# Molecular insights into the phototropin control of chloroplast movements

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## Highlight

The biochemical activity, structural features, localization, and expressional control of phototropins provide insight into their function in signalling to direct chloroplast movement.

## Abstract

Chloroplast movements are controlled by ultraviolet/blue light through phototropins. In *Arabidopsis thaliana*, chloroplast accumulation at low light intensity and chloroplast avoidance at high light intensity are observed. These responses are controlled by two homologous photoreceptors, the phototropins phot1 and phot2. Whereas chloroplast accumulation is triggered by both phototropins in a partially redundant manner, sustained chloroplast avoidance is elicited only by phot2. Phot1 is able to trigger only a small, transient chloroplast avoidance, followed by the accumulation phase. The source of this functional difference is not fully understood at either the photoreceptor or the signalling

pathway levels. In this article, we review the current understanding of phototropin functioning and try to dissect the differences that result in signalling to elicit two distinct chloroplast responses. First, we focus on phototropin structure and photochemical and biochemical activity. Next, we analyse phototropin expression and localization patterns. We also summarize known photoreceptor systems controlling chloroplast movements. Finally, we focus on the role of environmental stimuli in controlling phototropin activity. All these aspects impact the signalling to trigger chloroplast movements and raise outstanding questions about the mechanism involved.

**Keywords:** Arabidopsis, blue light, chloroplast movements, LOV domain, phosphorylation, photocycle, phototropin1, phototropin2, ultraviolet.

## Introduction

Light-induced chloroplast movements are observed in many species of aquatic and land plants (Senn, 1908; Yorinao and Kazuo, 1974), such as green algae (*Mougeotia* sp.) (Wagner and Karin, 1981; Gabrys *et al.*, 1985), mosses (*Physcomitrella patens*) (Kasahara *et al.*, 2004), liverworts (*Marchantia polymorpha*) (Komatsu *et al.*, 2014), ferns (*Adiantum capillus-veneris*) (Wada, 2007), and seed plants (e.g. *Lemna trisulca*, *A. thaliana*, *Nicotiana tabacum*, *Oryza* spp.) (Zurzycki *et al.*, 1983; Trojan and Gabrys, 1996; Anielska-Mazur *et al.*, 2009; Kihara *et al.*, 2020; Krzeszowiec *et al.*, 2020). The reader can refer to the section 'Insights from the diversity of phototropins', later in this review, for more examples. In the model plant *A. thaliana*, chloroplast movements are controlled by ultraviolet (UV)/blue light photoreceptors called phototropins (Christie, 2007). In this species, two types of chloroplast movements are triggered by two phototropins, phot1 and phot2, in a partially redundant manner (Christie, 2007). At low

intensities of blue light (0.01–20 μmol $m^{-2}$ $s^{-1}$), chloroplasts show an accumulation response (Fig. 1A, C). They gather at cell walls perpendicular to the direction of incident light to optimize the photosynthetic efficiency (Zurzycki, 1955; Gotoh *et al.*, 2018). Both phototropins elicit this reaction, but phot2 triggers chloroplast accumulation at a higher blue light intensity of 2 μmol $m^{-2}$ $s^{-1}$ (Jarillo *et al.*, 2001; Kagawa *et al.*, 2001; Sakai *et al.*, 2001). At blue light intensities >20 μmol $m^{-2}$ $s^{-1}$, chloroplasts gather close to the walls parallel to the direction of incident light. This chloroplast avoidance response is triggered only by phot2 (Fig. 1A, C) (Jarillo *et al.*, 2001; Kagawa *et al.*, 2001; Sakai *et al.*, 2001). It is usually assumed to have a photoprotective function at light intensities that saturate photosynthesis (Kasahara *et al.*, 2002a; Sztatelman *et al.*, 2010; Davis and Hangarter, 2012; Cazzaniga *et al.*, 2013; Pfündel *et al.*, 2018). However, a recent study contradicts these reports, implying that chloroplast avoidance is responsible only for increasing light penetration into deeper leaf mesophyll layers (Wilson and Ruban, 2020). At higher light intensities, phot1 elicits a biphasic response with short-lived, partial chloroplast avoidance preceding the accumulation phase (Luesse *et al.*, 2010; Łabuz *et al.*, 2015). Phot2 regulates the dark positioning of chloroplasts, which localize at the bottom of dark-adapted palisade cells of the Arabidopsis mesophyll. In dark-adapted *phot2* mutant leaves, chloroplasts gather at both the top and bottom periclinal walls of leaf mesophyll, thus the polarity of chloroplast positioning is lost (Suetsugu *et al.*, 2005a) (Fig. 1A).

Chloroplast movements operate on the scale of minutes and are local, as chloroplasts respond to light conditions in the part of the cell in which they are located. Partial irradiation of the cell with a blue light microbeam of 120 μmol $m^{-2}$ $s^{-1}$ causes

simultaneous chloroplast avoidance and accumulation. Chloroplasts that are directly irradiated move away from the light beam. Those outside the light beam show an accumulation reaction towards it, but do not cross the border of the irradiated cell fragment (Kagawa and Wada, 2000). Chloroplasts also respond to short blue light pulses (Fig. 1B): 0.1–1 s blue light pulses of 120 μmol $m^{-2}s^{-1}$ induce transient chloroplast accumulation. In the case of 10 s or 20 s pulses, chloroplasts show a biphasic response, with a brief avoidance response following the accumulation phase (Sztatelman *et al.*, 2016). Biphasic chloroplast reactions are also observed in continuous blue light of 16 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Luesse *et al.*, 2010; Łabuz *et al.*, 2015). Thus, signals leading to chloroplast accumulation and avoidance are simultaneously generated in the cell after irradiation; however, the signal leading to accumulation is characterized by a longer lifetime (Higa and Wada, 2015; Sztatelman *et al.*, 2016).

Apart from the actin cytoskeleton, which serves as the motile system (for review, see Wada and Kong, 2018), several proteins required for chloroplast movements have been identified. However, our understanding of their function, which is important for obtaining a picture of the subsequent signalling events, is still incomplete. This review does not focus on proteins involved in the control of chloroplast positioning, and the readers are advised to refer for more details to Banaś *et al.* (2012), Kong and Wada (2014, 2016), and Suetsugu and Wada (2020) for further information.

## The phototropin family

Phototropins contain two very similar, photosensory LOV (light, oxygen, or voltage-regulated) domains at the N-terminus, which bind flavin mononucleotide (FMN) as a

chromophore; these domains are called LOV1 and LOV2. LOV domains belong to a large family of PAS (Per, ARNT, Sim) domains, which bind cofactors and participate in protein interactions (Huala *et al.*, 1997). The C-terminal part of phototropins consists of a serine-threonine kinase domain (Christie, 1998), which belongs to the AGC kinase family (protein kinase A, cGMP-dependent protein kinase and protein kinase C) (Galván-Ampudia and Offringa, 2007). *PHOT* genes are present in green algae, bryophytes, ferns, and seed plants. They evolved from a single algal ancestral gene and underwent duplications independently in several plant lineages. The duplicated genes convergently evolved into specialized, low-light-sensing molecules of phot1 type, and more universal, but requiring higher light intensities, phot2-type photoreceptors (Li *et al.*, 2015). The number of phototropin genes in plant genomes varies from one in *Chlamydomonas reinhardtii* (Huang *et al.*, 2002), *Ostreococcus tauri* (Sullivan *et al.*, 2016), and *M. polymorpha* (Komatsu *et al.*, 2014), to seven in *P. patens* (Kasahara *et al.*, 2004; Li *et al.*, 2015). The analysis of LOV domain phylogeny suggests that LOV2 evolution simply reflects the differentiation of plant lines. By contrast, the phylogenetic tree of LOV1 domains obtained in Kato *et al.* (2021) does not match the evolutionary relationships between the species, which implies that it reflects convergent changes due to environmental pressures.

## The phototropin photocycle

The N-terminal part of the phototropin molecule is the photosensory module. The primary step of phototropin signalling is the absorption of light by LOV domains, which leads to kinase activation and receptor autophosphorylation (Christie, 1998). LOV

domains adapt a typical PAS domain fold, consisting of five antiparallel β-strands and two α-helices with additional N- and C-terminal helices, termed A'α and Jα, respectively (Christie, 2007; Nakasako *et al.*, 2008; Christie *et al.*, 2012; Halavaty and Moffat, 2013; Hart and Gardner, 2021). In the dark, each LOV domain non-covalently binds FMN (Christie *et al.*, 1999). Upon irradiation, a unique covalent bond forms between the FMN and a conserved cysteine residue in the LOV domain (Salomon *et al.*, 2000; Crosson and Moffat, 2001). This residue is crucial, as its mutation to alanine makes LOV domains photochemically inactive (Christie *et al.*, 2002). In darkness, the photoproduct returns to its original state. The kinetics of this process is temperature-controlled (Alexandre *et al.*, 2007). The light excitation and the subsequent dark reversal are described as the phototropin photocycle (Fig. 2). For both phototropins, the rate of photoproduct formation is faster for LOV2 domains than for LOV1 domains. The quantum yield of photoproduct formation is greater in the case of LOV2 domains, for both phot1 and phot2, compared with their LOV1 domains (Salomon *et al.*, 2000; Kasahara *et al.*, 2002b). The half-life of the cysteinyl adduct of an isolated LOV2 domain photoproduct of Arabidopsis phot1 is six times as long as that of the isolated LOV2 domain of phot2. It is prolonged in full-length proteins compared with isolated domains, due to the presence of LOV1 (Kasahara *et al.*, 2002b).

