A Chemical Genetic Approach to Engineer Phototropin Kinases for Substrate Labeling

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Running title: Gatekeeper Engineering of Phototropin

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Protein kinases (PKs) control many aspects of plant physiology by regulating signaling networks through protein phosphorylation. Phototropins (phots) are plasma membrane-associated serine/threonine PKs that control a range of physiological processes that collectively serve to optimize photosynthetic efficiency in plants. These include phototropism, leaf positioning and flattening, chloroplast movement and stomatal opening. Despite their identification over two decades ago, only a handful of substrates have been identified for these PKs. Progress in this area has been hampered by the lack of a convenient means to confirm the identity of potential substrate candidates. Here, we demonstrate that the kinase domain of *Arabidopsis* phot1 and phot2 can be successfully engineered to accommodate non-natural ATP analogues by substituting the bulky gatekeeper residue threonine for glycine. This approach circumvents the need for radioactivity to track phot kinase activity and follow light-induced receptor autophosphorylation *in vitro* by incorporating thiophosphate from N^6-benzyl-ATPγS. Consequently, thiophosphorylation of phot substrate candidates can be readily monitored when added or co-expressed with phots *in vitro*. Furthermore, gatekeeper-modified phot1 retained its functionality and its ability to accommodate N^6-benzyl-ATPγS as a phosphodonor when expressed in *Arabidopsis*. We therefore anticipate that this chemical genetic approach will provide new opportunities for labeling and identifying substrates for phots and other related AGC kinases under *in vitro* and near-native *in vivo* conditions.

Phosphorylation of tyrosine, threonine and serine residues is one of the most important post-translational modifications controlling protein structure, activity, turnover and subcellular localisation (1). Protein phosphorylation is achieved by protein kinases (PKs), one of the largest protein families in eukaryotes. PKs are particularly prevalent in plants and regulate a wide range of biological processes, including innate immunity (2), responses to environmental signals (3), in addition to many aspects of plant growth and development (4). *Arabidopsis thaliana* contains approximately 1,000 PKs (5), twice the number present in humans (6), and represents 4% of all protein-coding genes. Moreover, around 30% of the proteome in eukaryotes is phosphorylated (7). Therefore, dissecting the complexity of plant PK function, as well as determining their substrate relationships, represents a major challenge.

Recent methods have used small molecules to perturb the function of proteins in a way similar to genetic manipulation, hence the name chemical genetics. Such methods can be used to study the function of PKs, combining the specificity of genetics with the flexibility of chemistry, enabling the alteration of a PK in a conditional manner (8). These approaches rely on bio-orthogonal chemical reactions that are not found naturally to trace specific PK-substrate relationships. Specific substrate labeling can be performed by engineering the PK to accommodate bio-

orthogonal ATP analogues which can facilitate visualisation and identification of substrate targets by immunochemistry using antibodies recognising the bio-orthogonal label (9). In practice, mutation of the so-called gatekeeper residue within the ATP-binding pocket of the PK can be used to accommodate ATPγS analogues containing a bulky adenine substitution. This leads to thiophosphorylation of proteins by the gatekeeper engineered kinase and provides a selective means to distinguish specific PK substrates among a large and diverse phosphoprotein pool. We sought to implement this chemical genetic approach to label substrates targets of *Arabidopsis* phototropins (phots). Only a small number of substrate targets have been identified for these light-activated kinases despite the range of physiological responses that they regulate. Hence, chemical genetic approaches such as the gatekeeper system could shed new light on this poorly characterised PK signaling network, which is ultimately important for optimising photosynthetic productivity (10).

Phots are members of the AGCVIII kinase family (11) and bind to the intracellular side of the plasma membrane (12). *Arabidopsis* contains two phots (phot1 and phot2) which function as blue light-receptors for controlling a variety of processes that serve to promote growth particularly under low light conditions (12). These include stomatal opening, leaf blade flattening, leaf orientation, chloroplast movements and phototropism (13). Light-regulation of phot kinase activity is mediated by a photosensory region at the N-terminus that comprises two Light, Oxygen or Voltage sensing domains (LOV1 and LOV2) that bind flavin mononucleotide (FMN) as a UV/blue light absorbing cofactor (14). Phots can be viewed simply as molecular light switches where the activity of the C-terminal kinase domain is repressed in darkness and activated upon illumination (12). Light-sensing produces conformational changes associated with helical regions neighbouring the LOV2 core that leads to an uncoupling of this repression, ATP binding and consequently receptor autophosphorylation (12,15). Autophosphorylation occurs on multiple residues throughout the protein (16-18) and can be readily detected *in vitro* by phosphate incorporation from radiolabeled ATP (19,20). Alternatively, this process can be monitored *in vivo* by monitoring a reduced electrophoretic mobility of the protein following immunoblotting (18,21,22). Phosphorylation of two serine residues within the activation loop of the kinase domain of phot1 (S849 and S851) and phot2 (S761 or S763) are essential for their function and signaling (17,23). The majority of the remaining phosphorylation sites reside within either the N-terminal region upstream of LOV1 or in the linker sequence between LOV1 and LOV2 (12). Although the functional significance of these upstream phosphorylation sites is still largely unknown, phosphorylation at S350, S376 and S410 within the LOV linker region of phot1 can facilitate binding of 14-3-3 regulatory proteins (17,22). By contrast, 14-3-3 binding to phot2 is reported to involve S747 within the kinase domain. Mutational analysis of S747 suggests that 14-3-3-binding is required for phot2-mediated stomatal opening (24). However, the role of 14-3-3 binding to phot1 has yet to be determined since no impairment of stomatal opening was observed when S350 and S376 were mutated (17).

While the photochemical and biochemical properties of phots have been well studied, less information is available regarding the signaling events that couple light perception to various biological outcomes. Indeed, the range of physiological responses controlled by the phots is in stark contrast to the limited description of their substrates. So far, only four phosphorylation targets have been identified. ATP-binding Cassette B 19 (ABCB19) is phosphorylated by phot1 and appears to contribute to controlling polar auxin transport during hypocotyl phototropism (25). Phytochrome Kinase Substrate 4 (PKS4) is also phosphorylated by phot1 (26) and, together with other PKS proteins, is involved in phototropism, leaf flattening and positioning (27-29), whereas Blue Light Signaling 1 (BLUS1) and Convergence of Blue light and CO_2 1 (CBC1) are phosphorylated by phot1 and are involved in blue light-induced stomatal opening (30,31).

