicated. **(B)** Western blot analysis of phot1 and P1 L2K localisation in 3-day old etiolated seedlings. Total protein extract (total) was fractionated into soluble (sol) and membrane (mem) fractions by ultracentrifugation. Equal volumes of each fraction were probed with a C-terminal phot1 antibody (upper panels). Blots were probed with a UDP-glucose pyrophosphorylase (UGPase) antibody as a loading control and as a marker of membrane fraction purity (lower panels). **(C)** Autophosphorylation activity in membranes isolated from transgenic Arabidopsis expressing P1 L2K driven by the *35S* promoter (P1 L2K→*p1p2*) compared to *glabrous1* (WT) and *p1p2* controls. All manipulations were completed under dim red light. Samples were given a mock irradiation (D) or irradiated with white light (L) at a total photon fluence rate of 30,000 µmol $m^{-2}$ $s^{-1}$ prior to the addition of radiolabelled ATP in the presence of 1% Triton-X100. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown to the right. Phot1 (P1), phot1 degradation products (*) and P1 L2K are indicated. Molecular masses of marker proteins are indicated in kilodaltons (kDa).

**Figure 3.6 LOV2 kinase expression using a baculovirus transfection system. (A)** Diagram illustrating transfer vectors used to create baculovirus containing cDNA encoding the truncated protein phot1 LOV2-kinase. Amino acid positions are shown. H1 and H2 are homologous regions that allow recombination with linearised baculovirus to produce recombinant viral stocks **(B)** Autoradiograph showing light-induced autophosphorylation activity of phot1 LOV2-kinase (phot1 L2K/P1 L2K) prepared from insect cells. The kinase domain of phot1 L2K was inactivated using the D806N mutation and is shown alongside as a control (~~phot1 L2K~~). All manipulations were carried out under dim red light. Sample preparation and experimental procedures were performed as described in Figure 3.1B. Western blot analysis of phot1 L2K protein levels using a C-terminal anti-phot1 antibody is shown below. **(C)** Autoradiograph (left) comparing light-induced autophosphorylation activity of phot1 and phot1 L2K. Sample preparation and experimental procedures were performed as described in Figure 3.1B. Western blot analysis of phot1 (P1) and phot1 L2K protein levels using a C-terminal anti-phot1 antibody is shown to the right. **(D)** Diagram illustrating transfer vectors used to create baculovirus containing cDNA encoding the truncated protein phot2 LOV2-kinase (phot2 L2K). Amino acid positions are shown. H1 and H2 are homologous regions that allow recombination with linearised baculovirus to produce recombinant viral stocks **(E)** Autoradiograph showing light-induced autophosphorylation activity of phot2 L2K prepared from insect cells. The kinase domain of phot2 L2K was inactivated using the D720N mutation and is shown alongside as a control (~~phot2 L2K~~). Sample preparation and experimental procedures were performed as described in Figure 3.1B. Western blot analysis of phot2 L2K protein levels using a C-terminal anti-phot2 antibody is shown below.


A
phot1
1
996
LOV1
LOV2
Jα
Kinase
448
phot1 LOV2 Kinase (phot1 L2K)
AmpR
colE ori
H1
PolyH
6xHis
H2
pAcHLT-A
B
phot1 L2K
phot1 L2K
D
L
D
L
phot1 L2K
phot1 L2K
C
phot1
phot1 L2K
D
L
D
L
phot1
phot1 L2K
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phot2
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phot2 LOV2 Kinase (phot2 L2K)
E
phot2 L2K
phot2 L2K

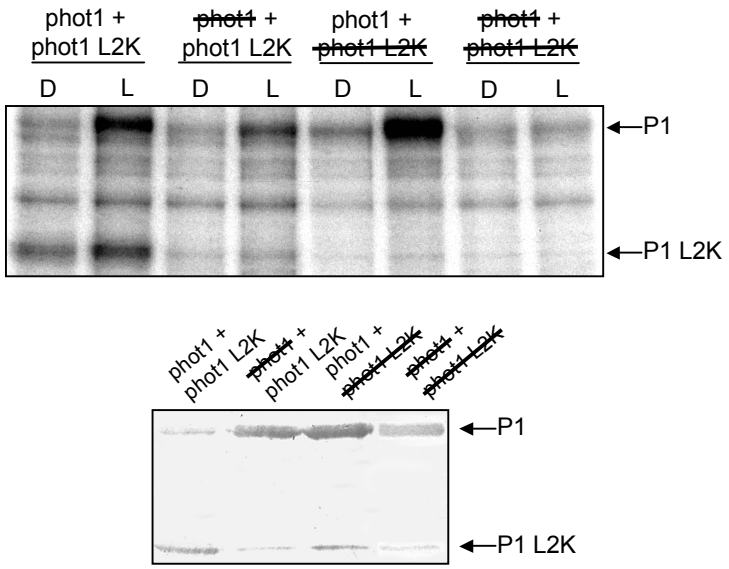


**Figure 3.7 Analysis of intermolecular phosphorylation between phot1 LOV2-kinase and phot1 *in vitro*.** Autoradiograph showing light-dependent phosphorylation of inactive phot1 in the presence of active phot1 LOV2 kinase (phot1 L2K/P1 L2K). Insect cells were co-infected with active or inactive versions of phot1 and phot1 L2K as indicated. Kinase inactive phot1 or p1 L2K is represented by a strike-though. The positions of phot1 (P1) and phot1 L2K are indicated. Sample preparation and experimental procedures were performed as described in Figure 3.1B. Western blot analysis of phot1 and phot1 L2K protein levels using a C-terminal anti-phot1 antibody is shown below.

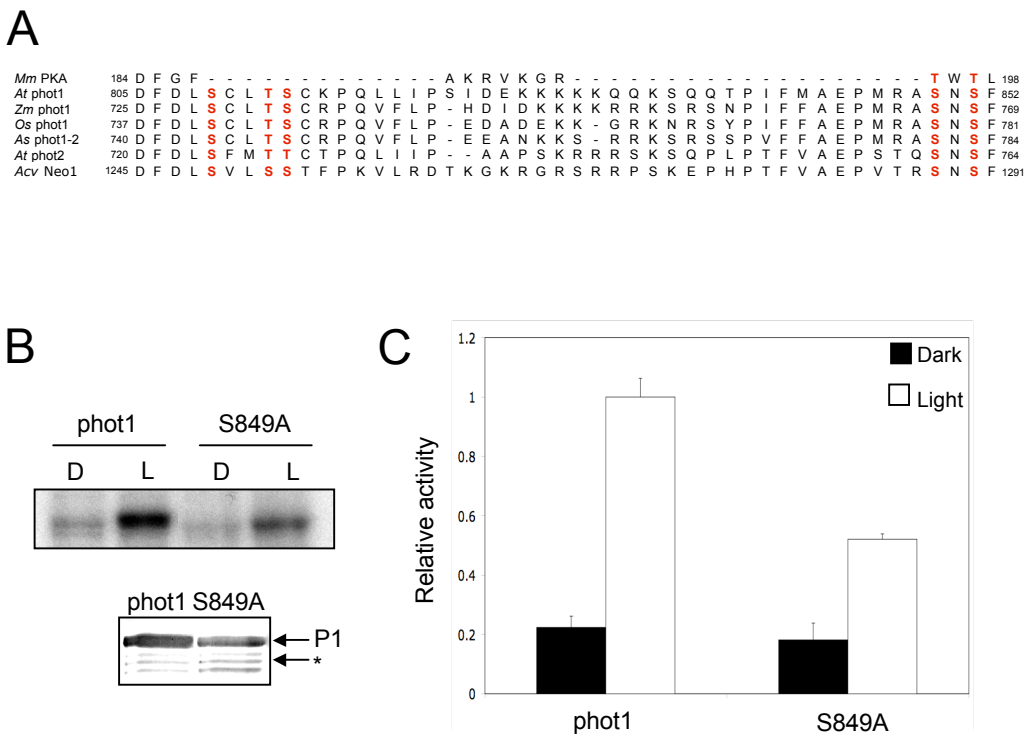


**Figure 3.8 Effect of S849A point mutation on the autophosphorylation activity of *Arabidopsis* phot1. (A)** Aligned amino acid sequences from *Mus musculus* protein kinase A (*Mm* PKA) and selected phototropins showing residue conservation within the activation loop Abbreviations as follows; *Arabidopsis thaliana*, *At; Zea mays,* zs; *Oryza sativa*, *Os; Avena sativa*, *As*, *Adiantum capillus-veneris, Acv*. Amino acid sequences were aligned using the program MegAlign (http://www.dnastar.com) using the ClustalW algorithm. Conserved potential phosphorylation sites are highlighted in red. **(B)** Autoradiograph showing the effect of the single S849A point mutation on phot1 autophosphorylation activity in soluble protein extracts prepared from insect cells. Sample preparation and experimental procedures were performed as described in Figure 3.1B Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. Phot1 (P1) and associated degradation products (*) are indicated. **(C)** Autophosphorylation activity of wild-type phot1 and the S849A mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal phot1 autophosphorylation (error bars indicate standard error, n=3).

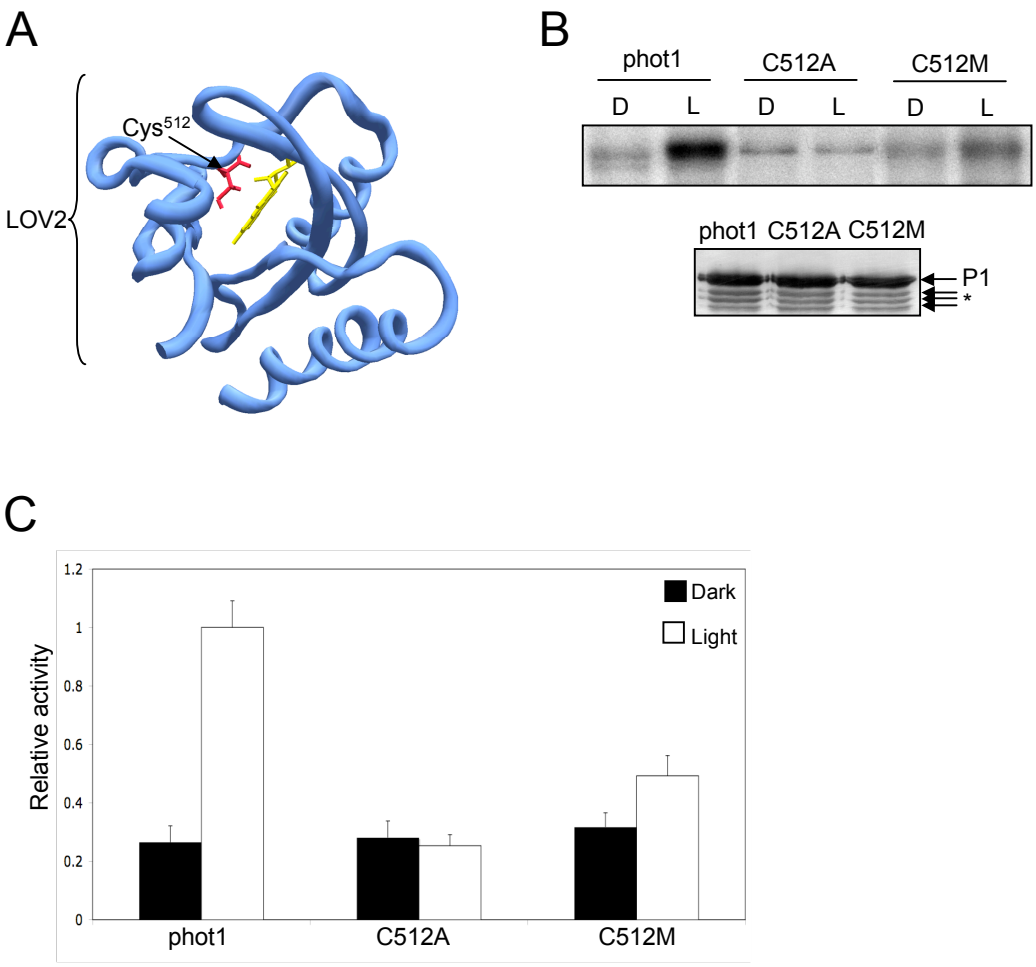


**Figure 3.9 Effect of C512M point mutation on the autophosphorylation activity of *Arabidopsis* phot1. (A)** Protein structure of the LOV2 domain of *Arabidopsis* phot1. The structure of *Arabidopsis* phot1 LOV2 was modelled using the program Swiss Model (http://www.expasy.org; Christie 2007). The backbone structures of α-helices, turns and β-sheets are coloured in blue. The FMN chromophore is shown in yellow, while the location of the reactive cysteine ($Cys^{512}$) required for LOV2 photochemical reactivity is shown in red. **(B)** Autoradiograph showing the effect of the single C512A and C512M point mutations on phot1 autophosphorylation activity in soluble protein extracts prepared from insect cells. Sample preparation and experimental procedures were performed as described in Figure 3.1B. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. Phot1 (P1) and associated degradation products (*) are indicated. **(C)** Autophosphorylation activity of wild-type phot1 and the C512A and C512M mutants in crude soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal phot1 autophosphorylation (error bars indicate standard error, n=3).

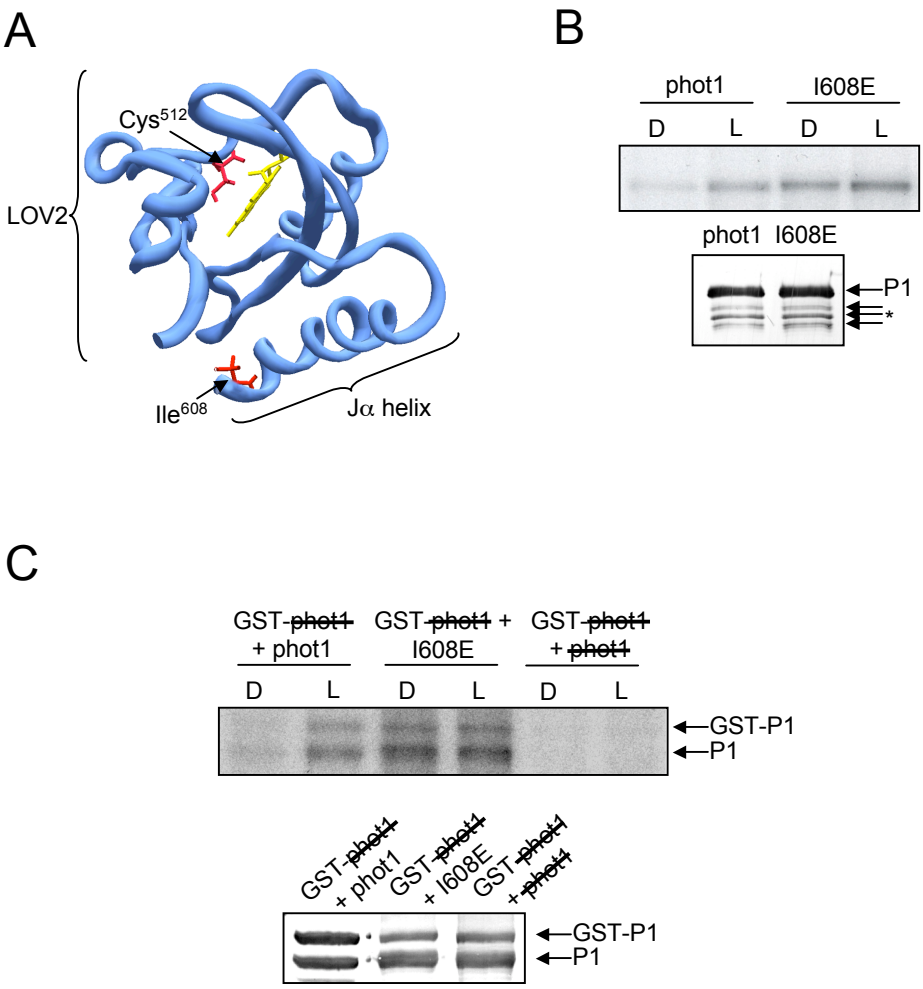


**Figure 3.10 Analysis of intermolecular phosphorylation between phot1 I608E and GST-phot1 *in vitro*.** **(A)** Protein structure of the LOV2 domain and associated Jα helix of *Arabidopsis* phot1. The structure of *Arabidopsis* phot1 LOV2 was obtained as described in Figure 3.9A. The backbone structures of α-helices, turns and β-sheets are coloured in blue. The FMN chromophore is shown in yellow, while the location of cysteine$^{512}$ (Cys$^{512}$) and isoleucine$^{608}$ (Ile$^{608}$) is shown in red. **(B)** Autoradiograph showing the effect of the single I608E point mutation on phot1 autophosphorylation activity in soluble protein extracts prepared from insect cells. Sample preparation and experimental procedures were performed as described in Figure 3.1B Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. phot1 (P1) and associated degradation products (*) are indicated. **(C)** Autoradiograph showing the effect of the single I608E mutation on intermolecular phot1 phosphorylation. Insect cells were co-infected with active or inactive versions of phot1 and GST-phot1 as indicated. Kinase-inactive phot1 is represented by a strike-though. Sample preparation and experimental procedures were performed as described in Figure 3.1B. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. Phot1 (P1) and GST-phot1 (GST-P1) are indicated.

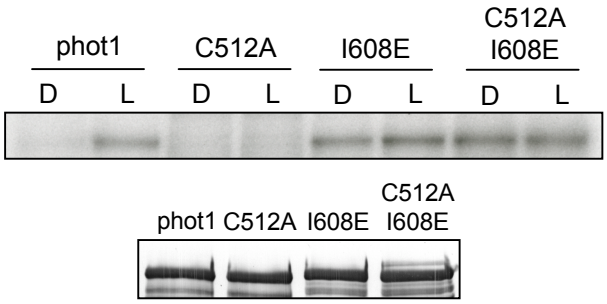


**Figure 3.11 Analysis of the effect of the combined C512A and I608E mutation on the autophosphorylation activity of phot1 expressed in insect cells.** Autoradiograph showing the effect of single C512A and I608E mutations and the combined double mutation on phot1 autophosphorylation activity in soluble protein extracts prepared from insect cells. Sample preparation and experimental procedures were performed as described in Figure 3.1B. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below.

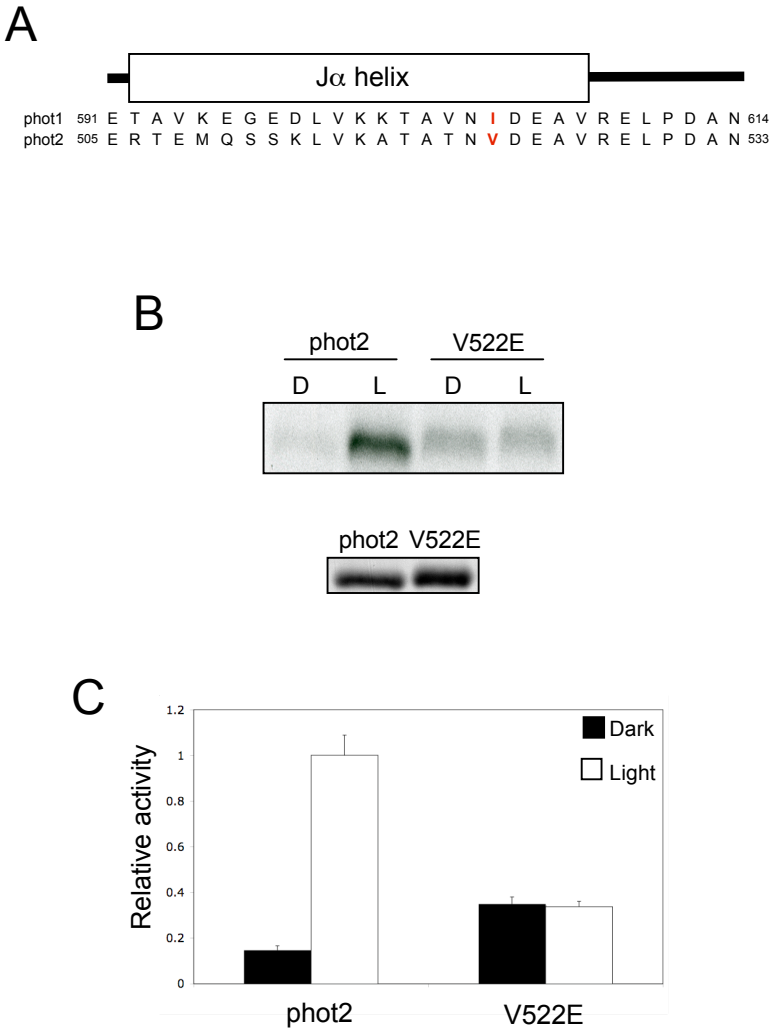


**Figure 3.12 Effect of V522E point mutation on the autophosphorylation activity of *Arabidopsis* phot2. (A)** Amino acid sequences of the Jα helix from phot1 and phot2. Amino acid sequences were aligned using the program MegAlign (http://www.dnastar.com) using the ClustalW algorithm. **(B)** Autoradiograph showing the effect of the single V522E mutation on phot2 autophosphorylation. Sample preparation and experimental procedures were performed as described in Figure 3.1B. Western blot analysis of phot1 protein levels using a C-terminal anti-phot2 antibody is shown below. **(C)** Autophosphorylation activity of wild-type phot2 and the V522E mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal phot2 autophosphorylation (error bars indicate standard error, n=5).

# Chapter 4: Mutational Analysis of Phototropin 1 Provides Insights Into The Mechanism Underlying LOV2 Signal Transmission

## 4.1 Introduction

As discussed in Chapter 3, LOV2 photochemistry is necessary and sufficient for phot1 autophosphorylation and function *in planta* (Cho *et al.*, 2007, Christie *et al.*, 2002). Photochemical reactions within LOV2 most likely result in Jα-helix disorganisation, which results in increased phot1 autophosphorylation (Harper *et al.*, 2004). However, the mechanism by which LOV2 photochemistry is linked to Jα-helix disorganisation remains obscure. X-ray crystallography analysis of LOV domain structures in both the dark and irradiated forms have provided valuable data regarding conformational changes within the domain upon light exposure (Crosson and Moffat, 2001, Crosson and Moffat, 2002, Fedorov *et al.*, 2003). Based on these studies two hypotheses have been proposed to link photochemical changes within the domain to conformational changes at the surface of the motif. In one scheme, structural changes between a conserved chain of amino acids causes disruption of a surface salt bridge (Crosson *et al.*, 2003). An alternative but not necessarily conflicting proposal suggests that steric changes in the location of the flavin chromophore within the domain stimulates movement within the β-sheets of the LOV2-core and causes subsequent structural alterations (Nozaki *et al.*, 2004). In this chapter point mutation analysis was used to examine these two leading hypotheses for LOV2 signal transmission, using phot1 autophosphorylation activity *in vitro* as an indicator of LOV2 signal transmission efficiency.

## 4.2 Results

### *4.2.1 Mutation of a conserved surface salt bridge reduces chromophore binding of Arabidopsis phot1 LOV2*

Both LOV1 and LOV2 motifs in higher plant phototropins contain a conserved salt bridge at the surface of the domain (Crosson and Moffat, 2001, Crosson and Moffat, 2002, Fedorov *et al.*, 2003). The crystal structure of neochrome1 LOV2 from

*Adiantum capillus-veneris* (*Adiantum*) suggests that this salt bridge is located at the end of a series of conserved amino acid residues that extend from the centre of the LOV2 domain and it has been hypothesised that light-dependent adduct formation within the LOV domain may cause salt bridge disruption via signal propagation through these conserved residues (Crosson *et al.*, 2003). If this were the case it would be expected that artificial disruption of this surface salt bridge would result in constitutive signalling from LOV2 to the kinase domain and cause an increase in phot1 autophosphorylation activity in mock-treated samples. Additionally, salt bridges are also thought to have an important role in maintaining the tertiary structure of proteins and mutation of residues necessary for their formation may inhibit correct protein folding (Kumar and Nussinov, 1999). Such incorrect folding may prevent light-induced phot1 autophosphorylation either by prevention of light-induced LOV2 domain structural alterations or by inhibition of chromophore co-factor association.

Individual LOV domains can be expressed and purified from *Escherichia coli* (*E. coli*) and this provides a rapid means of examining the effects of mutations on the spectral characteristics and flavin binding properties of the isolated domain (Christie *et al.*, 2007, Salomon *et al.*, 2000, Kottke *et al.*, 2003). In *Arabidopsis* phot1 LOV2, the conserved salt bridge is formed between $Glu^{506}$ and $Lys^{547}$ (Figure 4.1A). To assess the effect of disruption of the conserved surface salt bridge in *Arabidopsis* LOV2 one of these residues was mutated ($Glu^{506}$→Lys, E506K). Such a mutation presumably caused constitutive disruption of the salt bridge by preventing an ionic interaction between the two residues. Similar mutations have previously been used to study the effects of salt bridge disruption (Brodbeck *et al.*, 1993, Losi *et al.*, 2005). Incorporation of this mutation into the isolated LOV2 domain reduced protein yield compared to the wild-type LOV2 domain (data not shown) but sufficient amounts of mutant protein were obtained after protein purification to allow spectral analysis (Figure 4.1B). Wild-type LOV2 expressed and purifed from *E. coli* binds the chromophore flavin mononucleotide (FMN) and displays a characteristic LOV domain absorption profile with peaks in the UV-A and blue portions of the spectrum (Figure 4.1B). The introduction of the E506K point mutation into the LOV2 domain causes these spectral characteristics to be lost, indicating a loss of FMN binding. This result was confirmed by fluorescence spectroscopy (Figure 4.1C). Compared to wild-type, the LOV2 E506K mutant demonstrated a dramatic loss in fluorescence emission

after excitation (>95%). These data suggest that mutation of residues which constitute the surface salt bridge cause a loss of FMN chromophore within the isolated LOV2 domain.

### *4.2.2 The E506K substitution does not alter in vitro autophosphorylation activity of Arabidopsis phot1 expressed in insect cell culture*

Although the E506K substitution caused a loss of chromophore within the isolated LOV2 domain the effect of this mutation in full-length phot1 was examined as studies using the bacterial LOV domain-containing protein YtvA have shown that mutation of the equivalent surface salt bridge within the YtvA LOV domain did not abolish flavin chromophore binding in the full-length protein context (Losi *et al.*, 2005). These latter data suggest that extended protein constructs containing sequence outwith the canonical LOV domain may facilitate improved chromophore incorporation and compensate for the reduced flavin binding observed in the isolated LOV2 E506K mutant LOV2 domain.

To assess flavin binding in full-length phot1 containing the E506K mutation within LOV2 the mutated phot1 protein was expressed in the baculovirus/insect cell expression system. Levels of flavin association in insect cells expressing either wild-type phot1 or the E506K mutant variant were then compared. Although the majority of phot1 expressed in insect cells is insoluble and inactive this insoluble protein is still capable of binding chromophore, as detected by fluorescence spectroscopy (Christie *et al.*, 1998). Additionally, the amount of flavin released from insect cells infected with wild-type baculovirus lacking a recombinant insertion is negligible compared with those infected with wild-type phot1 (Figure 4.2A). Quantifying the amount of flavin released from infected insect cells therefore provides a convenient and rapid mechanism to assess flavin binding levels of the full-length phototropin protein expressed in insect cells.

After protein extraction and subsequent denaturation flavin levels in the cleared supernatant were assessed by fluorescence spectroscopy (Figure 4.2A). In contrast to studies completed on the isolated LOV2 domain (Figure 4.1) incorporation of the E506K mutation caused only a slight reduction (~10%) in flavin binding in the full-length phot1 protein. This suggests that the E506K mutation has different

consequences for chromophore association depending on whether the substitution is introduced into the isolated LOV2 domain or the full-length phot1 protein.

Once it had been confirmed that chromophore binding in the full-length phot1 E506K mutant was comparable to wild-type phot1 the *in vitro* autophosphorylation activity of phot1 E506K was characterised to investigate whether mutation of $Glu^{506}$ to lysine altered light-induced kinase activity. *In vitro* kinase assays indicated that incorporation of the E506K mutation did not prevent light-induced phot1 autophosphorylation (Figure 4.2B). As for the wild-type phot1 control, light-induced autophosphorylation activity of the phot1 E506K mutant was approximately four-fold that of mock-treated samples (Figure 4.2C). The slight reduction in flavin binding observed in phot1 E506K therefore does not appear to have a significant effect on the autophosphorylation activity of the mutated protein. These data suggest that mutation of residues forming the conserved LOV domain surface salt bridge does not mitigate light-induced phot1 autophosphorylation activity and therefore implies that disruption of the surface salt bridge is not required for transmission of the photochemical signal from LOV2.

### *4.2.3 Mutation of an amino acid within the LOV2 βE-sheet modifies the spectral properties of Arabidopsis phot1 LOV2*

The above findings suggest that disruption of the surface salt bridge does not alter LOV2 signal transmission. The second hypothesis regarding LOV2 signal transmission proposes that generalised β-sheet movement within the LOV2 domain following light-stimulated covalent adduct formation has a role in signal transmission (Nozaki *et al.*, 2004). Mutation of a glutamine residue within the βE-sheet of *Adiantum* neochrome1 LOV2 ($Gln^{1029}$→Leu) has been shown by fourier transform infra-red (FTIR) spectroscopy to reduce the amount of β-sheet movement induced by covalent adduct formation between the conserved cysteine residue within the LOV domain and the FMN chromophore (Nozaki *et al.*, 2004). It is therefore possible that this reduction in the conformational changes of LOV2 may mitigate the ability of LOV2 to induce phototropin kinase activation upon irradiation. In order to elucidate whether the corresponding mutation altered light-induced phot1 autophosphorylation *in vitro* the equivalent glutamine residue in *Arabidopsis* phot1 LOV2 was similarly substituted with leucine ($Gln^{575}$→Leu, Q575L).

Work with the *Adiantum* neochrome1 LOV2 domain has previously shown that both the spectral and photochemical properties of the mutant domain were altered by the Gln→Leu mutation (Iwata *et al.*, 2005, Nozaki *et al.*, 2004). Initially it was of interest to confirm whether the mutation had a similar effect on the spectral and photochemical properties of *Arabidopsis* phot1 LOV2. To examine these effects the Q575L mutation was incorporated into the isolated LOV2 domain. The mutated domain was expressed and purified from *E. coli* and comparable amounts of wild-type LOV2 and LOV2 Q575L protein were obtained after purification (Figure 4.3B). Absorbance and fluorescence emission spectroscopy were used to assess the spectral properties of the altered domain. The overall absorption spectrum of the Q575L variant was similar to wild-type LOV2 except that it showed a spectral blue shift of approximately 10 nm (Figure 4.3B). This is consistent with previously published data regarding *Adiantum* neochrome1 LOV2 and is likely caused by an alteration in the hydrogen bonding between the FMN chromophore and LOV2 in the mutated variant (Nozaki *et al.*, 2004, Iwata *et al.*, 2005). Whilst the absorption spectrum of the LOV2 Q575L mutant is similar to that of the wild-type LOV2 domain the level of peak absorbance observed was approximately 40% that of wild-type LOV2 (Figure 4.3B). This indicated a reduction of chromophore association for the LOV2 Q575L mutant. Fluorescence spectroscopy was used to further characterise this reduction in chromophore absorption (Figure 4.3C).

Consistent with the absorbance data obtained (Figure 4.3B), fluorescence emission spectra also displayed a blue spectral shift. Likewise, a severe reduction in chromophore fluorescence (approximately 80%) was also observed compared to that of wild-type LOV2. This reduced fluorescence observed for the Q575L mutant appears to be partly due to a loss of FMN-binding as denaturation of the wild-type and mutant proteins revealed that the LOV2 Q575L mutant binds approximately 30% less flavin than wild-type LOV2 as measured by relative flavin fluorescence emission (Figure 4.3D). Such a reduction is broadly comparable to that observed in the absorption spectra although a direct comparison is complicated by the increased background signal observed in the absorption spectra. This background signal is most likely caused by photon scattering by protein and trace contaminants present within each sample and this scattering subsequently prevents accurate determination of the absorption baseline of each of the samples. An alternate hypothesis to account for the greater difference in flavin fluorescence observed between the intact wild-type LOV2

and LOV2 Q575L mutant holoproteins is that incorporation of the Q575L mutation, in addition to affecting flavin chromophore binding, also modifies the nature of the chromophore environment within LOV2 so that the degree of chromophore fluorescence quenching is increased.

### *4.2.4 LOV2 Q575L photoproduct formation is marginally slower than that of wild-type LOV2*

Given the alteration in the chromophore environment observed in the LOV2 Q575L mutant (suggested by the shift in the observed absorption spectrum and fluorescence emission spectrum, and the apparent fluorescence emission quenching, Figure 4.3) it was of interest to examine the photochemical properties of the mutated domain as deficiencies in LOV2 photochemistry would also prevent light-induced phot1 autophosphorylation activity (Christie *et al.*, 2002). Upon light exposure LOV domains form a covalent adduct between a conserved active site cysteine residue and the associated FMN chromophore (Section 1.8; Salomon *et al.*, 2000). Formation of this covalent bond causes an alteration in the spectral properties of the domain, with photoproduct formation resulting in a loss in absorption in the blue portion of the spectrum around 450 nm and the appearance of a new absorbance peak at 390 nm. This photoproduct is typically defined as $LOV_{390}$ in reference to these altered spectral properties. The differing spectral characteristics of the LOV domain photoproduct allow photoproduct formation to be monitored indirectly by measuring the loss of absorption at 448 nm via absorbance spectroscopy during sample irradiation. As shown in Figure 4.4A, both LOV2 and the LOV2 Q575L mutant variant demonstrate a loss of absorbance in the blue portion of the spectrum (400-500 nm) upon irradiation with 300 μmol $m^{-2}$ $s^{-1}$ white light, suggesting that incorporation of the Q575L mutation does not prevent photoproduct formation.

Changes in absorbance were also monitored at a single wavelength during irradiation to further characterise the kinetics of photoproduct formation (Figure 4.4B). Purified LOV2 domains were irradiated with 300 μmol $m^{-2}$ $s^{-1}$ white light and changes in the peak of absorption in the dark state measured (Figure 4.4B). The wild-type LOV2 domain was monitored at 448 nm whilst the LOV2 Q575L variant was monitored at 440 nm to account for the observed absorption blue-shift (as indicated in Figure 4.3). These data allowed the rate constant of photoproduct formation ($K_{ppt}$) to

be calculated from semi-logarithmic graphs of photoproduct formation against time (Figure 4.4B; Salomon *et al.*, 2000). The observed changes in absorption indicate that the Q575L mutation causes a slight reduction in the initial rate of $LOV_{390}$ photoproduct formation ($K_{ppt}$ = 7.9 x $10^{-2}$ $s^{-1}$ and 5.9 x $10^{-2}$ $s^{-1}$ for LOV2 and LOV2 Q575L respectively). However, both LOV2 and LOV2 Q575L each reached photoproduct saturation within 30 s of light stimulation, suggesting that photoproduct formation in the isolated LOV2 domain is not significantly affected by the Q575L mutation.

### *4.2.5 LOV2 Q575L mutation does not alter the approximate photocycle quantum yield of isolated LOV2 domains*

The slight reduction in the rate of photoproduct formation observed for the LOV2 Q575L mutant (Figure 4.4B) may be potentially caused by a reduction in the photocycle quantum yield of the mutated LOV2 domain as a consequence of alterations in the chromophore environment. If this were the case an increased number of photons would be required to form photoproduct in the LOV2 Q575L mutant relative to wild-type LOV2. In order to test this hypothesis neutral density filters were used to modulate the light pulse provided by a strobe camera flash as previously described (Christie *et al.*, 2002). Density filters were selected that provided a linear relationship between the light intensity used and the observed changes in absorption in wild-type LOV2. This ensured that saturating levels of light were not used (data not shown). Additionally, sample protein levels were standardised so that similar amounts of holoprotein were used for wild-type LOV2 and the LOV2 Q575L mutant. This was achieved by equalising the peak flavin absorbance of wild-type LOV2 and the LOV2 Q575L variant (Figure 4.5A). As previously mentioned, absorption loss when protein samples were irradiated at different light intensities provided an indirect measure of relative LOV2 domain photoproduct formation. When the relative bleaching of LOV2 and the LOV2 Q575L mutant were compared (Figure 4.5B) it was apparent that the reduction in absorbance at each light intensity was similar. This suggests that the approximate quantum yield of the LOV2 Q575L photocycle is not significantly different from that of wild-type LOV2.

### *4.2.6 Introduction of the Q575L substitution greatly slows LOV domain photocycle recovery after irradiation*

Incorporation of the equivalent Gln→Leu substitution within *Adiantum* neochrome1 LOV2 has previously been reported to delay the photorecovery of the mutated domain after light stimulation (Nozaki *et al.*, 2004). The photorecovery of *Arabidopsis* phot1 LOV2 Q575L was therefore measured to determine whether a similar effect was observed. Changes in LOV2 absorbance during sample photo-reversion may also be used to estimate the photorecovery of LOV2 domains after irradiation. During dark reversion the peak of absorption (at 390 nm) of the irradiated sample diminishes whilst absorption at 350 nm and in the blue portion of the spectrum around 450 nm increases (Swartz *et al.*, 2001). Such data is typically presented by plotting dark recovery spectra, as shown in Figure 4.6A. By comparing wild-type LOV2 to the LOV2 Q575L variant (Figure 4.6A) it is clear that both LOV domain proteins complete a photorecovery reaction once returned to the dark. However, in contrast to the wild-type LOV2 domain, LOV2 Q575L did not fully recover to its initial resting absorbance spectrum within the time course presented here. These findings indicate that the photo-reversion of the LOV2 Q575L variant is slower than wild-type LOV2.

As for photoproduct formation, the kinetic rate of photo-reversion within the LOV2 photocycle may be estimated by monitoring changes in absorbance at a single wavelength (Figure 4.6B). The LOV2 domain photoproduct has previously been calculated to have a half-life of between 30 and 60 s once the sample has been returned to the dark (Salomon *et al.*, 2000, Kasahara *et al.*, 2002, Swartz *et al.*, 2001). To determine the rate of LOV2 domain photorecovery changes in absorption were monitored at 448 nm for wild-type LOV2 and 440 nm for the LOV2 Q575L mutant (Figure 4.6B). In contrast to the similar quantum yield and photoproduct formation kinetic measurements, the half-life of photo-reversion for the LOV2 Q575L mutant is significantly longer than that of wild-type LOV2 (16.7 min versus 40 s respectively).