LOV2 domains play a dominant role in receptor photochemistry and biological activity (Christie *et al.*, 2002). The duration of the LOV2 domain photocycle is determined by the amino acid residues surrounding the FMN binding site. Targeted mutagenesis of the LOV2 domain results in a decrease or increase in the duration of the photocycle *in vitro*, thus engineered variants of phototropins are characterized by a

reduced or extended lifetime of their activated state when expressed *in planta* (Fig. 3). For Arabidopsis phot1, the substitutions N476L, V478L, V525R, and L558I slow down the photocycle, whereas the substitutions V478T and I489V accelerate it. This has a direct impact on the photosensitivity of phot1 in triggering chloroplast movements. The slow-photocycle mutants are more sensitive to pulse irradiation in eliciting transient chloroplast accumulation, while the fast photocycle mutants are less sensitive. The point mutation V392L in the LOV2 domain of phot2 slows the photocycle, enhances the light sensitivity of the photoreceptor in plants, and lowers the light-intensity threshold for mediating chloroplast avoidance. These results suggest that a prolonged photocycle may block the signalling that directs chloroplast accumulation (Hart *et al.*, 2019).

In the dark, the LOV2 domain is associated with the kinase domain, and it has been postulated that its fragment functions as an inhibitor of the constitutive kinase activity (Matsuoka and Tokutomi, 2005; Tokutomi *et al.*, 2008). Light excitation of the phot1 LOV2 domain triggers conformational changes in this part of the protein and the adjacent conservative fragment of the so-called Jα-helix (Koyama *et al.*, 2009). The light-induced Jα-helix unfolding has been proposed to weaken the interaction between the LOV and kinase domains and, consequently, unlock the kinase activity (Crosson and Moffat, 2001; Harper *et al.*, 2003). Activation of the kinase domain is determined by the Aα-LOV2-Jα photosensory module and results in receptor autophosphorylation (Christie, 1998; Sakai *et al.*, 2001). A point mutation R472H localized in the A'α region, adjacent to the LOV2 domain of Arabidopsis phot1, constitutively activates the kinase but does not affect photoreceptor photochemistry. *In planta*, this phot1 mutein is autophosphorylated in darkness. However, the phot1 R472H variant expressed in the

phototropin double mutant requires light to initiate signalling and is poorly functional under low blue light intensities, as chloroplast accumulation is restored only at high blue light intensities (>40 μmol·$m^{-2}$·$s^{-1}$) (Petersen *et al.*, 2017).

The LOV2 domain is crucial for the activation of the kinase domain and cannot be replaced by the LOV1 domain *in planta*, as shown by domain swapping for phot1 (Kaiserli *et al.*, 2009). LOV1 modifies photoreceptor sensitivity by affecting the properties of LOV2 (Matsuoka and Tokutomi, 2005; Kaiserli *et al.*, 2009). The LOV1 domain of phot1 prolongs the dark recovery of the photoreceptor, increasing its light sensitivity (Kaiserli *et al.*, 2009). However, LOV1 is not required for inducing chloroplast responses. Truncated phot1 with only LOV2 and kinase domains is sufficient to induce chloroplast accumulation (Sullivan *et al.*, 2008). Plants bearing phot1 with LOV1 replaced by the LOV2 domain, expressed in the double phototropin mutant background, showed inhibition of chloroplast accumulation at lower light intensities compared with wild-type phot1. By contrast, transgenic plants expressing phot1 without the LOV1 domain, or with the reactivity of LOV1 abolished by mutation of the photoactive C residue to A, exhibited a more substantial accumulation response than plants expressing the whole phot1 molecule. Thus, the LOV1 domain of phot1 from Arabidopsis may take part in the reduction of chloroplast accumulation at high light intensities (Kaiserli *et al.*, 2009). The LOV1 domain of phot2 attenuates its photoactivation, which results in the reduced biological activity of the receptor at low light intensities (Matsuoka and Tokutomi, 2005). In Han *et al.* (2013), the expression of a mutein with the reactivity of LOV1 abolished by replacement of the photoactive C residue with A did not restore chloroplast movement in the *phot1phot2* mutant. However,

recombinant protein abundance was very low in that study and control plants expressing wild-type phot2 did not show chloroplast avoidance, thus bringing into question the conclusiveness of the results. Interestingly, a detailed analysis of numerous Arabidopsis *phot2* mutant alleles indicates that a phot2 variant defective in the LOV2 domain, *phot2-10*, retains slight photosensitivity. Phot2-10 bears an R427Q mutation, preventing the LOV2 domain from binding FMN (Fig. 3). Nevertheless, residual chloroplast avoidance is observed at blue light intensities of 20–50 μmol $m^{-2} s^{-1}$ in the double *phot1-5phot2-10* mutant, which points to a possible, although limited, role for LOV1 in this process (Suetsugu *et al.*, 2013).

LOV domains also play a role in phototropin interactions. Isolated LOV1 domains of both phototropins can form dimers (Nakasako *et al.*, 2004; Salomon *et al.*, 2004; Katsura *et al.*, 2009). Phot1 LOV1 dimerization may depend on a disulfide bridge formed by Cys261 residues protruding from the β-sheet surface, as observed in the crystal structure. Dimerization of LOV1 domains from phot1 seems to be mediated by Thr217 and Met232 (Nakasako *et al.*, 2008). Isolated LOV2 domains of phot1 are present in a blue-light-dependent monomer–dimer equilibrium, whereas phot2 LOV2 domains are monomeric (Nakasako *et al.*, 2008; Katsura *et al.*, 2009). Hence, phot2 dimerization appears to occur through the LOV1 domains (Oide *et al.*, 2018). Interactions between whole phototropin molecules have also been verified experimentally. The formation of all types of complexes, phot1–phot1 (Kaiserli *et al.*, 2009; Xue *et al.*, 2018), phot1–phot2, and phot2–phot2, has been shown (Sztatelman *et al.*, 2016). Phot1 dimerization is light-dependent but does not require the activity of the phototropin kinase (Xue *et al.*, 2018). Phototropin dimerization is proposed to fine-tune responses, in particular chloroplast

accumulation (Fig. 4). Dimer formation between phototropins can lead to signal amplification by the transactivation of phototropin molecules. Both phot1 (Kaiserli *et al.*, 2009) and phot2 (Cho *et al.*, 2006) can transphosphorylate phot1. Transphosphorylation can occur independently of the LOV1 domain, as it is observed even when LOV1 is inactivated or absent (Kaiserli *et al.*, 2009). Phot1 exhibits greater light sensitivity and higher kinase activity than phot2 (Aihara *et al.*, 2008). Therefore, at low light intensities, phot1 homodimers are thought to be more effective at transducing the signal than phot1–phot2 heterodimers. Thus, in the presence of phot2, the heterodimer formation would decrease the signalling from phot1 in a manner similar to the competitive inhibition of enzymes (Fig. 4A). Chloroplast movements observed in *phot1* and *phot2* mutants in response to low-intensity blue light (Luesse *et al.*, 2010) as well as short pulses of high-intensity blue light (Sztatelman *et al.*, 2016) confirm this hypothesis (Fig. 4B). The *phot1* mutant, in which only phot2 remains active and forms dimers, shows a weak reaction to the shortest pulses as well as a small amplitude of chloroplast accumulation at low light intensities compared with the wild type, in which all types of dimers are present. Consistently, the *phot2* mutant, in which only phot1 dimers are active, shows higher amplitudes of chloroplast accumulation compared with the wild type, both in weak light (Luesse *et al.*, 2010) and in response to short pulses (Sztatelman *et al.*, 2016). By contrast, the presence of phot1 increases the signal leading to the avoidance response in wild-type plants. Transient chloroplast avoidance triggered by blue light pulses of 10–20 s is stronger in wild-type Arabidopsis than in the *phot1* mutant (Sztatelman *et al.*, 2016) (Fig. 4B). Similarly, phot1 enhances the avoidance response at non-saturating blue light

conditions (20 μmol·m$^{-2}$·s$^{-1}$) in the wild type compared with *phot1* plants (Łabuz *et al.*, 2015).

The main role of the N-terminal part of phototropins is to translate the light signal into the chemical signal, through light-dependent control of the phototropin kinase. LOV2 is indispensable for light activation of the kinase at the levels sufficient for effective signalling to direct chloroplast movements, and the kinetics of its photocycle strongly affect the sensitivity of chloroplast responses. Although less prominent, LOV1 has also been shown to play a role in shaping chloroplast responses. The apparent ability of the LOV1 domain to control the light-dependent dimerization of phototropin molecules creates the possibility of a more complex signal transduction.