To date a robust and convenient means to identify phosphorylation targets for phots is still lacking. We therefore implemented a chemical genetic method to facilitate specific labeling of phot kinase substrates. We show that the kinase domain of *Arabidopsis* phots can be engineered successfully to incorporate and process the unnatural ATP analogue N^6-benzyl-ATPγS, which contains a bulky adenine modification. Mutation of the gatekeeper residue within the kinase domain does not significantly perturb the activity and biological function of phot1 in *Arabidopsis*, but facilitates thiophosphorylation of substrate targets *in vitro* in the presence of N^6-benzyl-ATPγS. Also, the activity of gatekeeper-engineered phot1 was rendered sensitive to a specific kinase

inhibitor. Finally, we show that thiophosphorylation activity of gatekeeper-engineered phot1 can be readily detected in plant extracts close to *in vivo* native conditions. Consequently, this methodology provides a sensitive and non-radioactive means to monitor and specifically inhibit phot kinase activity in a conditional way, as well as potentially identify new substrate targets for phots.

RESULTS

Gatekeeper identification in Arabidopsis phot1. The gatekeeper residue generally bears a large side chain that when mutated to a smaller amino acid creates an enlarged substrate binding pocket. This residue lines the bottom of the kinase active site and controls the sensitivity of the kinase to ATP-competitive inhibitors (32). Mutation of the gatekeeper residue within the kinase active site has emerged as a promising approach to incorporate unnatural ATP analogues, as well as alter kinase selectivity towards binding specific inhibitors (33). By contrast, the native kinase is resistant to binding such analogues owing to the presence of the larger gatekeeper residue. To identify the gatekeeper residue in the kinase domain of Arabidopsis phot1, we aligned its amino acid sequence with PK sequences from other organisms in which the identity of the gatekeeper residue had been determined previously. The gatekeeper is typically a methionine or threonine residue (34) that belongs to the ΦΦX+Φ motif (35,36) where Φ represents a hydrophobic side chain residue, X represents the gatekeeper and + represents a positively charged residue. Sequence homology to other PKs indicated that threonine 740 (T740) was the candidate gatekeeper in the kinase domain of *Arabidopsis* phot1 (Fig. 1A). From a structural perspective, the gatekeeper is localised at the end of the β5 strand in the N-lobe of the kinase domain (34) and interacts with a group of hydrophobic residues that form a cluster called the R-spine which regulates the kinase (37). Both these properties were observed for T740 of phot1 after homology modelling (Fig. 1B).

Engineering phot1 to process bulky ATP analogues. Mutation of the gatekeeper to alanine or glycine in over 80 PKs has been successful in accommodating N^6-benzyl-ATPγS (9,36). We therefore mutated T740 to alanine or glycine to determine whether this variant of phot1 could accept this ATP analogue. A cell-free *in vitro* transcription/translation system was used to express phot1 in the presence of FMN. Autophosphorylation activity was initially examined in the presence of radiolabeled ATP to assess the effect of different gatekeeper mutations. Light-induced autophosphorylation was detected for phot1 in this system but not for the kinase inactive mutant D806N (Fig. 1C). Both the T740A and T740G variants of phot1 displayed light-induced receptor kinase activity but at a reduced level relative to wild type (Fig. 1C). Additional active site mutations were therefore introduced (L700I and C737V) in an attempt to compensate for this decrease in activity, as has been reported previously for the gatekeeper engineering of other PKs (38). However, no activity enhancement was observed for phot1-T740A or phot1-T740G in the presence of these mutations (Fig. 1C). Our subsequent efforts therefore focused on phot1-T740G since it appeared to display higher activity than the T740A mutant (Fig. 1C). We rationalised that mutation of the gatekeeper should enable phot1 to accommodate the bulky ATP analogue N^6-benzyl-ATPγS and promote receptor thiophosphorylation, which can be detected by immunoblotting with anti-thiophosphoester antibody following chemical alkylation of the incorporated thiophosphates (9). Indeed, light-induced autophosphorylation was readily detected for phot1-T740G when N^6-benzyl-ATPγS was used as a phosphate donor (Fig. 2A). In contrast, no signal was evident in reactions containing wild-type phot1.

In addition to accommodating unnatural ATP analogues, the enlarged ATP-binding site created by the gatekeeper mutation also permits small molecule inhibitors to bind selectively to the kinase active site. The cell-permeable kinase inhibitor 1-NM-PP1 has been reported to effectively block the activity of gatekeeper-mutated kinases including members of the Src family of tyrosine kinases (39), the tomato resistance protein serine/threonine kinase Pto (40) and MAPK4 in *Arabidopsis* (41). Similarly, we found that thiophosphorylation of phot1 was reduced when treated with 1-NM-PP1 prior to blue light stimulation, demonstrating that this approach can also be used to reduce the activity of gatekeeper modified phot1 (Fig. 2B).

Phot-mediated thiophosphorylation of BLUS1. The above results confirmed T740 as the gatekeeper residue in phot1 and showed how mutation of this residue can be used to produce a low-background, non-radioactive means to monitor phot1 kinase activity in the presence of the bio-orthogonal ATP analogue, N^6-benzyl-ATPγS. We therefore named this phot1 variant Cerberus after the "keeper" of the underworld in Greek

mythology. We next determined whether phot1-Cerberus could be used to phosphorylate known substrate targets in addition to tracking receptor autophosphorylation. BLUS1 is a guard cell-specific PK which is phosphorylated by phot1 on serine 348, a signaling event that is required to regulate stomatal opening (31). BLUS1 was therefore expressed in *E. coli* as a glutathione S-transferase (GST) fusion, purified by affinity chromatography and used as a substrate target for *in vitro* kinase assays. In addition to phot1 autophosphorylation, thiophosphorylation of BLUS1 was clearly detected following blue light irradiation (Fig. 3A). Mutation of S348 in BLUS1 abolished its thiophosphorylation by phot1-Cerberus, whereas thiophosphorylation of the kinase inactive variant of BLUS1 (D157N) was still clearly evident (Fig. 3B). Together, these results are consistent with previous findings (31) and demonstrate that the engineered phot1-Cerberus retains native substrate specificity.

BLUS1 has also been shown to act as a substrate for phot2 kinase activity (42). To determine whether this substrate commonality between phot1 and phot2 could be detected using the gatekeeper system, we generated a Cerberus variant for phot2 (T654G). Light-induced autophosphorylation of phot2-Cerberus was observed using this approach, although the level of kinase activity in the dark was evidently higher compared to that observed for phot1 (Fig. 4A). Blue light-induced thiophosphorylation of GST-BLUS1 and its D157N variant were clearly evident in the presence of phot2-Cerberus (Fig. 4B). By contrast, no substrate phosphorylation was observed for BLUS1 S348A (Fig. 4B). Together, these results confirm that BLUS1 is a direct substrate of phot2 (42), and indicate that phot1 and phot2 share the same molecular mechanism to regulate BLUS1 activity through serine 348 phosphorylation.

Detection of phot substrate phosphorylation by co-expression. Our results so far demonstrate the utility of the gatekeeper system combined with *in vitro* transcription/translation to monitor phot substrate phosphorylation. However, a potential limitation of this approach is the requirement to produce and purify the substrate from *E. coli*. To circumvent the need for recombinant protein expression, we investigated whether substrate targets could be co-expressed along with phot1 in the cell-free expression system. We found that GST-BLUS1 was thiophosphorylated in a light-dependent manner when co-expressed with phot1-Cerberus (Fig. 3C).

