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Title page

Lipid anchoring of *Arabidopsis* phototropin 1 to assess the functional significance of receptor internalisation: Should I stay or should I go?

Tobias Preuten [1], Lisa Blackwood [2], John M. Christie [2] and Christian Fankhauser [1]

[1] Centre for Integrative Genomics, Faculty of Biology and Medicine, Génopode Building, University of Lausanne, CH-1015 Lausanne, Switzerland. [2] Institute of Molecular Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, Bower Building, University of Glasgow, Glasgow G12 8QQ, UK.

Author for correspondence: Christian Fankhauser

Email: christian.fankhauser@unil.ch

Tel: +41 21 692 3941

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Summary

- The phototropin 1 (phot1) blue light receptor mediates a number of adaptive responses including phototropism that generally serve to optimise photosynthetic capacity. Phot1 is a plasma membrane associated protein but upon irradiation, a fraction is internalised into the cytoplasm. Although this phenomenon has been reported for more than a decade, its biological significance remains elusive. Here, we use a genetic approach to revisit the prevalent hypotheses regarding the functional importance of receptor internalisation.
- Transgenic plants expressing lipidated versions of phot1 that are permanently anchored to the plasma membrane were used to analyse the effect of internalisation on receptor turnover, phototropism and other phot1-mediated responses.
- Myristoylation and farnesylation effectively prevented phot1 internalisation. Both modified photoreceptors were found to be fully functional in *Arabidopsis*, rescuing phototropism and all other phot1-mediated responses tested. Light-mediated phot1 turnover occurred as in the native receptor. Furthermore, our work does not provide any evidence for a role of phot1 internalisation in the attenuation of receptor signalling during phototropism.
- Our results demonstrate that phot1 signalling is initiated at the plasma membrane. They furthermore indicate that release of phot1 into the cytosol is not linked to receptor turnover or desensitisation.

Keywords: phototropin 1 (phot1), *Arabidopsis thaliana*, internalisation, phototropism, receptor turnover, subcellular localisation, myristoylation, farnesylation.

Introduction

Plants utilise light not only to produce energy through photosynthesis but also as a source of information about their surroundings. As sessile organisms, being able to determine and react to direction, intensity, quality and duration of incident light is of utmost importance for their survival. Consequently, plants have developed multiple classes of photoreceptors that absorb incoming light at different wavelengths (Christie, 2007; Franklin & Quail, 2010; Möglich *et al.*, 2010; Rizzini *et al.*, 2011; Chaves *et al.*, 2011; Hu *et al.*, 2012; Christie *et al.*, 2012; Ito *et al.*, 2012). The phototropin family of UV-A/blue light receptors mediates crucial photoresponses such as phototropism, leaf flattening, petiole positioning and chloroplast movement which collectively serve to optimise photosynthetic performance and thereby ultimately ensure competitiveness and reproduction (Whippo & Hangarter, 2006; Christie, 2007; Holland *et al.*, 2009; Demarsy & Fankhauser, 2009; Hohm *et al.*, 2013).

In higher plants, including *Arabidopsis*, two family members, phototropin 1 (phot1) and phot2 exist (Briggs *et al.*, 2001). They are highly similar in sequence and structure and have largely overlapping functions with phot1 being more sensitive and thus solely mediating responses to low intensities of blue light (Jarillo *et al.*, 2001; Sakai *et al.*, 2001; Christie, 2007). Over the last decade, phots have been studied extensively with respect to their photochemical, structural and biological properties (Christie, 2007; Tokutomi *et al.*, 2008; Christie & Murphy, 2013; Hohm *et al.*, 2013; Goyal *et al.*, 2013; Preuten *et al.*, 2013; Briggs, 2014; Yamamoto *et al.*, 2014). Phot1 is a plasma membrane-associated protein that consists of an N-terminal photosensory region and a C-

terminal serine/threonine kinase domain (Tokutomi *et al.*, 2008; Wan *et al.*, 2008). Upon blue light perception, the activity of the kinase domain is induced (Christie *et al.*, 2002; Matsuoka & Tokutomi, 2005), a step that has been shown to be essential for all tested phot1-mediated responses (Christie *et al.*, 2002; Kong *et al.*, 2007; Inoue *et al.*, 2008a). It is to date not fully understood how phot1 associates with the plasma membrane, but a short stretch of amino acids in the very C-terminal region of the protein has been implicated in this property (Kong *et al.*, 2013a).