# The phototropin kinase

Homology modelling of the structure of the phototropin kinase, using other kinases from the AGC family as templates, indicates that it consists of two lobes separated by a catalytic cleft, a basic structure that is shared with other protein kinases (Christie, 2007; Hart and Gardner, 2021). The core of the smaller N-lobe is formed by a β-sheet consisting of five antiparallel strands. The sequences of N-lobes of phototropins contain conserved motifs, known from other kinases to participate in ATP binding (Fig. 5A). The large C-lobe features several α-helices, forming its core, and short β-strands. It contains motifs common to protein kinases, which participate in binding protein substrates (P+1 loop) or are directly involved in the catalysis, such as the YRD motif in the catalytic loop. The topology of the phototropin kinase domain, predicted by the AlphaFold 2.0 (Jumper *et al.*, 2021) algorithm, resembles that of other AGC kinases, featuring all of the main secondary structure elements present in the well-studied murine PKA kinase (Knighton *et*

*al.*, 1991). A characteristic feature of the phototropin kinase domain (and of most other plant AGC kinases) is its unusually long activation loop. Most protein kinases require phosphorylation of at least one residue in the activation loop for activation. Protein kinases that require phosphorylation for activity contain an arginine residue before the catalytic aspartate in the catalytic loop (Johnson *et al.*, 1996; Nolen *et al.*, 2004). Phototropins satisfy this necessary (but not sufficient) condition.

AlphaFold 2 models of Arabidopsis phototropins, available from the EMBL-EBI AlphaFold Protein Database (Varadi *et al.*, 2022), feature multiple contacts between the N-terminal part of LOV2 and the kinase domains (residues that may participate in inter-domain hydrogen bonds are marked with black symbols in Fig. 5A). Interpretation of AlphaFold structures is not straightforward for photoactivated proteins, such as phototropins, as it is not immediately clear whether the structure corresponds to the form of the protein in dark or lit conditions. As the removal of the LOV domain abolishes the dark repression of the kinase domain, it is likely that the predicted contacts between LOV2 and the kinase domain are characteristic of the phototropin structure in darkness. In particular, they may be involved in dark repression and transmitting the light activation signal. These hypothetical interactions involve several residues located in key parts of the kinase domain, known to participate in the binding of both ATP and protein substrates in homologous protein kinases. AlphaFold predicts that residues located in the P+1 loop immediately after the C-terminal end of the activation loop (residues 853–854 in phot1; Fig. 5) contact the A'α helix in the N-terminal part of the LOV2 domain (residues 464–465 in phot1). The P+1 loop is known from other kinases to bind the residues of protein substrate in the vicinity of the phosphorylated site. Residues from LOV2 also appear to

contact regions of the kinase domain directly involved in ATP binding. For example, R461 is predicted to bind K790, located in the catalytic loop (Fig. 5C). The residue corresponding to K790 in murine PKA binds the γ-phosphate group of ATP (Bastidas *et al.*, 2012). Another prediction of AlphaFold is the close localization of R472, located in phot1 LOV2, and N850, S851, and I863. Phot1 mutein with R472 replaced with histidine is known to exhibit constitutive kinase activity (Petersen *et al.*, 2017), which results in impairment of the chloroplast accumulation response in low light. A similar observation has been reported for tomato phot1 (Sharma *et al.*, 2014). The potential interaction between R472 and S851 suggests a particular mechanism of inhibitory action of LOV2. As discussed below, phosphorylation of S851 appears to be necessary for the full activity of the kinase domain. The plausibility of predicted interactions between the N-terminal part of LOV2 and the kinase domain is indirectly supported by results obtained for *Chlamydomonas* phototropin (Crphot). Six conserved residues located within the A'α helix of Crphot LOV2 have been identified by Aihara *et al.* (2012) to reduce the attenuation of the kinase activity in darkness. Five of the corresponding residues in phot1 are predicted by AlphaFold to localize close to the activation segment of the kinase domain. Notably, AlphaFold does not predict direct contact between the Jα helix of LOV2 and the kinase domain. This would suggest that the A'α helix, and not the Jα helix, acts as the primary inhibitor of the kinase domain. The catalytic activity of the isolated Arabidopsis phot2 (Atphot2) kinase domain can be inhibited in darkness by LOV2 fragments that lack Jα but contain the A'α helix (Matsuoka and Tokutomi, 2005). If the AlphaFold structure is correct, a more complex mechanism is necessary to explain why

mutations in Jα, such as I539E in *Avena sativa* phot1 (Asphot1), lead to constitutive activation of the kinase (Harper *et al.*, 2004).

The activity of phototropin kinase is indispensable for chloroplast movements, as evidenced by observations of plants expressing phot1 or phot2 with a disrupted $Mg^{2+}$-binding motif in the double phototropin mutant (Kong *et al.*, 2007; Inoue *et al.*, 2008, 2011). Conversely, when the active phot2 kinase domain, lacking inhibitory LOV domains, is expressed in wild-type plants, chloroplasts assume avoidance positioning regardless of dark or light conditions (Kong *et al.*, 2007). The first target of the kinase domain of light-activated phototropin is the photoreceptor itself (Christie, 1998; Sakai *et al.*, 2001). Several autophosphorylation sites have been identified in phot1 and phot2 (Inoue *et al.*, 2008, 2011; Sullivan *et al.*, 2008; Boex-Fontvieille *et al.*, 2014; Deng *et al.*, 2014). Autophosphorylation of two conserved serine residues at the C-terminal end of the activation loop is essential for phot1 and phot2 signalling (Inoue *et al.*, 2008, 2011). These serine residues lie within a conserved part of the activation loop, known to undergo phosphorylation in other protein kinases, in which it stabilizes the catalytically active conformation of the protein. The importance of phototropin autophosphorylation sites for chloroplast movements has been determined by mutational analysis. Simultaneous substitutions of serine residues to alanine at positions 849 and 851 abolish the ability of phot1 to induce chloroplast accumulation when expressed in the *phot1phot2* mutant. Phosphomimic mutations of phot1 S849D S851D restore chloroplast accumulation (Inoue *et al.*, 2008). Other identified phosphorylation sites of phot1 (S58, S170, S185, S350, S376, S410, and T993) appear not to be important for phot1 signalling for chloroplast accumulation. Autophosphorylation at those sites modulates interactions

with other proteins, such as 14-3-3 proteins (Inoue *et al.*, 2008; Sullivan *et al.*, 2009). Chloroplast relocations in mutants of λ and κ 14-3-3 isoforms (including the double mutant) do not substantially differ from those observed in the wild type, although the lack of a clear phenotype may be due to redundancy between multiple isoforms of the 14-3-3 protein encoded in the Arabidopsis genome (Suetsugu and Wada, 2020). The S761 and S763 residues in phot2 are homologous to the phosphorylable phot1 sites located in the activation loop. Phosphorylation at these sites appears to be partly redundant with respect to chloroplast movements. Plants expressing a phot2 mutein with substitutions at S761A S763A in the *phot1phot2* background do not show chloroplast accumulation, while the avoidance response is induced only at high blue light intensity (90 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The phosphomimic mutations S761D S763D restore chloroplast movements. Plants bearing phot2 with the S761A mutation in the double phototropin background respond similarly to wild-type plants, while plants expressing phot2 S763A mutein show undisturbed chloroplast accumulation, although the magnitude of the avoidance response is reduced. Phosphorylation of the phot2 serine residues at positions 761 and 763 is also required for the dark positioning of chloroplasts (Inoue *et al.*, 2011). Another potentially important residue is threonine 767, which corresponds to a phosphorylated residue in the activation loop of the human PAS kinase (Inoue *et al.*, 2011). The mutation of T767 to alanine leads to kinase inactivation (Inoue *et al.*, 2011). In line with this, plants expressing phot2 bearing T767A (Inoue *et al.*, 2011) or T767I (as in the *phot2-2* allele; Suetsugu *et al.*, 2013) substitutions in the double mutant *phot1phot2* background do not show chloroplast responses. Plants with phot2 with a phosphomimic mutation at this site could not be obtained (Inoue *et al.*, 2011).

The choice of substrates for the phototropin kinase appears to determine the specificity of their physiological responses. Most of the identified substrates seem not to be involved in signalling to influence chloroplast movements (Christie *et al.*, 2011; Demarsy *et al.*, 2012; Kami *et al.*, 2014; Takemiya *et al.*, 2016; Hiyama *et al.*, 2017). Only recently has it been shown that NONPHOTOTROPIC HYPOCOTYL3 (NPH3) and ROOT PHOTOTROPISM2 (RPT2), members of the NRL family of BTB (broad complex, tramtrack, and bric-à-brac) domain proteins, are phosphorylated by phot1 (Sullivan *et al.*, 2021). RPT2 together with NCH1 (NRL PROTEIN FOR CHLOROPLASTMOVEMENT1) mediate chloroplast accumulation (Suetsugu *et al.*, 2016).