We have also shown previously that phot1 exhibits light-dependent dimerisation (43) and that receptor autophosphorylation can occur intermolecularly between two distinct phot molecules (43,44). Consistent with these findings, light-dependent thiophosphorylation of GST-phot1 was detected when co-expressed with phot1-Cerberus (Fig. 3C). Similar results were also found when a kinase inactive version of GST-phot1 (D806N) was used as substrate (Fig. S1). Taken together, these findings demonstrate the utility of co-expression in the cell free expression system combined with gatekeeper engineering to rapidly confirm the identity of phot substrate targets.

Phot1-Cerberus is functional in Arabidopsis. To determine whether phot1-Cerberus retains biological activity *in vivo*, we stably expressed this variant as a translational fusion to GFP in the *phot1 phot2* double mutant of *Arabidopsis* under the control of the native *PHOT1* promoter. Five independent homozygous lines were isolated for analysis and transgenic *Arabidopsis* expressing wild-type phot1-GFP was used as a control (21,45). The abundance of phot1 in three of the phot1-Cerberus lines (10, 14 and 21) was comparable to phot1 protein levels detected both in wild-type seedlings and seedlings expressing phot1-GFP (Fig. S2). Comparable levels of GFP fluorescence at the cell periphery of etiolated hypocotyls were also detected in lines expressing phot1-Cerberus (Fig. S3) consistent with the association of phot1 with the plasma membrane (46).

Examination of phot1 protein levels in extracts isolated from dark-grown (etiolated) seedlings showed that *in vivo* blue light treatment was sufficient to induce a reduced electrophoretic mobility shift for phot1-Cerberus (Fig. 5A), which is characteristic of receptor autophosphorylation (47). We therefore concluded that, despite the gatekeeper mutation, phot1-Cerberus is still capable of processing endogenous ATP as a phosphate donor. These findings are consistent with our *in vitro* analysis (Fig. 1C).

To further verify the functionality of phot1-Cerberus *in vivo*, we examined whether it could restore several different phot1-mediated responses in *Arabidopsis*. All lines expressing phot1-Cerberus displayed a robust hypocotyl phototropism response when irradiate at 10 μmol m^{-2} s^{-1} for 24 h (Fig. 5B) indicating that phot1-Cerberus retains functionality for

this response. In addition to its phototropic impairment, the rosette leaves of the *phot1 phot2* double mutant displays a downward curled or epinastic phenotype (46). Each of the phot1-Cerberus lines were able to complement the epinastic leaf phenotype of the *phot1 phot2* double mutant (Fig. 5C), once again demonstrating functionality. Phot1 and phot2 are also known to control chloroplast accumulation movement, which allows plants to maximise light capture for photosynthesis (48). This response can be visualised by the slit band assay, where a dark band appears on the exposed section of the leaf when irradiated with low fluence rates of blue light (1.5 μmol m^{-2} s^{-1}) through a 1 mm slit (49). While chloroplasts failed to accumulate to the upper cell surface in leaves from the *phot1 phot2* mutant, a dark band was readily observed in lines expressing phot1-Cerberus (Fig. 5D). Hence, these findings indicate that, as found for phototropism and leaf expansion, phot1-Cerberus retains an ability to utilise endogenous ATP to mediate this blue light response.

Monitoring phot1-Cerberus activity ex vivo. Given the functionality observed for phot1-Cerberus *in planta*, we rationalised that it was still capable of phosphorylating endogenous substrate targets *in vivo*. Phot1-Cerberus expressing lines could therefore offer a new means to monitor substrate phosphorylation. To explore this possibility, we performed *ex vivo* thiophosphorylation screening in protein extracts isolated from etiolated seedlings expressing phot1-Cerberus. Light-dependent autophosphorylation of phot1-Cerberus was readily detected in microsomal membrane fractions in the presence of N^6-benzyl-ATPγS, but was absent in microsomes isolated from phot1-GFP expressing seedlings (Fig. 6A). Thus, these findings demonstrate that phot1-Cerberus retains its ability to utilise the bulky ATP analogue as a phosphodonor when expressed in *Arabidopsis*.

We found that light-dependent thiophosphorylation of phot1-Cerberus could also be detected in total protein extracts isolated from etiolated seedlings (Fig. 6B). Additional light-dependent thiophosphorylation products were also observed in these reactions that ran immediately below phot1-Cerberus. Moreover, probing with antibody raised against the N- (19) or C-terminus of phot1 (18,50) suggested that these signals did not result from phot1 proteolysis (Fig. 6B), although we cannot exclude that these signals could correspond to internal fragments that lack both native termini. These findings therefore suggest that the chemical genetic approach described here has the potential to identify phot1 phosphorylation targets *ex vivo* in additional to tracking receptor phosphorylation.

DISCUSSION

Characterisation of signaling networks controlled by plant PKs is a major challenge because of their large number and their identical catalytic function, which obscures identifying specific kinase-substrate relationships. Our research is focused on identifying the underlying signaling processes associated with a small family of AGC kinases in plants known as phots (phot1 and phot2). Despite regulating a range of responses which collectively optimise photosynthetic productivity, minimal substrate targets have been identified for these light-activated kinases (12). Indeed, genetic screens in *Arabidopsis* have only been successful in identifying one substrate candidate for these kinases which is designated BLUS1 (31). Biochemical attempts to isolate further substrate candidates based on yeast-two-hybrid screening has met with minimal success (22,25). Identifying new substrates remains a major challenge not just for phots, but also for other AGC-related kinases.

In the present study, we adopted a chemical genetic approach to facilitate tracking phot autophosphorylation, as well as substrate phosphorylation. This approach relies on engineering the kinase domain of phots to accommodate the bio-orthogonal ATP analogue N^6-benzyl-ATPγS, which enables thiophosphorylation of substrate candidates. This was achieved by replacing the threonine gatekeeper residue within the ATP-binding site with an amino acid bearing a smaller side chain (Fig. 1). This synthetic approach has been successful in identifying kinase substrate relationships in several different organisms. For example, Banko *et al*. (51) have exploited this methodology to thiophosphorylate and label substrates of AMPKα2 in cultured mammalian cells. These thiophosphorylation targets were subsequently immunopurified and identified by mass spectrometry, uncovering 28 unknown substrates. More recently, Leissing *et al*. (52) have used this gatekeeper approach to identify substrate targets for *Arabidopsis* MAPKs.