Twelve years ago, Sakamoto & Briggs (2002) showed that a fraction of a green fluorescent protein (GFP)-labelled phot1 internalised from the plasma membrane when cells were irradiated with blue light. This phenomenon was analysed in more detail in the following years and a similar behaviour was observed for phot2 (Kong *et al.*, 2006; Wan *et al.*, 2008; Kaiserli *et al.*, 2009; Sullivan *et al.*, 2010). However, while phot2 was found to localise to Golgi vesicles after blue light irradiation (Kong *et al.*, 2006), no such co-localisation has been ascribed to phot1 which rather targets dynamic cytosolic structures (Sakamoto & Briggs, 2002; Wan *et al.*, 2008; Kaiserli *et al.*, 2009). In the case of phot1, re-localisation from the plasma membrane appears to be dependent on its kinase activity (Kaiserli *et al.*, 2009) while the situation seems to be more complex with phot2: kinase activity is necessary for internalisation from the plasma membrane and possibly subsequent degradation, but not for the association with Golgi vesicles (Kong *et al.*, 2006, 2007; Aggarwal *et al.*, 2014).

Only a relatively small fraction of phot1 (20 % in mustard; Knieb *et al.*, 2004) is internalised and there have been speculations about the possible function of this change in subcellular localisation ever since. The main

hypotheses that were brought forward over the years comprise (i) a potential role of endocytosis and/or trafficking in receptor signalling (Kaiserli *et al.*, 2009; Roberts *et al.*, 2011; Kong *et al.*, 2013b), (ii) internalisation as a prerequisite for protein turnover which has been shown to occur within hours under blue light (Sakamoto & Briggs, 2002; Kong *et al.*, 2006; Sullivan *et al.*, 2010; Roberts *et al.*, 2011) and (iii) internalisation as a mechanism for rapid receptor desensitisation (Wan *et al.*, 2008; Han *et al.*, 2008; Kaiserli *et al.*, 2009; Briggs, 2014). In favour of the latter, Han and co-workers (2008) found that internalisation of phot1-GFP was strongly impeded in hypocotyl cortical cells that were pre-treated with a pulse of red light before irradiation with blue light, a treatment that has long been known to enhance phototropic bending through phytochrome activation (Chon & Briggs, 1966; Janoudi & Poff, 1992; Parks *et al.*, 1996; Lariguet & Fankhauser, 2004; Rösler *et al.*, 2007; Briggs, 2014). The authors proposed that retention of phot1 at the plasma membrane accounts for enhanced phototropic sensitivity and that internalisation represents a means of desensitisation.

In this study, we aimed to determine the importance of the subcellular localisation of phot1 with respect to its function and regulation. To this end, we constructed transgenic *Arabidopsis* lines that express fluorescently labelled myristoylated and farnesylated phot1 proteins, respectively. Such lipid modifications are known to play an important role in targeting and attaching soluble proteins to the plasma membrane (Thompson & Okuyama, 2000). Indeed, addition of a myristoyl group has previously been successfully applied in *Arabidopsis* to constitutively tether a BRI1 kinase inhibitor 1 (BKI1)-yellow fluorescent protein (YFP) fusion protein to the plasma membrane, thereby preventing its internalisation (Wang & Chory, 2006). Using a similar approach,

we were able to systematically test the abovementioned hypotheses to better understand the functional relevance of phot1 subcellular localisation.