Dephosphorylation of the conserved serine residues in the kinase domain of phot1 appears to stop the signalling (Inoue *et al.*, 2008). PP2A scaffolding subunit A1, RCN1 (ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID 1), has been demonstrated to be responsible for phot2 dephosphorylation in Arabidopsis etiolated seedlings, resulting in enhanced phototropism in *rcn1* mutants (Tseng and Briggs, 2010). Studies using inhibitors of protein phosphatases have shown their involvement in signalling to induce chloroplast avoidance, too. Initially, the PP2A-2 catalytic subunit was postulated to act as a positive regulator, as the *pp2a-2* mutant displayed movement inhibition (Wen *et al.*, 2012). Other studies using *ppa-2* mutants of the PP2A-2 catalytic and regulatory B' subunits did not demonstrate impairment of chloroplast movements, thus questioning the role of PP2A-2 (Sztatelman *et al.*, 2016; Suetsugu and Wada, 2020). On the other hand, the *rcn1* mutant shows enhanced chloroplast avoidance in response to continuous light and a shift towards avoidance in response to light pulses, consistent with the role of

RCN1 in dephosphorylation and desensitization of phot2 (Wen *et al.*, 2012; Sztatelman *et al.*, 2016). However, its effect can be indirect, by influencing photoreceptor expression, as the phot2 level is higher in *rcn1* than in the wild type (Sztatelman *et al.*, 2016).

Phototropin kinase activity is crucial for signalling; however, its phosphorylation targets remain largely unidentified. The exact role of autophosphorylation signatures for downstream signalling is elusive. Only the role of phosphorylation sites that inactivate the kinase is evident. The dynamics of autophosphorylation and dephosphorylation at most of the identified sites are not understood.

## Phot1 and Phot2 domain swapping

Intriguing results have been obtained using plants expressing chimeric phototropins consisting of parts of phot1 and phot2 (reported in Aihara *et al.* 2008). Chimeric receptors bearing the N-terminal part of phot1 fused with the C-terminal part of phot2, or the N-terminal part of phot2 part fused with the C-terminal part of phot1, are capable of eliciting chloroplast avoidance when expressed in the double *phot1phot2* mutant. The simplest explanation of these results is that suppression of signalling to induce chloroplast avoidance observed for wild-type phot1 molecules is achieved through a specific way in which the N-terminal photosensory part interacts with the C-terminal kinase domain. This type of interaction is disrupted in chimeric molecules in which one part comes from phot2. In addition, the light sensitivity of both chloroplast accumulation and avoidance in plants expressing chimeric phototropins is determined by the N-terminal part, with molecules bearing the N-terminal part of phot1 being more sensitive. The expression of a phototropin molecule with the N-terminal part of phot2 is necessary

for the dark positioning of chloroplasts, but the kinase activity may be derived from any phototropin. Neither the N-terminal fragment nor the C-terminal fragment of phot2 expressed alone is sufficient to induce the dark positioning of chloroplasts (Aihara *et al.*, 2008).

## Expression and localization

The velocity of chloroplast avoidance movement depends not only on the fluence rate of blue light but also on the level of photochemically active phot2. This concentration dependence has been indicated by the reduced velocity observed in heterozygous *PHOT2/phot2* plants compared with the wild type (Kagawa and Wada, 2004). Experiments on transgenic plants expressing different levels of the phot2-green fluorescent protein (GFP) fusion protein in the double *phot1phot2* mutant background have also shown a correlation between the velocity of chloroplast avoidance and the level of the photoreceptor (Kimura and Kagawa, 2009). Interestingly, chloroplast accumulation has been observed even in lines expressing very little phot2, not detected by western blot, indicating a much lower protein threshold for this response (Kimura and Kagawa, 2009). Therefore, any factor influencing the abundance of phototropin in the cell may in turn influence the balance between chloroplast accumulation and avoidance. Phototropin protein levels in transgenic plants should always be taken into consideration for the correct interpretation of results.

Phototropins are regulated by light at the level of both gene expression and protein stability. Blue light leads to a decrease in the expression of *PHOT1* in etiolated Arabidopsis seedlings (Kang *et al.*, 2008) but increases the expression of *PHOT2* (Jarillo *et al.*, 2001; Kagawa *et al.*, 2001). This light-regulation pattern is conserved during

development and remains in mature leaves (Łabuz *et al.*, 2012). Similarly, the phot1 protein level drops upon irradiation with blue light of moderate intensity (20 μmol $m^{-2}$ $s^{-1}$) (Sakamoto and Briggs, 2002). Phot2 is constantly degraded in darkness but appears to be stabilized by blue light of both low and high intensity (2 μmol $m^{-2}$ $s^{-1}$ and 120 μmol $m^{-2}$ $s^{-1}$, respectively) (Aggarwal *et al.*, 2014). Such a pattern is consistent with the function of both phototropins. Phot1 is specialized in sensing light of very low fluence, whereas phot2 elicits photoprotective responses and as such is needed more at higher light intensities.

The phototropin protein level is dependent on post-translational modifications. Ubiquitination plays an important role in the light-dependent regulation of phot1 abundance. Low-intensity blue light (0.1 μmol $m^{-2}$ $s^{-1}$) triggers mono/multiubiquitination of phot1. Under high-intensity blue light (120 μmol $m^{-2}$ $s^{-1}$), phot1 is polyubiquitinated and marked for degradation. This is considered a means of receptor desensitization (Roberts *et al.*, 2011). Another type of post-translational modification that likely affects the abundance of phototropin protein is modification by SUMO (Small Ubiquitin-like MOdifier) proteins. Both phot1 and phot2 can be modified by SUMO in a bacterial system and interact with SUMO proteins *in planta*. One of the identified phot2 sumoylation sites is located within a sequence resembling a phosphorylation-dependent sumoylation motif (PDSM), suggesting that phot2 sumoylation can be regulated by phosphorylation. Indeed, phot2 has been demonstrated to be sumoylated in response to light in Arabidopsis leaves. This is correlated with the decreased level of phot2 and with increased amplitudes of chloroplast movements observed in mutants of the sumoylation

pathway. Taken together, these results point to the possibility that modification by SUMO can stabilize phot2 upon light treatment (Łabuz *et al.*, 2021).

Light not only affects the abundance of phototropins but also influences their localization inside the cell. Phototropins retain their activity when fused with fluorescent proteins. Phot1-GFP and phot2-GFP have been used to analyse the subcellular localization of the phototropins, as they are fully functional *in planta* and can mediate phototropin responses (Sakamoto and Briggs, 2002; Kong *et al.*, 2006). Inside the cell, phototropins are bound to the plasma membrane, despite lacking any transmembrane domains (Sakamoto and Briggs, 2002; Kong *et al.*, 2006). Their membrane localization depends on the short amino acid stretch at the C-terminus of the protein (Kong *et al.*, 2013a). Both phototropins have also been detected on the chloroplast outer membrane, with phot2 being more abundant than phot1 (Kong *et al.*, 2013b). Upon irradiation, a fraction of the photoreceptor changes its localization. Phot1 is released into the cytoplasm (Sakamoto and Briggs, 2002), whereas phot2 becomes associated with the Golgi apparatus (Kong *et al.*, 2006). These light-induced changes in localization have been proposed to play a role in phototropin function. Phot1 internalization may be involved in photoreceptor signalling (Kaiserli *et al.*, 2009) or desensitization (Han *et al.*, 2008), or be a prerequisite for degradation (Roberts *et al.*, 2011). The Golgi localization of phot2 correlates with chloroplast avoidance in terms of the timescale and light intensity required, which suggests a functional relationship (Kong *et al.*, 2006). However, phot1/phot2 chimeric proteins, which are not targeted to the Golgi, have still been able to elicit chloroplast avoidance (Aihara *et al.*, 2008). Despite intense efforts, the role of the cytoplasmic or Golgi localization of phototropins has not been demonstrated to date. The

plasma membrane has emerged as the predominant site of phototropin action. In particular, phot1 undergoes activation within sterol-rich membrane microdomains (Xue *et al.*, 2018). Engineered phot1 anchored to the plasma membrane by myristoylation or farnesylation is not translocated into the cytoplasm upon light irradiation. Expression of these lipidated versions of phot1 in the *phot1phot2* mutant background restores chloroplast accumulation (Preuten *et al.*, 2015). Likewise, phot2 anchored to the plasma membrane by myristoylation is fully active and can elicit both chloroplast accumulation and avoidance (Ishishita *et al.*, 2020). The last 42 amino acid residues of phot2, necessary for its membrane localization, are indispensable for inducing chloroplast avoidance (Kong *et al.*, 2013a). However, phot2 bearing a truncation of this sequence retains chloroplast accumulation in the *phot1phot2* background, albeit induced at higher light intensities. Phot2 lacking the last 65 amino acid residues is incapable of inducing chloroplast movements (Kong *et al.*, 2013a, b). Disturbances in phot2 localization that diminish the membrane-bound fraction alter the balance between chloroplast accumulation and avoidance, as observed for TAP-tagged variants (Kong *et al.*, 2013b). When phot2 is localized exclusively to the chloroplast outer membrane, it partially restores chloroplast avoidance but not accumulation. This suggests that phot2 present at the plasma membrane–chloroplast interface is predominantly active in regulating chloroplast avoidance (Ishishita 2020). Findings from microbeam irradiation of individual chloroplasts imply that they directly perceive light during the avoidance response (Tsuboi and Wada, 2010a, 2011). In contrast, the signal for chloroplast accumulation is mediated at long distances in the cell (Tsuboi and Wada, 2010a, 2011).