### *4.2.7 Incorporation of the Q575L mutation into full-length phot1 mitigates light-induced phot1 autophosphorylation in vitro*

The data discussed thus far suggests that although the photo-reversion of LOV2 is slowed by the incorporation of the Q575L mutation, photoproduct formation is not significantly altered. Such findings suggest incorporation of the Q575L mutation does

not inhibit the photosensitivity of the mutated LOV2 domain and rather that any observed perturbations in full-length phot1 autophosphorylation may be a consequence of impaired LOV2 signal transmission rather than deficient LOV2 photoproduct formation. In order to examine the consequences of Q575L mutation on phot1 autophosphorylation the Q575L substitution was introduced into full-length *Arabidopsis* phot1. Since the Q575L mutation caused a partial loss of chromophore binding when introduced into the isolated LOV2 domain (Figure 4.3) it was important to confirm whether flavin loss also occurred when the substitution was made in the full-length protein. Flavin levels in insect cells infected with phot1 Q575L were measured as previously described (Section 4.2.2). As shown earlier (Figure 4.2A) insect cells infected with wild-type baculovirus displayed low levels of flavin fluorescence, suggesting that the observed signal in phot1-expressing cells was derived from expressed phot1 protein. These studies showed only a slight reduction (~10%) in flavin binding in the phot1 Q575L mutant compared to wild-type phot1 (Figure 4.7A). This is similar to the flavin loss previously observed in the phot1 E506K mutant that displayed unaltered autophosphorylation activity (Figure 4.2C).

To assess the light-induced autophosphorylation of phot1 Q575L, the mutated protein was expressed using the baculovirus/insect cell expression system and the kinase activity of the mutated protein assessed *in vitro*. From these assays it appears that light-induced autophosphorylation activity of phot1 Q575L was severely attenuated compared with wild-type phot1 controls (Figure 4.7B and C). Wild-type phot1 displayed a five-fold increase in autophosphorylation whereas the activity of phot1 Q575L was approximately three-fold that of the mock-treated control (Figure 4.7C). Western analysis confirmed that the reduction in light-induced phosphorylation is not a consequence of reduced protein levels (Figure 4.7B) whilst assessment of flavin binding suggests that the deficiencies in light-induced autophosphorylation observed in the Q575L mutant are not a consequence of chromophore loss (Figure 4.7A). The reduced light-induced autophosphorylation activity of phot1 Q575L is consistent with the hypothesis that $Gln^{575}$ has a role in coupling LOV2 photostimulation with kinase activation. Despite this, the Q575L substitution did not abolish light-induced phot1 autophosphorylation, suggesting that mutation of $Gln^{575}$ does not completely inhibit LOV2 signal transmission.

### 4.2.8 *Mutation of the corresponding glutamine residue in Arabidopsis phot2 LOV2 also attenuates autophosphorylation activity in vitro*

As for phot1, LOV2 photochemical activity within phot2 is required for phot2 autophosphorylation *in vitro* and for mutant complementation in transgenic *Arabidopsis* (Sakai *et al.*, 2001, Cho *et al.*, 2007). This suggests that phot2 autophosphorylation activity is controlled by phot2 LOV2 in a manner analogous to that of phot1. As substitution of Glutamine$^{575}$ with leucine in *Arabidopsis* phot1 causes an attenuation of light-induced kinase activity (Figure 4.7) the effect of the equivalent glutamine substitution (Gln$^{489}$→Leu, Q489L) on phot2 autophosphorylation was examined to determine whether a similar effect was observed. The Q489L substitution was introduced into the full-length PHOT2 cDNA and the mutated protein expressed using the baculovirus/insect cell expression system. As previously described for phot1 the introduction of this mutation attenuated light-induced autophosphorylation (Figure 4.8A and B), with light-induced autophosphorylation of phot2 Q489L being approximately three-fold that of the dark control (Figure 4.10B). In comparison, wild-type phot2 autophosphorylation was approximately five-fold greater after light stimulation compared to mock-treated controls. These data suggest that the equivalent βE-sheet mutation is also capable of reducing phot2 light-induced kinase activation *in vitro* as was observed for phot1 (Figure 4.7).

### 4.2.9 *Residual autophosphorylation activity in the phot1 Q575L mutant is mediated by LOV2*

As described in Section 1.8, a single mutation (Cys$^{512}$→Ala; C512A) prevents LOV2 photochemistry through mutation of the cysteine residue within LOV2 which is required to form a covalent adduct with the flavin chromophore upon irradiation (Salomon *et al.*, 2000, Swartz *et al.*, 2001). In order to confirm that residual light-induced autophosphorylation activity observed in phot1 Q575L was caused by LOV2 photochemical activity a novel phot1 mutant was created containing both Q575L and C512A mutations. The inhibition of LOV2 photochemistry inhibits phot light-induced autophosphorylation activity when expressed in either insect cells or transgenic *Arabidopsis* (Chapter 3; Christie *et al.*, 2002, Salomon *et al.*, 2000, Cho *et al.*, 2007) and therefore the incorporation of this mutation into phot1 Q575L was hypothesised

to abolish the residual light-induced autophosphorylation observed in the phot1 Q575L mutant. The autophosphorylation activity of the phot1 C512A Q575L double mutant was assessed *in vitro* using the baculovirus/insect cell expression system (Figure 4.8A and B). In agreement with the necessity of LOV2 photochemistry for phot1 autophosphorylation (Christie *et al.*, 2002) incorporation of the C512A mutation in combination with Q575L abolished the residual light-induced increase in phot1 autophosphorylation *in vitro* (Figure 4.9A and B). Indeed, the lack of light-induced autophosphorylation observed in the phot1 C512A Q575L double mutant is similar to the phot1 C512A mutant. This suggests that the remaining light-induced activity of the Q575L single mutant can be attributed to residual LOV2 signal transmission.

### *4.2.10 Mutation of the Jα-helix in addition to Q575L restores phot1 autophosphorylation activity in vitro*

In Section 3.2.10 it was shown that a single point mutation ($Ile^{608}$→Glu, I608E) within the conserved Jα-helix immediately C-terminal of LOV2 was sufficient to restore increased levels of autophosphorylation activity when introduced in combination with the C512A mutation. Introduction of the I608E mutation therefore provides an artificial means to bypass the necessity of LOV2 photochemical activity for phot1 autophosphorylation activity *in vitro*. Considering data that suggests substitution of $Gln^{575}$ inhibits LOV2 signal transmission (Figures 4.7 and 4.9) it was hypothesised that the consequences of Q575L substitution would be superceded by introduction of the I608E mutation. To test this hypothesis a double mutant variant of phot1 was created containing both the Q575L and I608E substitutions. The phot1 Q575L I608E double mutant was expressed in insect cells and the autophosphorylation activity of the mutant assessed using *in vitro* kinase assays in comparison with wild-type phot1 and single mutant controls (Figure 4.10A and B). Consistent with the proposal that I608E mutation mimics the consequences of LOV2 light stimulation in the full-length phot1 protein (Chapter 3; Harper *et al.*, 2004), the phot1 Q575L I608E double variant displayed increased activity in the dark compared to the phot1 Q575L single mutant (Figure 4.10A). When the autophosphorylation activity of phot1 Q575L I608E double mutant was quantified it was apparent that phot1 Q575L I608E also had a comparable level of light-induced autophosphorylation

activity to wild-type phot1 and the phot1 I608E single mutant (Figure 4.10B). Interestingly, such quantification also revealed a residual degree of light-induced autophosphorylation in the phot1 I608E single mutant. These data suggest that phot1 autophosphorylation deficiencies observed in the phot1 Q575L single mutant can be overcome by the I608E substitution, but also imply that Jα-helix mutation does not fully mimic the light-activated signalling state of LOV2.

### *4.2.11 Phot1 Q575N autophosphorylation in vitro is indistinguishable from wild-type phot1*

Nuclear magnetic resonance (NMR) spectroscopy has been used previously to show that point mutation within the Jα-helix causes constitutive Jα-helix disorganisation which is correlated with an increase in phot1 autophosphorylation (Harper *et al.*, 2004, Harper *et al.*, 2003). Unpublished data communicated by K. Gardner (University of Texas, USA) has shown that a $Gln^{575}$→Asn (Q575N) substitution within an isolated LOV2 domain caused constitutive Jα-helix disorganisation as previously seen with mutations introduced into the Jα-helix such as I608E. This suggests that Q575N mutation within full-length phot1 may stimulate an increased level of autophosphorylation in the absence of a light stimulus as observed for phot1 I608E.

As previously described (Section 4.2.2) phot1 Q575N was expressed using the baculovirus/insect cell expression system and crude extracted protein used for *in vitro* kinase assays. Although NMR spectroscopy studies had suggested that incorporation of the Q575N mutation would mimic Jα-helix mutation and induce an increased level of autophosphorylation in the mock-treated phot1 Q575N sample such a phenotype was not apparent when assessed by *in vitro* kinase assays (Figure 4.11A). These data were confirmed when the activity of phot1 Q575N was quantified (Figure 4.11B). Although autophosphorylation of the mock-treated phot1 Q575N mutant was slightly raised compared with wild-type phot1 upon quantification such a difference is possibly a consequence of experimental error. Additionally, light-induced autophosphorylation was observed in the phot1 Q575N mutant (Figure 4.11). The four-fold increase in autophosphorylation activity observed in phot1 Q575N after light irradiation is broadly comparable to the five-fold increase in kinase activity typical of wild-type phot1 (Figure 4.11B) and suggests that the Q575N mutation does

not alter the light-regulated autophosphorylation of phot1 *in vitro*. To confirm this finding the equivalent Gln→Asn mutation was introduced into full-length *Arabidopsis* phot2. Consistent with the data shown here the autophosphorylation activity of the mutated phot2 protein was indistinguishable from wild-type phot2 (data not shown).

### *4.2.12 Phot1 Q575H does not display autophosphorylation activity in vitro*

Whilst phot1 Q575N did not display an alteration in autophosphorylation (Figure 4.11) it was possible that a more severe $Gln^{575}$ substitution would be more effective at inducing Jα-helix disorganisation and subsequent autophosphorylation in the mock-treated phot1 sample. Incorporation of a basic residue into the βE-sheet was more likely to disrupt the hydrophobic interactions between the LOV2-core and the Jα-helix (Harper *et al.*, 2004) and therefore the effects of a $Gln^{575}$→His (Q575H) substitution were assessed. Although the Gln→His substitution involves the incorporation of a basic amino acid in the place of an uncharged polar residue similar mutations have previously been incorporated into LOV2 domains without causing complete chromophore loss (Salomon *et al.*, 2000).

The phot1 Q575H protein was expressed using the baculovirus/insect cell system and its autophosphorylation activity assessed by *in vitro* kinase assays. From these assays it is clear that light-induced autophosphorylation activity is abolished in the phot1 Q575H mutant (Figure 4.12A and B). Such a loss of activity is similar to that observed for the phot1 C512A mutant (Figure 4.8; Christie *et al.*, 2002, Salomon *et al.*, 2000). In order to assess whether the observed inhibition of light-induced kinase activity was caused by a reduction of chromophore bound to phot1 the flavin levels of insect cells expressing phot1 Q575H were investigated as previously described (Section 4.2.2). These studies show that the flavin content of cells expressing the phot1 Q575H mutant is approximately 70% of the levels found in cells expressing wild-type phot1 (Figure 4.12C). This reduction in flavin binding is considerably more than that observed in the phot1 E506K and phot1 Q575L mutant variants previously assayed (Figure 4.2 and 4.7). Such data suggests that flavin binding is impaired in this mutant, particularly when it is considered that approximately half of the flavin released from phot1 is derived from LOV1 chromophore binding. Despite this, findings presented here indicate that LOV2 flavin

binding is not completely abolished in the full-length protein and therefore the possibility of the Q575H mutation having an additional effect on LOV2 signal transmission other than disruption of chromophore binding cannot be discounted.

## 4.3 Discussion

Data presented here suggest that substitution of a conserved glutamine residue within the βE-sheet of LOV2 with leucine in *Arabidopsis* phot1 and phot2 inhibits light-induced phot autophosphorylation *in vitro*. This glutamine residue is one of eleven residues within the LOV2 domain that are thought to interact directly with the FMN chromophore via hydrogen bonding based on X-ray crystallography data provided for *Adiantum* neochrome1 LOV2 (Crosson and Moffat, 2001). Interestingly it has also been suggested that this glutamine residue undergoes side-chain rotation upon illumination (Crosson and Moffat, 2002). Such a conformational change implies a mechanism by which photosensitivity of the LOV2 domain is linked with broader conformational changes in the full-length phot protein which ultimately result in phot autophosphorylation. Findings presented here allow further evaluation of this hypothesis.

### *4.3.1 Mutation of $Gln^{575}$ alters the spectral and photochemical properties of LOV2*

Substitution of $Gln^{575}$ with leucine (Q575L) in phot1 LOV2 caused a spectral blue shift of approximately 10 nm (Figure 4.3). As described, X-ray crystallography of *Adiantum* neochrome1 LOV2 shows that this conserved glutamine interacts directly with the FMN chromophore (Crosson and Moffat, 2001) and the observed spectral changes are therefore most likely a consequence of altered hydrogen bonding involving the FMN chromophore after Gln→Leu mutation, as suggested when the equivalent glutamine was mutated in *Adiantum* neochrome1 LOV2 (Nozaki *et al.*, 2004). The presented data also indicate an increase in fluorescence emission quenching in the LOV2 Q575L mutant compared to wild-type LOV2 (Figure 4.3). An approximation of the degree of fluorescence emission quenching may be calculated by correcting the observed flavin fluorescence emission using known absorbance values (Losi *et al.*, 2004, Losi *et al.*, 2002). Such calculations (detailed in Appendix II) suggest that fluorescence emission quenching of LOV2 Q575L is approximately

twice than that of wild-type LOV2, which would partially account for the discrepancies observed between the lack of chromophore indicated by fluorescence emission and the total chromophore subsequently released upon LOV2 denaturation (Figure 4.3C and D).

Changes in the chromophore environment raised the possibility that the photosensory functions of LOV2 were altered by the Q575L substitution and therefore both photoproduct formation and photorecovery were assessed by absorbance spectroscopy. Photoproduct formation in *Arabidopsis* phot1 LOV2 has previously been reported to occur with a rate constant ($K_{ppt}$) of 3.9 x $10^{-2}$ $s^{-1}$ (Kasahara *et al.*, 2002), which is approximately half of the value calculated in this study (7.9 x $10^{-2}$). This is likely a result of differences in experimental conditions; the most obvious discrepancy being that the previous study examined samples at 4°C (Kasahara *et al.*, 2002) whilst room temperature (~22°C) was used in this study. $K_{ppt}$ of the LOV2 Q575L variant was approximately 75% that of wild-type LOV2, suggesting that photoproduct formation was marginally slower in the mutant. The functional significance of this reduction is unclear, however, as $K_{ppt}$ varies between different phot LOV2 domains. For example, $K_{ppt}$ of *Arabidopsis* phot2 LOV2 is approximately 66% that of *Arabidopsis* phot1 LOV2 (Kasahara *et al.*, 2002). Despite this slower rate of photoproduct formation, phot2 LOV2 is equally important for phot2 autophosphorylation and function *in planta* (Cho *et al.*, 2007, Christie *et al.*, 2002). To further investigate whether there was a significant difference in $K_{ppt}$ between wild-type LOV2 and LOV2 Q575L samples at the saturating light intensities used for *in vitro* autophosphorylation assays photoproduct formation was examined using increased light fluences (15 000 μmol $m^{-2}$ $s^{-1}$ white light; data not shown). At these higher light levels $K_{ppt}$ of LOV2 and LOV2 Q575L were comparable ($K_{ppt}$ = 5.4 x $10^{-1}$ $s^{-1}$ for both LOV2 and LOV2 Q575L).

Although photoproduct formation was relatively unaffected by the Q575L substitution, incorporation of this mutation had a significant effect on the photorecovery of the LOV2 domain after light stimulation. The LOV domain photoproduct is considered to be the active signalling state of the domain and so a delay in photorecovery is unlikely to inhibit LOV2 signal transmission (Briggs, 2007). The data presented here using *Arabidopsis* phot1 LOV2 indicates photoproduct half-life was increased from 40 s to 16.7 min upon introduction of the Q575L

substitution (Figure 4.6), which is in broad agreement with previous studies with *Adiantum* neochrome1 LOV2 showing that the equivalent Gln→Leu mutation increased the half-life of the photoproduct from 90 s to 10.8 min (Iwata *et al.*, 2005).

An alternative explanation of the observed reduction in light-induced autophosphorylation activity in the phot1 Q575L mutant is that the Q575L substitution impaired the efficiency of the LOV2 domain photocycle. However, the relative bleaching experiments presented here (Figure 4.5) indicate that the LOV2 photocycle quantum yield is unaffected by the Q575L mutation. These data, in combination with the photochemical results presented are consistent with the notion that Q575L mutation impairs inter-domain signalling within the phot1 protein rather than acting to reduce the photosensitivity of the mutated LOV2 domain.

### *4.3.2 Role of the conserved surface salt bridge in LOV2 signal transmission*

Salt bridges have an important role in protein structure and function and in many cases are involved in stabilising the folded conformations of proteins (Kumar and Nussinov, 1999). All identified LOV domains contain a surface salt bridge that has been hypothesised to have a role in stabilising the tertiary structure of the domain (Crosson *et al.*, 2003, Losi, 2004). Data presented here suggests that the surface salt bridge is indeed important as a structural component in isolated LOV2 domains as mutation of the surface salt bridge caused near total chromophore loss in the truncated protein (Figure 4.1). Chromophore loss most likely derives from tertiary structure perturbation as studies indicated that secondary protein structure is unaltered in the LOV2 E506K variant as measured by circular dichroism spectroscopy (Jones *et al.*, 2007). Despite these data, mutational studies of phot1 expressed in insect cells indicate that chromophore loss is less apparent in the full-length phot1 protein (Figure 4.2). Such data suggests that the surrounding protein provides additional structural stabilisation of LOV2 and correlates with data from our laboratory that suggests removal of the Jα-helix from the LOV2 core sequence reduces FMN binding of the expressed fragment (data not shown), although further work is required to confirm this observation. Studies of the bacterial LOV domain-containing protein YtvA also indicate salt bridge mutation in the full-length protein did not compromise chromophore binding (Losi *et al.*, 2005). It is unclear whether the isolated YtvA LOV domain would also suffer flavin loss after salt bridge mutation as Losi *et al*. (2005)

restricted their mutational analysis to the full-length protein. However, the related FAD-binding protein Aer requires sequence in addition to the canonical LOV domain to stabilise co-factor binding (Watts *et al.*, 2006). Further comparative analysis of isolated LOV domains and their full-length protein counterparts is necessary to determine whether the observed discrepancy in flavin binding between isolated LOV domains and full-length protein is a consistent phenomenon.

In addition to an important structural function the LOV domain surface salt bridge has been postulated to have a role in signal transmission (Crosson *et al.*, 2003). Formation of a flavin-cysteinyl adduct within the LOV domain upon illumination was hypothesised to result in the disruption of the surface salt bridge, with the associated conformational changes providing an active signalling state. However, data provided here shows that a substitution which presumably causes constitutive salt bridge disruption by mutating one of the residues necessary for salt bridge formation ($Glu^{506}$) does not impair light-induced kinase activity, suggesting that this mechanism does not have a significant role in LOV2 signal transmission (Figure 4.2). This experimental evidence is supported by theoretical analysis of LOV domain light-induced structural changes upon irradiation (Freddolino *et al.*, 2006). These studies suggest that light stimulation causes surface salt bridge disruption only in LOV1 and therefore although salt bridge breakage does not alter signal transmission in LOV2 an equivalent mutation in LOV1 may impair signalling of the latter motif. Unfortunately the lack of a definitive light-dependent function of LOV1 (Salomon *et al.*, 2004) has prevented investigation of the role of salt bridge breakage in LOV1-derived signalling.

### *4.3.3 Phototropin light-induced autophosphorylation is altered by mutation of $Gln^{575}$*

Although the above findings indicate that the conserved surface salt bridge is not involved in LOV2 signal transmission mutational studies substituting $Gln^{575}$ with leucine had a considerable effect on light-induced kinase activation (Figure 4.7). Previous studies using FTIR spectroscopy, circular dichroism spectroscopy, small angle X-ray scattering, and pulsed laser-induced transient gating methods have all shown that irradiation of LOV2 fragments containing the Jα-helix results in significant structural changes at the surface of LOV2 (Corchnoy *et al.*, 2003, Eitoku *et al.*, 2005, Iwata *et al.*, 2003, Iwata *et al.*, 2002, Nakasako *et al.*, 2004, Nozaki *et al.*,

2004, Swartz *et al.*, 2002). In particular, FTIR spectroscopy has indicated that photoproduct formation within LOV2 induces progressive conformational changes that involve regions of loops, α-helices and β-sheets (Iwata *et al.*, 2005, Nozaki *et al.*, 2004). These changes presumably include the disassociation of the Jα-helix from the surface of the LOV2 domain which is sufficient for phot1 kinase activation (Harper *et al.*, 2004, Harper *et al.*, 2003). Indeed, LOV2 protein fragments lacking the Jα-helix sequence show greatly reduced structural changes upon illumination (Eitoku *et al.*, 2005, Nakasako *et al.*, 2004).

Previously, Nozaki *et al*. (2004) were able to show that the observed light-induced structural changes in the LOV2+Jα protein fragment isolated from *Adiantum* neochrome1 were limited by substitution of a conserved glutamine with leucine. Work here extends this observation by showing that the equivalent mutation within the LOV2 domain of *Arabidopsis* phot1 severely limits light-induced autophosphorylation without affecting flavin binding, whilst the equivalent mutation within *Arabidopsis* phot2 LOV2 also inhibits light-induced autophosphorylation (Figures 4.7 and 4.8). Whilst phot1 Q575L and phot2 Q489L display a slight light-induced increase in kinase activity this is consistent with residual β-sheet movement observed in the mutated *Adiantum* LOV2 fragment analysed by FTIR spectroscopy (Nozaki *et al.*, 2004). Analysis of the phot1 C512A Q575L double mutant confirms that phot1 LOV2 photochemistry is responsible for the observed residual autophosphorylation activity of the phot1 Q575L mutant (Figure 4.9). Such abolition of light-induced autophosphorylation activity may be ascribed to either loss of chromophore due to the incorporation of multiple mutations within the LOV2 domain or inhibition of flavin-cysteinyl adduct formation but in either case prevention of phot1 LOV2 photochemistry inhibits the residual levels of light-induced autophosphorylation observed. In support of these data, incorporation of the gain-of-function I608E mutation within the Jα-helix was able to bypass the inhibitory effect of the Q575L mutation on phot1 autophosphorylation activity regardless of the light conditions used to treat the phot1 Q575L I608E double mutant (Figure 4.10). This suggests that the Q575L mutation does not irreversibly inhibit phot1 kinase function, and that the effects of the I608E mutation supercede those caused by the Q575L substitution.

As an extension to this work alternate substitutions of $Gln^{575}$ were analysed. NMR spectroscopy of LOV2 had indicated that the Q575N substitution induced constitutive Jα-helix disorganisation, similar to that observed after Jα-helix mutation (K. Gardner, unpublished data, University of Texas, USA; Harper *et al.*, 2003). However, the Q575N substitution had no effect on phot1 autophosphorylation activity when introduced into full-length phot1 (Figure 4.11). The equivalent Gln→Asn mutation was also examined in phot2 and similarly the activity of the mutant protein was indistinguishable from wild-type phot2 (data not shown). Despite the lack of correlation between the different analyses it is important to note that the Q575N substitution does not prevent light-dependent autophosphorylation. This suggests that the presence of asparagine does not mitigate β-sheet movement in light-activated phot1 LOV2, which is perhaps unsurprising given that glutamine and asparagine differ by only a side-chain methyl group.

In an attempt to mimic the consequences of Jα-helix disorganisation through mutation of the βE-sheet a more severe mutation (Q575H) was introduced into full-length phot1. Incorporation of this basic amino acid abolished light-induced autophosphorylation activity in the full-length protein (Figure 4.12). This suggests that insertion of a histidine residue into the LOV2 βE-sheet is not sufficient to induce the constitutive Jα-helix disorganisation thought necessary for increased phot1 autophosphorylation (Harper *et al.*, 2004). Indeed, the data imply that the Q575H mutation limits LOV2 signal transmission. Although possibly a consequence of reduced LOV2 β-sheet movement following photoactivation, the observed loss of autophosphorylation in the phot1 Q575H mutant can at least partially be ascribed to a reduction in flavin chromophore association within the mutated LOV2 domain, thereby preventing LOV2 photochemistry and subsequent signal transmission (Figure 4.12C).

### *4.3.4 Mode of LOV2 signal transmission*

The mutant analysis completed in this chapter suggests a role for a conserved glutamine ($Gln^{575}$ in phot1) in LOV2 signal transmission. Substitution of this residue with leucine causes a deficiency in light-induced kinase activity in both phot1 and phot2 that is not caused by a loss of FMN chromophore in the former case. In addition to this, double mutant analysis places the effects of this substitution between LOV2

photoproduct formation and Jα-helix disorganisation. X-ray crystallography has shown that the conserved glutamine (which directly interacts with the FMN chromophore by hydrogen bonding) undergoes side chain rotation upon light-driven covalent adduct formation (Crosson and Moffat, 2001, Crosson and Moffat, 2002). Coupled with FTIR studies showing reduced β-sheet movement in a LOV domain containing the Gln→Leu mutation (Nozaki *et al.*, 2004), it is likely that the observed side chain rotation stimulates movement of the βE-sheet in which $Gln^{575}$ is a component. Given that the Jα-helix docks onto these β-sheets in the dark (Figure 4.3A; Harper *et al.*, 2003) it is plausible that flavin-cysteinyl adduct formation would induce conformational changes within the LOV2 β-sheets, triggering Jα-helix disorganisation and subsequent phot1 autophosphorylation. Support for this mechanism is found in another *Arabidopsis* LOV domain-containing protein (FKF1), where mutation of the corresponding glutamine mitigates light-dependent protein interactions *in vitro* (Sawa *et al.*, 2007). Furthermore several other studies have indicated the importance of β-sheet movement for intra- and inter-molecular signalling events in other members of the PAS domain superfamily (Losi *et al.*, 2005, Erbel *et al.*, 2003, Zoltowski *et al.*, 2007).

Results from this study also underscore the critical role of the Jα-helix in mediating light-dependent signalling from LOV2. The I608E mutation induces increased basal levels of kinase activity and double mutant analysis has shown that the mutation is capable of overcoming the deleterious effects of two separate substitutions (C512A and Q575L; Section 3.2.11 and Figure 4.7 respectively). Given the dominance of this mutation *in vitro* it was considered essential to assess the physiological effects of this mutated phototropin protein *in planta*. The consequences of phot1 I608E expression *in planta* will be discussed in Chapter 5.

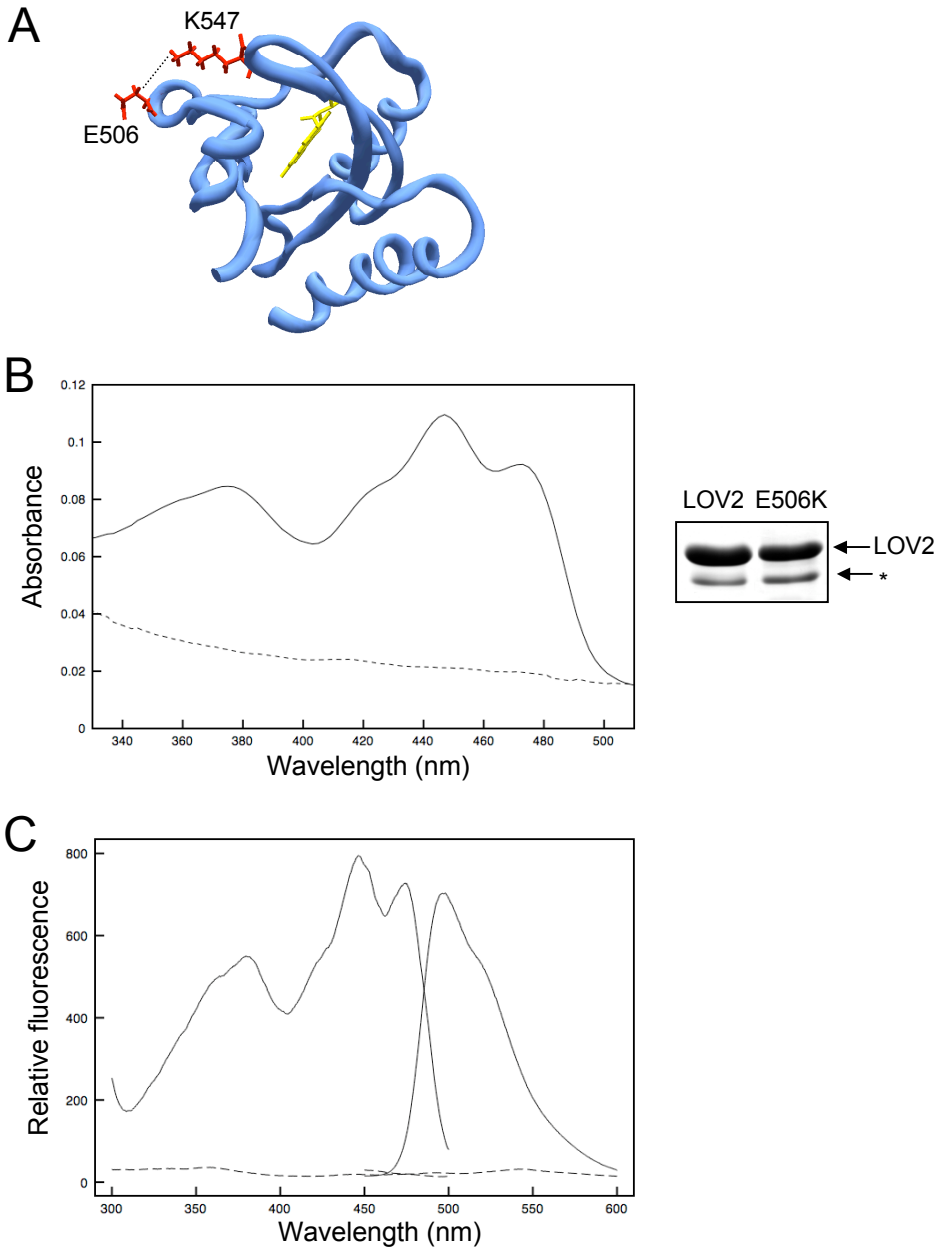


**Figure 4.1 Effect of salt bridge disruption on the spectral properties of *Arabidopsis* phot1 LOV2. (A)** Protein structure of the isolated LOV2 domain of *Arabidopsis* phot1. The structure of *Arabidopsis* phot1 LOV2 was modelled using the program Swiss Model (http://www.expasy.org; Christie 2007). The backbone structures of α-helices, turns and β-sheets are coloured in blue. The FMN chromophore is shown in yellow, while the location of $Glu^{506}$ (E506) and $Lys^{547}$ (K547) are shown in red. **(B)** Absorption spectra of wild-type LOV2 (solid line) and the LOV2 E506K mutant (dashed line) are shown on the left. Absorption spectra were recorded using equal concentrations (0.4 mg $ml^{-1}$) of wild-type and mutant proteins. SDS-PAGE analysis of wild-type LOV2 and the E506K salt bridge mutant expressed and purified from *E. coli* is shown on the right. Proteins (5 µg) were separated by 12.5% SDS-PAGE. LOV2 and a major degradation product (*) are indicated. **(C)** Fluorescence excitation spectra (left) and fluorescence emission spectra (right) of wild-type LOV2 (solid line) and the E506K mutant (dashed line). Fluorescence spectra were recorded using equal concentrations (0.1 mg $ml^{-1}$) of wild type and mutant proteins.

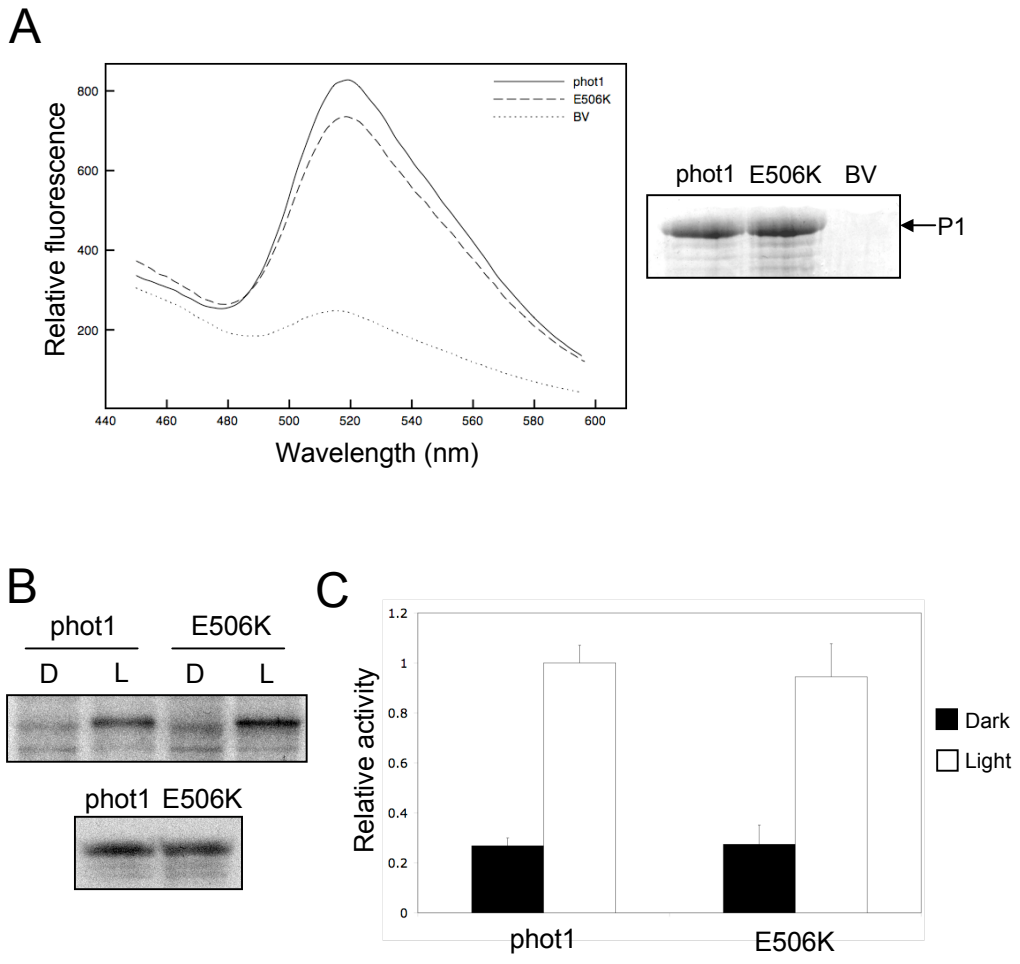


**Figure 4.2 Effect of the E506K point mutation on chromophore binding and autophosphorylation activity of *Arabidopsis* phot1. (A)** Estimation of flavin levels released upon denaturation of whole insect cells expressing wild type and E506K mutant forms of phot1. Fluorescence emission spectra of flavin levels released from equal amounts (1.5 mg protein) of insect cells infected with wild-type phot1 (phot1, solid line), E506K mutant (E506K, dashed line) and wild-type baculovirus (BV, dotted line) are shown on the left. SDS-PAGE analysis of wild-type phot1, phot1 E506K and wild-type baculovirus expressed in insect cells is shown on the right. Proteins (10 µg) were separated by 7.5% SDS-PAGE. The size of full-length phot1 is indicated (P1). **(B)** Autoradiograph showing the effect of the single E506K point mutation on phot1 autophosphorylation activity in soluble protein extracts prepared from insect cells. All manipulations were completed under dim red light. Samples were given a mock irradiation (D) or irradiated with white light (L) at a total photon fluence rate of 30,000 µmol $m^{-2}$ $s^{-1}$ prior to the addition of radiolabelled ATP. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. **(C)** Autophosphorylation activity of wild-type phot1 and the E506K mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal phot1 autophosphorylation (error bars indicate standard error, n=3).