Materials and Methods

Plasmid construction and transgenic lines

Plant transformation vectors used to create pPHOT1 and myristoylated phot1 lines were based on a pGreenII backbone (http://www.pgreen.ac.uk) and additionally contained a MCS/lacZ operon, a nos-BAR cassette (plant Basta resistance), the coding sequence of mCitrine (Shaner *et al.*, 2005), and an OCS terminator sequence. This vector was a gift from Julien Alassimone and Niko Geldner. The *PHOT1* endogenous promoter was obtained by amplifying 3.9 kb upstream of the initiation codon from the LEGT2 transformation vector (Sakamoto & Briggs, 2002). The full length coding sequence of PHOT1 was amplified from cDNA and inserted into the vector so that it was fused translationally with the *mCitrine* gene to give *pPHOT1::PHOT1-mCitrine*. To obtain the construct coding for the myristoylated phot1 fusion protein, a recognition sequence for the N-myristoyltransferase (MGICMSR, Wang & Chory, 2006) was added to the N-terminus of phot1 by PCR. Transformation vectors for farnesylated phot1 lines and the respective mutated farnesyl controls were constructed using the modified binary expression vector pEZR(K)-LN as described previously (Kaiserli *et al.*, 2009) to obtain a C-terminal GFP fusion. The *35S* promoter was removed using restriction sites SacI and HindIII and replaced with the native *PHOT1* promoter region to generate the plasmid pEZR-pPHOT1. To create the farnesyl tag, the C-terminal GFP from pEZR(K)-LN was removed using restriction sites BamHI and XbaI. pEZR(K)-LN was used as a template to amplify a modified GFP using the primers farnesyl F (GATGTGACATCTCCACTGACG) and farnesyl R

(GGCATGGACGAGCTGTACAAGTCTAAGGATGGAAAGAAGAAGAAGAAGAAGTCTAA GACTAAGTGTGTTATTATGTAATCTAGATATAT) to generate a GFP coding sequence with a C-terminal farnesylation sequence. This was cloned into pEZR-pPHOT1 using the restriction sites BamHI and XbaI to generate the plasmid pEZR-pPHOT1-GFPfarn. Amino acid changes in the mutated farnesyl construct were introduced by site-directed mutagenesis using KOD Hot-Start DNA Polymerase (Novagen) and the primers farnesyl C-A F (AAGAAGAAGAAGAAGTCTAAGACTAAGGCTGTTATTATGTAATCTAGAGTCC) and farnesyl C-A R (GGACTCTAGATTACATAATAACAGCCTTAGTCTTAGACTTCTTCTTCTTCTT).

Transgenic plants were generated by introduction of the plant expression constructs into the pSOUP-containing *Agrobacterium tumefaciens* strain GV3101. Double mutant *phot1-5phot2-1* plants (Kinoshita *et al.*, 2001) were transformed by floral dipping (Clough & Bent, 1998). Based on segregation of basta- or kanamycin-resistance, at least three independent homozygous T_3 lines of each genotype with a single transgene locus were selected.

Plant material and growth conditions

Seeds of the ecotype Columbia, *gl-1* (Col-0; wild type), the phot-deficient mutant *phot1-5phot2-1* and the *phot1-7* mutant containing a kinase inactive version of phot1 (D806N) have been described previously (Kinoshita *et al.*, 2001; Christie *et al.*, 2002; Sullivan *et al.*, 2010). Unless stated otherwise, surface-sterilised seeds were plated on nylon meshes placed on half-strength Murashige and Skoog medium with 0.8 % agar and kept at 4 °C in the dark for three days. Plates were then transferred to 21 ± 1 °C (Sanyo incubator) and exposed to 50 µmol m^{-2} s^{-1}

red light for 2-3 hours to induce germination prior to incubation in the dark at 21 ± 1 °C (Heraeus incubator) for 65-70 hours. The red light source was a light-emitting diode (λmax, 664 nm; CLF Plant Climatics GmbH). Light intensities were determined either with an IL1400A photometer equipped with an SEL033 probe with appropriate light filters (International Light) or with a Li-250A and quantum sensor (LI-COR).

Protein extraction for NPH3-dephosphorylation and phot1-degradation assays

Three-day-old, etiolated *Arabidopsis* seedlings were grown as described above and exposed to blue light from above at an intensity of 15 µmol m^{-2} s^{-1} (NPH3-dephosphorylation) and 120 µmol m^{-2} s^{-1} (phot1-degradation) for the indicated times. 50 seeds were sown for each time point. The blue light source was a light-emitting diode (λmax, 462 nm; CLF Plant Climatics GmbH). Total proteins were extracted at the indicated time points by grinding the seedlings in 100 µl 2x Laemmli buffer [0.125 M Tris pH 6.8; 4 % (w/v) SDS; 20 % (v/v) glycerol; 0.02 % (w/v) bromophenol blue; 10 % (v/v) β-mercaptoethanol].