Besides triggering photochemistry, light controls phototropins at the level of expression and subcellular localization. The effect of light on phototropin expression agrees well with their photochemical properties and physiological function, with the more light-sensitive phototropin1 being abundant in plants subjected to darkness or low light and the expression of phototropin2 being up-regulated in high light. The release of phototropins to the cytoplasm after blue light irradiation has been well documented, but its physiological role is not clear. The plasma membrane and, to a lesser extent, chloroplast envelope, which are closely associated in the leaf mesophyll (e.g. Łabuz *et al.*, 2016), are the main sites of phototropin signalling to elicit chloroplast movements. Recent work has shown that phot1 dimerization occurs in membrane microdomains (Xue *et al.*, 2018). It is essential to understand how the localization of photoreceptors determines the signalling outcome.

## Insights from the diversity of phototropins

Only a few plant phototropins have been characterized in terms of their capacity to elicit chloroplast movements. However, the expression of phototropins from different species in Arabidopsis provides further insights into the conservation of phototropin structure and their function during evolution.

In *P. patens*, several phototropin genes control chloroplast movements redundantly, showing different light sensitivity for inducing chloroplast accumulation and threshold intensity for triggering chloroplast avoidance. These phototropins fall into two groups, photA and photB; however, members of both groups can mediate chloroplast avoidance, similarly to Arabidopsis phot2 (Kasahara *et al.*, 2004). *Physcomitrella* is a useful, although not fully explored, model of phototropin signalling to elicit chloroplast

movements, due to its ease of transformation and unicellular protonema cells (Sato *et al.*, 2003).

Convenient models have been also established for chloroplast tracking in gametophytes of the fern *A. capillus-veneris* (Tsuboi and Wada, 2010b, 2011, 2012). *Adiantum* has two phototropin genes, *AcPHOT1* and *AcPHOT2*. A transient transformation assay using the *Adiantum phot2* mutant has provided valuable information about signalling to elicit chloroplast avoidance (Kagawa *et al.*, 2004). N-terminal and LOV1-domain truncations of Acphot2 show that these parts of the protein are not crucial for inducing chloroplast avoidance. However, the LOV1 domain prolongs the duration of Acphot2 activation. As is the case for Arabidopsis phot2, the last 40 amino acid residues of the C-terminal part of Acphot2 are required for inducing chloroplast avoidance (Kagawa *et al.*, 2004).

The phototropin from the green alga *C. reinhardtii* (Onodera *et al.*, 2005) controls multiple steps of the sexual life cycle (Huang and Beck, 2003) and phototactic behaviour (Trippens *et al.*, 2012). Crphot shows sequence similarity to angiosperm phototropins throughout the whole molecule except for the N-terminal extensions, which are characteristic of higher plant phototropins. When introduced into the *phot1phot2* Arabidopsis mutant, Crphot complemented chloroplast avoidance and accumulation. This indicates that the ability to trigger chloroplast movements, and in particular chloroplast avoidance, lies within the structure of Crphot. Autophosphorylation sites upstream of the LOV1 domain and within the hinge region are not conserved in Crphot, therefore they are not necessary for proper signal transduction. The N-terminal extension, a region that is highly divergent between phot1 and phot2, is missing in Crphot, so it is likely not

essential for phototropin function and specificity. Complementation of chloroplast movements has been observed only in lines with high Crphot protein abundance, but this elicited male sterility and additional adverse effects on development (Onodera *et al.*, 2005).

Another algal phototropin introduced into Arabidopsis was derived from the marine green picoalga *O. tauri*, the smallest free-living eukaryote (Sullivan *et al.*, 2016). Despite biochemical similarities to higher plant phototropins, it has been able to complement only some of the defects of the Arabidopsis *phot1phot2* mutant, including chloroplast accumulation, but not avoidance. Otphot protein levels in the examined plants were comparable with lines expressing phot1-GFP under the control of the *PHOT1* promoter. However, the LOV2 domain from Otphot has a lower protein quantum efficiency and therefore may have reduced receptor photosensitivity compared with Arabidopsis phototropins. This may shift the receptor photosensitivity threshold for chloroplast avoidance towards higher light intensities of >100 µmol $m^{-2}$ $s^{-1}$. However, this possibility was not examined in the study of Sullivan *et al.* (2016).

The genome of the liverwort *M. polymorpha* encodes a single *PHOT* gene, *MpPHOT*. Its product is involved in mediating chloroplast accumulation in low light and avoidance in high light, as well as chloroplast dark positioning (Komatsu *et al.*, 2014). The photochemical properties of Mpphot resemble those of Atphot2, as the quantum yield of the LOV2 domain is twice as high as that of the LOV1 domain. Conversely, the half-life of photoactive LOV2 of Mpphot is similar to that of Atphot1, with LOV1 being characterized by a longer half-life (Kato *et al.*, 2021). When introduced into Arabidopsis or *A. capillus-veneris phot* mutants, Mpphot can complement the defects in chloroplast

movements, which provides evidence for evolutionary conservation of its structural features (Komatsu *et al.*, 2014). When expressed in a *Marchantia* knockout line, Mpphot complements chloroplast movements as well. Mpphot with an inactive LOV1 domain induces chloroplast accumulation at low light intensities and chloroplast avoidance at high light intensities. However, Mpphot shows some distinct signalling features compared with Arabidopsis phototropins. Mpphot with an inactive LOV2 domain can trigger chloroplast accumulation regardless of the light intensity, indicating an active role of the LOV1 domain in signalling (Kato *et al.*, 2021).

Similarly to *Marchantia*, another thalloid liverwort, *Apopellia endiviifolia*, shows robust chloroplast movements. Due to its simple morphology, it may serve as new model species. In *Apopellia*, both the accumulation response at low blue light intensities and the avoidance response at high blue light intensities are controlled by a single phototropin, Aephot (Yong *et al.*, 2021).

*Welwitschia mirabilis*, a desert gymnosperm plant adapted to high light and limited water supply, has two phototropins, Wmphot1 and Wmphot2. This species exhibits some blue light responses, but not light-induced chloroplast movements. Wmphot1 can elicit chloroplast accumulation, but not avoidance, in the Arabidopsis phototropin mutant, proving functional conservation of phot1, even though it is not utilized by *Welwitschia*. It has not been examined whether Wmphot2 behaves like phot2 and can elicit chloroplast avoidance (Ishishita *et al.*, 2016).

Ghphot2 from cotton (*Gossypium hirsutum*), an important crop species, mediates chloroplast avoidance in cotton and, not surprisingly, complements all examined defects

of the double *phot1phot2* Arabidopsis mutant, including chloroplast accumulation and avoidance (Shang *et al.*, 2019).

In tomato (*Solanum lycopersicum*), two phototropin genes have been identified, *SlPHOT1* and *SlPHOT2.* An R495H mutation in the A'α-helix of Slphot1, corresponding to position R472H in Arabidopsis, has been identified in *Nps1,* a *Nonphototropic seedling1* mutant. The mutation results in the abolishment of blue-light-induced autophosphorylation and reduced levels of the Slphot1 protein compared with the wild type. However, *SlPHOT1* transcript levels remain unaffected, implying a loss of protein stability. Whereas the Slphot1-mediated accumulation response is impaired at low light intensities in the *Nps1* mutant, Slphot2-mediated chloroplast accumulation is still observed at higher intensities of blue light than in the wild type. Chloroplast avoidance and dark positioning are retained in *Nps1* plants (Sharma *et al.*, 2014).

The genome of *Phalaenopsis aphrodite*, an epiphytic monocot and an ornamental plant, encodes two genes, *PaPHOT1* and *PaPHOT2*, which group together with angiosperm *PHOT1* and *PHOT2*, respectively, in the phylogenetic analysis (Lin *et al.*, 2019). Virus-induced gene silencing experiments suggest that chloroplast avoidance and accumulation are controlled by both phototropins in *P. aphrodite*. When Paphot1 and Paphot2 are expressed in the Arabidopsis double phototropin mutant, only Paphot1 can restore the accumulation response. Paphot2 induces neither chloroplast accumulation nor avoidance in the tested light conditions. Paphot2 may be non-functional, as it fails to rescue other defects of the *phot1phot2* mutant (Lin *et al.*, 2019). This would make Paphot2 a unique phototropin, lacking sufficient conservation to function in a heterologous system. However, no data concerning protein expression levels in the

examined lines have been presented. The lack of activity may result from low protein accumulation, as has been demonstrated for lines expressing Crphot (Onodera *et al.*, 2005). Obtaining plant lines with a (modified or heterologous) phototropin expressed at a level comparable to the wild type is not always an easy task, and some constitutively active variants of Atphot1 cannot accumulate *in planta,* despite sufficient transcript levels (Kaiserli *et al.*, 2009).