Here, we demonstrate that engineering of the kinase domain of *Arabidopsis* phots can be successfully employed to create the analogue-sensitive variants designated phot1- and phot2-Cerberus. These variants are capable of light-induced receptor

thiophosphorylation in the presence of N^6-benzyl-ATPγS (Fig. 2-4). Moreover, our analysis in transgenic *Arabidopsis* has shown that phot1-Cerberus retains its functionality when expressed in the phot-deficient double mutant (Fig. 5) likely owing to its ability to still process natural ATP as a phosphodonor (Fig. 1C). Functionality was still observed in transgenic lines expressing low levels of phot1-Cerberus (Fig. S1; Fig. 5) These findings are consistent with previous reports showing very low levels of phot1 are capable of restoring receptor responsiveness in the *phot1 phot2* double mutant (21,50,53,54). Further detailed physiological analysis however would be required to determine whether lines expressing phot1-Cerberus exhibit quantitative differences in receptor activity compared to wild-type phot1.

While phot1-Cerberus is capable of using endogenous ATP to facilitate light-induced receptor autophosphorylation *in vitro* (Fig. 1C) and *in vivo* (Fig. 5A), it is also capable of processing N^6-benzyl-ATPγS as a thiophosphodonor *ex vivo* (Fig. 6). This chemical genetic approach should aid further probing of phot kinase-substrate relationships without the need for radioactivity. Furthermore, we found that the engineered kinase of phot1-Cerberus is able to accommodate the small molecule inhibitor 1-NM-PP1 and reduce its activity *in vitro* (Fig. 2B). These findings demonstrate the potential for using a chemical genetic strategy to inhibit phot1 function. Such inhibitors could be used in a conditional manner to facilitate spatial dissection of phot1 signaling *in planta* through localised application. Such an approach has shown to be particularly useful when kinase loss-of-function strategies are not possible owing to embryo lethality (55).

Our analysis demonstrates that the gatekeeper approach provides a means to conveniently monitor phot1 kinase activity in protein extracts from *Arabidopsis* (Fig. 6). These initial findings also suggest that thiophosphorylation of proteins other than phot1-Cerberus can be detected under these near native *in vivo* conditions. Future studies will now be focused on attempting to isolate and identity these thiophosphorylated products using immunoprecipitation and mass spectrometry approaches. Two purification strategies have been successfully employed for such purposes. The first involves immunoprecipitation using anti-thiophosphoester antibodies following thiophosphate alkylation (9). The second uses covalent capture of thiol-containing peptides with iodoacetyl resin (56). Although this approach can lead to the binding of cysteine-containing peptides in addition to thiophosphorylated peptides, the latter can be released preferentially through oxidation of the phosphate diester bond.

The power of the gatekeeper approach used here relies on the ability to directly and specifically label substrates of a single protein kinase in a complex protein mixture, an important step towards large-scale elucidation of PK-substrate relationships. While our studies on transgenic *Arabidopsis* were performed *ex vivo*, *in vivo* labeling strategies may be possible by employing non-ionic detergents to permeabilise the plasma membrane and facilitate the uptake of ATP analogues whilst maintaining cell viability (51,57). We anticipate that this approach will complement recent large-scale phosphoproteomic methods (30) to tackle the challenge of characterising phot kinase signaling in *Arabidopsis*. Notwithstanding, we envisage that the co-expression strategy adopted here will provide a rapid and robust means to confirm the identity of newly isolated substrate targets for phot and related AGC kinases.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions. Wild type (*gl-1* ecotype Columbia) and *phot1-5 phot2-1* mutants have been described previously (58,59), as have transgenic *Arabidopsis* expressing phot1-GFP (21). Seeds were planted on soil or on Murashige and Skoog (MS) salts with 0.8% agar (w/v). After 4 ºC treatment for two days, seeds were grown in a controlled environment room (Fitotron, Weiss-Gallenkamp) under 16/8 h, 22/18 ºC light/dark cycle.

Plasmid construction. Plasmids for cell-free expression were constructed using the plasmid pSP64 (Promega). A cDNA fragment encoding full-length *Arabidopsis PHOT1* was PCR amplified using plasmid containing *PHOT1* or *PHOT1-D806N* cDNA as a template. PCR fragments were inserted into the pSP64 vector using *Hind*III and *Pst*I restriction sites and the resulting constructs confirmed by DNA sequencing. The Haemagglutinin (HA) tag was encoded and included in the reverse oligonucleotide. Similarly, *PHOT2* cDNA fused to HA was PCR-amplified and inserted into the pSP64 polyA vector via *Hind*III and *Pst*I restriction sites. cDNA encoding GST-BLUS1 and GST-phot1 were PCR amplified from cDNA templates (31,43) and cloned into the pSP64 vector by Gibson assembly (New England Biolabs) via *Hind*III and *Pst*I

restriction sites. Mutation of the gatekeeper codon of was performed by sited-directed mutagenesis (Agilent). The plant transformation vector encoding *PHOT1-T740G-GFP* under the control of the *PHOT1* promoter was generated by replacing the coding sequence of wild-type *PHOT1* in the binary vector pEZR(K)-LN via *Eco*RI and *Bam*HI sites (21).

Transformation of Arabidopsis. Vectors for plant expression were transformed into the *phot1-5 phot2-1* double mutant using a modified version of the floral dip method (60). Homozygous plants lines containing a single transgene locus were selected for analysis, based on segregation of the kanamycin resistance.

Cell-free expression. Reactions were performed using the TnT® SP6 High-Yield Wheat Germ Protein Expression System (Promega) with 4-8 μg of vector for a 20 μl reaction. For co-expression 2-4 μg of each plasmid was used. Flavin mononucleotide (Sigma-Aldrich) was added to a final concentration of 10 μM. Protein expression was carried out in the dark at room temperature (20-25 ºC) for two hours.

Expression and purification of GST-BLUS1. GST-BLUS1, GST-BLUS1-D157N and GST-BLUS1-S348A were expressed as previously described (31) and purified from *E. coli* using the Glutathione HiCap kit (Qiagen) following the manufacturer's instructions. Purified proteins were dialized overnight at 4 ºC in a buffer containing 37.5 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM DTT. Dialysis was performed using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific).

Protein extraction from Arabidopsis. Proteins were extracted from 3-day-old etiolated seedlings under dim red light on ice in extraction buffer (50 mM Tris-HCl pH 7.5, 300 mM Sucrose, 150 mM NaCl, 10 mM potassium acetate, 5 mM EGTA, 1 mM DTT, 1 mM PMSF and 1 x complete protease inhibitor mixture (Roche)). Microsomal membranes were isolated by centrifugation at 45,000 rpm for 45 min and resuspended in extraction buffer.

In vitro phosphorylation assays. Radiolabeled kinase assays were performed as described previously in (61). Reactions were prepared under dim red light and performed in a final volume of 20 μl containing 10 μl of cell free expression extract or 10 μg protein extract from *Arabidopsis*. N^6-benzyl-ATPγS (Jena Bioscience) was used at a final concentration of 100 μM for phot1 autophosphorylation or 500 μM for substrate phosphorylation and for phosphorylation screening with plant protein extracts. Light-treated samples were illuminated for 20 s with white light at a total fluence of 60,000 μmol m^{-2}. Reactions were incubated at room temperature for 5 min and stopped by adding EDTA (pH 8.0) to a final concentration of 20 mM. Thiophosphorylated molecules were alkylated for 1-2 h at room temperature by adding p-nitrobenzyl mesylate (Abcam) to a final concentration of 2.5 mM. For inhibitor studies, 1-NM-PP1 (CAS 221244-14-0, Merck Millipore) at a final concentration of 100 μM or solvent (DMSO) was added to the reactions before light exposure.