SDS-PAGE and immunoblot analysis

Total proteins were separated on 8 % SDS-PAGE gels and transferred onto nitrocellulose membranes with 100 mM Tris-Glycine buffer [25 mM Tris, 192 mM glycine] + 10 % (v/v) ethanol. The blots were probed with anti-DET3, anti-phot1 and anti-NPH3 antibodies as described in (Lariguet *et al.*, 2006). Chemiluminescence signals were generated using Immobilon Western HRP Substrate (Millipore). Signals were captured with a Fujifilm Image Quant LAS

4000 mini CCD camera system and quantifications were performed with Image Quant TL software (GE Healthcare).

Measurement of hypocotyl curvature

For phototropism experiments, seedlings were grown on vertically orientated plates, either for three days in darkness (etiolated) or for three days at 50 µmol m^{-2} s^{-1} white light (de-etiolated) at 21 ± 1 °C. Subsequently, they were irradiated with unilateral blue light at the indicated light intensity. The blue light source was a light emitting diode (λmax, 462 nm; CLF Plant Climatics GmbH). In order to analyse the effect of red light pretreatment on phototropism, three-day-old, etiolated seedlings were irradiated with a pulse of red light (1800 µmol m^{-2}) and subsequently kept in the dark for one hour before exposure to unilateral blue light. Plates were photographed in infrared light at the indicated times after illumination. Hypocotyl angles relative to the growth direction before the start of blue light irradiation were measured using the National Institutes of Health ImageJ software version 1.38 (http://rsb.info.nih.gov/ij/).

Observation of leaf positioning

Seedlings were grown in a controlled growth room at 22 °C and 60 % humidity (Fitotron; Weiss-Gallenkamp). *Arabidopsis* seedlings were grown under white light at 70 µmol m^{-2} s^{-1} in a 16/8 hour light-dark cycle for seven days before being transferred to 10 µmol m^{-2} s^{-1} white light in a 16/8 hour light-dark cycle for a further seven days before representative seedlings were photographed. Petiole angles were measured using the ImageJ software.

Measurement of leaf flattening

Measurement of leaf flattening was carried out as described previously (Takemiya *et al.*, 2005). *Arabidopsis* plants were grown on soil under 70 μmol m^{-2} s^{-1} white light in a 16/8 hour light-dark cycle for three weeks. The fifth rosette leaves were detached and photographed. The leaves were then flattened manually and photographed again. Leaf area was measured using the ImageJ software. The leaf flattening index is designated as the ratio of unflattened leaf to flattened leaf.

Chloroplast accumulation response

Plants were grown on soil under 70 μmol m^{-2} s^{-1} white light in a 16/8 hour light-dark cycle for three weeks before rosette leaves were detached and placed on half strength Murashige and Skoog agar plates (0.8 %). Detached leaves were given either a low blue light treatment of 1.5 μmol m^{-2} s^{-1} or kept in darkness for three hours before chloroplasts in the palisade mesophyll cells were examined by confocal microscopy (Sullivan *et al.*, 2008; Kaiserli *et al.*, 2009).

Microscopy

Images of mCitrine and GFP-fluorescence in transgenic lines were taken with a 2-photon Zeiss LSM 710 NLO microscope equipped with a Chameleon Ultra II Ti:Sapphire laser at 960 and 900 nm, respectively, using the non-descanned detector (NDD) with a 500–550 nm bandpath filter. Cross-sections were reconstructed from z-stacks using the ImageJ software.

Results

Lipid modification of phot1 prevents its internalisation upon blue light irradiation

In darkness, phot1 is tightly associated with the plasma membrane (Fig. 1). Although a small region at the C-terminus of phot1 and phot2 appears to be important for membrane association (Kong *et al.*, 2013a), this mechanism of attachment has not been fully defined for phot1. Consequently, there is presently no convenient means to assess the functional role of internalised phot1 by preventing membrane attachment. It is however possible to constitutively anchor proteins to the plasma membrane. In eukaryotes, including plants, lipid modifications constitute an important post-translational means to facilitate plasma membrane localisation.