Taken together, features of phototropins responsible for their ability to elicit chloroplast movement appear to be highly conserved during evolution and are present even in those species in which chloroplast movements are not observed. The exceptions to this pattern, such as Osphot2, may be useful to identify the parts of the photoreceptors important for their specific function.

## Environmental stimuli

The quantity and quality of light that can be perceived by the phototropins differs, as phot1 is more light-sensitive than phot2 when responding to blue light (Harada and Shimazaki, 2007). The absorption spectrum of FMN shows two distinct peaks in the UV-A (315–380 nm) and blue range, and spreads into the UV-B range (280–315 nm) (Copeland and Spiro, 1986). Action spectra obtained for *L. trisulca* (Zurzycki, 1962) and *Funaria hygrometrica* (Zurzycki, 1967) show that chloroplast movements are very sensitive to UV-A. In Arabidopsis, UV-B radiation of 3.3 μmol $m^{-2}$ $s^{-1}$ triggers chloroplast accumulation in a phot1-dependent manner. Higher doses of UV-B act non-specifically and attenuate movements due to cell damage (Hermanowicz *et al.*, 2019). As sunlight contains UV-A and UV-B radiation, the balance between those wavelengths and blue light may alter the signalling outcome from phototropins.

Chloroplast movement induced by blue light can be also influenced by red light, in a manner dependent on red-light photoreceptors. A unique receptor, neochrome, was discovered in *A. capillus-veneris* and *Mougeotia scalaris*. Neochrome is a chimeric photoreceptor that is sensitive to both blue and red light. In addition to the kinase and two LOV domains, it has a phytochrome-type domain containing phycocyanobilin. Neochrome genes are found in Zygnematales, hornworts, and ferns (Li *et al.*, 2015). Neochrome had evolved independently in green algae and land plant lineages (Suetsugu *et al.*, 2005b), but then it was horizontally transferred from hornworts to ferns (Li *et al.*, 2014). In *Adiantum*, neochrome mediates the red-light-controlled movement of chloroplasts (Kawai *et al.*, 2003; Suetsugu *et al.*, 2005b). In *Physcomitrella*, a direct interaction of phot1 and phytochrome has been observed, which is important for signalling (Jaedicke *et al.*, 2012) and results in a phototropin-mediated chloroplast response to red light (Kasahara *et al.*, 2004). In Arabidopsis, red light is not active but has a stimulating effect on blue-light-induced chloroplast movements (Kagawa and Wada, 2000). The absence of phytochromes A and B, red and far-red light receptors, favours chloroplast avoidance, especially at light intensities that do not saturate this reaction (DeBlasio *et al.*, 2003). Phytochrome B modifies the signal leading to the avoidance response but does not affect chloroplast accumulation (Luesse *et al.*, 2010).

Temperature is another factor affecting chloroplast movements. Cold treatment (temperature of 5 °C) results in changes in chloroplast positions in *A. capillus-veneris* (Kodama *et al.*, 2008) and *M. polymorpha* (Tanaka *et al.*, 2017). In cold-treated plants, chloroplasts exhibit an avoidance response at light intensities low enough to induce the accumulation reaction at room temperature. A shift in the photosensitivity of chloroplast

avoidance to lower blue light intensities is also observed in Arabidopsis (Łabuz *et al.*, 2015). Chloroplast positioning in cold temperatures is dependent on *Adiantum* Acphot2 (Kodama *et al.*, 2008), Arabidopsis phot2, and *Marchantia* Mpphot (Fujii *et al.*, 2017), and *Apopellia* Aephot (Yong *et al.*, 2021). The phototropin photochemical reaction does not depend on temperature, but the dark reversal of the photoproduct is slower at 4 °C, as shown for isolated LOV domains of Asphot1 from *A. sativa* (Salomon *et al.*, 2000). Phot2 is characterized by a short dark-recovery lifetime (Kasahara *et al.*, 2002b), thus low temperature may promote the stability of the photoproduct, which preserves the signal. A valine to threonine substitution at position 594 (V594T) in the LOV2 domain of Mpphot accelerates the speed of dark reversion. Thus, the lifetime of the LOV2V594T photoproduct at 5 °C is similar to that of the LOV2 photoproduct at 22 °C. The dark reversal of the LOV2 but not the LOV1 domain seems to be crucial for mediating chloroplast positioning in cold temperatures in *Marchantia* (Kato *et al.*, 2021). Plants bearing phototropin with the V594T mutation expressed in the Mpphot knockout line misperceive 5 °C as 22 °C, and show chloroplast accumulation instead of avoidance. The autophosphorylation level of Mpphot at low blue light intensities is also higher at 5 °C than at 22 °C. This observation is attributed to the prolonged activated state of the LOV2 domain at low temperatures (Fujii *et al.*, 2017). Temperature also modulates the *in vitro* dimerization equilibrium between LOV2 domains and LOV2 domain interaction with the linker part lying adjacent to the Jα-helix of Arabidopsis phot1. The equilibrium of the interaction between the Jα-helix and LOV2 is shifted towards the photoreactive state at 5 °C, while the bound form prevails. At room temperature, dissociation of the LOV2 domain from the Jα-helix is observed, so that the number of non-reactive species is

increased (Nakasone *et al.*, 2008). Thus, phot1 has been proposed to function as a temperature receptor (Terazima, 2011). Studies in Arabidopsis show that phot1 photosensing is affected by low temperature, but results only in prolongation of the residual chloroplast avoidance response. Low temperature promotes chloroplast avoidance in Arabidopsis wild-type and *phot1* plants, but the amplitude of the residual avoidance response remained unaffected in the *phot2* mutant (Łabuz *et al.*, 2015).

The discussed studies give us clues to how chloroplast movements may be affected by environmental factors in the field. However, experiments in laboratory conditions in which light and temperature are tightly controlled do not allow us to assess the effect of diurnal and seasonal variation of physical factors in the environment. Experiments in the field must still be performed to achieve a better understanding of the ecophysiological role of chloroplast movements.

# Perspectives

Fundamental questions related to phototropin signalling to trigger chloroplast movement remain open. First, why is phot1 not capable of eliciting sustained chloroplast avoidance? Analysis of chloroplast responses in the *phot2* mutant indicates that phot1 can signal to elicit chloroplast avoidance, but cannot trigger a sustained reaction. A residual chloroplast avoidance response is observed after high-intensity continuous blue light (Luesse *et al.*, 2010; Łabuz *et al.*, 2015) or high-fluence blue light pulses (Sztatelman *et al.*, 2016). Domain-swapping studies also imply the suppression of phot1 signalling to trigger chloroplast avoidance, rather than its complete absence. The inability of phot1 to elicit sustained chloroplast avoidance may be a consequence of its evolution towards higher light sensitivity. The mechanism that aborts phot1 signalling to trigger chloroplast

avoidance is unknown. One possibility is that a protein interacting with phot2, but not phot1, is required for sustaining chloroplast avoidance. Alternatively, a protein interacting specifically with phot1 may actively suppress chloroplast avoidance. The lag time between the onset of the signal for chloroplast avoidance and its quenching by an external factor would allow the transient reaction to happen.

Second, how does phot2 switch the signalling pathway leading to chloroplast accumulation or avoidance? Phot2 can induce both chloroplast accumulation and avoidance responses, changing its signalling outcome according to the light intensity. Extensive structural studies of phototropins are needed to understand conformational changes of whole molecules in response to light and darkness. Analysis of the functions and structures of phototropins from different species may identify conserved tertiary structure features serving as protein interaction sites. Further autophosphorylation profiling is required to establish if sites connected exclusively to signal transduction to one of the chloroplast reactions are present.

Third, are phototropin dimers the primary signalling units? Phototropin can form both homo- and heterodimers. Interactions between phototropins are necessary for transphosphorylation, which may play a role in signal amplification. Such amplification could be achieved by increasing the number of activated molecules or by prolonging the time during which the molecules are in the active state, as the dephosphorylation is slower than the dark reversal of the phototropin photocycle. In particular, both amplification strategies may be employed if the transphosphorylated molecule is able to phosphorylate the phototropin molecule that underwent the initial light excitation. To demonstrate this, experiments that would distinguish between the potential in-*cis*

autophosphorylation of the light-activated phototropin and transphosphorylation by another phototropin molecule from the dimer are necessary. The second way of signal amplification requires that a non-excited transphosphorylated molecule is capable of propagating the signal efficiently. Light-excited phototropin molecules must be able to phosphorylate other phototropin molecules even with a non-phosphorylated activation loop. Is there a substrate specificity difference between light-activated and phosphorylation-activated phototropins? To understand the properties and signalling abilities of phototropins in light-excited and transphosphorylated states, the properties of a phototropin molecule with a phosphorylated activation loop and LOV2 in either the dark state or light-activated should be investigated.