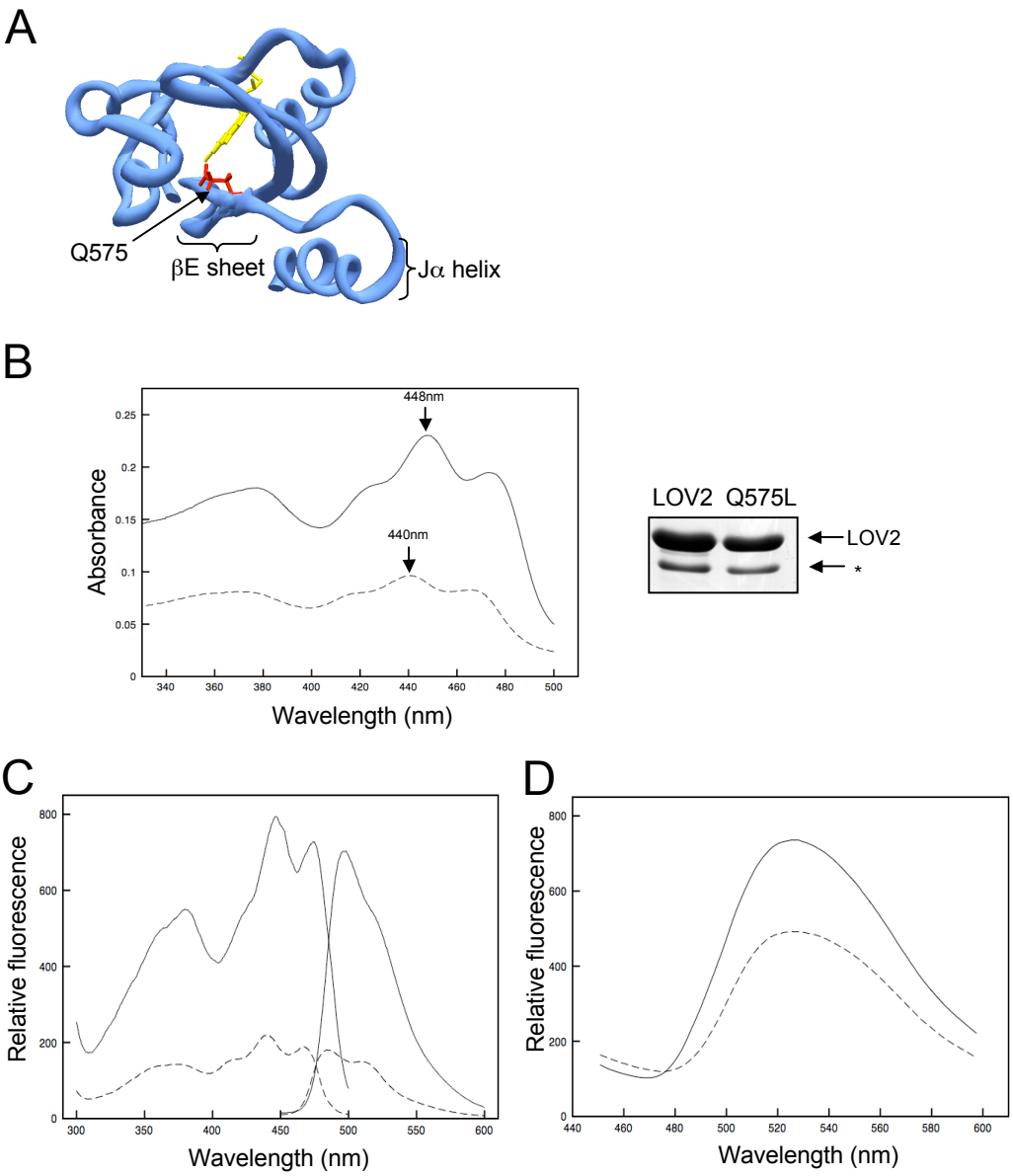


**Figure 4.3 Effect of the Q575L mutation on the spectral properties of *Arabidopsis* phot1 LOV2. (A)** Protein structure of the LOV2 domain and associated Jα helix of *Arabidopsis* phot1. The structure of *Arabidopsis* phot1 LOV2 was modelled using the program Swiss Model (http://www.expasy.org; Christie 2007). The backbone structures of α-helices, turns and β-sheets are coloured in blue. The FMN chromophore is shown in yellow, while the location of $Gln^{575}$ (Q575) within the βE-sheet is shown in red. For clarity a portion of the Jα-helix was omitted from this diagram. **(B)** Absorption spectra of wild-type LOV2 (solid line) and the Q575L mutant (dashed line) are shown on the left. Absorption spectra were recorded using equal concentrations (0.5 mg $ml^{-1}$) of wild type and mutant protein. Absorption maxima of wild type and mutant proteins are indicated. SDS-PAGE analysis of wild-type LOV2 and the Q575L mutant expressed and purified from *E. coli* is shown on the right. Proteins (5 µg) were separated by 12.5% SDS-PAGE. LOV2 and a major degradation product (*) are indicated. **(C)** Fluorescence excitation spectra (left) and fluorescence emission spectra (right) of wild-type LOV2 (solid line) and the Q575L mutant (dashed line). Fluorescence spectra were recorded using equal concentrations (0.4 mg $ml^{-1}$) of wild type and mutant proteins. **(D)** Fluorescence emission spectra of flavin levels released upon denaturation of equal amounts (125 µg) of wild-type LOV2 (solid line) and the Q575L mutant (dashed line).

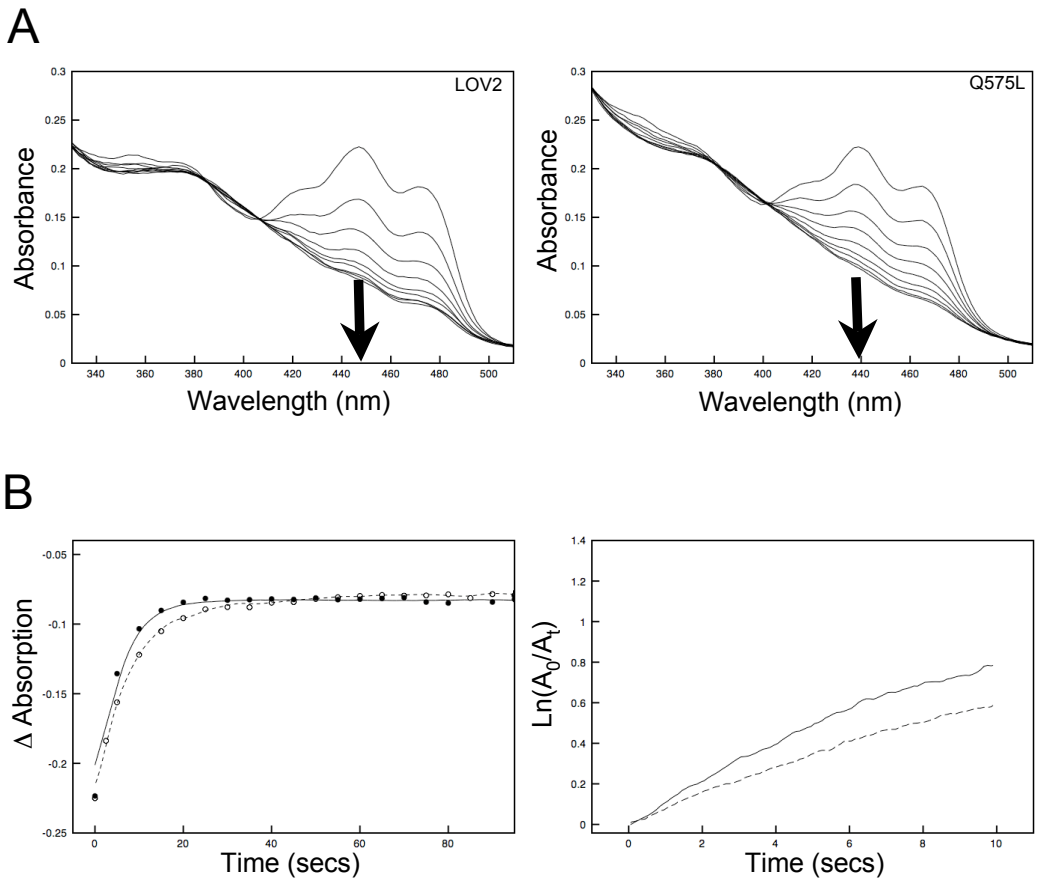


**Figure 4.4 Effect of Q575L mutation on LOV2 photoproduct formation kinetics. (A)** Absorption spectra illustrating photoproduct formation upon irradiation of sample with 300 µmol $m^{-2}$ $s^{-1}$ white light. Sample spectra are shown every 2.5 seconds. Photoproduct formation of LOV2 is shown on the left and that of the Q575L mutant on the right. Arrows indicate the loss of absorption as photoproduct formation occurs. **(B)** Reaction kinetics for photoproduct formation in response to 300 µmol $m^{-2}$ $s^{-1}$ white light. Changes in absorption at 448 nm or 440 nm were monitored for LOV2 (solid, black) and Q575L (dashed, white) mutant respectively and plotted on the left. Initial rates of photoproduct formation are shown on the right. $A_0$ represents the amount of unreacted LOV2 protein as indicated by the absorption detected immediately before light exposure. $A_t$ represents the corresponding amount of unreacted protein at time t, calculated from the amount of absorption at time t. Photoproduct kinetics of LOV2 (solid) and the Q575L mutant (dashed) are shown.

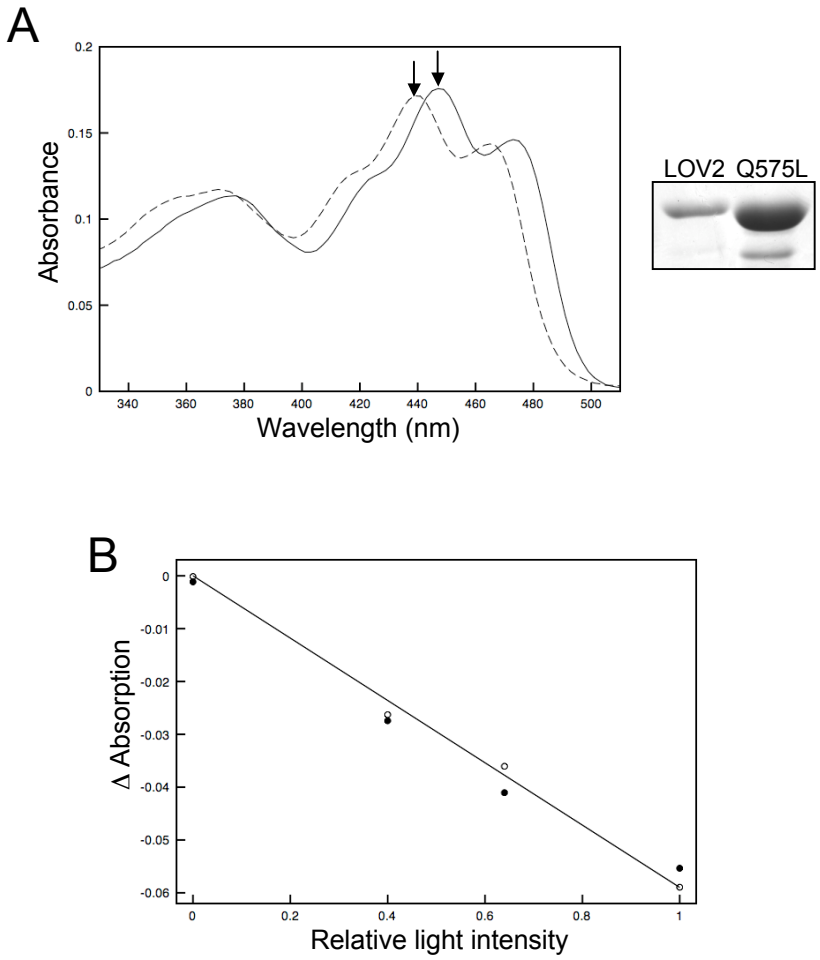


**Figure 4.5 Effect of Q575L mutation on LOV2 quantum yield. (A)** Absorption spectra of wild-type LOV2 (solid line) and the LOV2 Q575L mutant (dashed line). Protein levels were standardised using absorbance measurements at 448 nm and 440 nm for LOV2 and Q575L respectively. Final protein concentrations were 2.7 mg $ml^{-1}$ (LOV2) and 4.2 mg $ml^{-1}$ (Q575L). Proteins (12.5 µg LOV2 or 20 µg Q575L) were separated by 12.5% SDS-PAGE and are shown inset. Arrows indicate the absorbance peak of each sample. **(B)** Relative bleaching measurements for FMN-cysteinyl adduct formation using bacterially expressed phot1 LOV2 and the LOV2 Q575L mutant. Light-induced absorbance changes were monitored at 448 nm (LOV2, black) or 440 nm (Q575L, white) subsequent to irradiation with a strobe camera flash filtered with a neutral density glass. Light intensities indicated are relative to a strobe camera flash filtered through a 48% neutral density filter. The fitted line gives a measure of relative bleaching for both proteins. Relative bleaching values for each protein are shown and represent the average of two independent protein preparations.

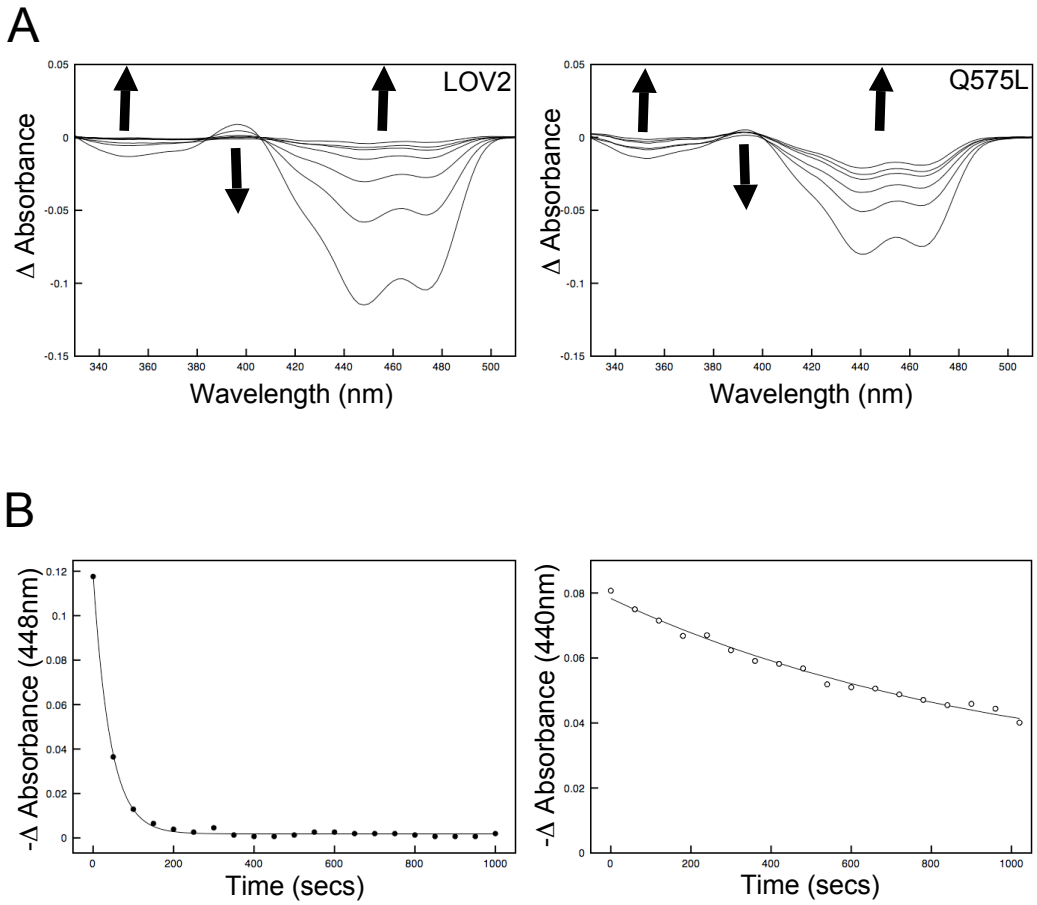


**Figure 4.6 Time-resolved light-induced absorption changes for LOV2 and the Q575L mutant. (A)** Light minus dark absorption difference spectra showing the return of LOV domains to the dark form following photoexcitation. Data for LOV2 are shown on the left (example spectra every 30 seconds) while data for the Q575L mutant are shown on the right (example spectra every 300 seconds). Arrows indicate the gain or loss of absorption as reversion to the initial resting state spectra occurs. **(B)** Absorption changes after light excitation measured at 448 nm or 440 nm for LOV2 (black, left) or the Q575L mutant (white, right) respectively.

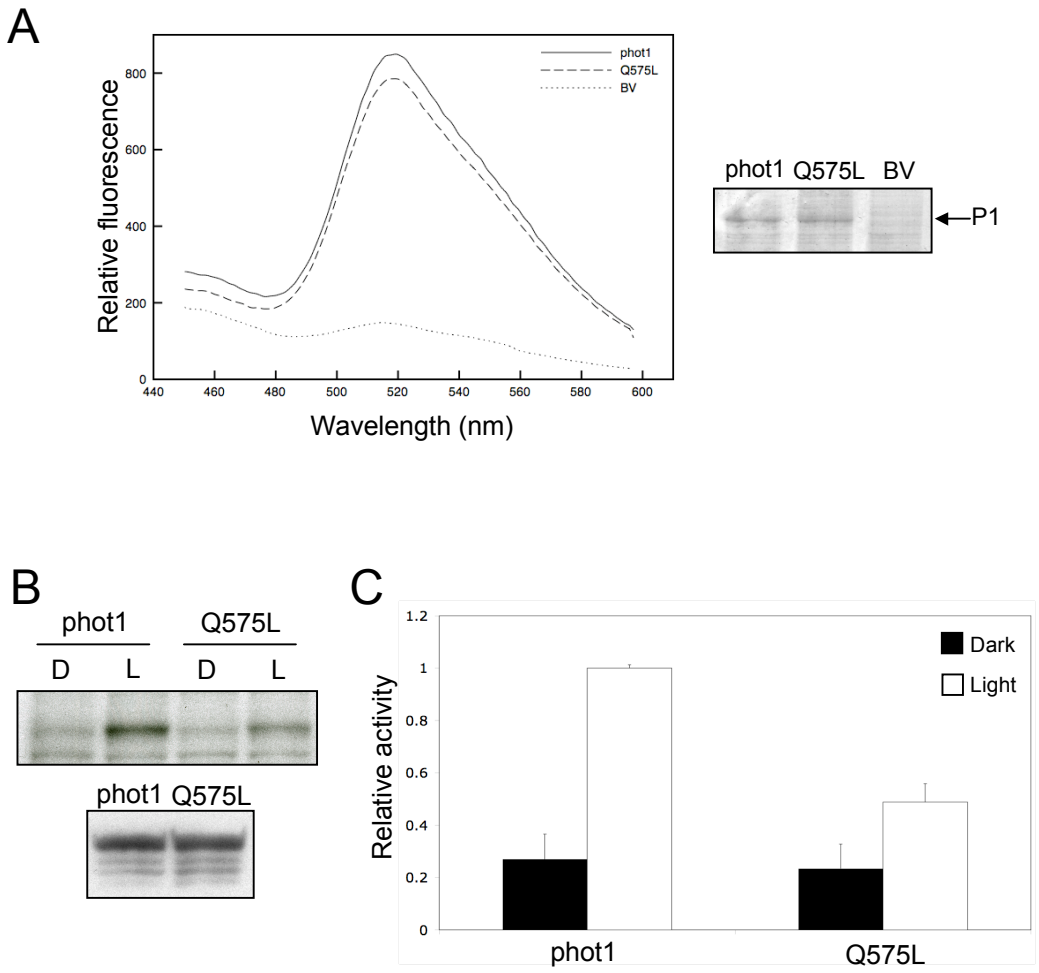


**Figure 4.7 Effect of the Q575L mutation on the autophosphorylation activity of *Arabidopsis* phot1 expressed in insect cells. (A)** Estimation of flavin levels released upon denaturation of whole insect cells expressing wild type and Q575L mutant forms of phot1. Fluorescence emission spectra of flavin amounts released from equal amounts (1.5 mg protein) of insect cells infected with wild-type phot1 (phot1, solid line), Q575L mutant (Q575L, dashed line) and wild-type baculovirus (BV, dotted line) are shown on the left. SDS-PAGE analysis of wild-type phot1, phot1 Q575L and wild-type baculovirus expressed in insect cells is shown on the right. Proteins (10 µg) were separated by 7.5% SDS-PAGE. The expected size of full-length phot1 is indicated (P1). **(B)** Autoradiograph showing light-dependent autophosphorylation activity of wild-type phot1 and the Q575L mutant in soluble protein extracts prepared from insect cells. All manipulations were carried out under dim red light. Sample preparation and experimental procedures were performed as described in Figure 4.2A. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. **(C)** Autophosphorylation activity of wild-type phot1 and the Q575L mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal phot1 autophosphorylation (error bars indicate standard error, n=3).

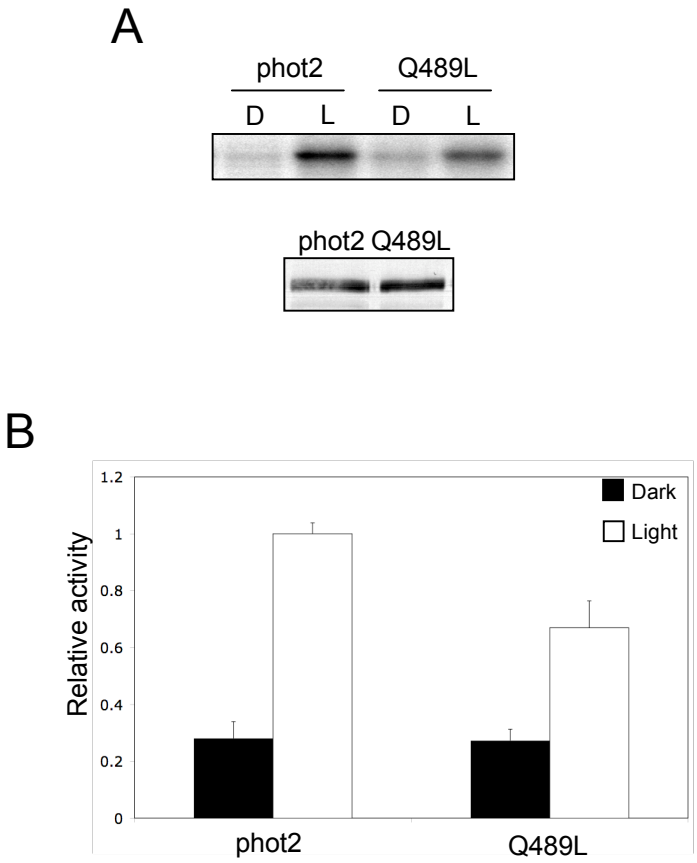


**Figure 4.8 Effect of the Q489L point mutation on the autophosphorylation activity of *Arabidopsis* phot2. (A)** Autoradiograph showing the effect of the single Q489L point mutation on phot2 autophosphorylation activity in soluble protein extracts prepared from insect cells. Sample preparation and experimental procedures were performed as described in Figure 4.2A. Western blot analysis of phot2 protein levels using a C-terminal anti-phot2 antibody is shown below. **(B)** Autophosphorylation activity of wild-type phot2 and the Q489L mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal phot2 autophosphorylation (error bars indicate standard error, n=3).

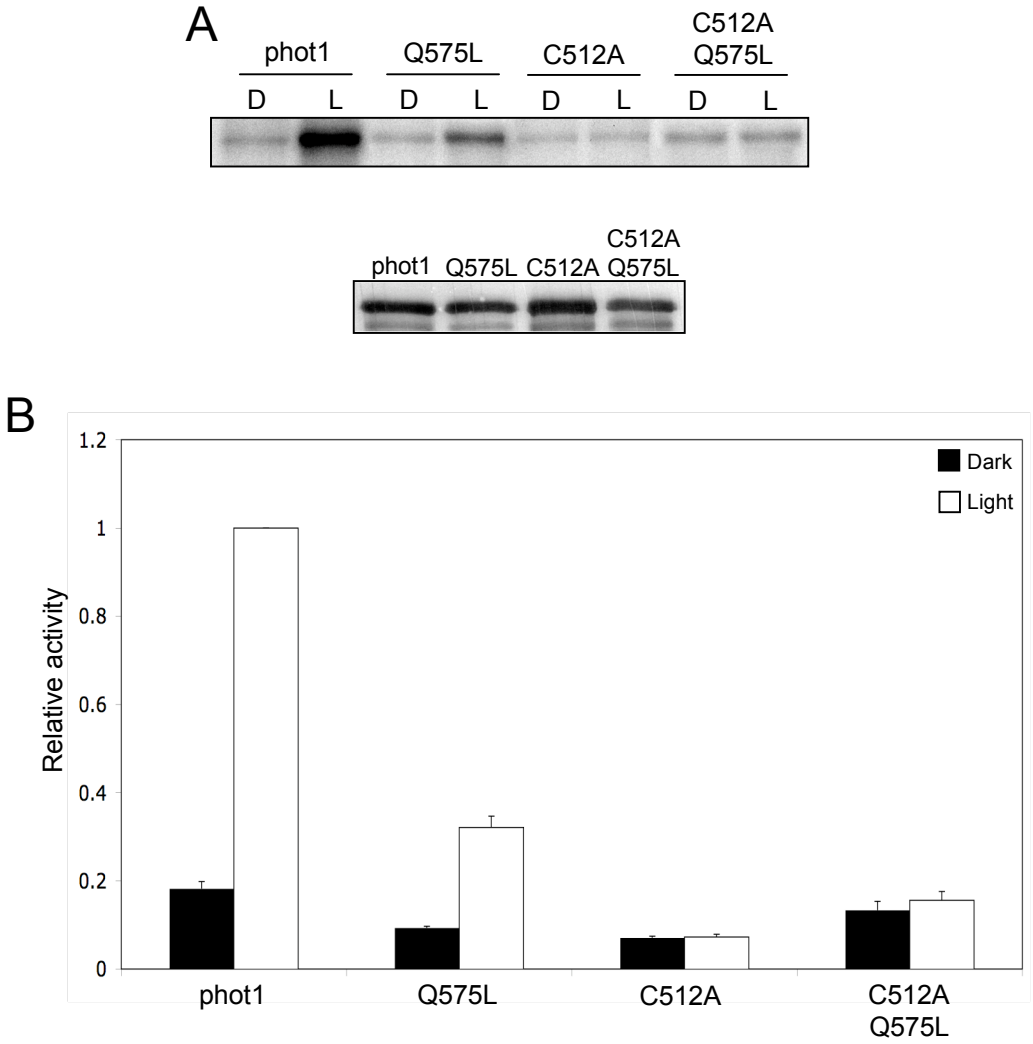


**Figure 4.9 Analysis of the effect of combined C512A and Q575L mutation on the autophosphorylation activity of *Arabidopsis* phot1 expressed in insect cells.** Sample preparation and experimental procedures were performed as described in Figure 4.2A. **(A)** Autoradiograph showing effect of single C512A and Q575L mutations and the combined double mutation on phot1 autophosphorylation activity in soluble protein extracts prepared from insect cells. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. **(B)** Quantification of autophosphorylation activity described in (A). Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal autophosphorylation (error bars indicate standard error, n=3).

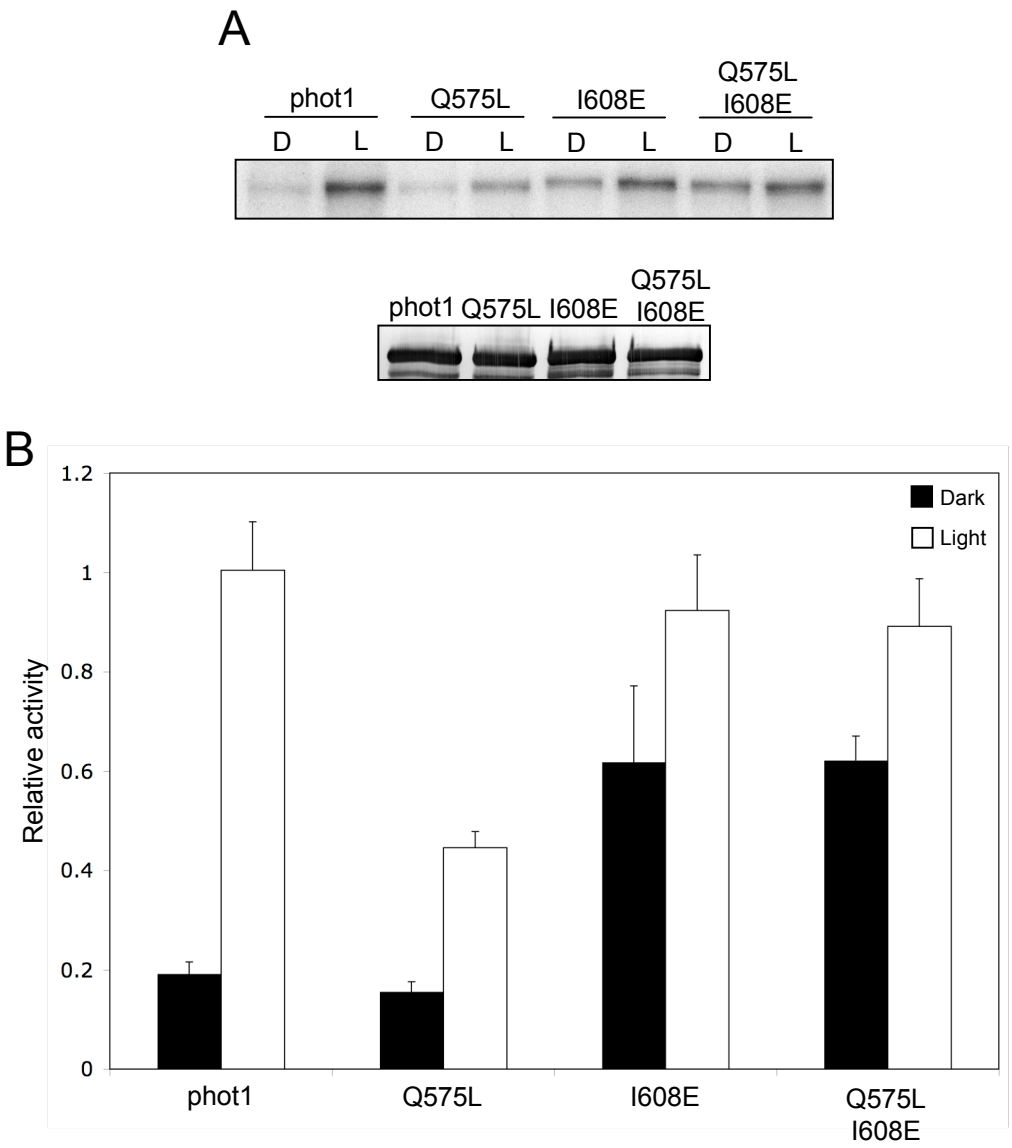


**Figure 4.10 Analysis of the effect of combined Q575L and I608E mutation on the autophosphorylation activity of *Arabidopsis* phot1 expressed in insect cells.** Sample preparation and experimental procedures were performed as described in Figure 4.2A. **(A)** Autoradiograph showing effect of single Q575L and I608E mutations and the combined double mutation on phot1 autophosphorylation activity in soluble protein extracts prepared from insect cells. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. **(B)** Quantification of autophosphorylation activity described in (A). Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal autophosphorylation (error bars indicate standard error, n=3).

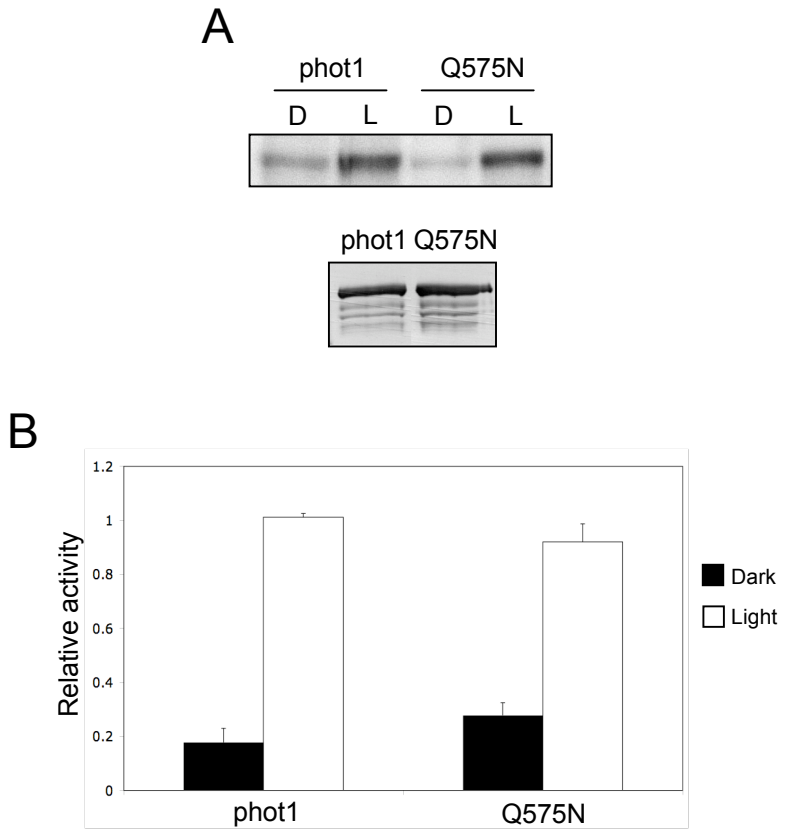


**Figure 4.11 Effect of the Q575N mutation on autophosphorylation activity of *Arabidopsis* phot1 expressed in insect cells. (A)** Autoradiograph showing light-dependent autophosphorylation activity of wild-type phot1 and the Q575N mutant in soluble protein extracts prepared from insect cells. Sample preparation and experimental procedures were performed as described in Figure 4.2A. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. **(B)** Autophosphorylation activity of wild-type phot1 and the Q575N mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal phot1 autophosphorylation (error bars indicate standard error, n=3).

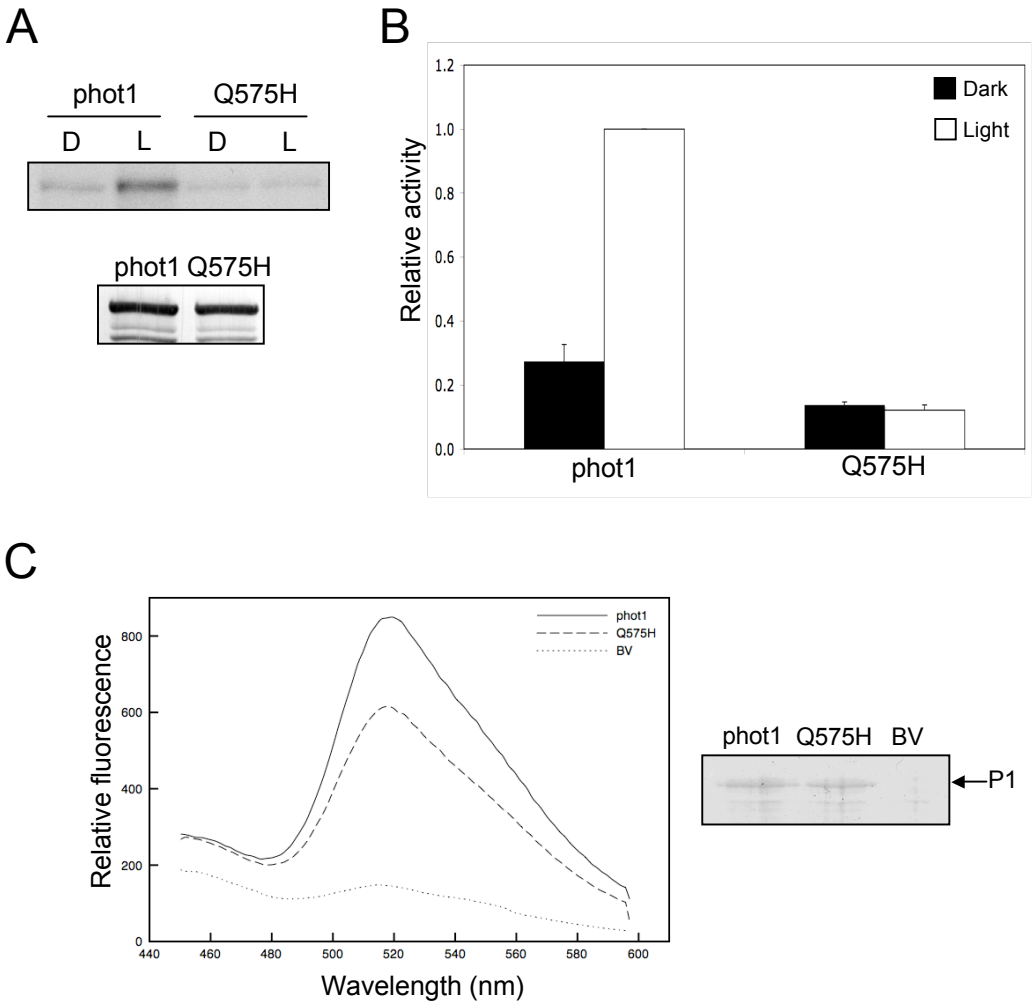


**Figure 4.12 Effect of the Q575H mutation on autophosphorylation activity of *Arabidopsis* phot1 expressed in insect cells. (A)** Autoradiograph showing light-dependent autophosphorylation activity of wild-type phot1 and the Q575H mutant in soluble protein extracts prepared from insect cells. Sample preparation and experimental procedures were performed as described in Figure 4.2A. Western blot analysis of phot1 protein levels using a C-terminal anti-phot1 antibody is shown below. **(B)** Autophosphorylation activity of wild-type phot1 and the Q575H mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a fraction of maximal phot1 autophosphorylation (error bars indicate standard error, n=3). **(C)** Estimation of flavin levels released upon denaturation of whole insect cells expressing wild type and Q575H mutant forms of phot1. Fluorescence emission spectra of flavin amounts released from equal amounts (1.5 mg protein) of insect cells infected with wild-type phot1 (phot1, solid line), Q575H mutant (Q575H, dashed line) and wild-type baculovirus (BV, dotted line) are shown on the left. SDS-PAGE analysis of wild-type phot1, phot1 Q575H and wild-type baculovirus expressed in insect cells is shown on the right. Proteins (10 µg) were separated by 7.5% SDS-PAGE. The expected size of full-length phot1 is indicated (P1).

# Chapter 5: Functional Characterisation of phototropin 1 I608E *in planta*

## 5.1 Introduction

As discussed in Chapters 3 and 4, previous work has shown that light detection by LOV2 is both necessary and sufficient for light-dependent kinase activation (Cho *et al.*, 2007, Christie *et al.*, 2002, Sullivan *et al.*, 2008). Signal transmission from LOV2 to the kinase domain is thought to occur primarily through light-dependent disorganisation of the highly conserved Jα-helix located immediately after the C-terminus of LOV2 (Harper *et al.*, 2004, Harper *et al.*, 2003). Artificial disruption of the Jα-helix via a single point mutation ($Ile^{608}$→Glu, I608E) induces increased basal levels of phot1 autophosphorylation as monitored by *in vitro* kinase assays using insect cells (Sections 3.2.10, 4.2.10; Harper *et al.*, 2004). As such, phot1 containing this mutation (phot1 I608E) is potentially a constitutively-active form of the phot1 photoreceptor and it was therefore of interest to investigate the physiological consequences of expressing this mutated form of phot1 *in planta*. Transgenic *Arabidopsis* were created which expressed the mutant phot1 I608E protein in both a *phot1-5 phot2-1* and wild-type background.