Western blot analysis. Proteins were detected by Western blot analysis on nitrocellulose membrane with rabbit anti-thiophosphoester monoclonal antibody (clone number 51-8, Abcam), anti-phot1 C-terminal antibody (50), anti-phot1 N-terminal antibody (19), rabbit anti-GFP-HRP monoclonal antibody (Miltenyi Biotech), rat anti-haemagglutinin monoclonal antibody (Roche), mouse anti-GST monoclonal antibody (Novagen). Blots were developed with anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Promega) or anti-rat HRP-conjugated secondary antibody (Dako Denmark A/S) and Pierce® ECL Plus Western blotting substrate (Thermo Fisher Scientific).

Measurement of phototropic curvature. Three-day-old etiolated seedlings grown vertically on Petri dishes containing MS agar were exposed to unilateral light provided by a fluorescent lamp filtered through one layer of blue Plexiglas for 24 h. Images of the seedlings were captured using a scanner and hypocotyl curvature was measured using ImageJ (http://rsb.info.nih.gov/ij/).

Chloroplast accumulation. Measurements of chloroplast positioning were performed as described (20). Rosette leaves detached from 3-week-old plants grown on soil were placed on agar plates and irradiated with 1.5 μmol m^{-2} s^{-1} blue light through a 1 mm slit, or placed into darkness for 1 h. The plates were placed on a white light transilluminator and photographed. Band intensities were quantified using ImageJ and the relative band intensities expressed as the ratio of the irradiated to the non-irradiated areas.

Leaf expansion. Measurement of leaf expansion was carried out as described (20). Plants were grown on soil under white light at 70 μmol m^{-2} s^{-1} for four weeks. The fifth rosette leaves were detached and photographed. The leaves were then uncurled manually and photographed again. Leaf areas were measured before and after uncurling and the ratio of the curled to uncurled area was designated the leaf expansion index. Leaf area was measured using ImageJ.

Confocal microscopy. Imaging of GFP-tagged proteins in Arabidopsis seedlings was visualised using a laser scanning confocal microscope (Zeiss LSM 510) as described previously (20).

Homology modelling of phot1 kinase domain. Modelling was performed using the automated online SwissModel server (62). Based on coverage and percentage of sequence identity, several templates were selected to build independent structural models. The template giving the best score for model quality was chosen (PDB ID 4gv1 for PKB alpha serine/threonine kinase).

CONFLICT OF INTERESTS STATEMENT

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

J.M.C. and J.S. designed and directed the research. J.S., P.H., T.W., G.G., and J.P. planned and performed experiments. All authors analysed data. J.M.C. and J.S. wrote the manuscript. All authors commented on the manuscript.

FOOTNOTES

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Abbreviations used are: FMN, flavin mononucleotide, GFP, green fluorescent protein, GST, glutathione S-transferase; KD, kinase dead; LOV, light oxygen or voltage; PK, protein kinase.

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FIGURE LEGENDS

Figure 1. Threonine 740 is the gatekeeper residue of *Arabidopsis* phot1. **A** Amino acid sequence alignment of the kinase domain of *Arabidopsis thaliana* (At) phototropin 1 with other protein kinases for which the gatekeeper residue has been identified (AtBRI1, AtFLS2 (35)) or for which the gatekeeper has been engineered to accommodate bulky ATP analogues (*Homo sapiens* (Hs) AMPKa2 (51), *Saccharomyces cerevisiae* (Sc) CDK1 (63)). **B** Structural model of the kinase domain of *Arabidopsis* phot1 generated by homology using SwissModel and displayed using PyMol. Secondary structures are displayed as a cartoon. The N-terminal lobe of the kinase domain is coloured blue while the C-terminal lobe is coloured pink. The β5 strand in the N-terminal lobe is shown

in red, and threonine 740 is shown in spheres coloured by atom (carbon is grey, oxygen is red, nitrogen is blue). **C** Cell-free expression and autophosphorylation analysis of wild-type phot1 and different gatekeeper mutants in the presence of [γ-^{32}P]ATP. Reactions were carried out in the absence (D) or presence of 10 s white light (L). Samples were separated between two SDS-PAGE gels but exposed to autoradiography simultaneously (top panel). The extent of autophosphorylation in the autoradiogram was quantified by ImageJ and the band intensity (%) relative to the phot1 light-treated sample is shown below each lane. Immunoblot of phot1 protein levels using anti-HA antibody is shown below.

Figure 2. Phot1 containing a modified gatekeeper residue (T740G) can accommodate N^6-benzyl-ATPγS and undergo thiophosphorylation *in vitro*. **A** Immunoblot of a kinase assay containing cell-free expressed wild-type phot1, phot1-D806N or phot1-T740G, in the presence of N^6-benzyl-ATPγS. Reactions were carried out in the absence (D) or presence of 20 s white light (L) and thiophosphorylation detected using anti-thiophosphoester antibody. Immunoblot analysis of phot1 protein levels using anti-HA antibody is shown below. **B** Phot1-T740G thiophosphorylation in the presence of the kinase inhibitor 1-NM-PP1 or DMSO as a control. Ponceau staining of cell-free expression reactions is shown below to indicate equal protein loading.

Figure 3. Phot1-Cerberus (p1-T740G) directly phosphorylates BLUS1 *in vitro*. **A** Thiophosphorylation analysis of *in vitro* kinase assays containing phot1-Cerberus (p1-Cerb) and GST-BLUS1 or the cell-free expression extract alone (-) with GST-BLUS1. Reactions were carried out in the absence (D) or presence of 20 s white light (L) and thiophosphorylation detected using anti-thiophosphoester antibody. Phot1-Cerberus thiophosphorylation is evident ~130 kDa whereas GST-BLUS1 is shown above 70 kDa. Blots were probed with anti-HA antibody to detect phot1 or anti-GST antibody to detect BLUS1 (shown below). **B** Thiophosphorylation analysis of *in vitro* kinase assays containing phot1-Cerberus together with GST-BLUS1-S348A or GST-BLUS1-D157N. **C** Thiophosphorylation analysis of *in vitro* kinase assays containing phot1-Cerberus (p1-Cerb) co-expressed with either GST-BLUS1 or GST-phot1 (GST-p1). Reactions were carried out in the absence (D) or presence of 20 s white light (L) and thiophosphorylation detected using anti-thiophosphoester antibody. Phot1-Cerberus thiophosphorylation is evident ~130 kDa whereas thiophosphorylation of GST-phot1 and GST-BLUS1 are indicated above 130 and 70 kDa, respectively with black arrows. Blots were probed with anti-HA antibody to detect phot1 and GST-phot1 or with anti-GST antibody to detect BLUS1 (shown below).