Major types of such lipid modifications include myristoylation and farnesylation (Thompson & Okuyama, 2000; Sorek *et al.*, 2009). In the former case, a myristoyl group is irreversibly added to an N-terminal glycine residue by the enzyme N-MYRISTOYLTRANSFERASE (NMT; Qi *et al.*, 2000), while the latter process comprises the covalent attachment of a farnesyl group to a C-terminal cysteine residue by PROTEIN FARNESYLTRANSFERASE (PFT; Johnson *et al.*, 2005). In both cases, the modifying enzyme recognises a short amino acid sequence (seven to ten amino acids in the case of NMT and four in the case of PFT; Thompson & Okuyama, 2000; Sorek *et al.*, 2009). Proteins modified in this way often associate with the plasma membrane due to the hydrophobic properties of the attached lipid residue. Indeed, myristoylation has been

278 previously used in plants to anchor proteins at the membrane (Wang & Chory,
279 2006).

280 Here, we made transgenic *Arabidopsis* expressing modified phot1 that is
281 tethered to the plasma membrane. Since phot1 activation is reliant on
282 conformational changes (Harper *et al.*, 2003; Christie, 2007), we adopted two
283 complementary approaches for membrane tethering in an attempt avoid any
284 steric issues that could render the protein inactive. The first transgene coded for
285 a myristoylated phot1 protein (myri; Fig. 1) with a myristoylation signal
286 sequence at the 5'-end of the *PHOT1* coding sequence fused to an mCitrine at the
287 C-terminus. The second transgene comprised a farnesylation signal sequence at
288 the 3'-end of a C-terminal *GFP* coding sequence, leading to the expression of a
289 farnesylated phot1-GFP fusion protein (farn; Fig. 1). Both transgenes were
290 expressed under the control of the endogenous *PHOT1* promoter in a phot-
291 deficient (*phot1phot2*) background. Using this strategy, we aimed to analyse the
292 importance of phot1 internalisation with regard to receptor function and
293 turnover.

294 Subcellular localisation of the modified proteins was analysed by 2-
295 photon microscopy alongside the appropriate controls. For myri-tagged phot1
296 we used lines expressing an unmodified phot1-mCitrine as a control (pPHOT1;
297 Preuten *et al.*, 2013). For farn-tagged phot1, control lines expressing phot1-GFP
298 harbouring a mutated farnesylation signal sequence (mut farn), no longer
299 recognised by PFT, were used. Both control transgenes were expressed under
300 the control of the endogenous *PHOT1* promoter in the *phot1phot2* background.
301 As expected, all four fusion-proteins associated with the plasma membrane in
302 darkness. For each of the controls, rapid emergence of fluorescence signals in the

303 cytoplasm was detected in cells irradiated with the blue laser of the microscope.
304 However, the fluorescence signal from modified phot1 was confined to the
305 plasma membrane even after prolonged exposure (Fig. 1; Fig. S1). We thus
306 conclude that lipid modification in both cases was successful in preventing
307 partial phot1 internalisation, thereby making the myri and farn lines suitable
308 tools to analyse the impact of this subcellular re-localisation on phot1 function
309 and turnover.
310
311 **Lipid-modified phot1 is active and mediates rapid responses at the plasma**
312 **membrane**
313 Autophosphorylation of phot1 instigates its internalisation from the plasma
314 membrane (Kaiserli *et al.*, 2009). Tethering phot1 to the plasma membrane
315 through N-terminal myristoylation or C-terminal farnesylation could interfere
316 with steric movements necessary for its activation (Harper *et al.*, 2003), thereby
317 impeding autophosphorylation and movement from the membrane. To test
318 whether the lack of internalisation observed for lipid-modified phot1 resulted
319 from abrogated kinase activity, we analysed receptor autophosphorylation by
320 monitoring its electrophoretic mobility. As shown in Figure 2, reduced mobility
321 after blue light treatment was detected in all lines, indicative of phot1
322 autophosphorylation (Knieb *et al.*, 2005; Sullivan *et al.*, 2008). We also analysed
323 the phot1-mediated dephosphorylation of NON-PHOTOTROPIC HYPOCOTYL 3
324 (NPH3), which is proposed to constitute an early event in phot1 signalling
325 (Pedmale & Liscum, 2007; Tsuchida-Mayama *et al.*, 2008). NPH3
326 dephosphorylation was observed in all transgenic lines, demonstrating the

presence of active phot1. These data therefore indicate that phot1 autophosphorylation and activation is not affected by lipid modification.