## 5.2 Results

### *5.2.1 Expression of phot1 I608E in a phot1 phot2 background*

Both phot1 and phot2 have previously been shown to be functional *in planta* when tagged with a C-terminal green fluorescent protein (GFP)-tag (Kong *et al.*, 2007, Sakamoto and Briggs, 2002, Wan *et al.*, 2007). These studies have additionally shown that a fraction of phot1-GFP is relocalised to the cytoplasm from the plasma membrane upon blue light irradiation (Sakamoto and Briggs, 2002, Wan *et al.*, 2007). If phot1 I608E were a constitutively-active version of phot1 *in planta* it may have an altered intracellular localisation in the absence of blue light and therefore lines were created expressing phot1 I608E with a C-terminal GFP-tag to allow convenient assessment of phot1 I608E-GFP sub-cellular localisation. *PHOT1* cDNA and *PHOT1* cDNA containing the I608E substitution were cloned into GFP-containing binary

vectors (Figure 5.1A) that were subsequently used to transform *phot1-5 phot2-1* (cv. Columbia, *gl1* background) double mutant plants (*p1p2*) to create transgenic *Arabidopsis* lines expressing phot1 and phot1 I608E with a C-terminal GFP tag under the control of a cauliflower mosaic virus *35S* promoter (*35S*). The *35S* promoter was used as phot1 fused to a His-tag and expressed under the control of a *35S* promoter has previously been used to successfully complement phot1-dependent phenotypes in either *phot1-5* or *p1p2* mutant *Arabidopsis* (Cho *et al.*, 2007, Christie *et al.*, 2002).

Homozygous T3 transgenic lines identified by kanamycin selection were assayed for wild-type phot1-GFP and phot1 I608E-GFP protein expression using anti-phot1 antibody (Figure 5.1B; Christie *et al.*, 1998). Such western blot analysis revealed a line expressing phot1-GFP at a comparable level to endogenous phot1 (35-P1G/*p1p2*-A, Figure 5.1B). Similar analysis of phot1 I608E-GFP expression in identified 35-P1G(I608E)/*p1p2* lines revealed that protein expression was much lower than endogenous phot1 levels in the three lines isolated (Figure 5.1B, two example lines shown). In an attempt to identify further independent transgenic lines expressing improved levels of phot1 I608E-GFP additional T2 lines were screened for protein expression (Figure 5.1C). Although phot1-GFP protein expression was detectable in multiple 35-P1G/*p1p2* lines, expression of phot1 I608E-GFP was not readily detectable by western analysis in the additional T2 lines examined (Figure 5.1C). Similarly, low levels of phot1 I608E protein expression were observed in transgenic lines expressing His-tagged phot1 I608E (Appendix III, Figure 9.1). In view of the presented data one high- and one low-phot1-GFP expressing line (35-P1G/*p1p2*-A and 35-P1G/*p1p2*-G respectively) were selected for physiological characterisation in the T3 generation along with the identified line which expressed detectable levels of phot1 I608E-GFP (35-P1G(I608E)/*p1p2*-A). DNA sequencing confirmed that each selected line contained the correct transgene (data not shown). As an initial stage of this analysis, the autophosphorylation activity and sub-cellular localisation of phot1 I608E-GFP was examined.

### *5.2.2 Phot1 I608E GFP has altered levels of autophosphorylation activity in vitro*

*In vitro* kinase assays have previously been used to show that phot1 I608E expressed using a baculovirus/insect cell expression system has an increased basal level of autophosphorylation activity *in vitro* in the absence of light (Section 3.2.10; Harper *et*

*al.*, 2004). To confirm whether a similar increase in activity was observed in transgenic *Arabidopsis* expressing phot1 I608E the *in vitro* autophosphorylation activity of phot1 I608E isolated from line 35-P1G(I608E)/*p1p2*-A was examined. Three-day-old etiolated seedlings were harvested under dim red light and microsomal membrane fractions isolated by ultra-centrifugation. *In vitro* kinase assays were then completed to assess the kinase activity of phot1-GFP and phot1 I608E-GFP after light induction in comparison to mock-irradiated samples (Figure 5.2A).

As previously reported (Sakamoto and Briggs, 2002), the incorporation of a C-terminal GFP-tag does not appear to prevent light-induced autophosphorylation activity of phot1-GFP *in vitro* (Figure 5.2A). Additionally, consistent with data obtained using the baculovirus/insect cell expression system (Harper *et al.*, 2004), an increased level of autophosphorylation was observed in mock-treated samples expressing phot1 I608E-GFP compared to the wild-type phot1-GFP control (Figure 5.2A). However, the autophosphorylation activity observed in the GFP-tagged lines is greatly reduced compared to wild-type phot1. Additionally, although the levels of light-stimulated kinase activity were comparable between phot1-GFP and phot1 I608E-GFP (Figure 5.2A) there was a large discrepancy between protein levels as assessed by western analysis (Figure 5.2B). To confirm equal loading, the levels of the membrane-localised protein NON-PHOTOTROPIC HYPOCOTYL 3-LIKE (NPH3-L) were assayed using an anti-NPH3-L antibody (Figure 5.2B; Thomson, 2008). NPH3-L is a protein with a highly conserved sequence compared to the known phot1-interacting protein NON-PHOTOTROPIC HYPOCOTYL 3 (Thomson, 2008). Immunoblot analysis indicates that similar levels of NPH3-L protein expression are present in each line, suggesting equal loading of protein samples. This suggests that the relative phosphorylation activity of phot1 I608E-GFP is greater than phot1-GFP. The observed discrepancy between autophosphorylation activity between these lines will be further discussed in Section 5.3.1.

### *5.2.3 Analysis of phot1-GFP and phot1 I608E-GFP relocalisation from the plasma membrane upon blue light stimulation*

Although differences in protein expression restrict data interpretation *in vitro* phosphorylation assays indicate that phot1 I608E-GFP retains light-induced kinase activity (Figure 5.2). This may suggest that phot1 I608E-GFP retains function *in*

*planta*. Along with phosphorylation, one of the immediate consequences of light exposure with regards phot1 is the relocalisation of the protein to the cytoplasm from the plasma membrane, which can be detected within 3 minutes of blue light irradiation (Sakamoto and Briggs, 2002, Wan *et al.*, 2007). Confocal microscopy was therefore used to determine the sub-cellular distribution of phot1-GFP and phot1 I608E-GFP expressed under the control of the *35S* promoter in three-day-old etiolated seedlings. As a positive control the movement of phot1-GFP expressed under the control of the native *PHOT1* promoter (*PHOT1*::phot1-GFP) was re-examined (Figure 5.3A). As reported previously (Sakamoto and Briggs, 2002, Wan *et al.*, 2007) the high-intensity blue laser light used to excite GFP was capable of inducing phot1-GFP movement away from the plasma membrane. To ensure that such movement was not a constitutive effect of prolonged blue light irradiation observed in GFP-tagged plasma membrane-localised proteins the movement of an additional GFP-tagged protein was examined as a negative control. GFP-LT16b has previously been described as a membrane-localised protein in *Arabidopsis* (Cutler *et al.*, 2000) and this protein did not display an altered distribution after 12 min blue light excitation. This suggests that phot1-GFP relocalisation is determined by the specific action of phot1-GFP rather than the GFP-tag itself as previously described (Sakamoto and Briggs, 2002). Redistribution of phot1-GFP after blue light stimulation was then examined in transgenic line 35-P1G/*p1p2*-A, where phot1-GFP is expressed under the control of the *35S* promoter (Figure 5.3B). As observed for *PHOT1*::phot1-GFP, *35S*::phot1-GFP also displays a redistribution away from the plasma membrane upon irradiation with high-intensity blue laser light. However, in contrast to *PHOT1*::phot1-GFP, an intra-cellular GFP signal was detected in 35-P1G/*p1p2*-A seedlings before light stimulation (Figure 5.3B, left).

Finally, the intracellular localisation of phot1 I608E-GFP was examined using 35-P1G(I608E)/p1p2-A seedlings (Figure 5.3C). Although a minimal GFP signal could be detected, redistribution of the protein upon irradiation was not observed. The greatly reduced GFP-signal observed is presumably a result of the much reduced protein expression observed in this line (Figure 5.1B). Whilst the faint GFP signal detected confirms GFP-tagged protein expression, the low signal strength prevented further analysis of the sub-cellular localisation of phot1 I608E-GFP using this methodology.

### *5.2.4 Phot1 I608E partially complements phototropic curvature when expressed in p1p2 mutant Arabidopsis*

Although the sub-cellular distribution of phot1 I608E-GFP could not be conclusively elucidated by confocal microscopy (Figure 5.3C), the *in vitro* autophosphorylation observed in microsomal extracts isolated from 35-P1G(I608E)/*p1p2*-A seedlings (Figure 5.2) suggested that phot1 I608E-GFP may retain a degree of functionality with regards phot1-mediated physiological responses. The ability of the mutant phot1 I608E-GFP protein to functionally complement known phot1-mediated responses *in vivo* (including phototropism, petiole positioning, leaf expansion and chloroplast movement) was therefore assessed.

The first physiological response examined was phototropism. Phot1 and phot2 have partially redundant roles in this response, with phot1 being the primary photoreceptor at lower light fluences (Sakai *et al.*, 2001). To determine whether phot1 I608E was capable of complementing this phot1-mediated phenotype, the ability of phot1 I608E-GFP to induce phototropism in response to low-fluence unilateral blue light was examined. Three-day-old etiolated seedlings were exposed to unilateral blue light at a fluence rate of 1 μmol $m^{-2}$ $s^{-1}$ for 24 hours and second positive hypocotyl curvature towards the light source measured as described (Lasceve *et al.*, 1999). Hypocotyl curvature was quantified and is shown in Figure 5.4A. Example images of irradiated seedlings are provided underneath. Wild-type *Arabidopsis* seedlings display a curvature of approximately 75° towards the light source after 24 hours whereas *p1p2* seedlings displayed negligible directional curvature (Figure 5.4A). Expression of phot1-GFP induced a restoration of the phototropic response when expressed in a *p1p2* background although phototropic curvature in 35-P1G/*p1p2*-G seedlings was less pronounced in agreement with the reduced protein levels detected in this line by immunoblot analysis (Figures 5.1B and 5.1C). Compared with these controls, it was apparent that expression of phot1 I608E-GFP facilitated a phototropic response to unilateral blue light, although the degree of hypocotyl curvature was greatly reduced compared to positive controls. Indeed, phototropic curvatures in this line was approximately a quarter of that observed in wild-type or 35-P1G/*p1p2*-A seedlings. Limited phototropic curvature was also observed in lines expressing low levels of His-phot1 I608E (Appendix III, Figure 9.2). These data indicate that phot1 I608E-GFP is capable of restoring phototropism *in planta*, although it is unclear from this

data whether the reduced hypocotyl curvature observed in lines expressing phot1 I608E-GFP is a consequence of reduced protein levels or whether the I608E mutation impairs the function of the mutant protein *in vivo*.

To further examine the ability of phot1 I608E-GFP to complement the phototropic response, three-day-old etiolated seedlings were exposed to higher intensities of unidirectional blue light. It was hypothesised that increased light fluence rates would induce a greater degree of phototropism if the transgenic seedlings were less sensitive to light either due to reduced levels of mutant phot1 I608E-GFP protein or a reduced functionality of the mutant protein. Phototropic curvature was assessed over an increasing range of photon fluence rates as indicated, with etiolated seedlings being irradiated with the indicated fluence of unilateral blue light for 24 hours (Figure 5.4B). Whilst curvature in *p1p2* seedlings remained minimal across all fluence rates examined, lines expressing phot1 I608E-GFP demonstrated increased phototropic curvature at higher light intensities (Figure 5.4B). Representative images of seedlings irradiated with 10 μmol $m^{-2}$ $s^{-1}$ for 24 hours are shown below. Despite this, phototropic curvature remained reduced in line 35-P1G(I608E)/*p1p2*-A compared with wild-type seedlings at all fluences measured, suggesting that the use of increased fluences only partially compensated for the impaired phototropism observed in these lines.

### *5.2.5 Phot1 I608E expression does not restore detectable NPH3 dephosphorylation in response to blue light irradiation in p1p2 seedlings*

As the phot1 I608E protein appears capable of partially complementing the phototropic response when expressed in *p1p2* seedlings the ability of phot1 I608E to restore signalling pathways associated with phototropism was examined. Work completed with etiolated oat coleoptiles has demonstrated that a gradient of phot1 autophosphorylation is created across the organ in response to a unilateral light source, with less autophosphorylation observed on the shaded side of the organ (Salomon *et al.*, 1996, Salomon *et al.*, 1997). This gradient of phot1 activity is inversely correlated with a redistribution of auxin across the hypocotyl in response to unilateral blue light irradiation (Haga *et al.*, 2005, Esmon *et al.*, 2006, Haga and Iino, 2006). Such an auxin gradient across the organ is thought necessary to induce differential growth and subsequent phototropic curvature (Esmon *et al.*, 2006,

Cholodny, 1927, Went and Thimann, 1937) and there is good genetic evidence that phots induce this auxin gradient (Liscum and Reed, 2002). A key protein required for the observed asymmetric redistribution of auxin is NONPHOTOTROPIC HYPOCOTYL 3 (NPH3) as the rice NPH3 ortholog COLEOPTILE PHOTOTROPISM 1 is necessary for both phototropic curvature and auxin distribution in this species (Haga *et al.*, 2005). NPH3 is required for phototropism (Liscum and Briggs, 1995), interacts directly with phot1 (Motchoulski and Liscum, 1999) and undergoes dephosphorylation upon blue light irradiation, a post-translational modification that causes an alteration in NPH3 electrophoretic mobility during SDS-PAGE and which requires phot1 activity (Pedmale and Liscum, 2007). NPH3 may therefore be an important intermediate linking phot1 activity to auxin redistribution. It was therefore of interest to determine whether the mutant phot1 I608E-GFP protein was capable of inducing NPH3 dephosphorylation.

The phosphorylation status of NPH3 was examined in 35-P1G(I608E)/*p1p2*-A seedlings as phototropic curvature was most pronounced in this line. NPH3 dephosphorylation causes the protein to migrate further through an SDS-PAGE gel during electrophoresis, and this electrophoretic mobility shift was used to indirectly monitor the phosphorylation status of NPH3 by immunoblot analysis. Etiolated three-day-old seedlings were irradiated with 10 μmol $m^{-2}$ $s^{-1}$ blue light for four hours to allow blue light-induced NPH3 dephosphorylation to occur before protein was extracted. Seedlings were irradiated with 10 μmol $m^{-2}$ $s^{-1}$ blue light as pronounced hypocotyl curvature had been observed for 35-P1G(I608E)/*p1p2*-A seedlings at this fluence rate (Figure 5.4). As previously described (Pedmale and Liscum, 2007), a band-shift indicative of NPH3 dephosphorylation was not observed in *p1p2* seedlings after *in vivo* irradiation, although the expression of phot1-GFP in a *p1p2* background was sufficient to alter NPH3 electrophoretic mobility in response to *in vivo* blue light irradiation as observed in wild-type seedlings (Figure 5.5). However, an alteration in NPH3 electrophoretic mobility was not apparent in seedlings expressing phot1 I608E-GFP (Figure 5.5). Such data may suggest that the phot1 I608E substitution prevents the mutated protein from inducing NPH3 dephosphorylation in response to blue light, although the reduced levels of phot1 I608E-GFP protein in this transgenic line may prevent detection of such a band-shift in these assays if only a portion of the available NPH3 was dephosphorylated by phot1 I608E-GFP.

### *5.2.6 Phot1 I608E expression does not complement initial petiole positioning in p1p2 seedlings*

As well as being required for phototropism, NPH3 is also necessary for other phot1-mediated responses such as the initial positioning of petioles in *Arabidopsis* seedlings (Inoue *et al.*, 2007). Correct petiole positioning allows *Arabidopsis* seedlings to optimise light harvesting for photosynthesis by appropriately orientating their leaves towards the available light source. This response is specifically controlled by blue light and is lacking in *p1p2* and *nph3* mutants (Inoue *et al.*, 2007). Although initially identified under monochromatic conditions, defects in petiole positioning are also apparent when seedlings are grown under 50 μmol $m^{-2}$ $s^{-1}$ white light for twelve days before examination as previously described (Sullivan *et al.*, 2008). As illustrated by wild-type seedlings (WT, Figure 5.6A), the wild-type phenotype is characterised by upward angled petioles. In comparison, the petioles of *p1p2* mutants are almost horizontal (Figure 5.6). The ability of phot1-GFP and phot1 I608E-GFP to complement this response was assessed.

Initially transgenic seedlings expressing wild-type phot1-GFP were examined. Representative seedlings are shown in Figure 5.6. Seedlings expressing increased levels of phot1-GFP (line 35-P1G/*p1p2*-A) demonstrated complementation for this response, which formally confirms a role for phot1 in petiole positioning. Compared with this control it is apparent that the expression of phot1 I608E-GFP (line 35-P1G(I608E)/*p1p2*-A) did not complement this response (Figure 5.6). However, the inability of phot1 I608E-GFP to complement this response may be a consequence of reduced protein levels as limited complementation of this response is observed in line 35-P1G/*p1p2*-G which expresses low levels of phot1-GFP (Figure 5.1C). Such data suggests that a threshold amount of wild-type phot1 expression is required to induce detectable complementation as was observed for phototropism (Figure 5.4) and raises the possibility that increased phot1 I608E-GFP protein expression may induce correct petiole positioning. Despite this possibility, the data obtained does not exclude the possibility that phot1 I608E-GFP is defective with regards this physiological response.

### *5.2.7 Expression of phot1 I608E is insufficient to complement phot1-mediated leaf expansion in p1p2 plants*

As well as mediating initial leaf positioning phots are also responsible for the expansion of the mature leaf blade in response to blue light (Takemiya *et al.*, 2005, Sakamoto and Briggs, 2002). Leaves from *p1p2* plants display an obvious curled phenotype compared to wild-type that can be restored by either phot1 or phot2 expression (Sakamoto and Briggs, 2002, Takemiya *et al.*, 2005, Kong *et al.*, 2006, Cho *et al.*, 2007). The ability of phot1 I608E-GFP to restore this response was therefore examined. Leaf curling may be quantified by comparing the leaf area directly exposed to light with the total leaf area (as measured after artificial flattening; (Cho *et al.*, 2007, Sullivan *et al.*, 2008). The ratio of these two measurements are typically expressed as an index of leaf expansion (LEI), with a completely flat leaf having a LEI of 1 and the LEI of *p1p2* plants typically being half that of wild-type (Sullivan *et al.*, 2008, Takemiya *et al.*, 2005). In order to assess the ability of phot1 I608E-GFP to complement this response plants were grown on soil for three weeks before the LEI of the fifth rosette leaf was measured and compared with controls (Figure 5.7A). Representative leaves are shown before artificial flattening (Figure 5.7B). The expression of wild-type phot1-GFP in a *p1p2* background results in restoration of wild-type levels of leaf expansion (line 35-P1G/*p1p2*-A, LEI=0.90), although plants expressing low levels of phot1-GFP appeared insufficient to complement the response as 35-P1G/*p1p2*-G plants had an LEI similar to that of the *p1p2* mutant (LEI=0.70 and 0.66 for 35-P1G/*p1p2*-G and *p1p2* respectively). This inability of plants expressing low levels of wild-type phot1-GFP to complement the leaf expansion mutant phenotype is consistent with the impaired phenotype complementation observed for phototropism and petiole positioning (Figures 5.4 and 5.6).

Characterisation of plants expressing phot1 I608E-GFP also revealed a phenotype comparable to *p1p2* plants (Figure 5.7A, LEI=0.63). This suggests that, as for petiole positioning, expression of low levels of the mutant phot1 I608E-GFP protein was insufficient to complement the leaf expansion mutant phenotype. Similarly, low levels of His-tagged phot1 I608E were not sufficient to complement this response (Appendix III, Figure 9.3). As previously described, this inability to complement the mutant phenotype may be attributable to either a lack of protein or an

inability of the mutant phot1 I608E-GFP protein to be functional in this response. Although western blot analysis of protein expression in three-day-old etiolated seedlings indicated that phot1 I608E-GFP expression was greatly reduced compared to phot1 expression in wild-type (Figure 5.1), the relative levels of protein expression in mature light-grown tissue was unclear. Western blot analysis was therefore completed to assess the levels of wild-type phot1 and phot1 I608E-GFP in three-week-old light-grown tissue (Figure 5.7C). As in etiolated seedlings (Figure 5.1) phot1 I608E-GFP expression was considerably lower than endogenous phot1 or that observed in 35-P1G/*p1p2*-A (Figure 5.7C). Expression levels of phot1 I608E-GFP in 35-P1G(I608E)/*p1p2*-A seedlings were broadly comparable to 35-P1G/*p1p2*-G, reinforcing the suggestion that reduced protein levels may account for the inability of phot1 I608E-GFP to complement this response (Figure 5.7C). Despite this, the hypothesis that phot1 I608E-GFP was not capable of complementing the leaf expansion response could not be discounted.

### *5.2.8 Phot1 I608E-GFP does not complement the chloroplast movement response*

The final phot1-mediated physiological response assayed to assess the functionality of phot1 I608E *in planta* was the orientation of chloroplasts within the cell in response to differing intensities of blue light. Both phot1 and phot2 have roles in controlling chloroplast localisation with differing light conditions inducing three alternate chloroplast positions (Kagawa, 2003). In the 'dark position', chloroplasts are loosely orientated to the lower regions of palisade mesophyll cells in a phot2-dependent fashion, while minimal illumination from above provides a directional stimulus and induces an 'accumulation' response at the mesophyll cell surface mediated by both phot1 and phot2 (Jarillo *et al.*, 2001, Kagawa *et al.*, 2001). Under high light conditions phot2 alone mediates the 'avoidance' response whereby chloroplasts are actively relocated to the side-walls of the palisade cells. The combination of these movements allows an optimisation of light capture under low light conditions and prevents chloroplast damage under intense light irradiation (Kagawa, 2003, Suetsugu and Wada, 2007, Kasahara *et al.*, 2002). *Arabidopsis p1p2* mutants show a random distribution of chloroplasts throughout the cell (Sakai *et al.*, 2001).

Monitoring chlorophyll autofluorescence in palisade mesophyll cells using confocal microscopy is a convenient method to assess chloroplast localisation. Leaves

from four-week-old plants were detached and either placed in the dark or irradiated with unidirectional blue light at a fluence rate of either 1 μmol $m^{-2}$ $s^{-1}$ or 10 μmol $m^{-2}$ $s^{-1}$ for three hours before examination as described (Sullivan *et al*., 2008, Onodera *et al.*, 2005). In wild-type leaves chloroplast accumulation and avoidance are apparent compared to the random orientation of chloroplasts incubated in the dark, while both accumulation and avoidance responses are absent in *p1p2* plants (Figure 5.8A; Sakai *et al.*, 2001). Representative images of chloroplast movement are provided in Figure 5.8B. Compared with controls, chloroplast movement was unaltered in the presence of phot1 I608E-GFP compared to *p1p2* (Figure 5.8A). This suggests that phot1 I608E-GFP is not capable of restoring chloroplast accumulation, even under higher light intensities (Figure 5.8A). It should be noted, however, that functional complementation was not observed in lines expressing low levels of wild-type phot1-GFP (Figure 5.7C, Figure 5.8A). High levels of phot1-GFP expression fully complemented the chloroplast accumulation response whilst chloroplast avoidance was not observed due to a lack of phot2 protein (35-P1G/*p1p2*-A, Figure 5.8A and B). Therefore an insufficient level of protein expression may account for the inability of phot1 I608E-GFP to complement the chloroplast accumulation response. However, as previously described it was also not possible to discount the hypothesis that incorporation of the I608E substitution within phot1 resulted in the mutated protein being non-functional *in planta* with regards this response.

### *5.2.9 Expression of phot1 I608E in a wild-type background*

Analysis of phot1 I608E functionality *in planta* by expressing either GFP- or His-tagged versions of phot1 I608E in a *p1p2* background proved inconclusive due to the low levels of phot1 I608E protein present in the identified transgenic lines (Figures 5.1-5.8; Appendix III). Despite this, the partial restoration of a phototropic response in etiolated seedlings (Figure 5.4) suggests the mutated phot1 I608E-GFP protein retained a degree of functionality *in planta*. Expression of photoreceptor mutants in a wild-type background has previously provided valuable insight into photoreceptor signalling mechanisms as inferences may be drawn from either enhancement or inhibition of endogenous photoreceptor activity (Yang *et al.*, 2000, Su and Lagarias, 2007). To further explore the function of phot1 I608E *in vivo*, the effect of phot1 I608E-GFP expression in a wild-type background was assessed.

Phot1 and phot1 I608E were transformed into wild-type *Arabidopsis* (cv. Columbia, *gl1* background) using the same expression constructs used to create 35-P1G/*p1p2* and 35-P1G(I608E)/*p1p2* lines (Figure 5.1A). The addition of a GFP-tag allowed phot1 I608E-GFP to be distinguished from endogenous phot1 when assessed by western blot. In common with the other transgenic lines created for this study, few independent lines expressing detectable levels of phot1 I608E-GFP were isolated. Although four independent homozygous T3 lines were identified by kanamycin selection after *Agrobacterium*-mediated transformation (data not shown) only one of these expressed detectable levels of protein when western blots were probed with anti-phot1 antibody. This line expressed low levels of phot1 I608E-GFP compared to endogenous phot1 levels (35-P1G(I608E)/WT-A; Figure 5.9A). A line expressing comparable levels of phot1-GFP was selected as an appropriate control for further characterisation (35-P1G/WT-A; Figure 5.9A). In order to confirm expression of GFP-tagged protein in these lines confocal microscopy was used to examine one-week-old light-grown seedlings (Figure 5.9B). In agreement with the immunoblot analysis of etiolated seedlings broadly comparable levels of GFP signal were detected in lines 35-P1G/WT-A and 35-P1G(I608E)/WT-A, thereby confirming expression of the GFP fusion protein in each line (Figure 5.9). DNA sequencing confirmed that each selected line contained the appropriate transgene (data not shown).

### *5.2.10 Phot1 I608E has increased basal levels of kinase activity when expressed in a wild-type background*

As an initial stage of characterisation of these transgenic seedlings the autophosphorylation activity of endogenous phot1 and the introduced phot1 I608E-GFP protein were assessed *in vitro*. Phot1 I608E has increased basal levels of kinase activity *in vitro* using protein derived from the baculovirus/insect cell system (Section 3.2.10; Harper *et al.*, 2004) although low levels of protein expression prevented conclusive determination of the *in vitro* kinase activity of phot1 I608E-GFP when expressed in a *p1p2* background (Figure 5.2). Additionally, characterisation of phot1 phosphorylation using protein expressed using the baculovirus/insect cell system indicated that phot1 I608E is capable of intermolecular phosphorylation *in vitro* (Section 3.2.10). It was therefore of interest to establish whether phot1 I608E-GFP expressed in a wild-type *Arabidopsis* background demonstrated altered kinase activity

and additionally whether this activity altered endogenous phot1 autophosphorylation activity in the absence of a light stimulation.

Phot1 autophosphorylation was monitored via *in vitro* kinase assays using 35-P1G/WT-A and 35-P1G(I608E)/WT-A seedlings. Three-day-old etiolated seedlings were harvested under dim red light and crude microsomal membrane fractions isolated by ultra-centrifugation before these membrane fractions were used for *in vitro* kinase assays (Figure 5.10A). Phot1 I608E-GFP isolated from transgenic *Arabidopsis* displayed an increased level of autophosphorylation compared to the phot1-GFP mock-irradiated control (Figure 5.10A). This reaffirms the suggestion that phot1 I608E-GFP has increased basal levels of autophosphorylation activity *in vitro* compared to phot1-GFP. However, the altered autophosphorylation of phot1 I608E-GFP observed in 35-P1G(I608E)/WT-A seedlings did not appear to affect the autophosphorylation of endogenous phot1 compared to the 35-P1G/WT-A control. Such data suggest that either intermolecular phosphorylation is not occurring in these samples or that the relatively low levels of phot1 I608E-GFP protein expression relative to endogenous phot1 (Figure 5.10B) prevent detection of any increase in intermolecular phosphorylation. Interestingly, the activity of endogenous phot1 in mock-treated samples appeared to be increased in the presence of either phot1-GFP or phot1 I608E-GFP compared with the untransformed control (Figure 5.10A). However, as a similar effect was observed in both 35-P1G/WT-A and 35-P1G(I608E)/WT-A seedlings it would appear that such an effect is not caused by the incorporation of the I608E substitution.

### *5.2.11 Phot1 I608E-GFP expression does not alter the magnitude of the phototropism response when expressed in a wild-type background*

As phot1 I608E retains autophosphorylation activity *in vitro* when expressed in both a *p1p2* and wild-type background it was of interest to determine whether the expression of phot1 I608E-GFP altered the ability of endogenous phot1 to mediate appropriate physiological responses. The first response examined was phototropism. As the expression of phot1-GFP and phot1 I608E-GFP under the control of a *35S* promoter has previously been shown to restore phototropism when expressed in a *p1p2* background (Figure 5.4; Cho *et al.*, 2007, Christie *et al.*, 2002) it was hypothesised

that phot1-GFP and phot1 I608E-GFP expression may enhance phototropism in a wild-type background.

To assess whether phot1 I608E-GFP was capable of altering the phototropic response when expressed in a wild-type background phototropism was initially assessed at a photon fluence rate of 1 μmol $m^{-2}$ $s^{-1}$ for 24 hours (Figure 5.11A). Representative treated seedlings are shown below. Phototropic curvature in 35-P1G(I608E)/WT-A seedlings was indistinguishable from that observed in the control 35-P1G/WT-A line, although both of these lines demonstrated enhanced phototropic curvature compared to wild-type (Figure 5.11A). To further characterise the phototropic response of these seedlings hypocotyl curvature was further examined over a range of increasing light intensities (Figure 5.11B). As discussed previously (Section 5.2.4) etiolated seedlings were irradiated with the indicated fluence of unilateral blue light for 24 hours. At increased fluence rates curvature of both 35-P1G/WT-A and P1G(I608E)/WT-A seedlings was slightly enhanced, suggesting that expression of either phot1-GFP or phot1 I608E-GFP under the control of a *35S* promoter increases phototropism in these transgenic lines relative to wild-type seedlings. However, at each fluence tested the phototropic curvature of 35-P1G(I608E)/WT-A seedlings was similar to that observed for 35-P1G/WT-A, suggesting that the expression of phot I608E-GFP in a wild-type background has a similar effect as the expression of phot1-GFP.

### *5.2.12 Phot1 I608E-GFP expression mitigates wild-type petiole positioning in wild-type seedlings*

Given the ability of phot1 I608E-GFP to restore and enhance the phototropic response in *p1p2* and wild-type plants respectively (Figures 5.4 and 5.11) it was expected that similarly moderate differences would be observed when petiole positioning was examined in 35-P1G(I608E)/WT-A seedlings. Previously presented data has shown that low levels of phot1 I608E-GFP expression were unable to complement the mutant phenotype when expressed in a *p1p2* background (Figure 5.6), although this was possibly caused by low protein expression levels. 35-P1G/WT-A and 35-P1G(I608E)/WT-A seedlings were grown alongside wild-type and *p1p2* controls under 50 μmol $m^{-2}$ $s^{-1}$ white light for twelve days as described (Sullivan *et al.*, 2008). Representative seedlings are shown in Figure 5.12A. As previously described, the

wild-type phenotype is characterised by upward angled petioles which are approximately 55° from horizontal whilst the petioles of *p1p2* seedlings have a greatly reduced angle (Figure 5.12A and B; Inoue *et al.*, 2007). Interestingly, the petioles of 35-P1G(I608E)/WT-A seedlings are angled approximately 30° from horizontal (Figure 5.12A and B) whilst 35-P1G/WT-A seedlings are indistinguishable from wild-type. Such data suggests that phot1 I608E-GFP expression is specifically capable of mitigating the wild-type petiole orientation response as the expression of phot1-GFP under the control of a *35S* promoter does not alter petiole positioning. Therefore although phot1 I608E-GFP appears capable of restoring phototropic curvature in 35-P1G(I608E)/*p1p2* seedlings (Figure 5.4A) the expression of phot1 I608E-GFP in a WT background appears to inhibit petiole positioning. This novel effect will be discussed in further detail in Section 5.3.3.

### *5.2.13 Phot1 I608E-GFP expression reduces leaf expansion in wild-type plants*

The inability of phot1 I608E-GFP to complement the leaf expansion phenotype when expressed in a *p1p2* background (Figure 5.7) suggests that either transgenic protein levels are insufficient to complement this response or that phot1 I608E-GFP is non-functional in relation to leaf expansion. To further evaluate the role of phot1 I608E-GFP in this response 35-P1G(I608E)/WT-A seedlings were examined for a leaf expansion phenotype. As described previously, leaf expansion can be quantified by comparing the area of the leaf directly exposed to the light with the total area of the leaf, which is expressed as a leaf expansion index (LEI; Sullivan *et al.*, 2008, Takemiya *et al.*, 2005). 35-P1G/WT-A and 35-P1G(I608E)/WT-A plants were grown for three weeks under 50 μmol $m^{-2}$ $s^{-1}$ white light before the LEI of the fifth rosette leaf was measured (Figure 5.13A). Representative leaves before artificial flattening are shown in Figure 5.13B. Whilst the expression of wild-type phot1-GFP under the control of a *35S* promoter did not alter leaf expansion compared with wild-type (LEI=0.78 and 0.85 for 35-P1G/WT-A plants and wild-type respectively), as previously observed for petiole positioning 35-P1G(I608E)/WT-A plants displayed an inhibition of leaf expansion (LEI=0.48). Indeed, leaf expansion in this transgenic line was similar to that observed in the *p1p2* mutant (LEI=0.46). To further assess this response, phot1 and phot2 protein levels in these lines were characterised, as it was possible that the expression of phot1 I608E-GFP using the *35S* promoter prevented or

reduced endogenous phot protein accumulation. As shown in Figure 5.13C however, it appears that neither phot1 nor phot2 protein levels were altered in the transgenic lines. This suggests that an alternate mechanism is responsible for the inhibition of leaf expansion in these lines.

### *5.2.14 Phot1 I608E expression does not alter chloroplast movement in a wild-type background*

Given that phot1 I608E expression in a wild-type background alters petiole positioning and leaf expansion in light grown tissue (Figures 5.12B and 5.13A) whilst promoting phototropism in three-day-old etiolated seedlings (Figure 5.11A) it was of interest to characterise chloroplast movement in 35-P1G/WT-A and 35-P1G(I608E)/WT-A. As previously described (Section 5.2.8), confocal microscopy was used to assess chloroplast localisation. Leaves from four-week-old plants were detached and either placed in the dark or irradiated with unidirectional blue light at a photon fluence rate of either 1 μmol $m^{-2}$ $s^{-1}$ or 10 μmol $m^{-2}$ $s^{-1}$ for three hours before examination as described (Sullivan *et al.*, 2008, Onodera *et al.*, 2005). As previously mentioned (Section 5.2.8), chloroplast accumulation and avoidance are apparent in wild-type leaves compared to the orientation of chloroplasts in dark-treated tissue (Figure 5.14A). These responses are absent in *p1p2* plants, with chloroplasts being randomly orientated regardless of the light conditions used. Representative images of chloroplast movement are shown in Figure 5.14B. Compared with controls, chloroplast movement observed in 35-P1G(I608E)/WT-A plants was indistinguishable from either wild-type plants or 35-P1G/WT-A plants (Figure 5.14A). This suggests that the expression of phot1 I608E-GFP in a wild-type background does not alter chloroplast accumulation in response to the light fluence rates assessed.

## 5.3 Discussion

### *5.3.1 Phot1 I608E-GFP extracted from plant tissue has altered levels of autophosphorylation activity*

Insect-expressed phot1 I608E displays increased basal levels kinase activity *in vitro* compared with wild-type phot1 (Section 3.2.10; Harper *et al.*, 2004). To confirm

whether phot1 I608E-GFP expressed in transgenic plants also displayed this phenotype crude microsomal extracts were isolated from transgenic plants and phot1 autophosphorylation activity monitored after light irradiation in comparison to mock-treated samples. Consistent with the insect-expressed protein results (Harper *et al.*, 2004), phot1 I608E-GFP expressed *in planta* retains kinase activity as shown by the autophosphorylation observed in Figures 5.2A and 5.10A. Also in agreement with insect-expressed protein results (Harper *et al.*, 2004), phot1 I608E-GFP has higher levels of *in vitro* autophosphorylation activity in mock-treated samples compared to phot1-GFP controls (Figures 5.2A and 5.10A).

The increased basal levels of activity observed in these independent transgenic *Arabidopsis* lines further validates the use of insect-expressed protein for assessing the mode of phot1 activation. As discussed in Chapter 3, however, the ability to incorporate $^{32}$P-ATP *in vitro* suggests that the mutant phot1 I608E-GFP protein is not completely phosphorylated *in vivo*. This apparent contradiction may either be explained by inhibition of phot1 I608E autophosphoryation *in vivo* through an unknown mechanism or by the presence of a phosphatase which acts to dephosphorylate phot1 *in planta*. Such a phosphatase may act to retain at least a portion of phot1 I608E-GFP in an unphosphorylated state. The existence of a phot1 phosphatase has been suggested previously, although a candidate protein for this function has yet to be identified (Salomon *et al.*, 2003, Kinoshita *et al.*, 2003).

One apparent inconsistency between the activity of phot1 I608E-GFP extracted from 35-P1G(I608E)/*p1p2*-A plants compared to that of 35-P1G(I608E)/WT-A seedlings and phot1 I608E expressed in insect cells was the increased degree of light-induced kinase activity observed in 35-P1G(I608E)/*p1p2*-A extracts (Figures 5.2A and 5.10A). Examination of kinase activity in 35-P1G(I608E)/*p1p2*-A seedlings was hindered by a lack of T3 seed and this assay was therefore only completed twice, although results were consistent between these experiments. Whilst a greater degree of light-induced kinase activity was observed in this line it should be noted that the relative activity of phot1 I608E-GFP was higher when compared to phot1-GFP, consistent with the notion that the I608E mutation increases phot1 kinase activity. To further determine the degree of light-induced kinase activity in *Arabidopsis* membrane extracts it will be necessary to identify further independent transgenic lines expressing phot1 I608E-GFP in both *p1p2* and wild-type backgrounds.