Figure 4. Phot2-Cerberus phosphorylates BLUS1 at S348. **A** Thiophosphorylation analysis of *in vitro* kinase assays containing wild-type phot2 (p2) or phot2-Cerberus (p2-Cerb). Reactions were carried out in the absence (D) or presence of 20 s white light (L) and thiophosphorylation detected using anti-thiophosphoester antibody. Phot2-Cerberus thiophosphorylation is evident above 100 kDa. Blots were probed with anti-HA antibody to detect phot2 protein levels (shown below). **B** Thiophosphorylation analysis of *in vitro* kinase assays containing phot2-Cerberus together with GST-BLUS1-S348A or GST-BLUS1-D157N. Reactions were carried out as in A. Thiophosphorylation of GST-BLUS1 is shown above 70 kDa whereas phot2 thiophosphorylation is evident above 100 kDa.

Figure 5. Phot1-Cerberus is functional when expressed in the *phot1 phot2* double mutant. **A** Immunoblot analysis of phot1 protein abundance in transgenic *Arabidopsis* expressing phot1-GFP (p1-GFP) and 3 independent lines expressing phot1-Cerberus fused to GFP (p1-Cerb-GFP). Protein extracts were isolated from 3-day-old etiolated seedlings either maintained in darkness (D) or irradiated with 20 μmol m^{-2} s^{-1} of blue light for 15 min (L) and probed with anti-GFP antibody. The dashed line indicates the lowest mobility edge. **B** Phototropic responses of 3-day-old etiolated wild-type (*gl-1*) seedlings, the *phot1 phot2* double mutant (*p1p2*), plants expressing p1-GFP and 5 independent lines expressing p1-Cerb-GFP. Measurements represent the angle made by the tip of the hypocotyl with the vertical. Representative seedling images are shown above. **C** Leaf flatness index (ratio of leaf area before and after leaf uncurling) for wild-type (*gl-1*), *phot1 phot2* double mutant (*p1p2*), plants expressing p1-GFP and 5 independent lines expressing p1-Cerb-GFP. **D** Chloroplast accumulation measurements in the same genotypes as in A. Detached leaves were illuminated with 1.5 μmol m^{-2} s^{-1} of blue light through a 1 mm slit for 90 min and the slit band intensity quantified. Relative band intensity shown is expressed as the ratio of the irradiated to the non-

irradiated areas. Ratios above 1 indicate accumulation. For B-D, quantification of plant responses are represented as the median (horizontal line) and individual data points (open circles).

Figure 6. Phot1-Cerberus thiophosphorylation in plant protein extracts. **A** Thiophosphorylation analysis of *in vitro* kinase assays on microsomal extracts isolated from transgenic *Arabidopsis* expressing phot1-Cerberus fused to GFP (p1-Cerb-GFP) or phot1-GFP (p1-GFP). Reactions were carried out in the absence (D) or presence of 20 s white light (L) and thiophosphorylation detected using anti-thiophosphoester antibody. Phot1-Cerberus thiophosphorylation is evident above 130 kDa. Blots were probed with anti-GFP antibody to detect phot1 protein levels (shown below). **B** Thiophosphorylation analysis of *in vitro* kinase assays on total protein extracts isolated from p1-Cerb-GFP or p1-GFP lines. Reactions were carried out as described in A, except that only the light-treated p1-GFP sample was used as a negative control. Phot1-Cerberus thiophosphorylation is evident above 130 kDa. Additional thiophosphorylation products are indicated with the black arrow. Blots were also probed with anti-phot1 N-terminal (p1-N) or C-terminal (p1-C) antibodies to detect phot1 and its proteolytic cleavage products.

Figure 1

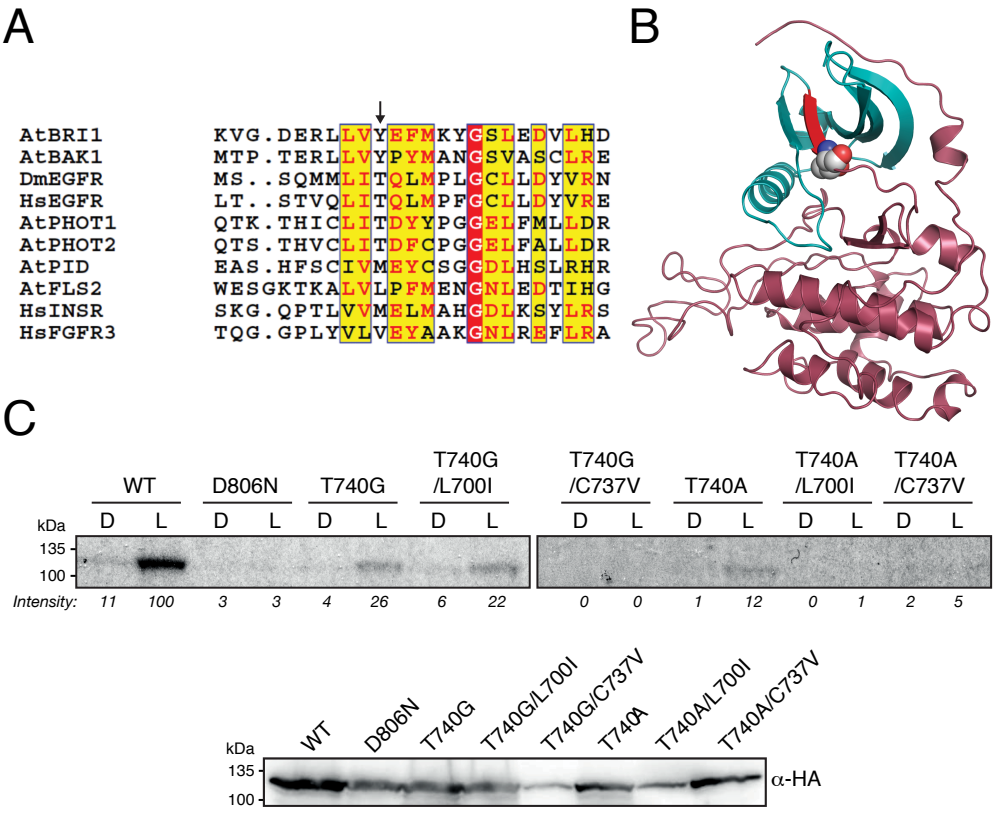
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Figure 2