Finally, which proteins are phosphorylated by phototropins in mesophyll cells, and what is their role in chloroplast movement signalling and how the signal from the photoreceptor reaches the actin cytoskeleton? Several phototropin substrates have been identified to date, but none of them is involved in signalling to elicit chloroplast movements. It is possible that distinct substrate specificity could be responsible for the functional difference between phot1 and phot2. Signalling through NRL family members is crucial for triggering phototropin-mediated responses (Christie *et al.*, 2017), and only recently have those proteins emerged as new targets of the phot1 kinase (Sullivan *et al.*, 2021).

## Author contributions

JŁ and OS wrote the manuscript; PH wrote the kinase section, prepared graphical data. and edited the manuscript.

## Conflict of interest

The authors declare no conflict of interest.

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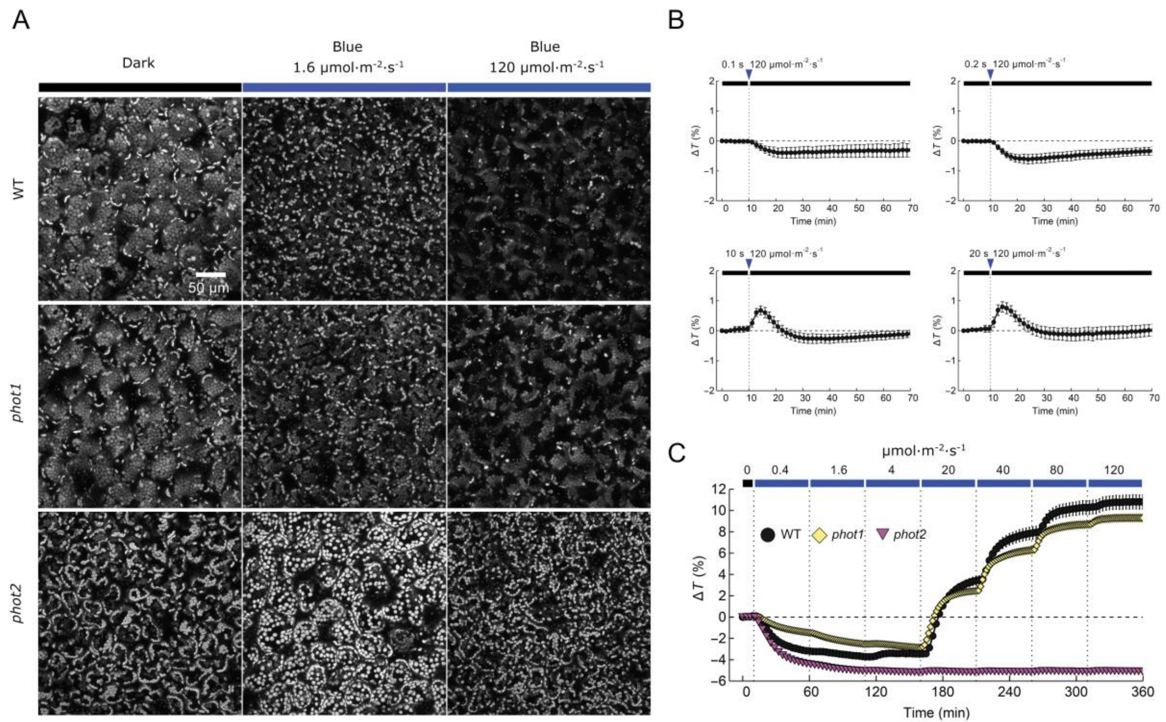


**Fig. 1.** Blue-light-induced chloroplast relocations, studied (A) using optical microscopy and (B) by monitoring of changes in leaf transmittance. (A) Chloroplast arrangement in palisade cells of Arabidopsis rosette leaves of wild-type (WT), *phot1*, and *phot2* mutant plants. Leaves detached from 4-week-old dark-adapted plants were either kept in darkness (mock irradiation) or irradiated for 1 h with blue light (455 nm) at 1.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Chloroplast arrangements were imaged with a laser scanning confocal microscope, using chlorophyll autofluorescence. Maximum intensity projections were calculated from Z-stacks spanning the epidermis and palisade parenchyma. Reproduced from Łabuz *et al.* (2021). (B) Changes in WT leaf transmittance at 660 nm, induced by a pulse of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ blue light (455 nm), of varying duration (0.1–20 s, as indicated in the figures). Pulses of short duration induce a transient accumulation response, while longer pulses induce fast avoidance followed by a slower, transient accumulation response. Error bars show the standard deviation. Raw data from

Sztatelman *et al.* (2016), replotted. (C) Changes in leaf transmittance induced by continuous blue light (455 nm) of increasing intensity, from 0.4 µmol $m^{-2} \cdot s^{-1}$ to 120 µmol $m^{-2} \cdot s^{-1}$, recorded for WT, *phot1*, and *phot2* plants. Error bars show the standard error. Raw data from Łabuz *et al.* (2021), replotted. A decrease in leaf transmittance corresponds to chloroplast accumulation; an increase corresponds to chloroplast avoidance.

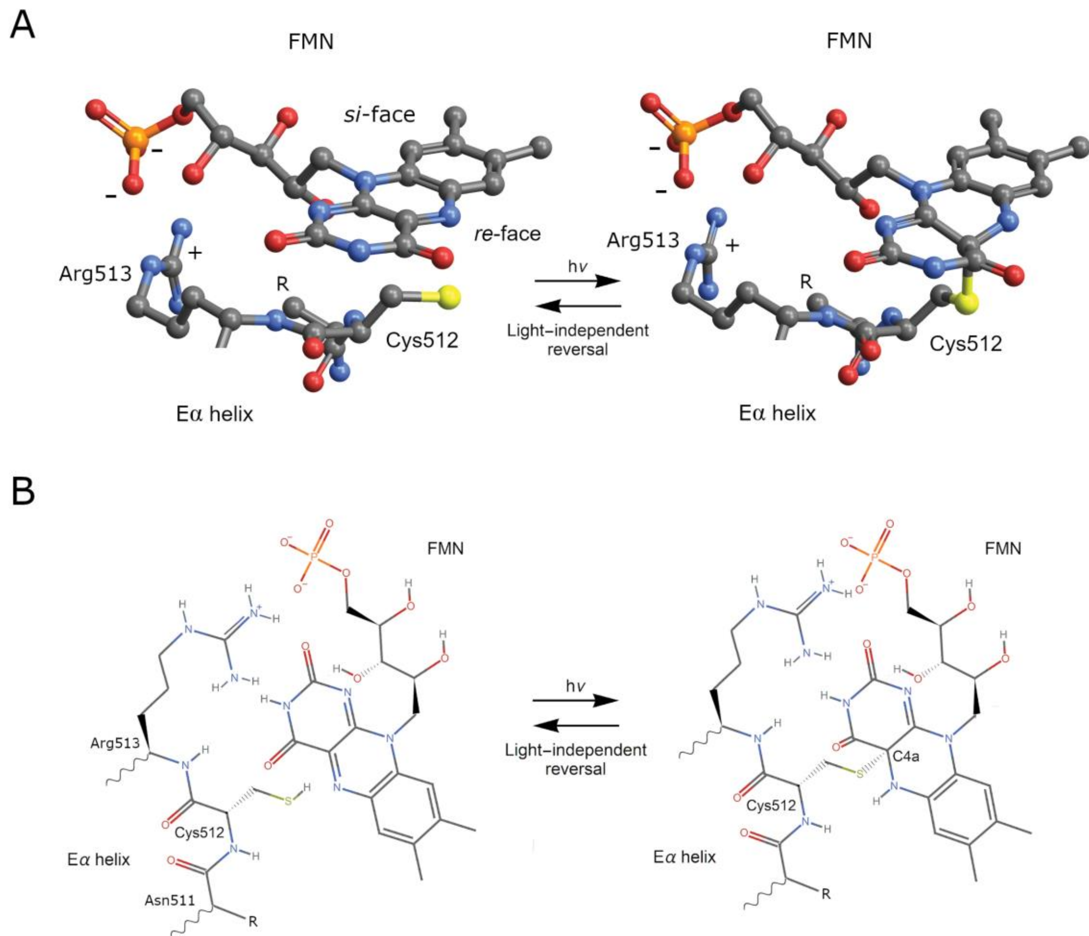


**Fig. 2.** Formation of the photo-adduct between the photoactive cysteine residue (Cys512 in Arabidopsis phot1) and the flavin mononucleotide (FMN) chromophore, induced by the absorption of a photon of blue or UV light by FMN. Arginine 513 stabilizes the negatively charged phosphate group of FMN. The mutation of an analogous arginine residue in phot2 LOV2 blocks the binding of FMN and disrupts chloroplast movements in Arabidopsis (Suetsugu *et al.*, 2013). The atomic coordinates were taken from the crystal structures of the dark-adapted and light-trapped LOV2 domain of phototropin1 from *Avena sativa* (Halavaty and Moffat, 2007).