A curious discrepancy in this dataset is the reduced light-inducible autophosphorylation activity of phot1-GFP when expressed in a *p1p2* background (Figure 5.2). The decreased autophosphorylation activity of phot1-GFP is particularly apparent when compared with the typical light-induced *in vitro* autophosphorylation observed for endogenous phot1 isolated from wild-type etiolated seedlings (Figure 5.2A). Despite this reduced *in vitro* autophosphorylation 35-P1G/*p1p2*-A seedlings were able to fully complement all characterised phot1 phenotypes (Figures 5.4-5.8), suggesting that the impaired kinase activity does not prevent complementation of physiological responses. Whilst the reason for this discrepancy is unknown at present a similar decrease in light-induced kinase activity has previously been observed in lines expressing phot1-GFP (Sakamoto and Briggs, 2002). It is therefore possible that addition of a C-terminal GFP-tag limits phot1 autophosphorylation without preventing function *in planta*.

### *5.3.2 Phot1 I608E expression is capable of partially complementing the phototropic response*

The expression of phot1 I608E-GFP allowed a partial restoration of phototropism when expressed in a *p1p2* background (Figure 5.4). Such data suggests that phot1 I608E is capable of complementing the phototropic response at light fluence rates at which phot1 is the sole functional photoreceptor in wild-type plants (Christie, 2007). Phototropic curvature in 35-P1G(I608E)/*p1p2*-A seedlings was improved at higher light intensities, although interpretation of these data is complicated by the consideration that the *phot2-1* allele used in this analysis is not a complete null and has previously been shown capable of expressing very low levels of phot2 protein in etiolated seedlings (Cho *et al.*, 2007). The observed enhancement in phototropic curvature observed at higher fluences in 35-P1G(I608E)/*p1p2*-A seedlings may therefore be partially accounted for by intermolecular phosphorylation between phot1 I608E-GFP and residual phot2 as previously hypothesised to account for residual phototropism observed in a blinded version of phot1 at intermediate light fluences (Cho *et al.*, 2007). It should be noted however that the increased curvature observed in 35-P1G(I608E)/*p1p2*-A seedlings at higher fluences was greater than that observed in 35-P1G/*p1p2*-G seedlings (which express low levels of phot1-GFP). This suggests that the involvement of any residual phot2 is minimal.

A further phenotype observed in lines expressing phot1-GFP under the control of a *35S* promoter in either a *p1p2* or wild-type background was an increase in phototropic curvature compared with wild-type seedlings which was particularly apparent at higher fluence levels (Figures 5.4B and 5.11B). Such a response has been reported previously in transgenic lines expressing phot1 in a *p1p2* background (Cho *et al.*, 2007) and is most likely a consequence of ectopic phot1 expression caused by the use of the *35S* promoter.

In order to further evaluate the role of phot1 I608E in mediating a phototropic response the phosphorylation status of NPH3 was assessed after *in vivo* blue light irradiation compared with mock-treated controls as it has been proposed that NPH3 dephosphorylation is a necessary component of the phototropic response (Pedmale and Liscum, 2007). Whilst etiolated seedlings expressing phot1 I608E retained a degree of phototropism (Figure 5.4), it was not possible to detect an alteration in NPH3 electrophoretic mobility in 35-P1G(I608E)/*p1p2* seedlings compared to a *p1p2* negative control. Expression of wild-type phot1-GFP induced an observable alteration in NPH3 electrophoretic mobility in response to *in vivo* light stimulation (Figure 5.5). Such data may correlate with the possible cross-phosphorylation of residual phot2 by phot1 I608E-GFP to induce phototropism as discussed above as previous work has shown that phot2 induces phototropism in the absence of NPH3 dephosphorylation (Pedmale and Liscum, 2007). These data suggest that phototropism may occur in the absence of a detectable change in NPH3 electrophoretic mobility although the low levels of phot1 I608E-GFP protein expression in the transgenic lines assessed prevent exclusion of the possibility that NPH3 dephosphorylation occurs at a level which is undetectable by immunoblot analysis in these lines.

### *5.3.3 Petiole positioning and leaf movement are altered by phot1 I608E-GFP expression in a wild-type background*

Although the necessity of phots for phototropism has been well-defined (Huala *et al.*, 1997, Christie *et al.*, 2002, Liscum and Briggs, 1995) it is only relatively recently that the role of phots in initial leaf positioning has been characterised (Inoue *et al.*, 2007). In the absence of blue light *Arabidopsis* petioles extend in a horizontal position which is phenocopied by the *p1p2* mutant under all light conditions (Inoue *et al.*, 2007). Recent work has described two apparently independent signalling pathways which act

at control petiole positioning (Inoue *et al.*, 2007). Under low levels of blue light (0.1 μmol $m^{-2}$ $s^{-1}$) petioles are re-orientated so that they angle upwards towards the blue light source, allowing optimal light harvesting (Inoue *et al.*, 2007). This low-intensity blue light pathway requires both phot1 and NPH3 as *Arabidopsis* seedlings deficient in either do not display the described leaf positioning. Intriguingly, there also appears to be an independent medium-fluence sensing pathway that requires phot2 but neither phot1 nor NPH3 (Inoue *et al.*, 2007). Under medium-intensity blue light (5 μmol $m^{-2}$ $s^{-1}$) both *phot1* and *nph3* mutants displayed correct petiole positioning which was not observed in the *p1p2* mutant (Inoue *et al.*, 2007). Such data is consistent with the known differing light sensitivities of phot1 and phot2 (Christie, 2007) and suggests that multiple signalling pathways converge to allow correct petiole positioning. The data presented here suggests that phot1 I608E-GFP is unable to complement leaf positioning under white light when expressed in a *p1p2* background (Figure 5.6), although the low levels of transgenic protein expressed in these lines preclude definitive analysis and it is possible that higher light fluences may allow phenotype complementation. However, the altered phenotypes observed in 35-P1G(I608E)/WT plants suggest that phot1 I608E may act to inhibit petiole positioning rather than promote this response (Figure 5.12). This is surprising given the ability of phot1 I608E-GFP to functionally complement phototropism when expressed in a *p1p2* background (Figure 5.4). However, this inhibitory effect was repeatedly observed in this line. To further confirm this phenotype it will be necessary to identify and characterise additional 35-P1G(I608E)/WT lines.

An additional role of phots in mature *Arabidopsis* is in leaf expansion. In a similar fashion to petiole positioning, phot1 I608E-GFP expression was not able to complement leaf expansion when expressed in a *p1p2* background (Figure 5.7) whilst expression of phot1 I608E-GFP in a wild-type background appeared to inhibit leaf expansion (Figure 5.13). Similar to the altered petiole positioning observed in 35-P1G(I608E)/WT-A seedlings this effect was unexpected but reproducible. Wild-type *Arabidopsis* grown under red light display a distinctive leaf curling phenotype that is phenocopied by the *p1p2* mutant under all light conditions (Takemiya *et al.*, 2005, Sakai *et al.*, 2001). Expression of phot1 or phot2 is sufficient to restore wild-type leaf expansion (Sakamoto and Briggs, 2002, Takemiya *et al.*, 2005). As for petiole positioning, the roles of phot1 and phot2 in regulating leaf expansion can be separated into low-blue and high-blue signalling pathways, with phot1 (in combination with

NPH3) being responsible for low-blue mediated leaf expansion (Inoue *et al.*, 2007). Phot2 acts via an NPH3-independent pathway at higher levels of blue light (Inoue *et al.*, 2007).

Given that NPH3 has a role in phot1-mediated petiole positioning and leaf expansion, it is possible that a common mechanism is responsible for the disruptions observed in both of these physiological responses. However, as little is known regarding the components of these phot-induced signalling cascades it is difficult to interpret the presented data. One possibility is that phot1 I608E-GFP acts to restrict phot-mediated $Ca^{2+}$ import and $Ca^{2+}$ release from intracellular stores. An increase in cytosolic $Ca^{2+}$ as a component of phot-initiated signal transduction is required for other physiological responses (such as phototropism and stomatal opening) and phot1-mediated repression of phot2 signalling has previously been suggested to occur during phot1-mediated $Ca^{2+}$ import (Harada and Shimazaki, 2007). However, at present experimental evidence is lacking for $Ca^{2+}$ signalling involvement in either petiole positioning or leaf expansion.

It has also been suggested that leaf positioning and leaf expansion phenotypes are mediated by modulation of auxin levels across the plant organ in a mechanism similar to the auxin-mediated differential growth which results in phototropism (Esmon *et al.*, 2006, Inoue *et al.*, 2007). Indeed, auxin-insensitive mutants also have curled leaves which has been attributed to differential growth between the upper and lower sides of the leaf (Stowe-Evans *et al.*, 1998, Watahiki and Yamamoto, 1997, Hobbie and Estelle, 1995). The related *Arabidopsis* protein kinase PINOID positively regulates auxin transport (Benjamins *et al.*, 2001) and has a role in regulating the localisation of PIN-FORMED proteins which are thought to have a role in auxin transport (Friml, 2004). It will therefore be of interest to determine whether auxin distribution is altered in 35-P1G(I608E)/WT plants. This could be achieved by crossing the homozygous 35-P1G(I608E)/WT line isolated as part of this study with a transgenic line containing an auxin-responsive reporter gene (Friml *et al.*, 2002). However, if auxin distribution were altered in the identified 35-P1G(I608E)/WT line it is unclear why phototropism was unaffected by phot1 I608E expression in a wild-type background. The functional activity of phot1 I608E-GFP *in planta* will be further discussed in Section 5.3.6.

### *5.3.4 Phot1 I608E expression did not alter chloroplast movement responses in either p1p2 or wild-type plants*

As for other phot-mediated responses, chloroplast movement is mediated by phot1 at lower light fluences whilst phot2 is the dominant photoreceptor at increased light levels (Wada *et al.*, 2003). The inability of phot1 I608E-GFP to alter chloroplast movements in 35-P1G(I608E)/WT lines (Figure 5.14A and B) in contrast to the observed effects on leaf positioning and leaf expansion is possibly a consequence of different signalling intermediates being required for chloroplast movement (Wada *et al.*, 2003, Inoue *et al.*, 2007). Given that chloroplast movement occurs at a sub-cellular level (Wada *et al.*, 2003) it is perhaps unsurprising that different proteins are involved in the signalling pathway associated with this response. In addition, 35-P1G(I608E)/*p1p2* plants did not demonstrate a chloroplast accumulation response under the light conditions examined (Figure 5.8A and B), suggesting that low levels of phot1 I608E-GFP expression were insufficient to restore the chloroplast accumulation response.

The lack of complementation observed in 35-P1G(I608E)/*p1p2* lines may be a consequence of low protein levels observed in mature leaf tissue (Figure 5.7C), although it is not possible to discount the possibility that the lack of detectable chloroplast accumulation is a consequence of the relative simplicity of the methodology used. Similarly, alterations in chloroplast positioning in 35-P1G(I608E)/WT-A plants may be apparent with the use of more sophisticated analysis. Such further work should include the irradiation of individual cells with higher fluences of blue light as increased fluences of light may be required to induce a detectable response in these transgenic plants in a similar manner to that observed for phototropic responses (Figure 5.4B). Such work could be completed using microbeam irradiation as previously described (Sakai *et al.*, 2001).

### *5.3.5 Characterisation of phot1 I608E function in additional phot1-mediated physiological functions*

In addition to the physiological responses described in this chapter, phot1 is also responsible for a range of other blue-light responsive phenotypes including hypocotyl growth inhibition and stomatal opening (Christie, 2007). If phot1 I608E were constitutively active *in planta* it would be expected that these responses would also be

altered by phot1 I608E expression. For example, expression of the phot2 kinase domain in isolation reduces hypocotyl elongation in etiolated seedlings via an unknown mechanism which requires kinase activity within the expressed domain (Kong *et al.*, 2007). Phot1-mediated hypocotyl growth inhibition is a rapid, transient blue light response which is controlled in the longer term by cryptochrome function (Folta and Spalding, 2001). To assess the role of phot1 I608E-GFP in this response the length of three-day-old etiolated 35-P1G(I608E)/*p1p2*-A seedling hypocotyls were measured. However no marked differences in hypocotyl length were observed between *p1p2* and 35-P1G(I608E)/*p1p2*-A (data not shown). It therefore appears that phot1 I608E-GFP expression does not alter either hypocotyl length although it is possible that the low expression levels of phot1 I608E-GFP in the transgenic lines may have prevented characterisation of an altered phenotype. In order to further assess the role of phot1 I608E-GFP in the rapid, transient inhibition of hypocotyl expansion it will be necessary to grow seedlings under repeated brief pulses of blue light as previously described (Folta and Spalding, 2001). Unfortunately, the growth room conditions and the high resolution imaging techniques required for this assay (Folta and Spalding, 2001) were not readily available for use in this study.

Attempts were also made to investigate the effect of phot1 I608E-GFP expression on stomatal opening. Stomatal opening is controlled by a range of different factors including sub-stomatal $CO_2$ concentration, sugar levels and relative levels of red and blue light (Shimazaki *et al.*, 2007), with blue light responses being primarily mediated by phots (Kinoshita *et al.*, 2001). In contrast to wild-type, the stomata of *p1p2* plants do not extrude protons in response to blue light and this inhibition of proton pumping prevents blue-light induced stomatal opening (Kinoshita *et al.*, 2001, Dietrich *et al.*, 2001). Proton extrusion in wild-type plants is mediated by a $H^+$-ATPase which is activated indirectly by phot1 (Dietrich *et al.*, 2001, Takemiya *et al.*, 2006). It was therefore hypothesised that phot1 I608E-GFP expression may promote stomatal opening in the absence of a blue light stimulus. Preliminary experiments comparing stomatal opening in dark-incubated plants with those irradiated with 10 μmol $m^{-2}$ $s^{-1}$ blue light indicated that there was no difference in stomatal opening between 35-P1G(I608E)/*p1p2* plants and *p1p2* controls. Future experiments should focus on whether 35-P1G(I608E)/*p1p2* plants have increased stomatal apertures under red light compared to controls as this may increase the sensitivity of the assay.

### *5.3.6 Activity of phot1 I608E in planta*

In this chapter I have attempted to characterise the role of phot1 I608E when expressed *in planta*. Whilst a partial complementation of phototropism was observed in 35-P1G(I608E)/*p1p2* seedlings, phot1 I608E expression did not appear to complement other phot-mediated responses. Although analysis of this data has been greatly impeded by the reduced levels of phot1 I608E expression observed in transgenic lines the presented data suggests that phot1 I608E is not constitutively active *in planta*. A truncated form of phot2 lacking both LOV domains (phot2 kinase) constitutively phosphorylates casein *in vitro* (Matsuoka and Tokutomi, 2005) and induces constitutive phot2 responses *in planta* (Kong *et al.*, 2007). Interestingly, phototropic curvature in wild-type plants was inhibited by the expression of phot2 kinase, suggesting that constitutive phot2 activity prevents phototropic curvature (Kong *et al.*, 2007). This presumably occurs by preventing the creation of a gradient of phot2 activity across the hypocotyl after blue light stimulation. Given that phot1 I608E restores phototropism in *p1p2* seedlings (Figure 5.4) and does not inhibit phototropism when expressed in a WT background (Figure 5.11) it is plausible that light irradiation stimulates phot1 I608E signalling *in planta*, in contrast to the constitutive activity observed for phot2 kinase. How this correlates with the increased autophosphorylation activity of phot1 I608E observed *in vitro* in the absence of light (Figures 5.2 and 5.10) is unclear. However, phot-mediated physiological responses are apparent at photon fluences under which phot autophosphorylation is not observed (Briggs *et al.*, 2001) and additionally phots are phosphorylated at different residues in a hierarchical manner dependent on the light intensity used for irradiation (Salomon *et al*., 2003). This suggests that phosphorylation acts to modulate phot signalling at high fluences rather than acting as a primary signal (Salomon *et al.*, 2003). Indeed, the data presented here is broadly consistent with the hypothesis that phot1 I608E displays increased autophosphorylation without constitutively activating phot1-signalling pathways in the absence of light. However, it remains necessary to reconcile the apparently inhibitory role of phot1 I608E-GFP in petiole positioning and leaf expansion in P1G(I608E)/WT-A plants with the partial restoration of phototropism observed in 35-P1G(I608E)/*p1p2*-A seedlings. One possibility is that phot1 autophosphorylation has differing roles in the regulation of each of these physiological responses and therefore the mutated phot1 I608E protein retains activity

in only a subset of phot1-mediated responses. However, further investigation is required to assess the validity of this speculation. Recent work expressing a mutated form of phytochrome A (phyA Y242H) in wild-type and *phyA* mutant backgrounds has revealed that this mutated form of phyA (containing a single point mutation) retains a weak light-independent activity resulting in a mild constitutively photomorphogenic (COP) phenotype when expressed in a *phyA* background but which inhibits the action of endogenous phyA in a wild-type background (Su and Lagarias, 2007). Such an example illustrates how the activity of a mutated plant photoreceptor may have differing consequences when expressed in different genetic backgrounds. The consequences of such a possibility will be discussed in further detail in Chapter 6.

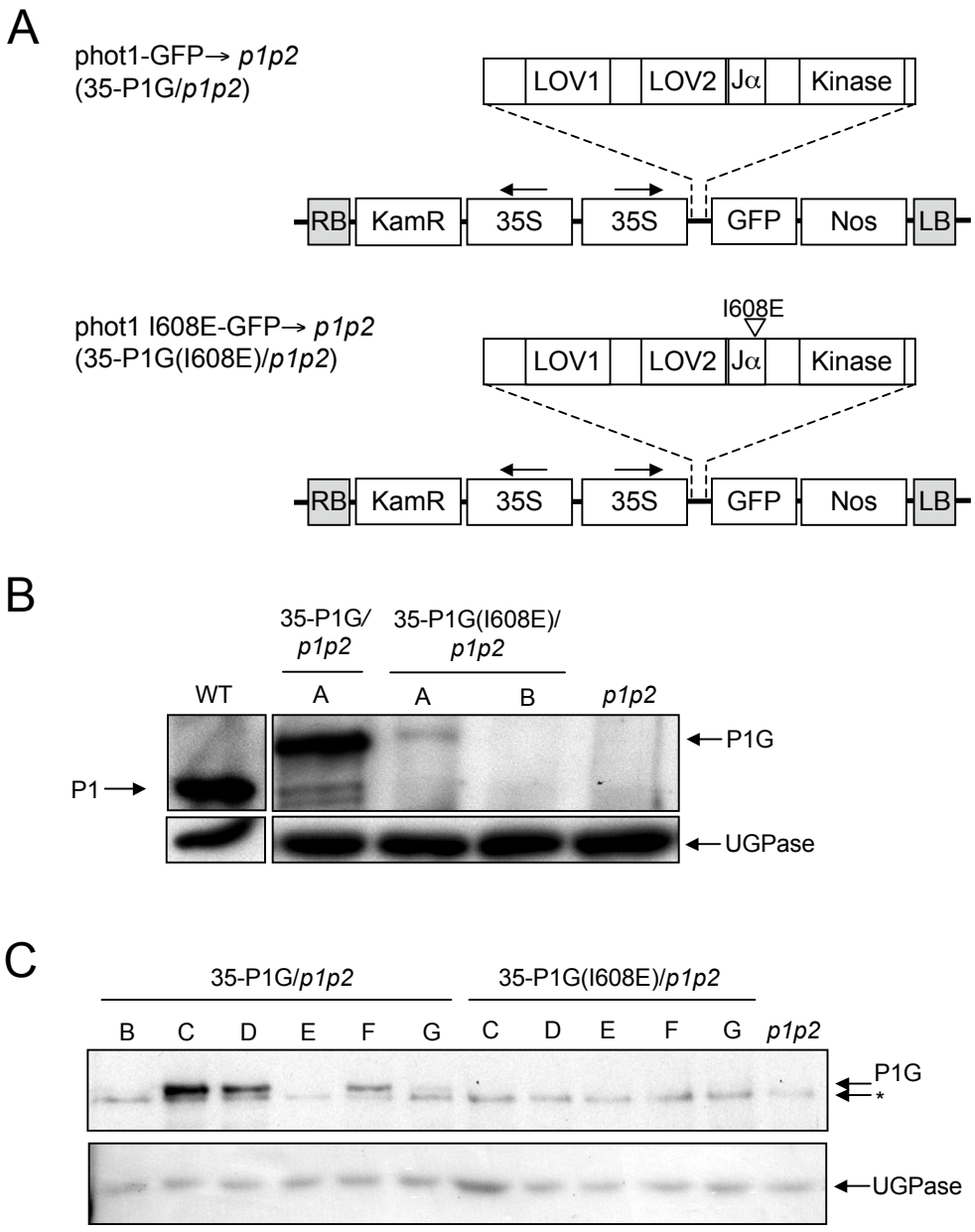


**Figure 5.1 Expression of phot1-GFP and phot1 I608E-GFP in *phot1-5 phot2-1* mutant plants. (A)** Schematic diagram showing construction of expression vectors used to transform *phot1-5 phot2-1* (*p1p2*) plants. The I608E point mutation within the Jα-helix is indicated. Transgenic lines were created expressing either phot1-GFP (P1G) or phot1 I608E-GFP in a *p1p2* background (35-P1G/*p1p2* and 35-P1G(I608E)/*p1p2* respectively). **(B)** Western blot analysis of P1G and P1G I608E expression in T3 transgenic lines compared with *p1p2* and wild-type (WT) controls. Crude soluble protein extracts (30 μg) from three-day-old etiolated seedlings were probed with anti-phot1 antibody (upper panel). The lane containing crude protein extract from WT was separated from the remaining samples for clarity of exposure, although exposure times in each case were identical. P1G and wild-type phot1 (P1) are indicated. Blots were probed with a UDP-GLUCOSE PYROPHOSPHORYLASE (UGPase) antibody as a loading control (lower panel). **(C)** Western blot analysis of P1G expression in additional heterozygous T2 transgenic lines. Western blots were probed as described in (B). P1G and a non-specific band (*) are indicated.

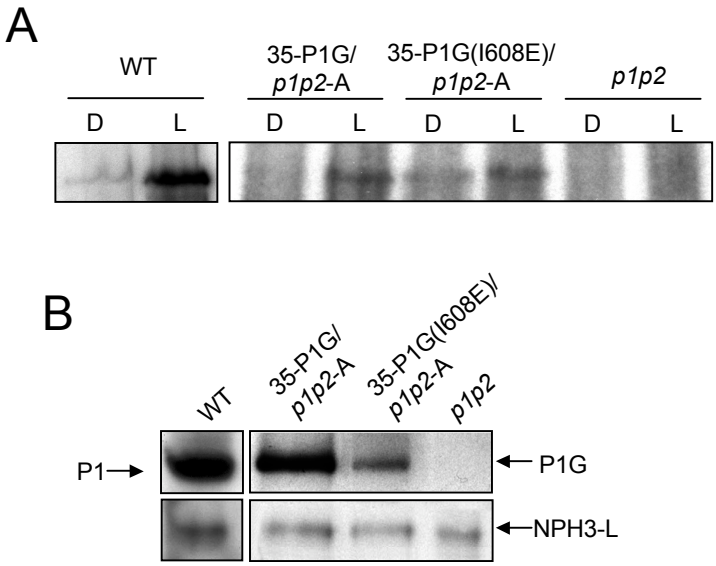


**Figure 5.2 Autophosphorylation analysis of phot1-GFP and phot1 I608E-GFP expressed in a *phot1-5 phot2-1* background. (A)** Autophosphorylation activity in membranes isolated from T3 transgenic *Arabidopsis* expressing phot1-GFP (35-P1G/*p1p2*-A) or phot1 I608E-GFP (35-P1G(I608E)/*p1p2*-A) was compared with activity from *phot1-5 phot2-1* seedlings. Etiolated three-day-old seedlings were used and all manipulations were completed under dim red light. Samples were given a mock irradiation (D) or irradiated with white light (L) at a total photon fluence rate of 30,000 μmol $m^{-2}$ $s^{-1}$ prior to the addition of radiolabelled ATP. Light-induced phosphorylation of wild-type phot1 protein extracts (WT) is shown as a comparison. For clarity lanes containing WT sample were run on a separate gel. However, length of exposure to X-ray film was identical in each case. **(B)** Western blot analysis of phot1 (P1) and phot1-GFP (P1G) protein levels using anti-phot1 antibody (upper panel). The lane containing crude microsomal extract from WT was separated from the remaining samples for clarity of exposure, although exposure times in each case were identical. As a protein loading control the blot was reprobed with anti-NON-PHOTOTROPIC HYPOCOTYL 3-LIKE (NPH3-L) antibody (lower panel).

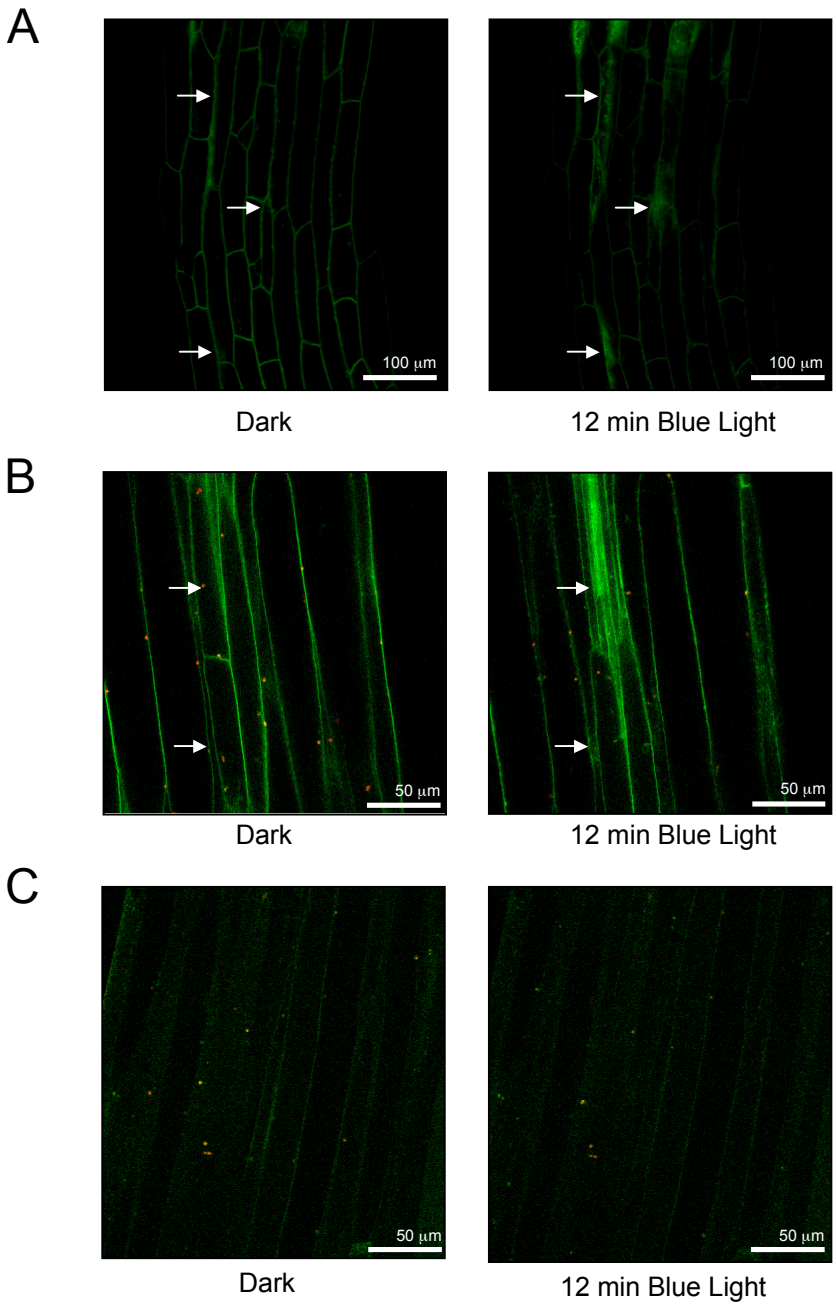


**Figure 5.3 Analysis of blue light-induced movement of phot1-GFP and phot1 I608E-GFP expressed in a *phot1-5 phot2-1* background. (A)** Analysis of blue light-dependent movement of phot1-GFP expressed under the *PHOT1* promoter. Confocal images of single optical sections of hypocotyl epidermal cells from an etiolated seedling before and after 12 min blue light exposure are shown. Blue light was provided by the excitation laser scan. Increased GFP signal in the cytoplasm is indicated (arrows). **(B)** Analysis of blue light-dependent movement of phot1-GFP in homozygous T3 35-P1G/*p1p2*-A seedlings. Confocal images of single optical sections of hypocotyl epidermal cells from an etiolated 35-P1G/*p1p2*-A seedling before and after 12 min blue light. Increased GFP signal in the cytoplasm after irradiation is indicated (arrows). **(C)** Analysis of phot1 I608E-GFP localisation in homozygous T3 35-P1G(I608E)/*p1p2*-A seedlings. Confocal images of single optical sections of hypocotyl epidermal cells from an etiolated 35-P1G(I608E)/*p1p2*-A seedling are shown before and after 12 min blue light.

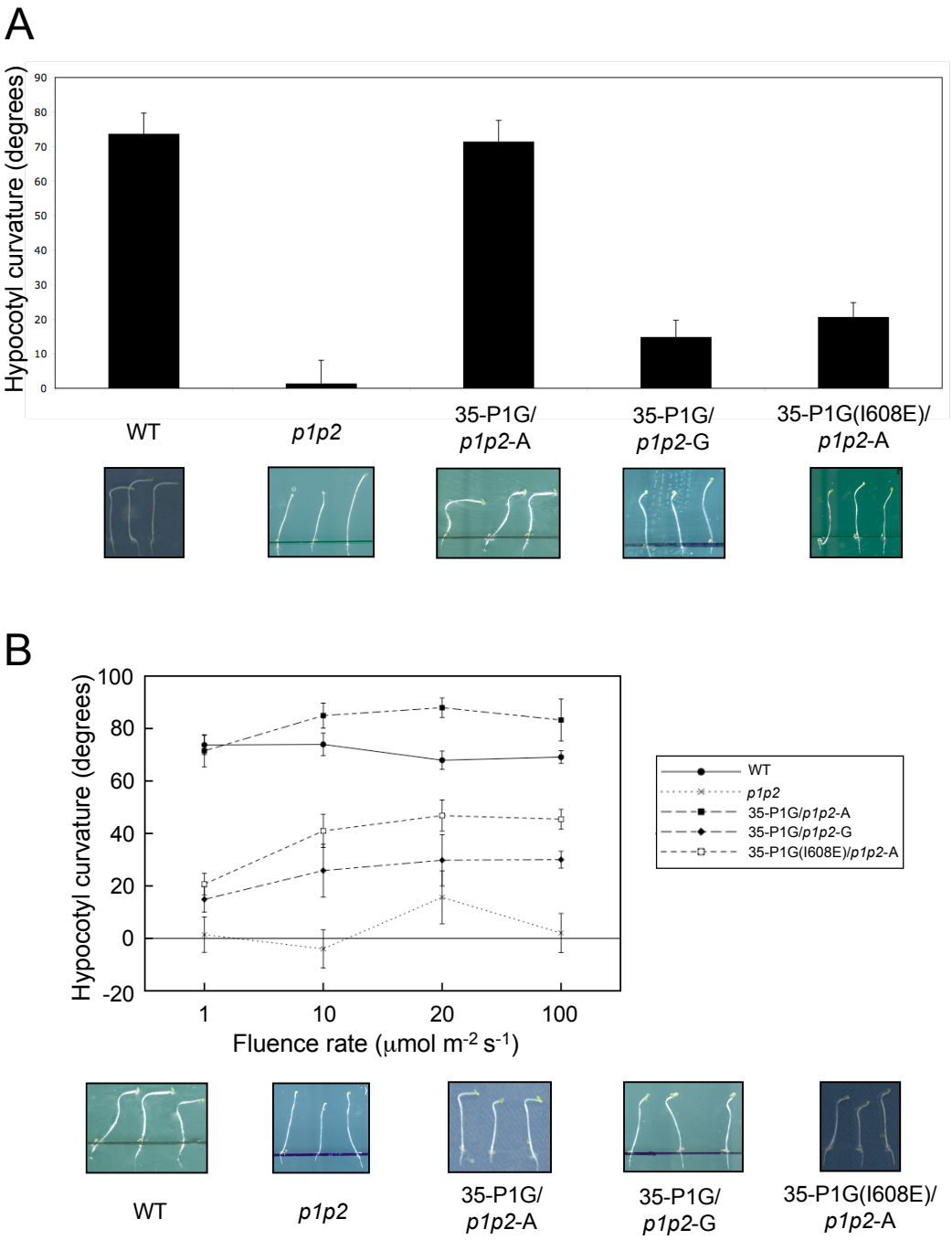


**Figure 5.4 Analysis of phototropic curvature in etiolated seedlings expressing phot1-GFP or phot1 I608E-GFP in a *phot1-5 phot2-1* background. (A)** Phototropic curvature of wild type (WT), *phot1-5 phot2-1* (*p1p2*) or transgenic *Arabidopsis* seedlings expressing either phot1-GFP (35-P1G/*p1p2*) or phot1 I608E-GFP (35-P1G(I608E)/*p1p2*) were examined. Seedlings were grown in the dark for three days before being exposed to 1 μmol $m^{-2}$ $s^{-1}$ unilateral blue light for 24 hours. Error bars indicate standard error (n≥15). Representative images are shown below. **(B)** Phototropism fluence rate response curves for lines described in (A). Seedlings were grown in the dark for three days before being exposed to unilateral blue light at the indicated fluence for 24 hours. Error bars indicate standard error (n≥15). Representative images of seedlings illuminated with 10 μmol $m^{-2}$ $s^{-1}$ unilateral blue light are shown below.

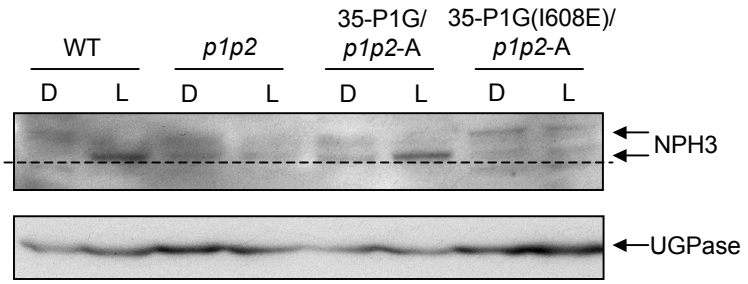


**Figure 5.5 Assessment of light-induced shift in the electrophoretic mobility of *Arabidopsis* NPH3.** Three-day-old etiolated *Arabidopsis* seedlings expressing phot1-GFP (35-P1G/*p1p2*-A) or phot1 I608E-GFP (35-P1G(I608E)/p1p2-A) were mock-treated (D) or irradiated 10 μmol $m^{-2}$ $s^{-1}$ blue light for four hours (L) alongside wild-type (WT) and *phot1-5 phot2-1* (*p1p2*) controls. Crude soluble protein was extracted and analysed by immunoblotting with an anti-NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3) antibody (upper panel). NPH3 antibody recognises a doublet in mock-treated three-day-old etiolated WT seedlings and a singlet in blue light-treated WT seedlings (Pedmale and Liscum, 2007). The position of these forms is indicated (arrows). The dashed line indicates the highest mobility edge of NPH3. Blots were probed with a UDP-GLUCOSE PYROPHOSPHORYLASE (UGPase) antibody as a loading control (lower panel).


**Figure 5.6 Characterisation of petiole positioning in *phot1-5 phot2-1* plants expressing phot1-GFP or phot1 I608E-GFP.** Plants were grown for 12 days under 50 μmol $m^{-2}$ $s^{-1}$ white light in long day conditions (16 hours light, 8 hours dark). Wild-type (WT), *phot1-5 phot2-1* (*p1p2*), 35-P1G/*p1p2*-A, 35-P1G/*p1p2*-G and 35-P1G(I608E)/*p1p2*-A seedlings were examined.