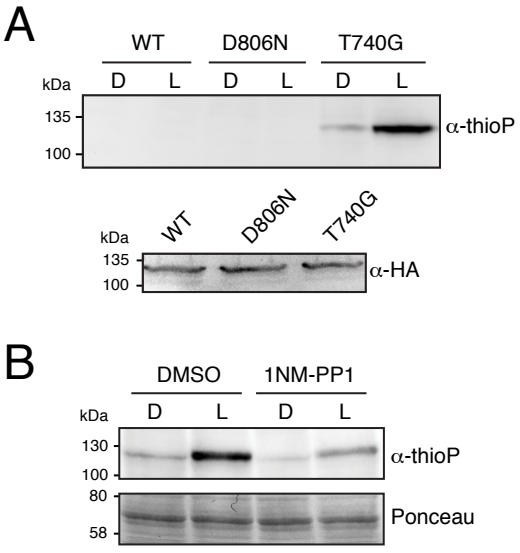
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Figure 3

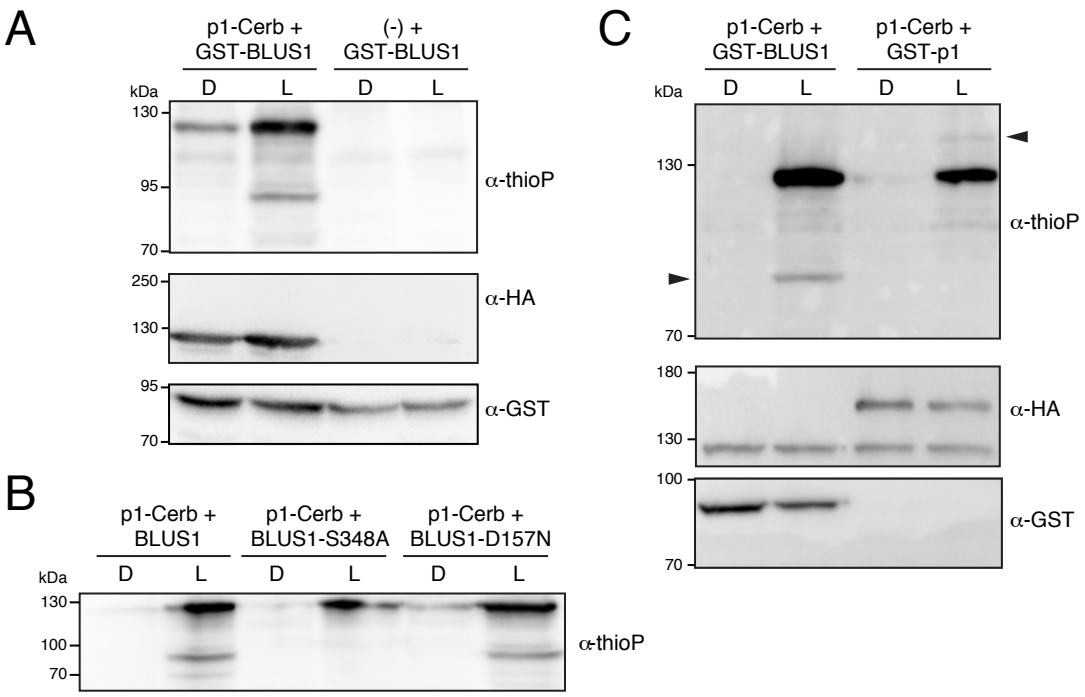
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Figure 4

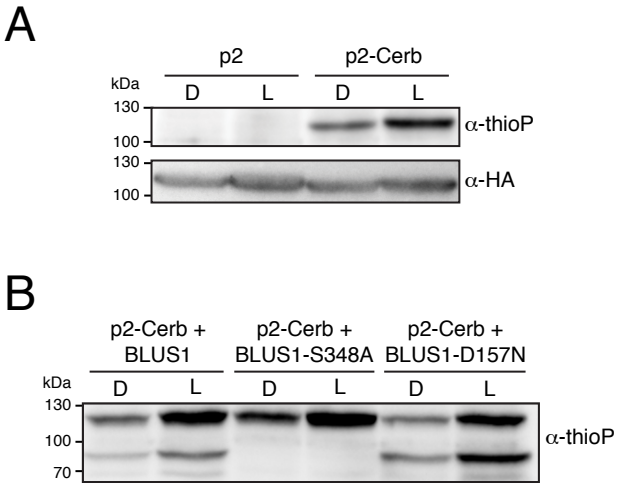
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Figure 5

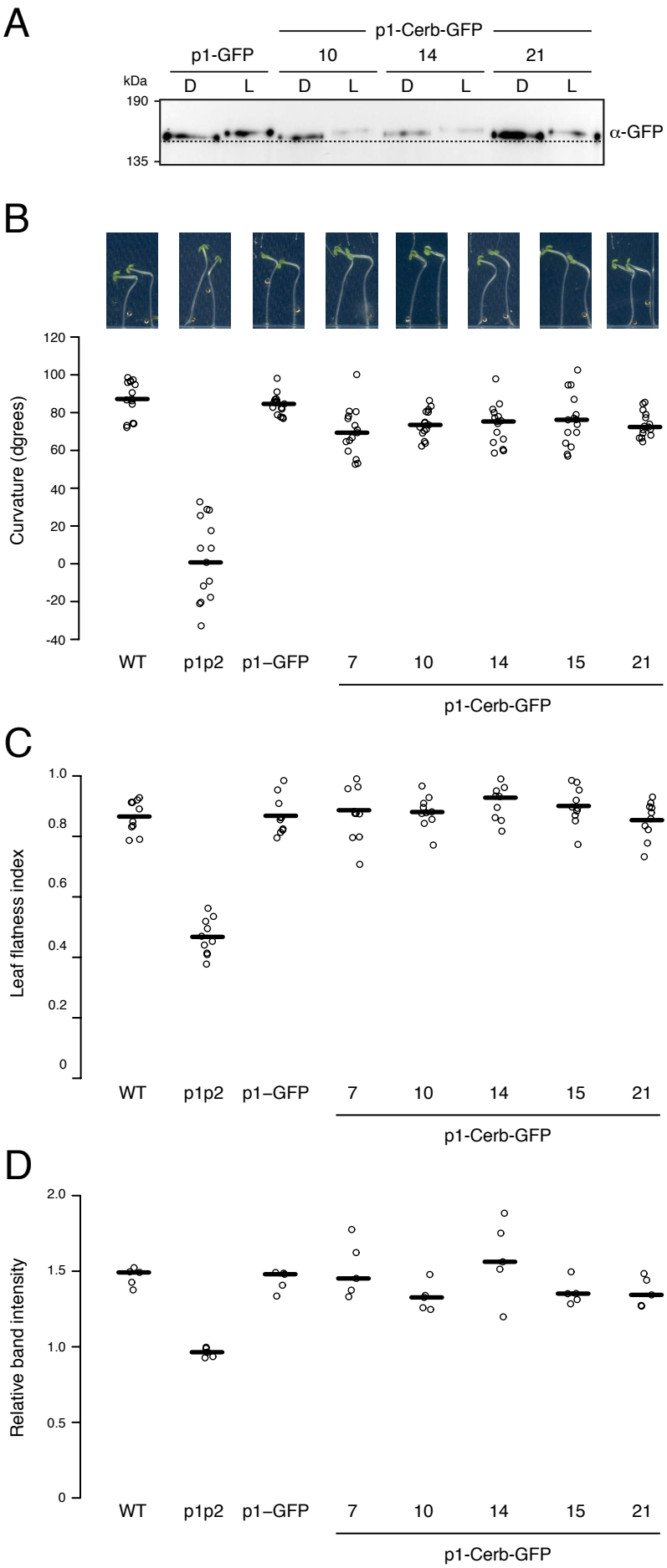
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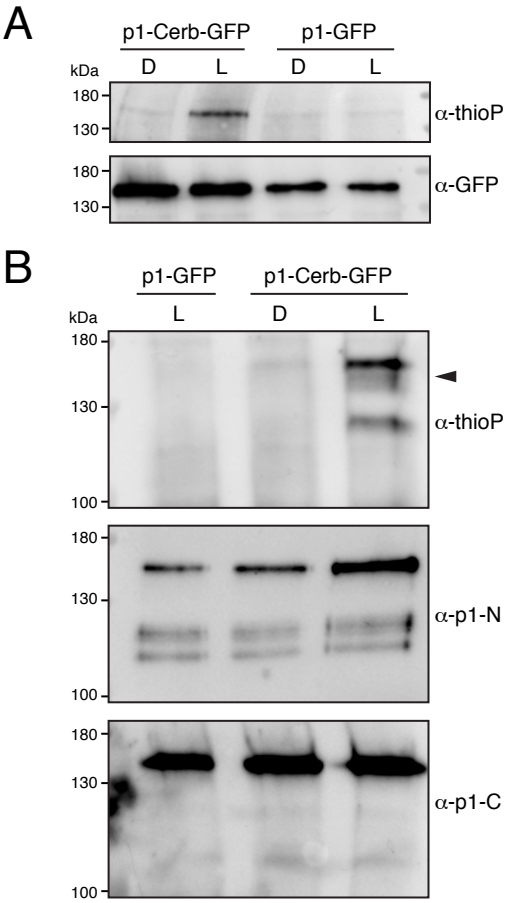
Figure 6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L
180
130
α-thioP
180
130
α-GFP
B
p1-GFP
p1-Cerb-GFP
kDa
L
D
L
180
130
100
α-thioP
180
130
100
α-p1-N
180
130
100
α-p1-C