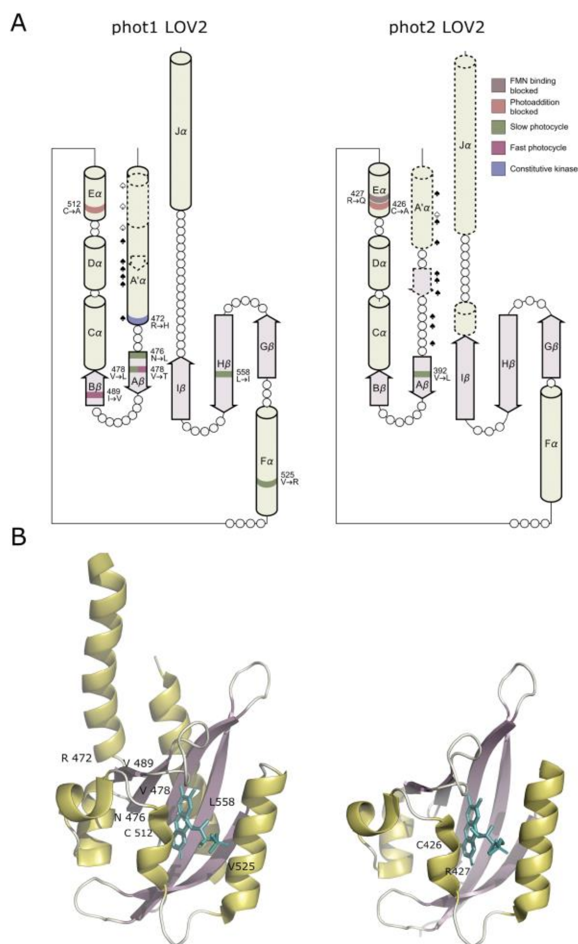


**Fig. 3.** Mutations in the LOV2 domain of Arabidopsis phot1 and phot2 affect chloroplast responses. (A) The topology of LOV2 domains was based on the published crystal structures [4HHD for phot1 (Halavaty and Moffat 2013), 4EEP for phot2 (Christie *et al.*, 2012)]. Part of the phot2 LOV2 domain missing from the crystal structure was drawn using the AlphaFold2 prediction (dashed outlines). The AlphaFold-based model (Jumper *et al.*, 2021; Varadi *et al.*, 2022) indicates that multiple residues of the N-terminal part of LOV2 may contact residues located in the kinase domain. The possible interactions are marked with black symbols (♠) if the expected position error is smaller than 10 Å, and with white symbols (♤) if the expected error is larger. The secondary structure predictions (dashed) for the N-terminal part of phot1 LOV2 differ from the crystal

structure of isolated LOV2 (solid lines). (B) Structures of LOV2 domains rendered with PyMol.

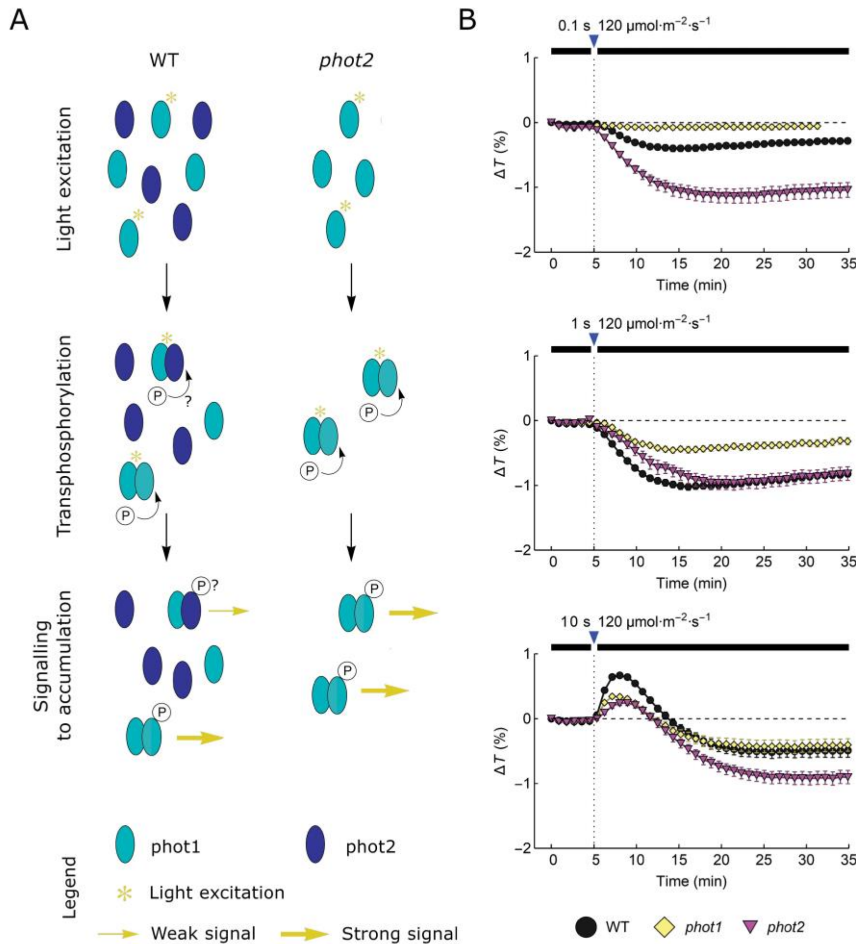


**Fig. 4.** (A) A model of hypothetical phototropin signalling to direct chloroplast movements through the use of dimers. At very low light intensities, the phot2 molecule serves as a competitive inhibitor for phot1. Phot1 dimer formation is known to depend on the presence of light; the same is assumed for the heterodimer and phot2 homodimer formation. In low light, only phot1 is activated. In wild-type (WT) plants, each phot1 molecule may interact either with phot1 or with phot2. In the *phot2* mutant, each activated phot1 molecule binds another phot1 molecule. The activated phot1 molecule

transphosphorylases its counterpart in the dimer. The phosphorylated phototropin transduces the signal to chloroplast accumulation, with phot1 being more efficient than phot2. (B) Chloroplast responses to blue (455 nm) light pulses (120 μmol·m$^{-2}$·s$^{-1}$) of different duration for WT, *phot1*, and *phot2* rosette leaves. Error bars show the standard error. Raw data from Sztatelman *et al.* (2016), replotted.

**Fig. 5.** The AlphaFold 2 algorithm (Jumper *et al.*, 2021; Varadi *et al.*, 2022) predicts multiple contacts between the N-terminal part of LOV2 and key regions of the kinase domain. (A, B). Topology (A) and ternary structure (B) of the kinase domain of phot1, predicted by AlphaFold. Like other protein kinases, it consists of two lobes, separated by

a catalytic cleft. The core of the N-terminal lobe is composed of a β-sheet, while the C-terminal lobe is dominated by α-helices. The order of secondary structure elements strongly resembles that found in the murine PKA kinase (Knighton *et al.*, 1991), and the elements are named accordingly. The residues predicted to contact LOV2 are marked with black symbols (♠) if the expected position error is smaller than 10 Å, and with white symbols (♤) if the expected error is larger. In the $Mg^{2+}$-binding loop, mutation of aspartate 806 (grey) to asparagine blocks kinase activity, disrupting chloroplast movements (Kong *et al.*, 2007). Serine residues 849 and 851 (purple), located in the N-terminal part of the activation loop, appear to undergo blue-light-induced phosphorylation, which is necessary for signalling to direct chloroplast accumulation. (C) Predicted interactions between the N-terminal part of LOV2 and the kinase domain of phot1. Protein regions are coloured as in (B). Based on the AlphaFold model, arginine 472 may form hydrogen bonds with serine 851, located in the activation loop. The mutein R472H exhibits constitutive kinase activity, which suggests that it plays a role in the dark repression of the kinase domain by LOV2 (Petersen *et al.*, 2017). S851 corresponds to the primary phosphorylation site of other protein kinases and its blue-light-dependent phosphorylation is required for normal phototropin signalling (Inoue *et al.*, 2008). The side-chain of S851 is predicted to be positioned in the vicinity of R787, a residue located in the catalytic loop, within the conserved YRD motif. In several protein kinases, the interaction between the corresponding catalytic loop arginine and the primary phosphorylation site in the activation loop is necessary for the enzyme to assume active conformation (Johnson *et al.*, 1996). The aspartate residue, which, by homology, appears to be directly involved in the catalysis, is immediately adjacent to R787. The second

residue of the catalytic loop predicted to interact with LOV2 is K790, which appears to contact R461. The residue corresponding to K790 binds γ-phosphate of ATP in the crystalized murine PKA kinase (Bastidas *et al.*, 2012). For simplicity, the side chains of most of the residues that are predicted to participate in inter-domain contacts are not shown. The regions of the kinase domain are delineated after Nolen *et al.* (2004).