Plasma membrane-associated phot1 triggers phototropism

Next, we tested whether the constitutively plasma membrane-attached phot1 receptor was functional for phototropism. We first analysed phototropic bending in etiolated seedlings at different blue light intensities. At a fluence rate of 1 μmol m^{-2} s^{-1}, both myri and farn seedlings showed fully restored phototropism with bending kinetics almost identical to those in control lines (Fig. 3a,c). These findings demonstrate that lipid modification does not affect phot1 function under these conditions. Both myri and farn lines responded similar to controls at lower fluence rates (0.01 μmol m^{-2} s^{-1}) reaching the same final bending angle after a 24-hour irradiation period. Curvature was slightly slower for the myri and farn lines under these conditions (Fig. 3b,d).

Previous studies have shown that the time of onset and the extent of phot1 internalisation are sensitive to blue light intensity (Wan *et al.*, 2008). A greater difference in internalisation would be expected between phot1 and its lipid-modified versions at higher fluence rates of blue light. However, the observation that myri and farn lines exhibit decelerated phototropism at very low light intensities indicates that our data cannot easily be explained by altered internalisation. We therefore also tested the bending response of de-etiolated seedlings, since one would expect more phot1 to be internalised under these conditions given their pre-exposure to light. However, de-etiolated seedlings expressing lipid-modified versions of phot1 showed fully rescued phototropism with very similar bending as the controls (Fig. S2).

352

353 **Lipid-anchoring phot1 does not impair petiole positioning, leaf flattening**
354 **and chloroplast accumulation movement**

355 Phots not only mediate phototropism but a variety of other adaptive responses
356 (Christie, 2007). Leaf flattening and petiole positioning are both phot-mediated
357 processes that optimise photosynthetic light capture (Takemiya *et al.*, 2005;
358 Inoue *et al.*, 2008b; de Carbonnel *et al.*, 2010). At a subcellular level, chloroplasts
359 have to be optimally positioned to either maximise photosynthetic activity or
360 minimise photodamage. At low blue light intensities, chloroplasts accumulate at
361 the upper surface of mesophyll cells in a phot-dependent manner (Suetsugu &
362 Wada, 2007). We therefore analysed whether lipid anchoring of phot1 to the
363 plasma membrane could impact its ability to mediate these responses.

364 The petiole angles of two-week-old myri and farn lines were very similar
365 to those observed for the control lines (between approximately 40 and 45 °; Fig.
366 4a,b) and slightly lower than those of wild type (52 °; Fig. S2a,b). By comparison,
367 petioles of the *phot1phot2* mutant pointed downward with an angle of about 7 °;
368 Fig. S2a, b). Hence, constitutive membrane tethering of phot1 does not affect its
369 ability to mediate petiole positioning relative to controls.

370 Three-week-old plants of all transgenic lines examined showed a similar
371 leaf flattening index (the ratio of leaf size before and after artificial flattening, i.e.
372 an index of 1 would represent a perfectly flat leaf) of around 0.8 with no
373 significant differences between control lines and lines expressing lipid-modified
374 phot1 (Fig. 4c). By comparison, wild type plants showed a leaf flattening index of
375 approximately 0.9 while the *phot1phot2* mutant has strongly curled leaves with a
376 leaf flattening index of 0.51 (Fig. S2c,d). In conclusion, anchoring phot1 at the

plasma membrane was sufficient to rescue leaf flattening in the *phot1phot2* mutant background.