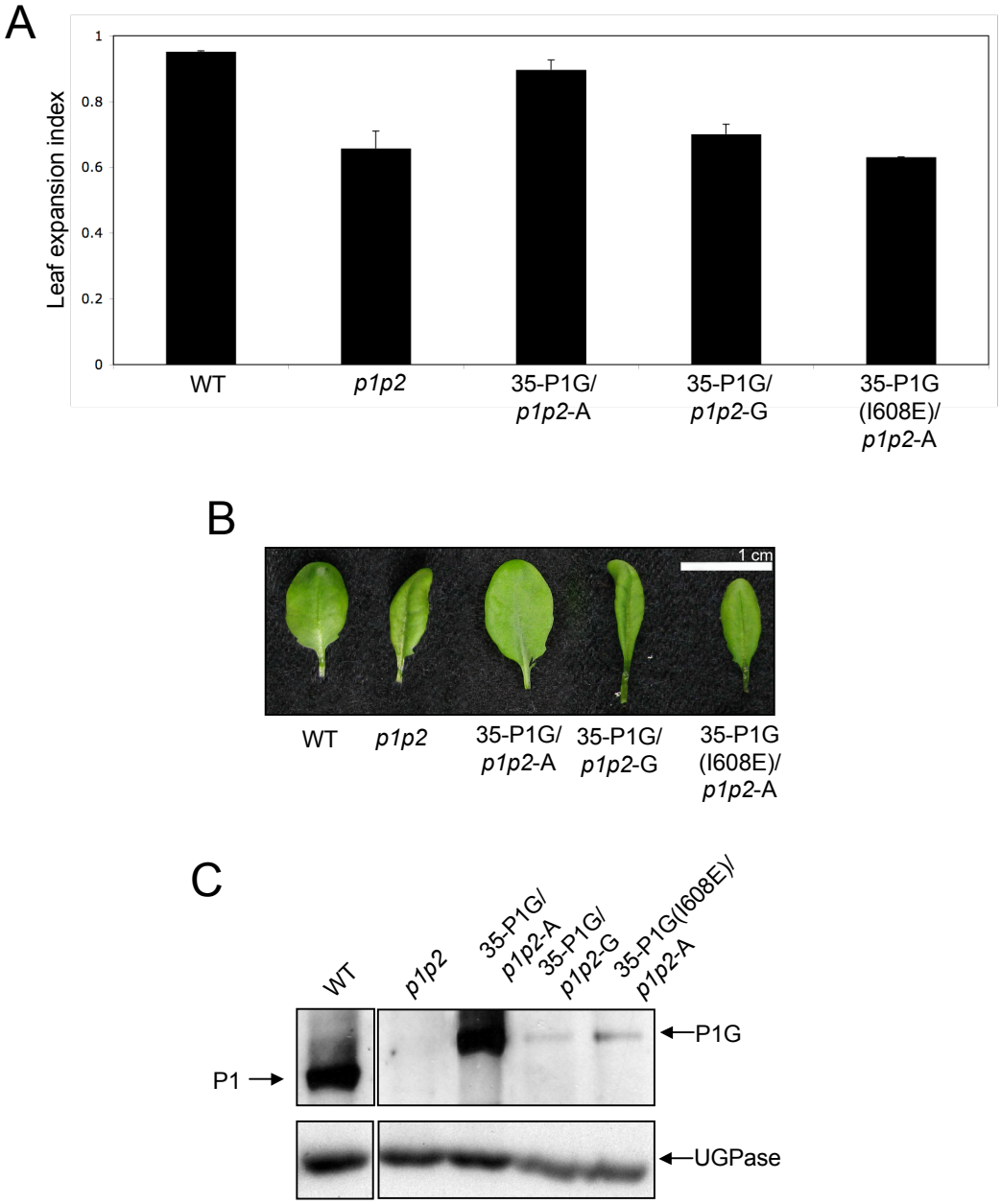


**Figure 5.7 Analysis of leaf expansion in *phot1-5 phot2-1* plants expressing phot1-GFP or phot1 I608E-GFP. (A)** Leaf expansion index of the fifth rosette leaves of wild type (WT), *phot1-5 phot2*-1 (*p1p2*), 35-P1G/*p1p2*-A, 35-P1G/*p1p2*-G and 35-P1G(I608E)/*p1p2*-A plants. Plants were grown on soil for three weeks under 50 μmol $m^{-2}$ $s^{-1}$ constant white light. The leaf expansion index is expressed as the ratio of leaf area before and after artificial flattening. Error bars indicate standard error (n=10). **(B)** Representative fifth rosette leaves from plants described in (A). **(C)** Western blot analysis of phot1 expression levels in three-week-old plants described in (A). Crude soluble protein extracts (30 μg) were probed with anti-phot1 antibody (upper panel). The lane containing crude protein extract from WT was separated from the remaining samples for clarity of exposure, although exposure times in each case were identical. Phot1-GFP (P1G) and phot1 (P1) are indicated. Blots were probed with a UDP-GLUCOSE PYROPHOSPHORYLASE (UGPase) antibody as a protein loading control (bottom panel).

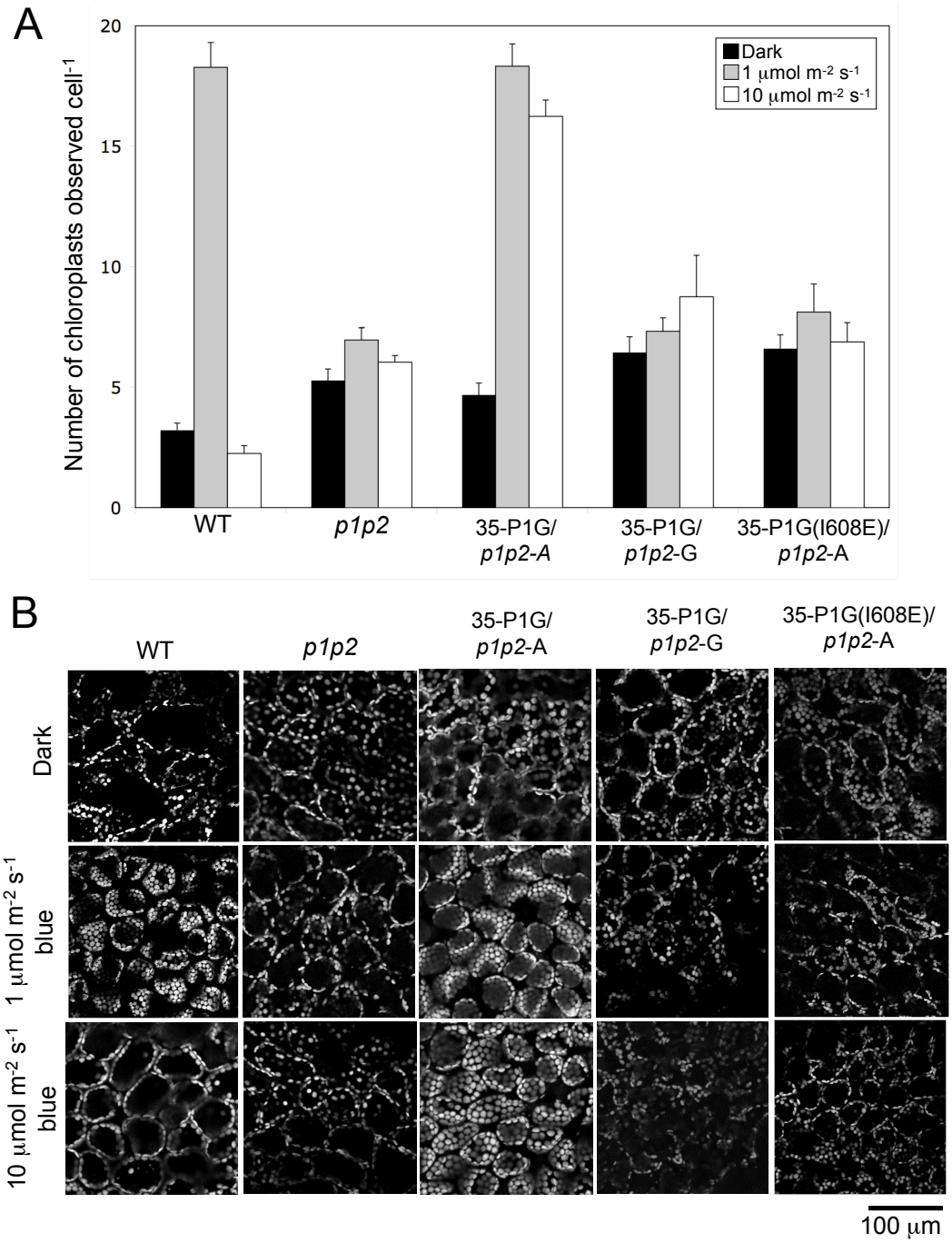


**Figure 5.8 Analysis of chloroplast positioning in *phot1-5 phot2-1* plants expressing phot1-GFP or phot1 I608E-GFP. (A)** Chloroplast positioning in *Arabidopsis* expressing phot1-GFP or phot1 I608E-GFP in a *phot1-5 phot2-1* (*p1p2*) background. Wild-type (WT), *p1p2,* 35-P1G/*p1p2*-A, 35-P1G/*p1p2*-G *and* 35-P1G(I608E)/*p1p2*-A plants were examined. Plants were grown on soil for four weeks under 50 μmol $m^{-2}$ $s^{-1}$ white light in long days (16 hours light, 8 hours dark) before rosette leaves were detached and transferred to either dark, 1 μmol $m^{-2}$ $s^{-1}$, or 10 μ mol $m^{-2}$ $s^{-1}$ unilateral blue light for three hours. Chloroplast positioning was visualised via chlorophyll autofluorescence by confocal microscopy. Shown values are the average number of chloroplasts at the upper face of palisade mesophyll cells. Error bars indicate standard error (n=25). **(B)** Example images illustrating the number of chloroplasts at the upper face of the palisade mesophyll cells as described in (A).

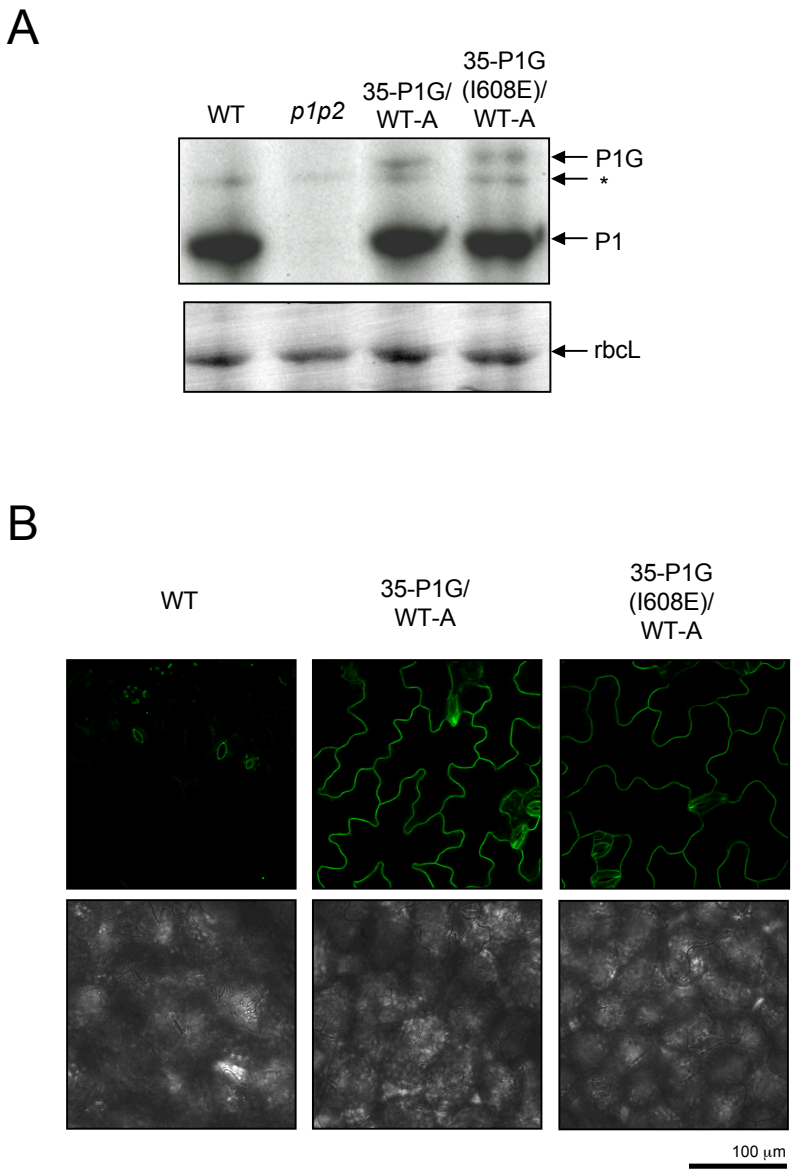


**Figure 5.9 Expression of phot1-GFP and phot1 I608E-GFP in wild-type plants.** **(A)** Western blot analysis of phot1 expression in wild-type (WT), *p1p2* and T3 transgenic lines expressing phot1-GFP (35-P1G/WT) or phot1 I608E-GFP (35-P1G(I608E)/WT). Crude soluble protein extracts (30 µg) from three-day-old etiolated seedlings were probed with anti-phot1 antibody (upper panel). Phot1-GFP (P1G), phot1 (P1) and a non-specific band (*) are indicated. A ponceau stain highlighting the large subunit of RIBULOSE-BISPHOSPHATE CARBOXYLASE (rbcL) is shown below as a protein loading control. **(B)** Examination of phot1-GFP and phot1 I608E-GFP expression in WT, 35-P1G/WT-A and 35-P1G(I608E)/WT-A lines by confocal microscopy. Confocal images of single optical sections of cotyledon tissue from seven-day-old light-grown seedlings are shown (upper panels) along with corresponding bright-field images (lower panels).

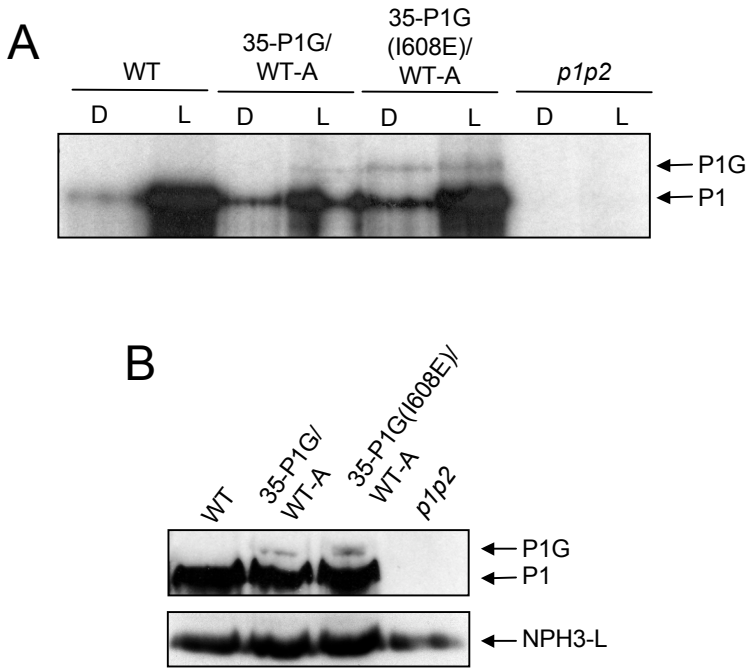


**Figure 5.10 Autophosphorylation analysis of phot1 I608E-GFP expressed in wild-type plants. (A)** Autophosphorylation activity in membranes isolated from wild-type (WT), *p1p2* mutant or transgenic *Arabidopsis* expressing phot1-GFP (P1G/WT-A) or phot1 I608E-GFP (P1G (I608E)/WT-A) were examined. Etiolated three-day-old seedlings were used and all manipulations were completed under dim red light. Samples were given a mock irradiation (D) or irradiated with white light (L) at a total photon fluence rate of 30,000 μmol $m^{-2}$ $s^{-1}$ prior to the addition of radiolabelled ATP. **(B)** Western blot analysis of phot1 protein levels using anti-phot1 antibody (upper panel). As a loading control the blot was reprobed with anti-NON-PHOTOTROPIC HYPOCOTYL 3-LIKE (NPH3-L) antibody (lower panel).

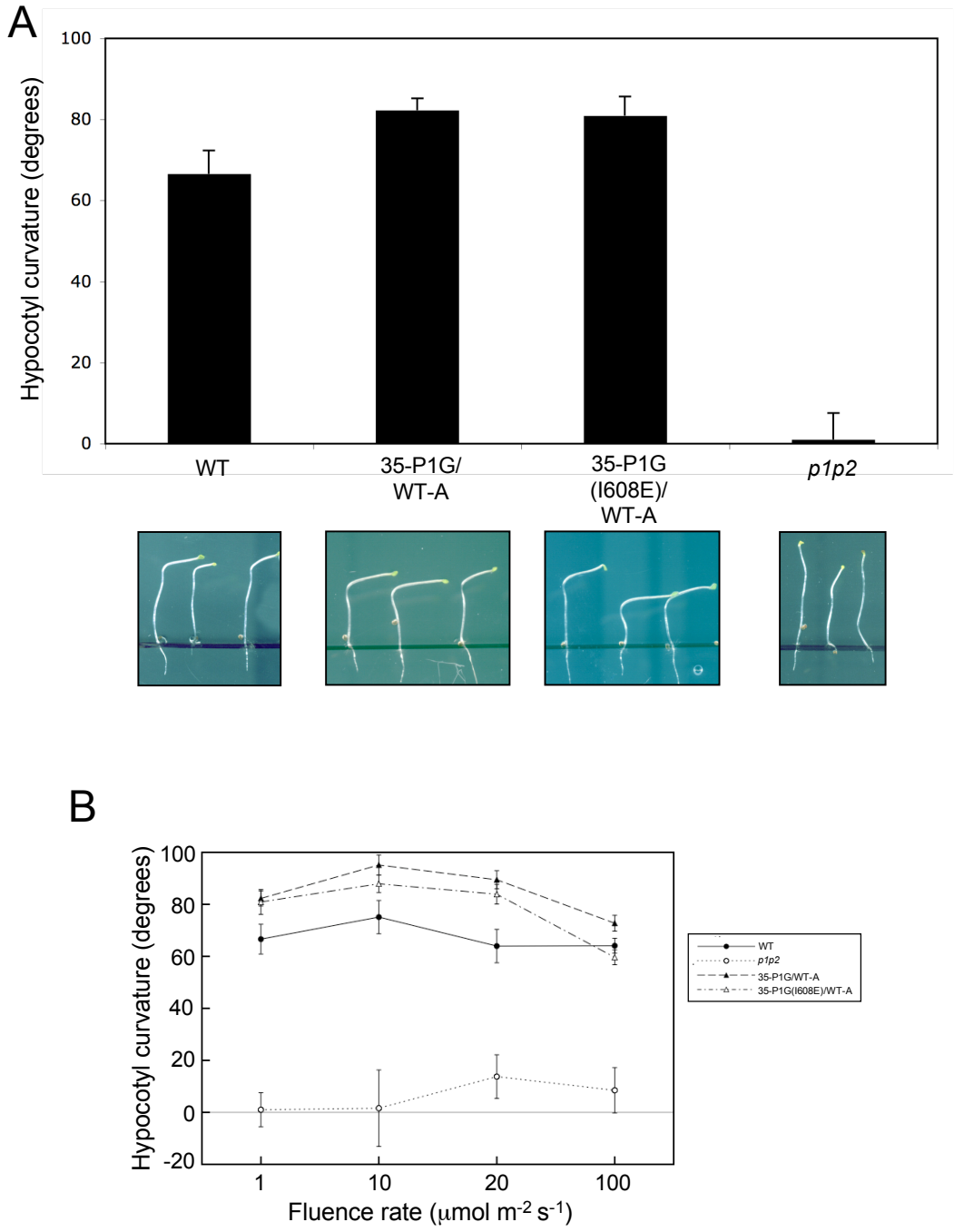


**Figure 5.11 Analysis of phototropic curvature in plants expressing phot1-GFP or phot1 I608E-GFP in a wild-type background. (A)** Phototropic curvature of wild-type (WT), *p1p2* or transgenic *Arabidopsis* seedlings expressing phot1-GFP (P1G/WT-A) or phot1 I608E-GFP (P1G(I608E)/WT-A) was examined. Seedlings were grown in the dark for three days before being exposed to 1 μmol $m^{-2}$ $s^{-1}$ unilateral blue light for 24 hours. Error bars indicate standard error (n≥19). Representative images are shown below. **(B)** Phototropism fluence rate response curves for etiolated seedlings. Seedlings were grown in the dark for three days before being exposed to unilateral blue light at the indicated fluence for 24 hours. Error bars indicate standard error (n=20).

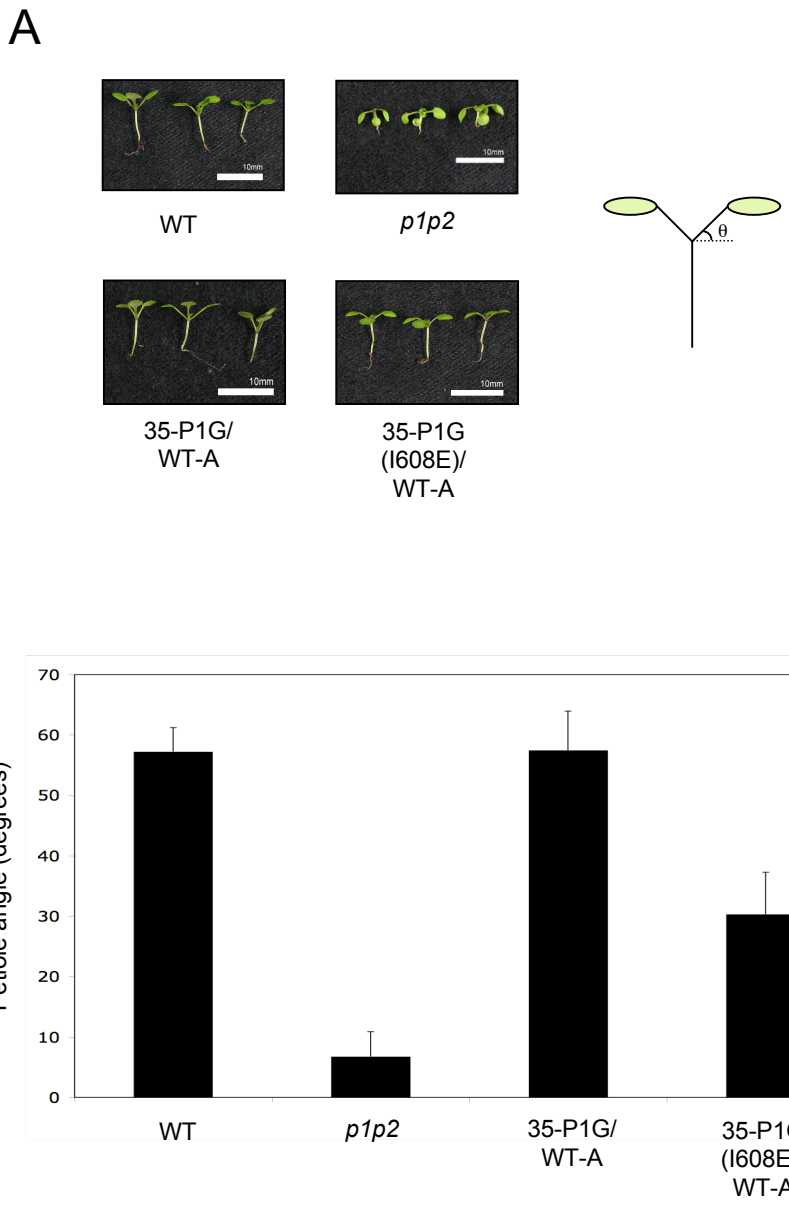


**Figure 5.12 Analysis of leaf positioning in plants expressing phot1-GFP or phot1 I608E-GFP in a wild-type background. (A)** Representative seedlings showing positioning of first true leaves. Wild-type (WT), *phot1phot2* (*p1p2*) and transgenic *Arabidopsis* seedlings expressing phot1-GFP (P1G/WT-A) or phot1 I608E-GFP (P1G(I608E)/WT-A) were examined. Plants were grown for 12 days under 50 μmol $m^{-2}$ $s^{-1}$ white light in long day conditions (16 hours light, 8 hours dark). Of particular interest is the petiole angle from horizontal (θ), as indicated on the diagram on the right. **(B)** Quantification of petiole angle from horizontal (θ). Error bars indicate standard error (n=10).

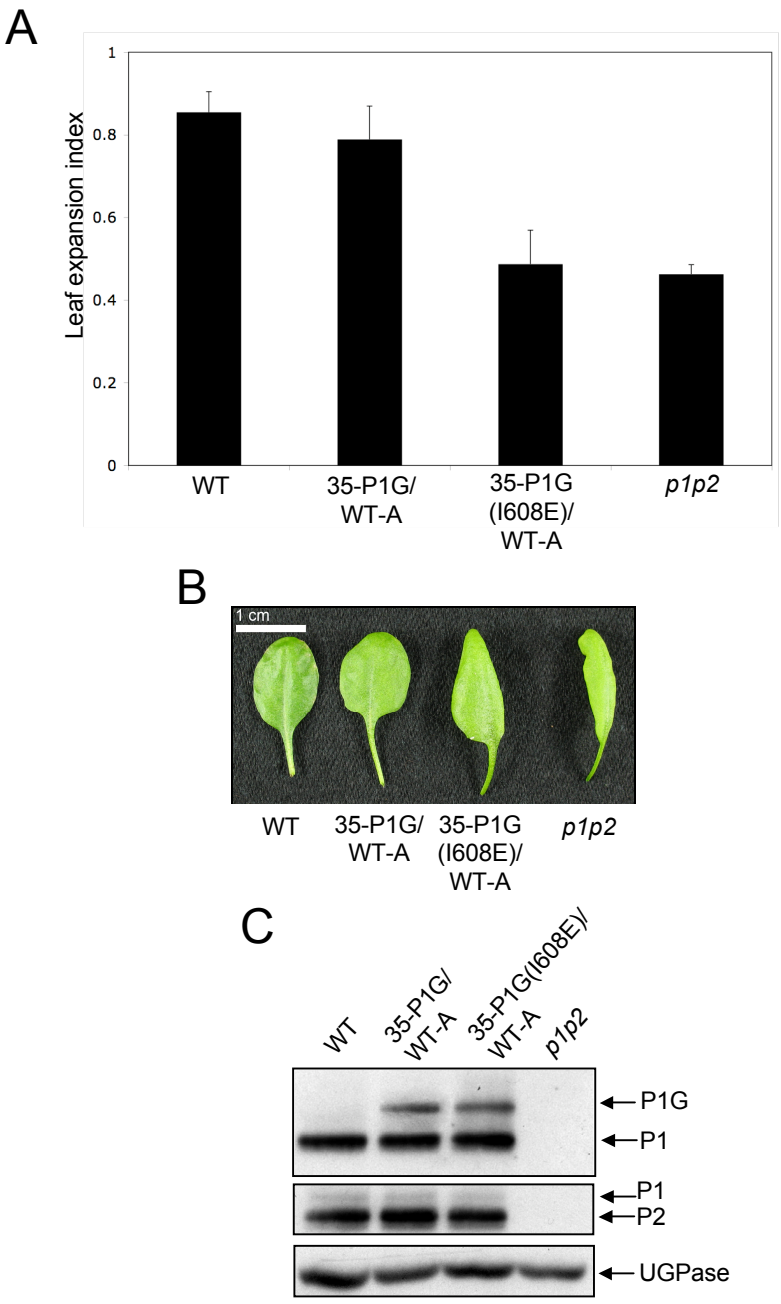


**Figure 5.13 Analysis of leaf expansion and fresh weight in *Arabidopsis* expressing phot1-GFP or phot1 I608E-GFP in a wild-type background. (A)** Leaf expansion index of the fifth rosette leaves of wild-type (WT), *phot1 phot2* (*p1p2*) or transgenic *Arabidopsis* seedlings expressing phot1-GFP (35-P1G/WT-A) or phot1 I608E-GFP (35-P1G(I608E)/WT-A) in a WT background. Plants were grown and analysed as per Figure 5.7A. Error bars indicate standard error (n=10). **(B)** Representative fifth rosette leaves from plants described in (A). **(C)** Western blot analysis of phot1 and phot2 expression levels in three-week-old plants described in (A). Crude soluble protein extracts (30 μg) were probed with anti-phot1 antibody (upper panel). Phot1-GFP (P1G) and phot1 (P1) are indicated. The blots were stripped and reprobed using a C-terminal phot2 antibody (middle panel). Phot2 (P2) and residual phot1 signal (P1) are indicated. UDP-GLUCOSE PYROPHOSPHORYLASE (UGPase) levels were monitored as a protein loading control using a UGPase antibody (bottom panel).

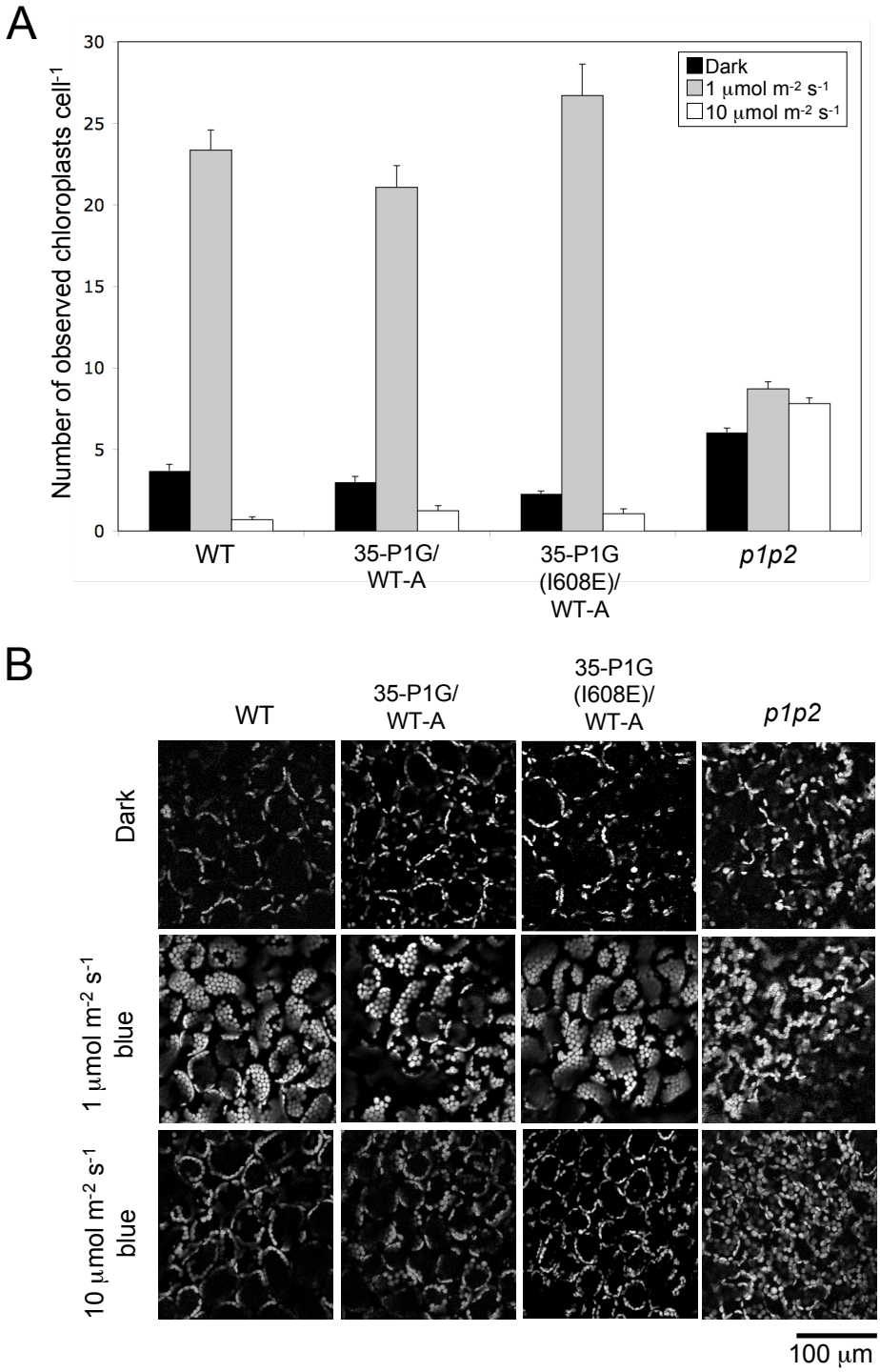


**Figure 5.14 Analysis of chloroplast positioning in plants expressing phot1-GFP or phot1 I608E-GFP in a wild-type background. (A)** Chloroplast positioning in wild-type (WT), *phot1 phot2* (*p1p2*) or transgenic *Arabidopsis* seedlings expressing phot1-GFP (35-P1G/WT-A) or phot1 I608E-GFP (35-P1G(I608E)/WT-A) were examined. Plants were grown on soil for four weeks under 50 μmol $m^{-2}$ $s^{-1}$ white light in long days (16 hours light, 8 hours dark) before rosette leaves were detached and transferred to either dark, 1 μmol $m^{-2}$ $s^{-1}$, or 10 μmol $m^{-2}$ $s^{-1}$ unilateral blue light for three hours. Chloroplast positioning was visualised via chlorophyll autofluorescence by confocal microscopy. Shown values are the average number of chloroplasts at the upper face of palisade mesophyll cells. Error bars indicate standard error (n=25). **(B)** Example images illustrating the number of chloroplasts at the upper face of the palisade mesophyll cells as described in (A).

# Chapter 6: General Discussion

## 6.1 Introduction

The role of phototropins (phots) as blue light-regulated autophosphorylating protein kinases mediating a variety of physiological responses after a directional light stimulus is well characterised (Christie, 2007). The cumulative effect of phot-mediated physiological responses allows plants to optimise their photosynthetic efficiency, which has clear beneficial consequences for plant growth under limiting levels of photosynthetically active radiation (Takemiya *et al.*, 2005). Whilst phot blue-light dependent autophosphorylation was first characterised 20 years ago (Gallagher *et al.*, 1988) the mechanism by which phot kinase regulation occurs and the precise role of this light-induced phosphorylation *in vivo* are unclear, although important advances have been made. During this project point mutation analysis was used in combination with heterologous protein expression system to further examine the mechanism of light-regulated phot1 autophosphorylation *in vitro*. Furthermore, a mutant form of phot1 that had previously been shown to stimulate increased levels of autophosphorylation *in vitro* (Harper *et al.*, 2004a) was transformed into *Arabidopsis* to determine the functional consequences of this mutation *in vivo*. In this final chapter, the data from preceding chapters are discussed and avenues for future research identified.

## 6.2 Mechanism of phot plasma membrane localisation

Whilst phots have long been characterised as plasma membrane-associated proteins (Briggs *et al.*, 2001) the mechanism by which this is achieved is unclear. Although neither phot1 nor phot2 contain an apparent transmembrane domain, aqueous two-phase partitioning experiments and studies using phot1-GFP suggest that unphosphorylated phot1 is associated with the plasma membrane (Sakamoto and Briggs, 2002, Salomon *et al.*, 1996, Short *et al.*, 1993, Palmer *et al.*, 1993b). Phot2-GFP is also associated with the plasma membrane in dark-grown seedlings (Kong *et al.*, 2006). Further studies using right-side-out plasma membrane vesicles have suggested that phot1 is associated solely with the inner surface of the plasma

membrane (Short *et al.*, 1993), while the characterisation of transgenic *Arabidopsis* lines expressing phot1-GFP or phot2-GFP has revealed both of these proteins undergo relocalisation away from the plasma membrane upon blue light exposure (Kong *et al.*, 2006, Sakamoto and Briggs, 2002). Such blue light-induced movement may be a signal transduction event or may desensitise phot signalling to allow perception of prolonged or increased irradiation as observed for other plant photoreceptors (Christie, 2007, Li and Yang, 2007, Rockwell *et al.*, 2006).

Plasma membrane association is typically mediated via either a docking protein or by a direct interaction with the lipid bilayer. Given that phot1 is associated with microsomal extracts both *in planta* and when expressed using the baculovirus/insect cell expression system (Briggs *et al.*, 2001 and Section 3.2.1 respectively) it would appear that such membrane association occurs by a mechanism conserved between these two expression systems. Whilst this does not exclude the possibility that phot1 interacts with the plasma membrane via a docking protein common to both plant and insect cells it would appear more likely that phot localisation is mediated by a direct interaction with the lipid bilayer. Although phots do not contain a prominent lipid-interacting motif, insight into this mechanism may be gained from examining related proteins. Phots are defined by homology as being members of the AGC superfamily of protein kinases (Bogre *et al.*, 2003), of which founding members include cAMP-dependent kinase (PKA) and calcium/phospholipid-dependent kinase C (PKC; Hanks and Hunter, 1995). Previous work has shown that PKC similarly translocates between the cytoplasm and the plasma membrane, although in this case co-factor binding induces localisation of PKC to the plasma membrane from the cytoplasm (Newton, 1995) rather than the relocalisation away from the plasma membrane observed for phots upon light exposure (Kong *et al.*, 2007, Sakamoto and Briggs, 2002).

PKC plasma membrane localisation occurs via two separate domains within the regulatory portion of the protein (Newton, 1995). In each case the binding of activating ligands to the C1 and C2 domains (by diacylglycerol and $Ca^{2+}$ respectively) enhance the affinity of PKC for the plasma membrane (Newton, 1995). Whilst binding of diacylglycerol to the C1 domain is thought to promote plasma membrane interactions by masking hydrophilic residues at the surface of C1 without inducing further conformational changes within PKC (Zhang *et al.*, 1995), $Ca^{2+}$ interaction with the C2 domain is thought to induce structural changes within the motif to re-

orientate a short sequence of positively charged residues and allow direct interaction between PKC and negatively charged lipids present in the plasma membrane (Bazzi and Nelsestuen, 1987, Newton and Keranen, 1994, Newton, 1995). Interestingly, it has been noted that some isoforms of PKC contain a modified C2 domain which does not require $Ca^{2+}$ to promote membrane association (Newton, 1995). In these latter cases it is hypothesised that the C2 domain retains a conformation permissive of lipid interactions in the absence of $Ca^{2+}$ (Newton, 1995).

Whilst phots do not contain diacylgylcerol or $Ca^{2+}$ binding motifs, analysis of the phot protein sequence reveals an insertion within the activation- or T-loop of the phot kinase domain which contains multiple positively-charged residues (Christie, 2007, Tokutomi *et al.*, 2008). A similar insertion within the related plant AGC kinase PINOID (PID) is sufficient to mediate plasma membrane-localisation of this latter protein (Zegzouti *et al.*, 2006b), suggesting that this insertion is responsible for the membrane localisation of PID. Additionally, protein truncation analysis using the phot2 kinase domain fused to GFP indicates that the C-terminal kinase domain, rather than the N-terminal regulatory motif, is necessary and sufficient for membrane association (Kong *et al.*, 2007). Indeed, western blotting similarly suggests that a truncated form of phot1 lacking the N-terminal sequence before LOV2 (phot1 LOV2 kinase) retains a microsomal localisation when expressed in *Arabidopsis* (Section 3.2.5). This positively-charged T-loop insertion within the phot kinase domain is therefore a strong candidate for the motif responsible for phot plasma-membrane association, although further analysis is required to confirm this hypothesis. The baculovirus/insect cell expression system may prove to be an invaluable tool for this investigation as this would allow the consequences of either point mutations or larger insertions and deletions within the T-loop insertion sequence on phot1 microsomal localisation to be evaluated prior to more detailed analysis *in planta*.