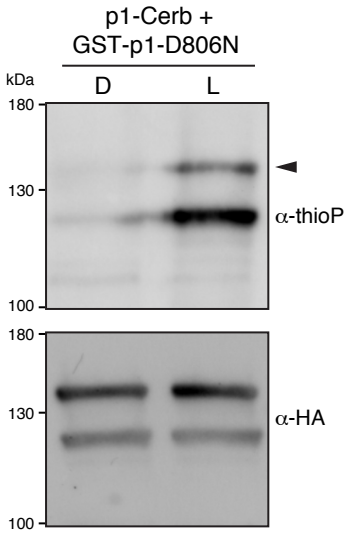

Figure S1. Phot1-Cerberus can cross-phosphorylate a kinase dead variant of GST-phot1 following co-expression. Phot1-Cerberus and GST-phot1-D806N were co-expressed in a cell-free system and used for in vitro kinase assays containing N6-benzyl-ATPγS. Reactions were carried out in the absence (D) or presence of 20 s white light (L) and analysed by immunoblotting using anti-thiophosphate antibodies. The arrowhead indicates the position of GST-p1-D806N. Immunoblots with anti-HA antibody are shown below to detect both phot1 and GST-phot1 protein levels.

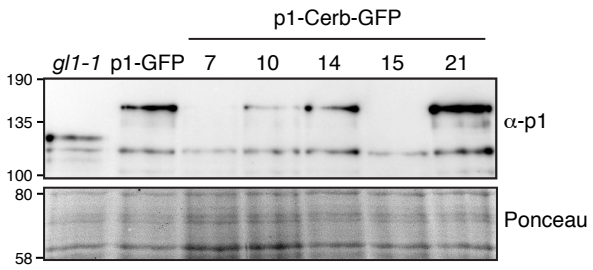

Figure S2. Phot1 protein levels in transgenic *Arabidopsis* expressing phot1-Cerberus. Immunoblot analysis of phot1 protein abundance in wild-type (*gl-1*) seedlings and in transgenic *Arabidopsis* expressing phot1-GFP (p1-GFP) or 5 independent lines expressing phot1-Cerberus fused to GFP (p1-Cerb-GFP). Protein extracts were isolated from 3-day-old etiolated seedlings and probed with anti-phot1 antibody. Ponceau staining is shown below to indicate equal protein loading.

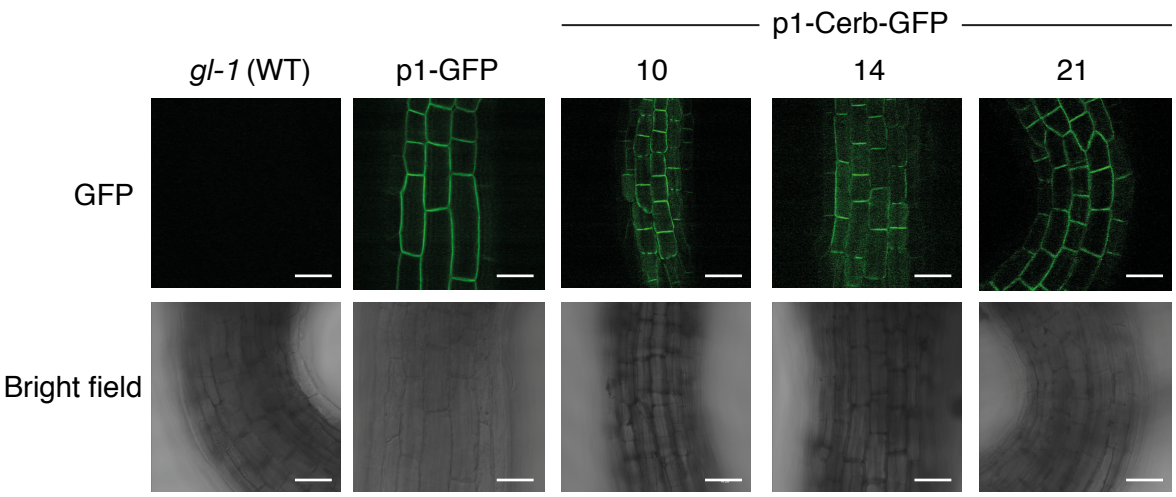

Figure S3. Phot1-Cerberus is localised at the cell periphery. The subcellular localisation of phot1-GFP (p1-GFP) or phot1-Cerberus fused to GFP (p1-Cerb-GFP) was investigated by laser-scanning confocal microscopy in the apical hook region of 3-day-old etiolated *Arabidopsis* seedlings. Bar, 40 μm.