To analyse effects on chloroplast accumulation, we compared the number of chloroplasts situated at the upper surface of mesophyll cells between leaves that were kept in darkness and those exposed to low intensity blue light for three hours (Fig. 5). Mesophyll cells of wild type and both control lines used throughout this study showed a typical chloroplast accumulation response, which was absent in the *phot1phot2* mutant (Fig. 5; Fig. S4). Both myri and farn lines exhibited normal chloroplast accumulation (Fig. 5), demonstrating that phot1 initiates this response from the plasma membrane. These results are in accordance with a mechanism involving long-distance signal transfer from the plasma membrane (Tsuboi & Wada, 2010, 2011; Kong *et al.*, 2013b).

Taken together, our physiological characterisation shows that phot1 is active at the plasma membrane and that receptor internalisation is not required for mediating the above tested responses.

Retention of phot1 at the plasma membrane does not account for the red light enhancement of phototropic sensitivity

As mentioned, one of the prevalent hypotheses regarding the importance of phot1 internalisation came from the discovery that red light treatment before unidirectional blue light stimulation not only enhanced phototropic bending but also caused retention of phot1 at the plasma membrane in specific cells of the *Arabidopsis* hypocotyl (Han *et al.*, 2008). Enhanced phototropism could therefore result from phot1 retention at the plasma membrane. If so, internalisation would represent a means of desensitisation through an attenuation of receptor

signalling. However, our results indicate that membrane-anchored phot1 *per se* does not lead to increased phototropic bending but rather is less sensitive under very low fluence rates of blue light (Fig. 3, Fig. S1).

To investigate further the relationship between phot1 subcellular localisation and red light mediated promotion of phototropic sensitivity, we carried out phototropism experiments with or without red light pre-treatment. For this purpose, three-day-old, etiolated seedlings were given a pulse of red light and transferred to darkness for one hour prior to phototropic stimulation. As expected, all control lines showed significantly enhanced phototropic bending when given a pulse of red light before phototropic stimulation (Fig. 6a,c). Interestingly, both myri and farn lines still showed significantly enhanced phototropic sensitivity in the same range as the controls (Fig. 6b,d). Hence, these findings suggest that the enhancing effect of red light on phototropism is not dependent on phot1 retention at the membrane. A role for internalisation in the attenuation of receptor signalling also seems highly unlikely in light of these results.

Plasma membrane-localised phot1 is targeted for turnover in response to high intensity blue light

Earlier studies have shown that phot1 is degraded within hours upon transfer of etiolated seedlings to high intensity blue light (Sakamoto & Briggs, 2002; Kong *et al.*, 2006; Sullivan *et al.*, 2010; Roberts *et al.*, 2011). Since phot1 is also internalised under these conditions, it was proposed that re-localisation from the plasma membrane is a prerequisite for receptor turnover (Knieb *et al.*, 2004; Roberts *et al.*, 2011). In order to test this hypothesis we illuminated three-day-

old, etiolated seedlings from each of our transgenic lines with high intensity blue light for four hours, extracted total proteins and performed Western blot analyses with an antibody against phot1. We then quantified the relative amount of phot1 protein in each sample in relation to the internal standard DE-ETIOLATED 3 (DET3) whose protein level is unaltered by blue light (Demarsy *et al.*, 2012). In our control lines, phot1 levels decreased within four hours to 33 – 35 % compared to those measured before irradiation (Fig. 7a,b). Similarly, phot1 levels decreased considerably to 23 – 25 % in both myri and farn lines following high intensity blue light treatment (Fig. 7a,b). Hence, we conclude that release into the cytosol is not necessary for phot1 turnover but rather that membrane-localised receptor molecules are targeted for degradation. In support of this conclusion we found that turnover of a kinase inactive mutant of phot1 (D806N; *phot1-7;* Christie *et al.*, 2002) displayed comparable rates of turnover (Fig. 7c; Sullivan *et al.*, 2010). Given that blue light-dependent autophosphorylation is required for phot1 internalisation (Kaiserli *et al.*, 2009), these findings provide further evidence that plasma membrane-localised phot1 is targeted for turnover.