Whilst the existence of a novel positively-charged motif within the phot kinase domain is a tantalizing candidate sequence for membrane localisation, additional factors may also be required. Another possible mechanism which would facilitate a direct interaction between phots and the plasma membrane is post-translational lipidation (Nadolski and Linder, 2007). The three most common lipid modifications that occur in the cytoplasm include N-myristoylation, prenylation and S-palmitoylation (palmitoylation) and each of these modifications may be sufficient to confer membrane association (Nadolski and Linder, 2007). Whilst phots lack the

prerequisite protein motifs to permit N-myristoylation or prenylation (Resh, 1999, Zhang and Casey, 1996), a consensus motif required for palmitoylation has yet to be identified (Nadolski and Linder, 2007). Unlike other lipid alterations, palmitoylation is a reversible modification which links palmitate, a saturated fatty acid, to surface cysteine residues (Resh, 2006a) and this modification has previously been shown to have a role in protein sorting in eukaryotic cells (reviewed in Greaves and Chamberlain, 2007). Palmitoylation-mediated protein sorting is best illustrated by the small human GTPase Ras (Greaves and Chamberlain, 2007). Ras is localised either to the plasma membrane or to the Golgi apparatus dependent upon its palmitoylation status (Goodwin *et al.*, 2005). It is therefore possible that phots associate with the plasma membrane subsequent to this post-translational modification and then disassociate from the membrane following a reversal of palmitoylation. However, it should be noted that palmitoylation most often acts in combination with other weak membrane-association signals to modulate protein localisation (Greaves and Chamberlain, 2007) and it is therefore likely that other localisation signals are required for correct phot localisation. The palmitoylation status of proteins is routinely assessed by monitoring the incorporation of either radioactively- and chemically-labelled palmitate (Resh, 2006b). Once again, phot proteins over-expressed using the baculovirus/insect cell system will prove a useful tool for this initial characterisation as the signal generated by such assays are weak and require protein over-expression and prolonged radiograph development for visualisation (Resh, 2006b).

The final possibility to account for the plasma membrane localisation of phot1 is the existence of an anchoring protein which tethers phot1 to the plasma membrane. A recent yeast two-hybrid screen completed in our laboratory identified two members of the *Arabidopsis* ADP-Ribosylation Factor (ARF) family which interact with both phot1 and phot2 in a blue light-dependent fashion in this system (Thomson, 2008). Preliminary mass spectroscopy analysis completed in our laboratory using phot1-GFP immuno-purified from dark-grown transgenic *Arabidopsis* has also identified an ARF protein which co-purifies with phot1-GFP (Sullivan and Christie, unpublished data), although further characterisation of this interaction has yet to be completed. ARF proteins are best known as GTP-dependent switches which are necessary for vesicle budding and fusion (Gebbie *et al.*, 2005) but contain an N-terminal myristoylated α-helix involved in GTP-dependent interactions with membranes (Nielsen *et al.*, 2006).

As such these proteins may sequester phots to the plasma membrane. As the phot/ARF interaction only occurs in the absence of blue light in yeast (Thomson, 2008), it is plausible that light exposure *in planta* inhibits the interaction, resulting in the relocation of phots away from the plasma membrane. If this interaction were necessary for the correct localisation of phot to the plasma membrane such data would have to be reconciled with the observed association of phot1 with the plasma membrane of infected insect cells (Section 3.2.1). However, previous work has shown that *Arabidopsis* ARFs can functionally complement an *arf* mutant strain of *Saccharomyces cerevisiae* (Takeuchi *et al.*, 2002), suggesting that a degree of functionality is retained by ARFs between eukaryotic species. It will now be necessary to assess the localisation of phot1 in *Arabidopsis arf* mutants to determine whether they display an altered localisation.

## 6.3 Phot autophosphorylation occurs at additional sites to those mapped by mass-spectroscopy

The most obvious biochemical response of phots to blue light stimulation is autophosphorylation (Christie, 2007). However, the mechanism by which this phosphorylation occurs has not previously been characterised in detail. The closely related *Arabidopsis* kinase PID is autophosphorylated by an intramolecular mechanism which results in an enhancement of the ability of PID to phosphorylate the artificial substrate casein (Zegzouti *et al.*, 2006a). In contrast, work presented here suggests that, at least *in vitro*, phot1 is capable of intermolecular autophosphorylation between separate phot1 molecules (Section 3.2.4). This is in agreement with previous work demonstrating that artificial mixing of plasma membrane samples from different species results in phot cross-phosphorylation (Reymond *et al.*, 1992) and work completed using insect cell-expressed phot1 and phot2 which demonstrated the ability of phot2 to phosphorylate inactive phot1 *in vitro* (Cho *et al.*, 2007). Such intermolecular phosphorylation between phot1 molecules *in vitro* suggests that phot1 may function as a multimer, although attempts to verify this interaction by characterising the ability of His-phot1 to associate with GST-phot1 bound to a glutathione column following co-expression in the baculovirus/insect cell expression system failed to detect His-phot1 via western blot analysis (data not shown).

Although mass-spectroscopy and phosphopeptide mapping have identified *in vivo* phot1 phosphorylation sites in the N-terminal region of phot1 and within the linker sequence between LOV1 and LOV2 (Sullivan *et al.*, 2008), data presented here suggests that additional phosphorylation sites are located in the C-terminal region of the protein downstream of LOV2 (Section 3.2.6). Truncated forms of both phot1 and phot2 (phot1 LOV2 kinase and phot2 LOV2 kinase) retain a degree of light-dependent autophosphorylation activity *in vitro* when expressed in the baculovirus/insect cell system (Section 3.2.6). Predictive phosphorylation site programs (such as http://www.cbs.dtu.dk/services/NetPhos/; Blom *et al.*, 1999) identify serine residues within the phot1 kinase T-loop as the most likely sites of phosphorylation within the C-terminal region of phot1. Given the reduction in light-induced autophosphorylation activity observed when a residue within the phot1 kinase T-loop was mutated (Section 3.2.8) further mass-spectroscopy analysis of phosphorylation sites within the truncated LOV2 kinase protein is awaited with interest to confirm whether phot1 autophosphorylation occurs within the kinase T-loop. An alternative experimental procedure would be to replace each of the predicted phosphorylation sites within phot1 LOV2 kinase with alanine. However, such alterations would involve mutation within the phot1 kinase domain and therefore any detrimental effect of these mutations on phot1 LOV2 kinase autophosphorylation may be associated with alternate kinase defects (such as gross conformational changes) other than removal of phosphorylation sites.

## 6.4 Insights into the mechanism of phot1 autophosphorylation

The AGC kinase family, of which phots are a member, have a highly conserved catalytic domain structure when in an active conformation, although a variety of mechanisms have evolved to regulate their activity which allows tight control of kinase activity in response to a variety of stimuli (Huse and Kuriyan, 2002). The most direct method of inhibiting kinase activity involves the use of inhibitory proteins to prevent substrate binding (Soderling, 1990). Protein sequences which inhibit substrate binding often resemble the motifs recognised by the regulated kinase domain except with alanine in the place of the typically phosphorylated serine and are typically referred to as 'pseudo-substrates'. Such a competitive mechanism of kinase regulation was initially suggested for the chicken protein myosin light chain kinase (Kemp *et al.*,

1987), and has since been shown to be a common method of kinase regulation (Kobe and Kemp, 1999). Indeed, both PKA and PKC are regulated in this fashion (Soderling and Stull, 2001, Taylor, 1989). Whilst the majority of AGC kinases (including PKC and phots) are single proteins containing both regulatory and catalytic domains, PKA consists of a hetero-tetramer containing two distinct regulatory (R) subunits bound to two catalytic subunits (Taylor *et al.*, 1990). Upon binding cAMP the R subunits disassociate as a dimer, releasing the catalytic subunits as active monomers (Taylor *et al.*, 1990). Conversely, PKC is regulated by an intramolecular mechanism, with an integral autoinhibitory sequence within bovine PKC-α encoding a PKC pseudo-substrate motif (House and Kemp, 1987).

As the kinase activity of phototropins is up-regulated by light and both proteins encode integral light-sensing domains (Christie *et al.*, 1998, Huala *et al.*, 1997, Jarillo *et al.*, 2001) it remains possible that light stimulation causes a release of phot kinase repression as initially proposed (Christie *et al.*, 2002). Such light-induced activity is most likely a consequence of autoregulation as both phot1 and phot2 display light-dependent activity either when isolated from plant extracts (Gallagher *et al.*, 1988) or when heterologously expressed in insect cells (Christie *et al.*, 1998, Sakai *et al.*, 2001). Whilst studies presented here indicate that intermolecular phosphorylation may occur between phot1 molecules *in vitro* (Section 3.2.4) it remains unclear whether light-mediated kinase regulation occurs via an intra- or inter-molecular mechanism. However, if phot kinase activity is regulated in a similar fashion to related kinases a pseudo-substrate motif may be encoded within the protein sequence. Protein truncation analysis has revealed that the C-terminal region of phot2 containing the kinase domain constitutively phosphorylates the artificial substrate casein *in vitro* (Matsuoka and Tokutomi, 2005), whilst expression of a similar phot2 kinase fragment induces constitutive phot2 responses when expressed in transgenic *Arabidopsis* (Kong *et al.*, 2007). This suggests that phot2 kinase regulation is mediated by the N-terminal domain containing the LOV domains.

Several lines of evidence suggest that phot kinase regulation is achieved through the function of the LOV2 domain. Mutational analysis of *Arabidopsis* phot1 and phot2 to selectively prevent photochemistry within either LOV domain revealed the predominant role of LOV2 photochemistry in light-dependent kinase stimulation (Christie *et al.*, 2002), whilst data presented here suggests that a phot fragment lacking the protein sequence N-terminal of LOV2 retains light-dependent

autophosphorylation *in vitro* when expressed in insect cells (Section 3.2.6). This inhibitory role of LOV2 has been elegantly examined *in vitro* using phot2 fragments expressed in *E. coli* (Matsuoka and Tokutomi, 2005). In this study, Matsuoka and Tokutomi were able to show that incubation of the isolated phot2 kinase domain in the presence of separately expressed phot2 LOV2 reduced the phosphorylation of casein. Such an effect was not observed when casein phosphorylation was monitored in the presence of phot2 LOV1 (Matsuoka and Tokutomi, 2005). Whilst attempts to duplicate these results using phot1 expressed via the baculovirus/insect cell system to phosphorylate casein *in vitro* were unsuccessful (data not shown) it is plausible that such a regulatory mechanism is conserved between phots.

If a component of LOV2 were acting in a homologous manner to that of the PKA R subunits or the integral PKC pseudo-substrate, it would be expected that a protein sequence motif similar to that recognised by the phot kinase would be present within LOV2. Studies of both PKA and PKC have characterised short peptide sequences which act to inhibit kinase function. A short peptide sequence of 13 amino acids within bovine PKC-α acts to competitively inhibit PKC activity (House and Kemp, 1987), whilst X-ray crystallography studies of PKA have revealed an unrelated 5-residue motif within a known inhibitor peptide (PROTEIN KINASE A INHIBITOR; PKI) which interacts directly with the substrate-binding groove within the PKA catalytic subunit (Cheng *et al.*, 1985, Knighton *et al.*, 1991). The PKA inhibitory motif (RRXA*I, where the asterisk indicates the alanine replacing the substrate serine in the pseudo-substrate) is conserved between PKI and the regulatory R subunits of PKA, although one of these R subunits contains a serine in place of the typical pseudo-substrate alanine (Taylor, 1989). Whilst R subunits also have secondary points of contact with the catalytic domain (Herberg *et al.*, 1994), the RRXA*I motif represents a template motif which may allow an interaction between LOV2 and the phot kinase domain. Indeed, it has recently been shown that LOV2 contains a similar sequence (IRXA*I) on the surface of the LOV2 domain within the Cα-helix (Tokutomi *et al.*, 2008) which corresponds with the phot1 kinase substrate recognition motif suggested by recent phosphorylation site mapping (RXS, Sullivan *et al.*, 2008). This 5-residue motif is not conserved in phot LOV1 and therefore may partially account for the inability of LOV1 photoreactivity to modulate phot kinase activity compared to LOV2 (Tokutomi *et al.*, 2008).

The proposed role of LOV2 in regulation of the phot kinase domain is paralleled by the suggested mechanism of regulation within the human PAS kinase (Rutter *et al.*, 2001). In a similar fashion to phots, PAS kinase contains two PAS motifs in parallel with an autoregulated kinase domain, with this regulation primarily mediated by one of the integral PAS domains (PAS-A; Rutter *et al.*, 2001). As observed for isolated LOV2 and phot2 kinase (Matsuoka and Tokutomi, 2005), addition of PAS-A expressed in *E. coli* is sufficient to inhibit PAS kinase catalytic activity, suggesting a direct interaction between PAS-A and the catalytic domain (Amezcua *et al.*, 2002, Rutter *et al.*, 2001). By assaying the effects of kinase domain titration on the NMR spectra of the isolated PAS-A it has been possible to identify the region of PAS-A involved in the interaction between PAS-A and the catalytic domain of PAS kinase (Amezcua *et al.*, 2002). This mapped region includes the PAS domain Cα-helix (referred to as 'Fα' by Amezcua *et al.*, 2002) which contains the putative pseudo-substrate motif that has been proposed to have a role in the LOV2/phot kinase interaction (Tokutomi *et al.*, 2008). It will now be necessary to confirm whether the LOV2 Cα-helix is sufficient to repress phot kinase activity. Previous investigations have used short polypeptide sequences to competitively inhibit kinase activity (Kemp *et al.*, 1987) and a similar approach may provide conformation of the role of the LOV2 Cα-helix fragment in phot kinase regulation.

Although recent modelling data suggests that LOV2 may act to competitively inhibit phot kinase substrate binding (Tokutomi *et al.*, 2008) it remains possible that LOV2 binding to the kinase domain additionally induces conformational changes within phot kinase that reduce the favourability of phosphotransfer within the kinase domain (Huse and Kuriyan, 2002). Examples of this additional layer of regulation include the human $Ca^{2+}$-regulated kinases twitchin and titin (Hu *et al.*, 1994, Mayans *et al.*, 1998). In each case, X-ray crystallography has been used to demonstrate that C-terminal extensions outwith the kinase domain alter the position of residues necessary for catalytic activity in addition to acting as a pseudo-substrate. The affected residues include those thought to be structurally coupled to the T-loop (Huse and Kuriyan, 2002). A similar combination of regulatory mechanisms to control the activity of phot kinase would correlate with data suggesting a role for phot kinase T-loop phosphorylation in the regulation of phot catalytic activity (Section 3.2.8 and Tokutomi *et al.*, 2008) in common with other PKA-related proteins (Adams, 2003).

## 6.5 Point mutation of phot1 provides insights into the signalling mechanism linking LOV2 photochemistry with phot light-regulated autophosphorylation

The predominant role of LOV2 in regulating phot autophosphorylation (Christie *et al.*, 2002) allowed examination of LOV2 signal transmission by monitoring the effects of specific point mutations on phot autophosphorylation *in vitro*. One important discrepancy highlighted by this study was the observed difference between flavin binding in mutated isolated LOV domains expressed in *E. coli* and full-length protein containing the equivalent mutation expressed using the baculovirus/insect cell expression system (Sections 4.2.1-3, 4.2.7). This suggests that data gathered from isolated LOV domains should be treated with caution and verified in the full-length protein context when possible. Despite this caveat, work presented here describes informative correlations between photochemical and conformational perturbations observed in isolated LOV domains and altered autophosphorylation activity of full-length phot1 *in vitro*. Data presented in this study reaffirms the requirement of LOV2 photochemistry in regulating phot1 autophosphorylation by demonstrating that substitution of the photochemically-active cysteine within LOV2 with methionine ($Cys^{512}$→Met) does not completely abolish light-induced phot1 autophosphorylation *in vitro* (Section 3.2.9), in agreement with studies using isolated LOV domains which indicate that a photoproduct was formed in spite of the mutation (Kottke *et al.*, 2003). Therefore, although the photoproduct formed within the mutated domain is different to that formed within wild-type LOV2 (a methionyl-flavin adduct rather than the usual cysteinyl-flavin adduct) it appears that this alternate bond is sufficient to induce a degree of phot1 light-induced kinase activation.

Studies completed during this project have shown that although mutation of residues involved in formation of a surface salt bridge does not alter light-induced phot1 autophosphorylation (Section 4.2.2) mutation of a residue within the LOV2 domain βE-sheet ($Gln^{575}$→Leu, Q575L) reduces phot1 autophosphorylation *in vitro* (Section 4.2.7). Such an observation correlates with fourier transform infra-red (FTIR) spectroscopy studies where an equivalent mutation within *Adiantum* neochrome 1 LOV2 was shown to limit conformational changes within this latter LOV domain after light exposure (Nozaki *et al.*, 2004) and other mutational studies

whereby the blue light-dependent effects of LOV domain photochemistry are mitigated by the Gln→Leu substitution in *Arabidopsis* FKF1 and *Neurospora* VIVID proteins (Sawa *et al.*, 2007, Zoltowski *et al.*, 2007). In the case of FKF1, the equivalent Gln→Leu substitution prevented blue light-dependent interactions between the FKF1 LOV domain and GIGANTEA *in vitro* (Sawa *et al.*, 2007) whilst the Gln→Leu mutation in VIVID limited conformational changes within the VIVID LOV domain after irradiation (Zoltowski *et al.*, 2007). However, it is apparent from the presented data that the Q575L mutation of the LOV2 βE-sheet does not completely abolish signal transmission from LOV2 to the kinase domain (Section 4.2.7). Mutational analysis of neochrome 1 LOV2 has recently identified a residue within an alternate β-sheet ($Phe^{1010}$, within the LOV2 Cβ-sheet) that also reduces conformational changes within the isolated neochrome 1 LOV2 domain as monitored by FTIR spectroscopy (Yamamoto *et al.*, 2008), whilst similar mutations within the LOV domain Cβ-sheet of the bacterial YtvA protein also inhibit light-induced signal transmission (Losi *et al.*, 2005). It therefore appears that although $Gln^{575}$ of phot1 LOV2 is involved in signal propagation following photoproduct formation additional residues within the LOV2 domain also have a function. It will be of future interest to determine whether these additional residues, such as the *Arabidopsis* phot1 equivalent of neochrome 1 $Phe^{1010}$ ($Phe^{556}$), have a similar inhibitory effect on light-induced kinase activation when mutated.

Previous investigations have shown that mutation of the Jα-helix associated with LOV2 ($Ile^{608}$→Glu, I608E) results in increased phot1 autophosphorylation of mock-treated controls *in vitro* compared with wild-type (Harper *et al.*, 2004a), suggesting that the Jα-helix has a role in LOV2 signal transmission. Interestingly, wild-type levels of phot1 autophosphorylation were restored when this mutation within the Jα-helix was incorporated in combination with Q575L (Section 4.2.10). This suggests that the effects of the Jα-helix mutation superceded the consequences of the Q575L substitution within the βE-sheet. Although disruption of the Jα-helix was sufficient to stimulate increased levels of phot1 autophosphorylation (Harper *et al.*, 2004a) it is becoming apparent that additional protein structures outwith LOV2 also undergo conformational changes upon LOV2 irradiation (Halavaty and Moffat, 2007). This recent study used X-ray crystallography techniques to examine the structural changes induced by light stimulation in an extended *Avena sativa* phot1 LOV2

fragment that included both the C-terminal Jα-helix, and also sequences N-terminal to LOV2 (Halavaty and Moffat, 2007).

In contrast to previous studies (Harper *et al.*, 2003, Chen *et al.*, 2007, Harper *et al.*, 2004b) Halavaty and Moffat (2007) observed only minimal structural changes within the Jα-helix upon light stimulation. This discrepancy may in part be caused by the X-ray crystallography techniques used in this most recent study, as examination of crystal packing in this latest study reveals extensive intermolecular contacts within the C-terminal region of the protein fragment containing the Jα-helix (Halavaty and Moffat, 2007). These intermolecular contacts may restrict light-induced Jα-helix disorganisation in contrast to that observed in solution by NMR spectroscopy (Harper *et al.*, 2004b, Harper *et al.*, 2003). Despite this discrepancy, the study of conformational changes within *Avena* phot1 LOV2 reaffirms previous data suggesting that LOV2 signal transmission occurs primarily via structural rearrangement within β-sheet structure of the LOV domain (Anderson *et al.*, 2004, Ihee *et al.*, 2005, Harper *et al.*, 2004a, Nozaki *et al.*, 2004) although alterations in α-helix structure are also observed (Salomon *et al.*, 2001). The latest findings show that conformational changes are observed in the extended N-terminal region included in this LOV domain construct as well as within the Jα-helix. Whilst care must be taken in data interpretation, the role of additional structures in LOV2 signal transmission may correlate with the minimal light-induced activity retained by phot1 I608E mutant proteins observed when autophosphorylation activity of the single phot1 I608E mutant was quantified *in vitro* (Section 4.2.10).

The study by Halavaty and Moffat (2007) also provides additional information relating to residues within the Jα-helix that interact with the core region of the LOV2 domain protein sequence. Interestingly, the *Avena* phot1 LOV2 Jα-helix interacts via hydrogen bonding with both the core LOV2 protein sequence and the extended N-terminal region used in this study. The residues necessary for these interactions are conserved between *Avena* phot1 and *Arabidopsis* phot1 and phot2, suggesting a conservation of function. These conserved residues were not included in the initial mutagenic series created to assess the effects of Jα-helix mutation on phot1 autophosphorylation (Harper *et al.*, 2004a) but these newly characterised residues are now prime candidates for substitution with hydrophilic residues to assess signal transmission from LOV2. Such analysis would be particularly informative for

*Arabidopsis* phot2 as attempts to induce increased phot2 activity *in vitro* by Jα-helix mutation resulted in only marginal increases in autophosphorylation activity in this study (Section 3.2.12).

## 6.6 The role of LOV1 in phot-mediated signalling remains elusive

As discussed, phots are unique amongst LOV domain-containing proteins as they contain two LOV domains (Briggs, 2007, Losi, 2004). The presence of an additional motif is particularly puzzling given that only the photoactivity of the LOV2 domain is required for complementation of phot-dependent phenotypes *in planta* (Cho *et al.*, 2007, Christie *et al.*, 2002), while analysis of phot1 LOV2 kinase and phot2 LOV2 kinase reveals the truncated proteins retain light-regulated autophosphorylation *in vitro* when expressed in insect cells (Section 3.2.6). Studies in our laboratory have revealed that phot1 LOV2 kinase is capable of complementing a subset of phot1-dependent phenotypes when expressed in a *p1p2* background, despite microsomal extracts from these transgenic *Arabidopsis* lacking detectable light-induced phosphorylation of the truncated protein (Section 3.2.5 and Sullivan *et al.*, 2008). Similarly, a truncated LOV2 kinase fragment isolated from *Adiantum* phot2 functionally complements phot2-dependent chloroplast movements when transiently expressed in this species (Kagawa *et al.*, 2004).

The ability of phot1 LOV2 kinase to partially complement phot1-dependent phenotypes when expressed in *Arabidopsis p1p2* mutants suggests that sequence N-terminal of LOV2 (including LOV1) is dispensable for a subset of phot1-mediated signalling (Sullivan *et al.*, 2008). Another suggested role for LOV1 is as a homodimerisation site between phot molecules. LOV1 was first suggested to act as a homodimerisation motif based upon the apparent dimerisation of *Avena* phot1 LOV1 fragments *in vitro* in contrast to the monomeric form of similar phot1 LOV2 fragments when these proteins were expressed in *E. coli* (Salomon *et al.*, 2004). However, the ability of phot1 LOV2 kinase to cross-phosphorylate full-length phot1 *in vitro* suggests that homodimerisation between LOV1 domains is not necessary for intermolecular phosphorylation between phot1 molecules (Section 3.2.7).

Other than having a role in primary signalling events LOV1-mediated heterodimerisation may have a role in modulation of the light-induced signalling cascade. The *Arabidopsis* LOV-domain containing proteins ZTL and FKF1 have

recently been shown to have a blue light-enhanced affinity for an interacting protein (GIGANTEA; Kim *et al.*, 2007, Sawa *et al.*, 2007) and protein truncation analysis reveals that this blue light-dependent interaction is mediated by the LOV domain (Kim *et al.*, 2007, Sawa *et al.*, 2007). Such data reaffirms the suggestion that LOV domains may act as blue-light dependent dimerisation motifs. Interestingly, the blue-light activation of FKF1 and ZTL also alters the stability of partnering proteins (Imaizumi *et al.*, 2005, Kim *et al.*, 2007), with blue light additionally enhancing the stability of ZTL in a blue light-dependent manner that requires a photosensitive LOV domain (Kim *et al.*, 2007). The LOV domains of FKF1 and ZTL share greater sequence homology with phot LOV1 rather than LOV2 (Crosson *et al.*, 2003) and it is therefore plausible that phot LOV1 facilitates blue light-dependent interactions with proteins which regulate the stability of phots following irradiation.

An alternative role for phot1 LOV1 is suggested by photochemical analysis of bacterially-expressed phot1 N-terminal regions containing both LOV1 and LOV2 domains (Song *et al.*, 2005, Kasahara *et al.*, 2002). Analysis of this phot fragment revealed that the presence of LOV1 extended the half-life of the LOV2 signalling state, although LOV1 photosensitivity was not required for this activity (Kasahara *et al.*, 2002). This may suggest a role for LOV1 in extending the active signalling state of the full-length phot protein (Kagawa *et al.*, 2004, Christie *et al.*, 2002) rather than LOV1 being required for the initial light-dependent increase in phot1 autophosphorylation *per se* (Christie, 2007, Matsuoka and Tokutomi, 2005). Such a hypothesis correlates with the increased blue light fluences required to induce phototropic bending in transgenic seedlings expressing phot1 LOV2 kinase compared with wild-type (Sullivan *et al*., 2008). If this were the case, it may be predicted that phot1 LOV2 kinase would remain in the light-induced 'kinase-active' conformation for a shorter period than full-length phot1. However, initial attempts to determine whether phot1 LOV2 kinase was less capable of incorporating $^{32}$P-ATP following light stimulation and a subsequent dark incubation prior to radiolabel addition proved inconclusive (data not shown).

## 6.7 Mutation of the phot1 Jα-helix induces constitutive autophosphorylation *in vitro* but has an obscure role *in vivo*

Although previous work (Harper *et al.*, 2004a) and results presented as components of this thesis suggest that phot1 I608E has increased levels of autophosphorylation in mock-treated insect cell samples *in vitro* (Section 3.2.10) it remains undetermined whether phot1 I608E is constitutively active *in planta* with regards to the physiological responses characterised in this study (Sections 5.3.2-4). Data interpretation was severely hampered by the inability to isolate multiple transgenic lines expressing phot1 I608E-GFP in each of the genetic backgrounds examined, and therefore the conclusions drawn should be treated with due caution. Whilst there are several explanations for the limited number of transgenic lines obtained expressing phot1 I608E one possibility is that the cauliflower mosaic virus *35S* (*35S*) promoter was less efficient at driving expression of the transgenic gene than the native promoter.

The *35S* promoter was selected for use in this study primarily because it had previously been used successfully to drive the expression of both phot1 and phot2 cDNA and mutant variants thereof (Cho *et al.*, 2007, Christie *et al.*, 2002, Kong *et al.*, 2006, Kong *et al.*, 2007, Sullivan *et al.*, 2008). Whilst in retrospect it would have been beneficial to express phot1 I608E under the control of the native *PHOT1* promoter the use of the native promoter is complicated by the fact that it has yet to be fully defined. Although a large region of DNA 5' of *PHOT1* has been used to drive phot1-GFP expression in *Arabidopsis* this study also incorporated DNA 3' of *PHOT1* in addition to using full-length genomic *PHOT1* rather than the cDNA sequence used elsewhere (Sakamoto and Briggs, 2002). The extent of the native *PHOT1* promoter is therefore not immediately apparent. In any case, the expression levels of phot1-GFP in one of the transgenic lines characterised as part of this study (Section 5.2.1; line 35-P1G/*p1p2*-A) suggest that the *35S* promoter is capable of driving *PHOT1* transcription at an appropriate rate.

Whilst it is difficult to draw firm conclusions from the data presented it appears that expression of low levels of phot1 I608E-GFP are insufficient to induce constitutive phot1 signalling. Recent work characterising expression of a truncated form of *Arabidopsis* phot2 encoding only the kinase domain in a *p1p2* background reveals a number of mutant phenotypes that correspond with the predicted effect of

constitutive phot kinase activity (Kong *et al.*, 2007). For example, the expression of phot2 kinase in a wild-type background inhibits phototropism, a phenotype that is not observed in wild-type plants expressing inactive phot2 kinase. Coupled with data showing that phot2 kinase expressed in *E. coli* is able to constitutively phosphorylate casein *in vitro* (Matsuoka and Tokutomi, 2005) such data imply that the phot2 kinase domain is constitutively active *in planta*. An additional mutant phenotype caused by phot2 kinase expression in a wild-type background is dwarfism, which is possibly accounted for by a misregulation of auxin transport (Kong *et al.*, 2007). Interestingly, preliminary data from our laboratory indicates that isolated phot1 kinase expression in a wild-type background causes a similar dwarf phenotype in the T2 generation that was not observed in wild-type *Arabidopsis* expressing an inactive phot1 kinase fragment (Thomson, 2008). This suggests that the expression of either phot1 kinase or phot2 kinase in wild-type *Arabidopsis* has comparable consequences. Given that phototropism was not mitigated by phot1 I608E-GFP expression in a wild-type background (Section 5.2.11) and that a dwarf phenotype was not apparent in transgenic lines expressing phot1 I608E-GFP (data not shown) it would appear that phot1 I608E does not have constitutive signalling activity *in planta*, although the partial complementation of phototropism by phot1 I608E expression in a *p1p2* background suggests that the mutated protein retains a degree of light-induced activity (Section 5.2.4).

One of the most intriguing components of the data presented here regards the expression of phot1 I608E-GFP in a wild-type background. Whilst phototropism under the conditions monitored was unaffected by phot1 I608E-GFP expression, correct leaf positioning and leaf expansion appeared to be inhibited by expression of the phot1 I608E-GFP protein (Sections 5.2.12-13). Such a phenotype may be a consequence of the I608E mutation causing an impairment of phot1-and phot2-mediated signalling. The ability of mutated plant photoreceptors to interfere with the signalling of their endogenous counterparts has recently been documented (Su and Lagarias, 2007). In this study Su and Lagarias (2007) interpret the impaired HIR response of a transgenic line expressing a mutated version of phyA (phyA Y242H) in a wild-type background as a consequence of dimerisation between endogenous phyA and the signalling-defective transgenic protein. Comparison with this study is particularly informative as the phyA Y242H mutant appears to have a residual signalling ability which induces light-independent photomorphogenesis when phyA

Y242H was expressed in a *phyA* mutant background (Su and Lagarias, 2007). If phot1 I608E is deficient in phot1-mediated signalling it is plausible that a similar effect occurs between endogenous phot1 and phot1 I608E, resulting in impaired signal transmission which has a dominant-negative effect when expressed in a wild-type background but which is sufficient to restore limited phot1-mediated signalling in *p1p2* plants. However, the results presented here should be treated with caution as only a single independent transgenic *Arabidopsis* line expressing phot1 I608E in a wild-type background was identified (Section 5.2.9). Consequently, the possibility that the observed deficiencies observed in leaf positioning and expansion are a result of an insertion event within another gene critical for these responses cannot be discounted.

If the mutated phot1 I608E protein is deficient in signalling *in planta* it will be important to determine the mechanism by which this occurs, as such data would allow further elucidation of phot-induced signalling. The inability of phot1 I608E-GFP to induce constitutive signalling *in planta* may be reflective of either a lack of phot1 I608E-GFP phosphorylation *in planta* or suggest that phot1 I608E-GFP autophosphorylation is insufficient to induce phot1 light-induced signalling. As an initial stage of this characterisation it will be important to determine whether phot1 I608E is constitutively phosphorylated *in planta*. Phot1 *in vivo* autophosphorylation typically results in a reduced electrophoretic mobility of the phosphorylated protein (Short and Briggs, 1990) and whilst altered electrophoretic mobility of phot1 I608E-GFP was observed intermittently (Figures 5.1B and 5.9A) such an altered migration was not consistently reproducible (data not shown). The ability of phot1 I608E-GFP isolated from transgenic *Arabidopsis* to incorporate $^{32}$P-ATP *in vitro* (Section 5.3.1) is also indicative of a portion of phot1 phosphorylation sites remaining unphosphorylated *in planta* in these transgenic lines. This data is in contrast with the effect of prolonged blue light irradiation *in vivo* which resulted in reduced *in vitro* $^{32}$P-ATP incorporation of *Pisum sativum* membrane extracts, presumably as a consequence of all available phosphorylation sites being filled with unlabelled ATP (Gallagher *et al.*, 1988). An alternative explanation of the retention of $^{32}$P-ATP incorporation *in vitro* is the activity of an as yet unidentified phot1 phosphatase. The activity of this phosphatase allows restoration of $^{32}$P-ATP incorporation *in vitro* if pea, broad bean, or oat seedlings are incubated in the dark for several hours following light exposure *in vivo* (Salomon *et al.*, 2003, Kinoshita *et al.*, 2003, Short *et al.*, 1992)

and the activity of this enzyme may be sufficient to maintain at least a portion of phot1 I608E-GFP phosphorylation sites unfilled *in vivo*. Phosphatase activity may also account for the retention of *in vitro* kinase activity in phot1 I608E expressed in insect cells (Section 3.3.5). Future work should focus on confirming the phosphorylation status of phot1 I608E-GFP *in planta* using transgenic *Arabidopsis* lines accumulating greater levels of phot1 I608E-GFP as the low levels of phot1 I608E-GFP expression in line 35-P1G(I608E)/*p1p2*-A greatly hampered interpretation of data.

If phot1 I608E is constitutively phosphorylated *in planta* it may be that such phosphorylation is insufficient for signal transmission. Protein phosphorylation is known to have multiple consequences including an alteration of protein catalytic activity, protein stability, sub-cellular localisation or binding affinities with interacting proteins (Hardie, 1999) and indeed it has been demonstrated that phosphorylation at different sites may have differing effects on protein kinase activity (Keranen *et al.*, 1995). In this study, Keranen *et al*. showed that an isoform of PKC (PKC-βII) is phosphorylated at three different sites in turn, with each having a defined role in the activation and subsequent regulation of the enzyme. It has previously been speculated that sites of phot autophosphorylation can be separated into those required to induce signalling and those involved in photoreceptor desensitisation (Briggs *et al.*, 2001) as such a division would account for the apparent discrepancy between light fluences required for a detectable phototropic response in comparison to the increased fluences required to induce a detectable phot autophosphorylation response (Palmer *et al.*, 1993a, Salomon *et al.*, 1997). In support of this notion phosphopeptide mapping has shown that phot1 is phosphorylated at additional sites when irradiated with higher fluences of light (Salomon *et al.*, 2003). In relation to the observed activity of phot1 I608E-GFP discussed above it is possible that the phot1 I608E mutation induces constitutive autophosphorylation of sites typically phosphorylated at higher fluences rather than those required for the initiation of phot-mediated signalling, thereby inhibiting phot1 signalling rather than inducing constitutive activity. To further evaluate this hypothesis it will be necessary to identify the phosphorylation sites which are phosphorylated by phot1 I608E in dark-treated samples and compare these to those sites phosphorylated in wild-type phot1 after light exposure. Mass spectroscopy could be used to assess this phosphorylation as previously described (Sullivan *et al.*, 2008).

Another role of phot1 autophosphorylation may be to induce relocalisation of phot1 away from the plasma membrane (Christie, 2007). Confocal microscopy studies assessing the redistribution of wild-type phot1 and phot2 upon blue light stimulation have shown that both phots demonstrate an altered sub-cellular distribution upon irradiation (Kong *et al.*, 2006, Sakamoto and Briggs, 2002, Wan *et al.*, 2007). Phot2 relocalisation requires a catalytically-active kinase domain for this relocalisation as phot2-GFP lacking kinase activity does not demonstrate light-dependent relocalisation (Kong *et al.*, 2006). Although the low level of phot1 I608E-GFP expression observed in the lines isolated for this study prevented conclusive analysis of the sub-cellular localisation of phot1 I608E-GFP (Section 5.2.3) it is hoped that the identification of lines expressing increased levels of the transgenic protein will facilitate characterisation of phot1 I608E-GFP sub-cellular localisation. Western blot analysis could also be used to assess phot1 I608E-GFP localisation in the presence or absence of light stimulation.

Another key concept in the regulation of protein kinases is recruitment of these enzymes to signalling complexes by scaffolding proteins (Smith *et al.*, 2006). Such signalling complexes are thought to have multiple functions; they may act to localise kinase activity to specific regions of the cell and can also improve the efficiency of signal transmission through a kinase cascade by recruiting appropriate substrates (Smith *et al.*, 2006). The sequestion of kinases to distinct signalling complexes also allows an additional layer of specificity to be conferred upon kinase activity. Indeed, the recruitment of phot1 to different signalling complexes may account for the ability of phot1 to regulate multiple physiological functions (Briggs and Christie, 2002) and may partially explain the differing consequences of phot1 I608E expression.

One of the best-characterised families of kinase scaffolding proteins are the A-Kinase Anchoring Proteins (AKAPs; Wong and Scott, 2004). AKAPs form a highly structurally diverse family that were initially identified as proteins which interact with PKA (Theurkauf and Vallee, 1982), although since this initial characterisation several AKAPs have been identified that interact with alternate kinases in addition to PKA (Smith *et al.*, 2006). For example, both PKA and PKC bind to human AKAP79 (Klauck *et al.*, 1996). AKAPs are multifunctional proteins that contain distinct localisation motifs in addition to binding sites for separate components of a signalling cascade (Tasken and Aandahl, 2004). Whilst little is known regarding the signalling

complexes in which phots participate, individual proteins which interact with phot1 have been identified. These proteins include NONPHOTOTROPIC HYPOCOTYL 3 (NPH3) and ROOT PHOTOTROPISM 2 (RPT2; Inada *et al.*, 2004, Motchoulski and Liscum, 1999). Both of these proteins are members of the NPH3/RPT2-like (NRL) family which have been speculated to act as scaffolds to enhance interactions between phots and downstream signalling factors (Celaya and Liscum, 2005). Interestingly, co-immunoprecipitation assays using PHYTOCHROME KINASE SUBSTRATE1 (PKS1) tagged with GFP and expressed in transgenic *Arabidopsis* have demonstrated that PKS1 interacts with both NPH3 and phot1 *in vivo*, suggesting that these proteins form part of the same signalling complex (Lariguet *et al.*, 2006). As each of these proteins have been suggested to have a role in phototropism it is therefore reasonable to assume that this signalling complex may have a role in mediating the phototropic response (Lariguet *et al.*, 2006, Liscum and Briggs, 1995). Such a signalling complex may potentially facilitate the integration of the phytochrome-mediated enhancement of phototropism (Janoudi *et al.*, 1997, Parks *et al.*, 1996), as PKS1 is phosphorylated by oat phyA *in vitro* and undergoes light-dependent phosphorylation *in vivo* (Fankhauser *et al.*, 1999). As such it will be interesting to confirm whether phytochromes co-localise with NPH3 and phot1 in addition to PKS1 *in vivo*.

An alternative role of NPH3 and RPT2 may be to regulate the light-dependent proteolytic turnover of proteins associated with phot1 signal transduction as both of these proteins contain BTB/POZ domains which are commonly associated with proteolytic degradation (Celaya and Liscum, 2005, Pintard *et al.*, 2004). Light-regulated protein degradation induced by phosphorylation has previously been observed for both phyA and cry2 (Seo *et al.*, 2004, Shalitin *et al.*, 2002) and it has been suggested that light-dependent photoreceptor degradation subsequent to phosphorylation acts to fine-tune photoreceptor signalling (Hoecker, 2005). Whilst there is no indication that NPH3 or RPT2 have a role in phot1 degradation, blue light stimulation has previously been shown to reduce phot1 protein levels in etiolated seedlings (Sakamoto and Briggs, 2002). However, phot1 protein expression is not completely abolished as indicated by the requirement of phot1 for a host of physiological functions in light-grown tissue (Briggs and Christie, 2002, Christie, 2007, Inoue *et al.*, 2007) and additionally phot1 protein is readily detectable in mature light-grown tissue (Section 5.2.13; Sullivan *et al.*, 2008). If phot1 phosphorylation enhances protein degradation the inability to isolate transgenic lines expressing high

levels of phot1 I608E may indirectly suggest phot1 I608E *in vivo* phosphorylation subsequently induces increased protein turnover in comparison to wild-type phot1. Such a hypothesis may be investigated through the use of proteosome inhibitors such as MG132 (Lee and Goldberg, 1998). MG132 acts to inhibit chymotrypsin-like activity within the 26S proteosome (Rock *et al.*, 1994) and this inhibitor has been used previously to demonstrate the light-dependent proteolytic degradation of *Chlamydomonas* cryptochrome (Reisdorph and Small, 2004). It will therefore be interesting to determine whether the application of MG132 enhances the accumulation of phot1 I608E-GFP in the transgenic lines created.

An additional phot1-interacting protein which may have a role in phot1-mediated signal transduction is the *Arabidopsis* 14-3-3 protein, 14-3-3λ (Thomson, 2008). 14-3-3 proteins have been shown to bind the heavily phosphorylated linker region between the LOV1 and LOV2 domains of *Vicia faba* phot1 (Kinoshita *et al.*, 2003), whilst the truncated protein phot1 LOV2 kinase does not interact with 14-3-3λ *in vitro* (Thomson, 2008). This latter result suggests that 14-3-3 binding similarly occurs at a site N-terminal of *Arabidopsis* phot1 LOV2. 14-3-3 proteins are conserved amongst eukaryotic systems (Aitken *et al.*, 1992) and typically act by binding to phosphorylated proteins to facilitate signal transduction (Sehnke *et al.*, 2002). The role of 14-3-3λ binding to *Arabidopsis* phot1 has yet to be fully characterised although 14-3-3 binding to *Vicia* phot1 is required for stomatal opening in this species (Kinoshita *et al.*, 2003). Alternatively, 14-3-3 binding has also been reported to increase protein instability of phosphorylated proteins such as nitrate reductase in spinach extracts (Weiner and Kaiser, 1999). If this latter example were applicable to phot1 it may be that 14-3-3 protein binding has a role in the mediating phot1 deactivation and degradation subsequent to its autophosphorylation.

## 6.8 Conclusions

During this project I have attempted to further characterise the mechanism by which phot1 autophosphorylation occurs using the baculovirus/insect cell expression system as a convenient means of evaluating the autophosphorylation of phot1 and phot2 *in vitro*. Transgenic *Arabidopsis* were also used to determine whether a single point mutation sufficient to induce constitutive autophosphorylation *in vitro* altered phot1 signalling when expressed *in vivo*.

Data presented in this thesis suggests that phot1, in contrast to closely-related protein kinases such as PINOID, is capable of intermolecular autophosphorylation. However, whilst progress has been achieved in evaluating the mechanism of phot1 autophosphorylation *in vitro* it remains to be seen whether such a mechanism occurs *in planta*. The second portion of this thesis further investigated the signalling mechanism linking LOV2 photochemistry with light-induced autophosphorylation. Consistent with previously reported conformational changes induced within the LOV2 domain upon light irradiation it appears that a mutation observed within the LOV2 βE-sheet enclosing the flavin chromophore inhibits signal transmission from the LOV2 domain to the integral kinase. Such data provides important *in vitro* verification of the relevance of conformational changes within the isolated LOV domain and suggests a mechanism for LOV2 signal transmission. Finally, the role of a constitutively phosphorylated version of phot1 (phot1 I608E) was evaluated *in planta*. Whilst conclusive analysis of this data was hampered by the low level of phot1 I608E-GFP expression observed in the isolated transgenic lines the results obtained suggest that this mutant phot1 is not capable of inducing constitutive phot1-mediated responses *in vivo*. Instead, phot1 I608E possibly acts to inhibit some phot signalling pathways such as petiole positioning and leaf expansion whilst being functional for others such as phototropism. Whilst such contradictory data may be explained by phot1 having differing roles in each of the signalling cascades required for these physiological responses, more detailed analysis is required to further evaluate the function of phot1 I608E *in planta*. For example, it is possible that the time period over which phototropism occurs is altered in lines expressing phot1 I608E-GFP compared to those expressing phot1-GFP. Time courses to assess the kinetics of phototropism in 35-P1G(I608E)/*p1p2* and 35-P1G(I608E)/WT lines should therefore be completed. Future work should focus on further evaluation of the role of phot1 I608E phosphorylation and its subsequent activity *in planta* using transgenic lines showing enhanced levels of phot1 I608E expression.

## 6.9 Future work

Although the data obtained during the course of this study further characterises the initial signalling steps induced within phot1 and phot2 subsequent to light stimulation our understanding of phot light detection and initial signal transduction remains

incomplete. Whilst significant progress has been made in characterising the role of LOV2 in the activation of *Arabidopsis* phot kinases (Cho *et al.*, 2007, Christie *et al.*, 2002), a functional role for LOV1 has yet to be unambiguously determined. Similarly, although light-dependent autophosphorylation of phot1 and phot2 is well established, it is becoming apparent that phosphorylation of the sites recently identified by mass spectroscopy (Sullivan *et al.*, 2008) is not required for initial signal transmission, as indicated by the functional complementation provided by the phot1 LOV2 kinase protein (Sullivan *et al.*, 2008). It therefore appears likely that additional sites within phots are autophosphorylated and that so far uncharacterised phot kinase substrates exist. Identification of these elusive phot kinase substrates and the mapping and functional characterisation of additional phot phosphorylation sites remains a high priority. The final, perhaps most interesting question posed by the data presented here relates to the dominant negative phenotypes observed in transgenic *Arabidopsis* lines expressing phot1 I608E in a wild-type background.

Given that LOV1 may have a role in modulating signal transmission initiated by LOV2 photoactivation (Kasahara *et al.*, 2002) it will be of interest to investigate whether such a role is mediated through the alteration of protein light-induced conformational changes. X-ray crystallography, NMR spectroscopy and FTIR spectroscopy have all previously been used to probe structural changes induced within individual LOV1 and LOV2 domains (Crosson and Moffat, 2001, Crosson and Moffat, 2002, Fedorov *et al.*, 2003, Halavaty and Moffat, 2007, Harper *et al.*, 2003, Iwata *et al.*, 2003, Iwata *et al.*, 2005), and the next challenge will be to examine light-induced structural changes in an extended construct containing both LOV1, LOV2 and the intervening linker sequence (LOV1+LOV2). However, such an investigation is likely to pose considerable technical challenges and a more amenable alternative in the short-term may involve the use of size-exclusion chromatography to indirectly monitor changes in apparent molecular weight induced by light exposure. Such a technique has previously been used to indicate light-induced alterations in LOV domain structural conformation (Zoltowski *et al.*, 2007). Zoltowski *et al.* were able to show that a fragment of the fungal LOV domain-containing protein VIVID had a greater hydrodynamic radius after light exposure compared with a dark-incubated sample, with the observed shift in apparent molecular weight less than that expected if the alteration were attributable to dimerisation. Similar investigation of a phot LOV1+LOV2 fragment in combination with mutant variants deficient in either LOV1

or LOV2 photochemistry may provide further information regarding the role of LOV1 in modulating LOV2 signal transmission. Such an investigation may also determine whether this N-terminal phot fragment is capable of dimerisation.

Further insight into the role of phot LOV1 domains may also be drawn from comparison with other *Arabidopsis* LOV-domain containing proteins. The *Arabidopsis* genome contains an additional four genes along with phot1 and phot2 that encode proteins containing LOV domains (Crosson *et al.*, 2003), with three of these proteins comprising the ZTL/ADO family (Section 1.5). The remaining LOV domain-containing protein encoded by the *Arabidopsis* genome is PAS/LOV Protein (PLP; At2g02710). As its name suggests, PLP contains an N-terminal PAS domain coupled to a C-terminal LOV domain (Appendix IV, Figure 10.1A). Three different gene models have been predicted for *PLP* transcription *in planta* (http://www.arabidopsis.org/), resulting in three potential mRNA splice variants of PLP. One of these variants encoded a truncated version of PLP lacking the LOV domain whilst the other two isoforms differed by a two amino acid insertion within the LOV domain. Initial work to determine whether the PLP variants containing LOV domains were capable of binding a flavin chromophore revealed trace chromophore binding in full-length PLP protein expressed in *E. coli*, (Appendix IV, Figure 10.1B). Interestingly, flavin binding was altered between the two predicted full-length isoforms of PLP, suggesting that the different splices have differing flavin binding affinities *in vitro*. A similar trace chromophore interaction between isolated PLP LOV domains and flavin has recently been reported by an independent research group (Ogura *et al.*, 2007). Ogura *et al.* (2007) also demonstrated blue light-dependent interactions between PLP and potential binding partners using a yeast-two hybrid assay. However, the relevance of these interactions *in vivo* is unclear. To further assess PLP chromophore interactions and photochemical activity it will be necessary to isolate PLP from transgenic *Arabidopsis* over-expressing this protein. Alternately a heterologous expression system such as the baculovirus/insect cell expression system used for this study could be used, while recent work by Kim *et al*. (2007) has demonstrated that LOV domain-containing proteins may be successfully isolated from tobacco following transient protein expression (Kim *et al.*, 2007).

Another area of pressing concern in phot signalling is the identification of phot substrates other than the phots themselves, as the major sites of phot1 autophosphorylation mapped by mass spectroscopy are not necessary for functional

complementation (Sullivan *et al.*, 2008). The ability to purify catalytically-active GST-tagged phot1 from the baculovirus/insect cell expression system (Section 3.2.3) may facilitate identification of substrates if dark-grown *Arabidopsis* tissue were incubated with heterologously-expressed GST-phot1 in the presence of $^{32}$P-ATP *in vitro*. However, such an approach is complicated by the presence of endogenous *Arabidopsis* kinases which may mask signal derived from the light-induced activity of phot1 kinase. Such a difficulty may be overcome through the introduction of a novel modification into the phot1 kinase domain to preferentially incorporate an ATP analogue. It has previously been demonstrated that substitution of a single residue within the ATP-binding pocket of a tyrosine kinase allows the modified kinase domain to catalyse the phosphorylation of proteins with a bulky N6-modified ATP analogue (Shah *et al.*, 1997), presumably by increasing the volume of the ATP-binding motif within the kinase domain. Recent work has demonstrated that a similar mutation within PKA allows identification of novel substrates (Schauble *et al.*, 2007). In this latter case, substrate proteins labelled with the radioactive ATP analogue were separated by 2D-electrophoresis before being identified by mass-spectroscopy. The application of a similar technique using an appropriately modified phot1 kinase may greatly enhance the sensitivity of substrate-identification assays.

Possibly the most enigmatic data presented in this thesis concerns the observed dominant negative effect of phot1 I608E expression in a wild-type background (Sections 5.2.12-13). As data presented here indicates that endogenous phot1 and phot2 protein expression is unaffected by phot1 I608E expression in mature leaf tissue (Section 5.2.13) it would appear that an alternate mechanism interferes with signal transduction from endogenous phot1 and phot2. Such a mechanism may involve an inhibition of binding affinities between endogenous phots and their interacting proteins. One method to evaluate the retention of these interactions by phot1 I608E would be to characterise the co-precipitation of known interacting proteins (such as NPH3, RPT2, PKS1 and 14-3-3λ; Inada *et al.*, 2004, Lariguet *et al.*, 2006, Motchoulski and Liscum, 1999, Thomson, 2008) with phot1 I608E-GFP immunoprecipitated from transgenic *Arabidopsis* expressing the mutated protein in a *p1p2* background using a GFP antibody as described previously (Lariguet *et al.*, 2006). However, the relatively low levels of phot1 I608E-GFP expressed in the lines characterised in this study may preclude sensitive analysis (Section 5.2.1). As an alternative, phot1 I608E may be used as a bait for a yeast two-hybrid (Y2H) screen.

Initial results indicated that phot1 I608E did not auto-activate when expressed in this Y2H system and it will now be of interest to examine whether known phot1-interacting partners retain an interaction with the mutated phot1 I608E protein in this system.

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# Appendix I

| | |
|---|---|
| polyhedrin primer2 | AAATGATAACCATCTCGC |
| phot1 bait del 3L | GCGCTGCAGCTGAGACCACAAACGTTT |
| phot1 seq1 | TCAACCCCGACCACAACAAGA |
| NPHD36 | CGCAGAGAAACTCGCAAAACT |
| NPHU6 | CTTCCGCCGCCAGAGTTTCTT |
| NPHD26 | GATGCAGCGAATCAACGAAAT |
| NPHD31 | TGATAACCAAACCGAAGTGAC |
| phot1 seq6 | CCGATTGGTTTGAAGCATTTC |
| phot1 seq7 | AACAGATTACTATCCAGGAGG |
| phot1 seq8 | AACTCATTTGTTGGCACTGAA |
| phot2 Seq1 | CCTTTGAATGATGCGGAGTC |
| phot2 Seq2 | TCCACGTTGCAGCAGACTTTTGTT |
| phot2 Seq3 | TCCTTGATTCGATATGATGCTC |
| phot2 Seq4 | AAGAATTTCGTCATCAGTG |
| phot2 Seq5 | ACGGCAACAAATGTAGATGAA |
| phot2 Seq6 | ACCTCTACGCACGTCTGTTTGA |
| phot2 Seq7 | TGAAGAATACATTGCGCCTGAGAT |
| pCAL-n-5' | GAATTTCATAGCCGTCTCAGC |
| pCAL-n-3' | CTAGTTATTGCTCAGCGG |

**Table 7.1 Primers used for DNA sequencing reactions.** DNA oligonucleotides used to prime PCR and DNA sequencing reactions are listed. All primer sequences are given 5’ – 3’.

| | |
|---|---|
| phot1-7 U (D806N) | ATATCTCTTTGTCGAATTTTGATCTGTCTTGC |
| phot1-7 L (D806N) | GCAAGACAGATCAAAATTCGACAAAGAGATAT |
| phot1 S849A U | GCTGAACCAATGCGTGCATCAAACGCATTTGTTGGCACTGAAGAGTACATT |
| phot1 S849A L | AATGTACTCTTCAGTGCCAACAAATGCGTTTGATGCACGCATTGGTTCAGC |
| AtLOV2 C39M U | CTTGGAAGAAATATGAGGTTTCTACAAGG |
| AtLOV2 C39M L | CCTTGTAGAAACCTCATATTTCTTTCCAAG |
| Atphot1 l608E U | TGAAAAAAACAGCTGTGAATGAGGATGAAGCGGTTCGAGAACT |
| Atphot1 l608E L | AGTTCTCGAACCGCTTCATCCTCATTCACAGCTGTTTTTTTCA |
| phot2 V522E U | TGAAAGCTACGGCAACAAATGAAGATGAAGCTGTCAGAGAGC |
| phot2 V522E L | GCTCTCTGACAGCTTCATCTTCATTTGTTGCCGTAGC |
| phot1 LOV2 C39A U | AAGAAATTCTTGGAAGAAATGCCAGGTTTCTACAAGGTCCAG |
| phot1 LOV2 C39A L | TCTGGACCTTGTAGAAACCTGGCATTTCTTCCAAGAATTTC |
| phot2 kinase D-N upper | GATGGACACATAGTATTGGCTAACTTTGATTTATCATTCATGACG |
| phot2 kinase D-N lower | CGTCATGAATGATAAATCAAAGTTAGCCAATACTATGTGTCCATC |
| phot1 LOV2 E33K U | CTCACGGAATATAGCCGTGAAAAAATTCTTGGAAGAAATTGCAGG |
| phot1 LOV2 E33K L | CCTGCAATTTCTTCCAAGAATTTTTTCACGGCTATATTCCGTGAG |

| | |
|---|---|
| phot1 Q575L U | CAATACTTTATTGGAGTTCTACTAGACGGGAGCAAGCACG |
| phot1 Q575L L | CGTGCTTGCTCCCGTCTAGTAGAACTCCAATAAAGTATTG |
| phot1 Q575H U | CAATACTTTATTGGAGTTCATCTAGACGGGAGCAAGCACG |
| phot1 Q575H L | CGTGCTTGCTCCCGTCTAGATGAACTCCAATAAAGTATTG |
| phot1 Q575N U | CAATACTTTATTGGAGTTAATCTAGACGGGAGCAAGCACG |
| phot1 Q575N L | CGTGCTTGCTCCCGTCTAGATTAACTCCAATAAAGTATTG |
| phot2 Q489L U | GCTTCAATACTTCATCGGTGTGTTACTTGATGGAAGTGATCATGTAGAGC |
| phot2 Q489L L | GCTCTACATGATCACTTCCATCAAGTAACACACCGATGAAGTATTGAAGC |

**Table 7.2 Primers used for site-directed mutagenesis reactions.** DNA oligonucleotides used to prime PCR and DNA sequencing reactions are listed. All primer sequences are given 5' – 3'.

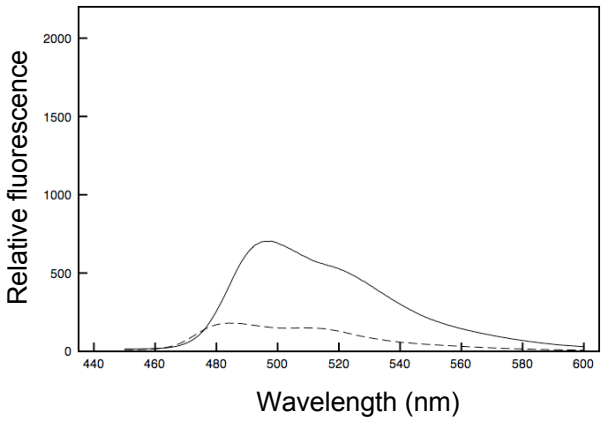
# Appendix II

In order to compensate for differences in chromophore fluorescence emission quenching between samples observed flavin fluorescence emission values were transformed using known absorbance values via the following equation;

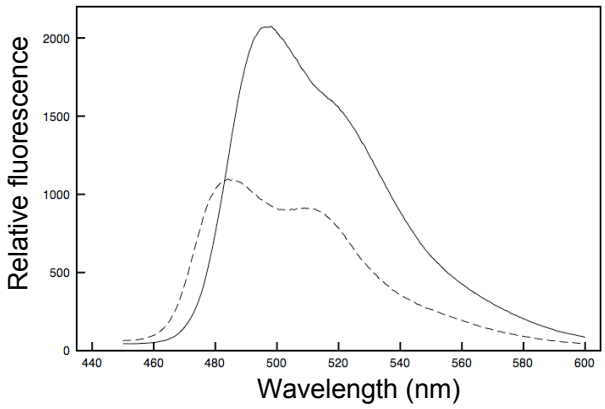
$$F_{correct} = \frac{F_{Observed}}{1 - 10^{-Abs}}$$

where $F_{correct}$ represents the corrected value, $F_{observed}$ is the observed fluorescence emission and Abs represents the absorbance of the sample at 380 nm. Transformation of the data presented in Figure 4.3 using this equation is illustrated below (Abs = 0.18 and 0.078 for LOV2 and LOV2 Q575L respectively). Such a transformation suggests that the fluorescence emission quenching of LOV2 Q575L is approximately twice that of wild-type LOV2.

## Untransformed data




## Transformed data



# Appendix III

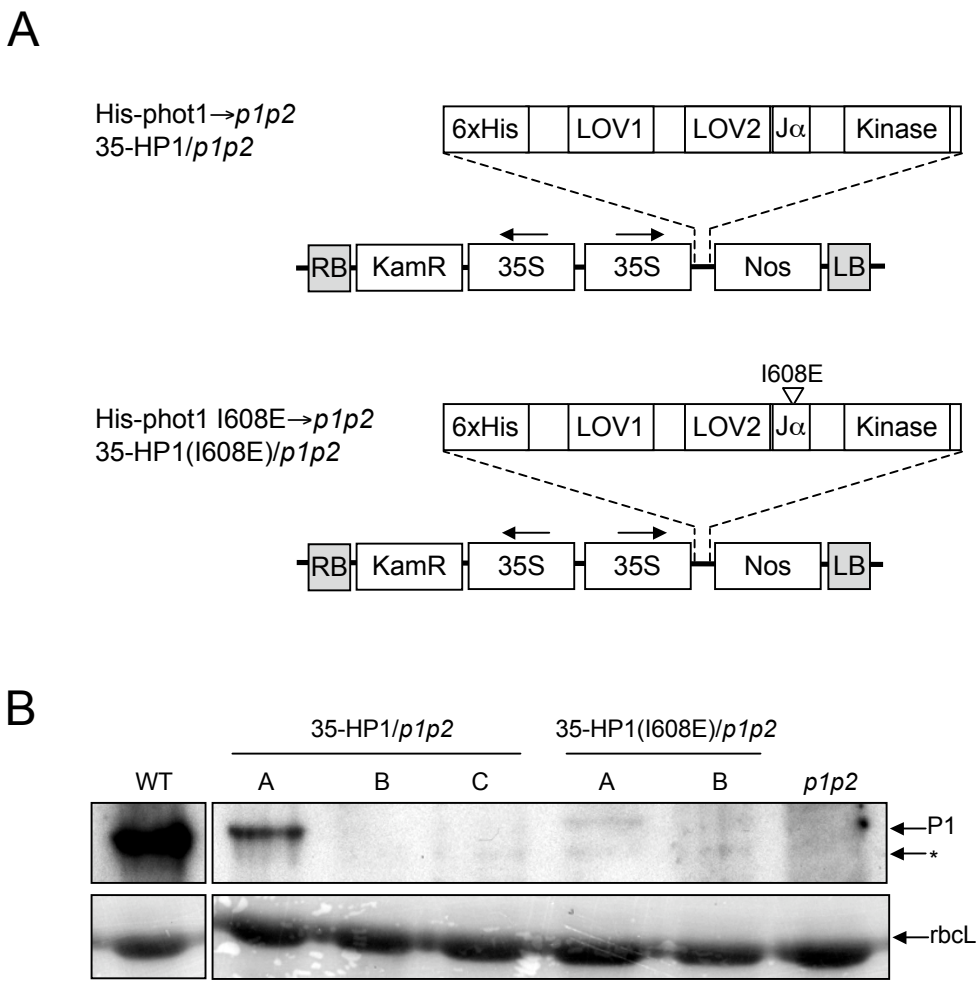



**Figure 9.1 Expression of His-phot1 and His-phot1 I608E in *phot1-5 phot2-1* seedlings. (A)** Schematic diagram showing construction of expression vectors used to transform *phot1-5 phot2-1* (*p1p2*) plants. The I608E point mutation within the Jα-helix is indicated. Transgenic lines were created expressing either His-phot or His-phot1 I608E in a *p1p2* background (35-HP1/*p1p2* and 35-HP1(I608E)/*p1p2* respectively). **(B)** Western blot analysis of His-phot1 and His-phot1 I608E protein expression in homozygous T3 transgenic lines compared to the *p1p2* double mutant and wild-type (WT). The lane containing crude protein extract from WT was separated from the remaining samples for clarity of exposure, although exposure times in each case were identical. Transgenic phot1 has a larger molecular weight due to the inclusion of a 6x His tag. Crude soluble protein extracts (30 μg) from three-day-old etiolated seedlings were probed with anti-phot1 antibody. Phot1 (P1) and a non-specific band (*) are indicated. A ponceau stain highlighting the large subunit of RIBULOSE-BISPHOSPHATE CARBOXYLASE (rbcL) is shown below as a loading control.

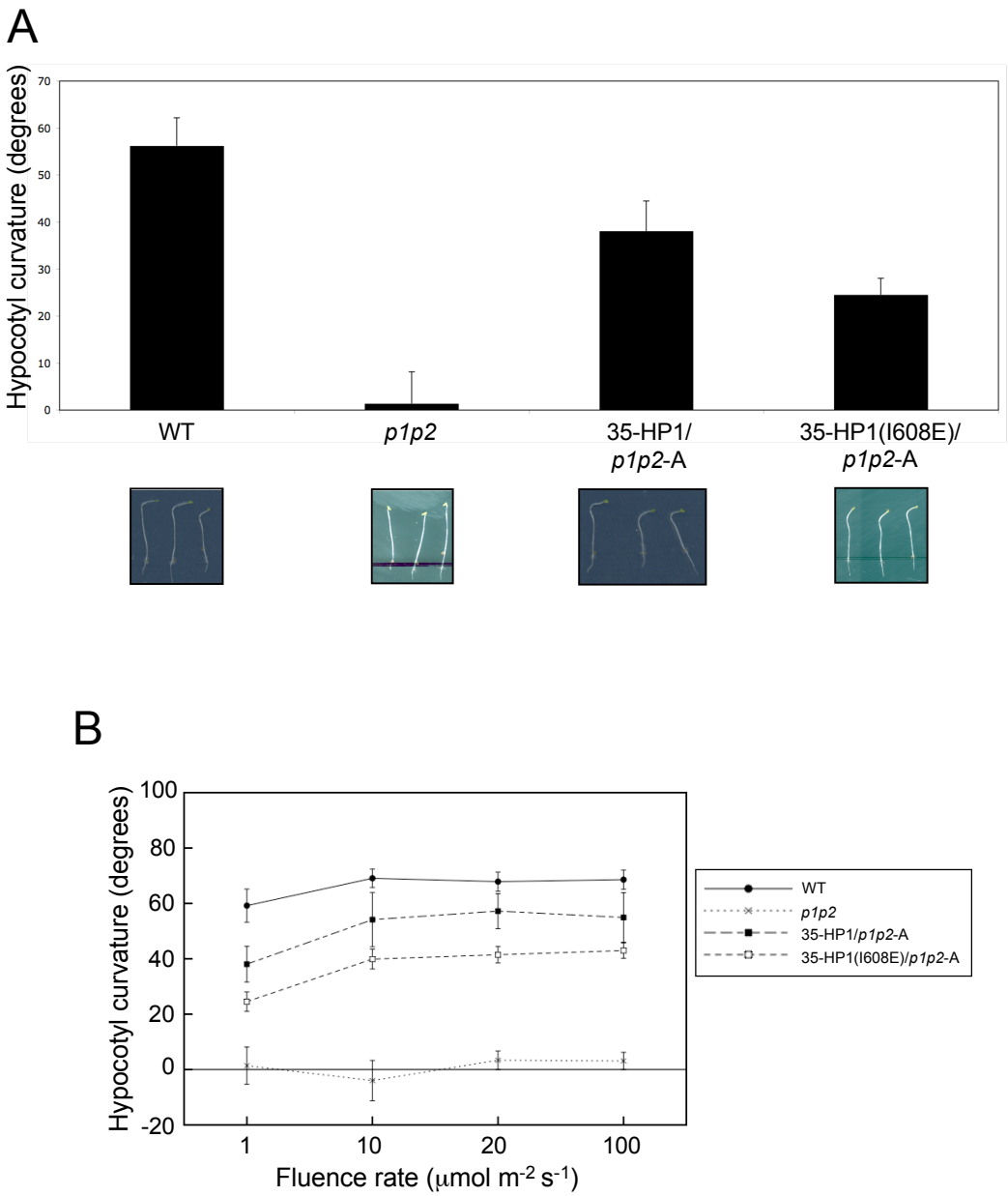



**Figure 9.2 Analysis of phototropic curvature in etiolated seedlings expressing His-phot1 or His-phot1 I608E in a *phot1-5 phot2-1* background. (A)** Phototropic curvature of wild-type (WT), *p1p2* or transgenic *Arabidopsis* seedlings expressing His-phot1 (35-HP1/*p1p2*) or His-phot1 I608E (35-HP1(I608E)/*p1p2*) were examined. Seedlings were grown in the dark for three days before being exposed to 1 μmol $m^{-2}$ $s^{-1}$ unilateral blue light for 24 hours. Error bars indicate standard error (n≥15). Representative images are shown below. **(B)** Phototropism fluence response curves for WT, *p1p2,* 35-HP1/*p1p2* and 35-HP1(I608E)/*p1p2* seedlings. Seedlings were grown in the dark for three days before being exposed to unilateral blue light at the indicated fluence for 24 hours. Error bars indicate standard error (n≥15).

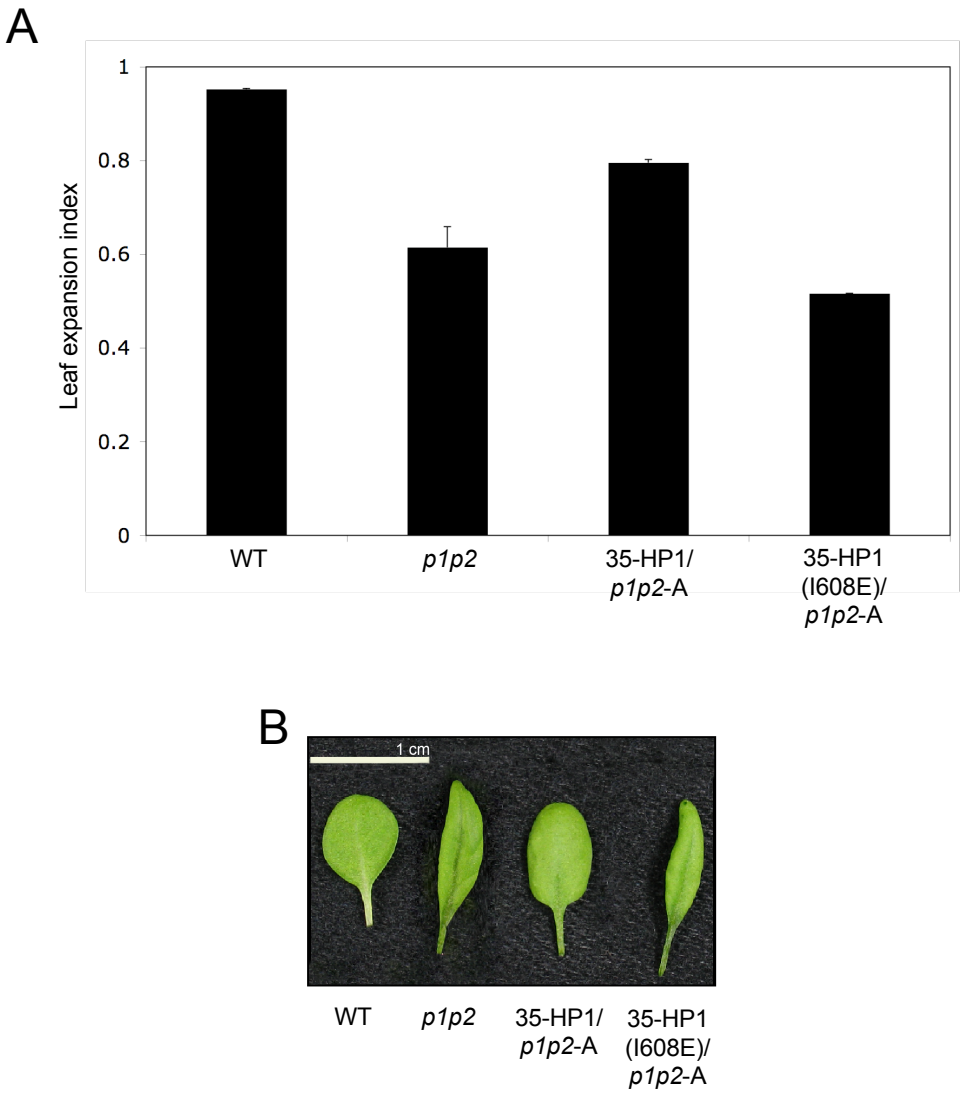



**Figure 9.3 Analysis of leaf expansion in *phot1-5 phot2-1* plants expressing His-phot1 or His-phot1 I608E. (A)** Leaf expansion index of the fifth rosette leaves of wild type (WT), *phot1-5 phot2*-1 (*p1p2*), 35-HP1/*p1p2*-A and 35-HP1(I608E)/*p1p2*-A plants. Plants were grown on soil for three weeks under 50 μmol $m^{-2}$ $s^{-1}$ constant white light. The leaf expansion index is expressed as the ratio of leaf area before and after artificial flattening. Error bars indicate standard error (n=10). **(B)** Representative fifth rosette leaves from plants described in (A).

# Appendix IV

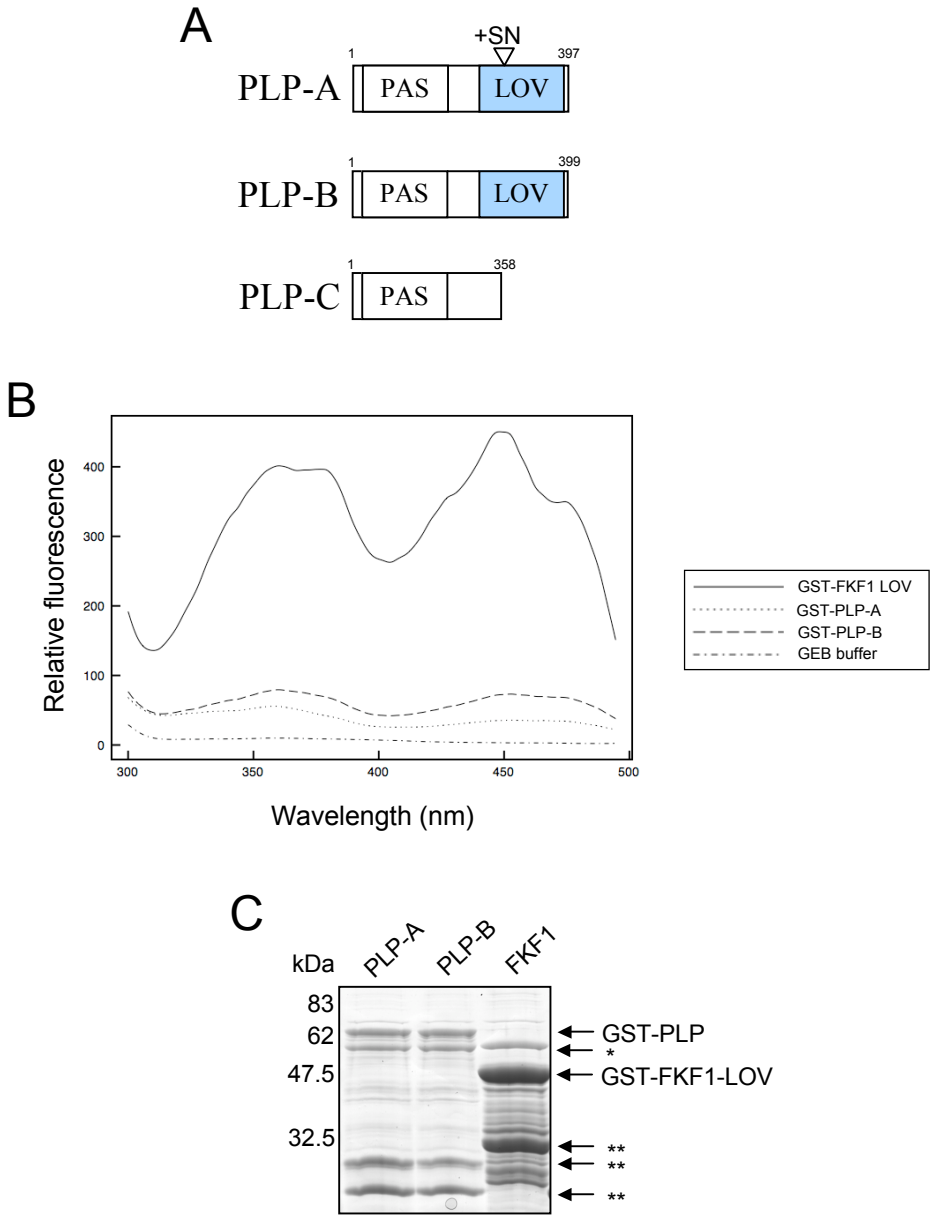



**Figure 10.1 Assessment of flavin binding levels of PLP protein expressed in *E. coli*. (A)** Schematic diagram illustrating the three different variants of PLP predicted to be expressed in *Arabidopsis*. The approximate location of the serine and asparagine insert within the PLP-A LOV domain is shown. Amino acid positions are shown. **(B)** Fluorescence excitation spectra of GST-tagged FKF1 LOV domain (GST-FKF1-LOV), PLP-A (GST-PLP-A) and PLP-B (GST-PLP-B). Proteins were purified from *E.coli* and fluorescence spectra were recorded using equal concentrations (0.3 mg $ml^{-1}$) of each purified sample. Glutathione elution bugger (GEB) is shown as a negative control. (**C)** SDS-PAGE analysis of proteins assayed in (B). Proteins (10 µg) were separated by 12.5% SDS-PAGE. Expected protein sizes, contaminating bacterial proteins (*) and presumed major degradation products (**) are indicated.