Discussion

Plasma membrane-localised phot1 mediates all tested blue light responses

Higher plants, including *Arabidopsis*, show a number of specific short-term adaptations in response to blue light irradiation. Most of these responses ultimately serve to optimise photosynthetic performance. A well-known adaptive response to light is phototropism, which allows plants to align their photosynthetic tissues with incoming light. Phototropism in *Arabidopsis* is predominantly mediated by the UV-A/blue light receptor phot1 (reviewed e.g. in (Christie & Murphy, 2013; Hohm *et al.*, 2013; Briggs, 2014; Liscum *et al.*, 2014). This receptor also triggers, amongst others, leaf flattening, petiole positioning and chloroplast movement that also serve to optimise photosynthesis (Christie, 2007). It has long been known that phot1 is a plasma membrane-associated protein, although the mechanism of attachment is still not fully elucidated (Sakamoto & Briggs, 2002; Wan *et al.*, 2008; Kong *et al.*, 2013a). Moreover, phot1 changes its subcellular localisation when cells are irradiated with blue light, leading to a fraction of the receptor being internalised into the cytosol (Sakamoto & Briggs, 2002; Wan *et al.*, 2008; Kaiserli *et al.*, 2009).

In this study, we examined the functional relevance of phot1 subcellular re-localisation by constitutively anchoring the receptor to the plasma membrane (Fig. 1). This was achieved by the addition of a short signal sequence either to the N- or C-terminal regions of phot1 (Fig. 1). We used two different types of modification, myristoylation and farnesylation, reported to play important roles in localising proteins to the plasma membrane (Thompson & Okuyama, 2000; Sorek *et al.*, 2009). These modified phot1 receptors were labelled with a

fluorescent reporter (mCitrine and GFP, respectively) to track subcellular localisation. The lipid-modified versions of phot1 associate with the plasma membrane and are unable to partially re-localise in response to blue light (Figs 1 and S1). Importantly, this absence of phot1 internalisation does not result from defective receptor autophosphorylation (Fig. 2). Our N- and C- terminal-anchoring strategy therefore provided a means to study the functional importance of phot1 subcellular re-localisation.

In animal systems, endocytosis of membrane-associated receptors does not only play a role in signal attenuation but provides an important means to regulate and control signalling. Signal transduction may be crucially influenced by or depend on the subcellular localisation of the involved receptors. Most importantly, endosomal membranes have been identified as sites of signal initiation in multiple cases (Chen, 2009; Sadowski *et al.*, 2009; Hwang *et al.*, 2014). Although plants seem to be ideally suited for similar regulative mechanisms and several findings suggest their existence, evidence for endocytotic control of signalling is still scarce (Irani & Russinova, 2009; Bar & Avni, 2014). The receptor for the fungal elicitor ethylene-inducing xylanase (EIX) in tomato, LeEix2 (Ron & Avni, 2004), showed endosomal signalling to some extent (Sharfman *et al.*, 2011). Likewise, *Arabidopsis* FLS2, a receptor for the bacterial elicitor flagellin (Gómez-Gómez & Boller, 2000) was suggested to partially signal from the endosome (Robatzek *et al.*, 2006; Bar & Avni, 2014).

Tethering phot1 to the membrane did not impact its ability to mediate several responses tested, including phototropism, petiole positioning, leaf flattening and chloroplast accumulation movement (Figs 2-4). Our findings show that phot1 was fully functional despite being artificially tethered to the

membrane. More importantly, these findings indicate that signalling is initiated by membrane-associated phot1 while generation of the internalised, cytosolic protein does not appear to be necessary for function as has been proposed with respect to phototropism (Roberts *et al.*, 2011). We did observe slightly slower phototropic bending in transgenic lines expressing lipid-modified versions of phot1 at very low light intensities (Fig. 3b,d). However, given this small apparent difference was not observed under higher light intensities, it seems unlikely that an absence of receptor internalisation was responsible for this effect. Nonetheless, it would be of interest to examine whether artificial attachment of phot1 to the plasma membrane could attenuate pulse-induced or first positive phototropism (Janoudi & Poff, 1993).

Given that phototropism is dependent on the establishment of a lateral auxin gradient in the bending region (Christie & Murphy, 2013; Hohm *et al.*, 2013) and that auxin transport related proteins implicated in this process (e.g. PINFORMED 1 (PIN1), PIN3, PIN4, PIN7, PINOID (PID) and ATP-BINDING CASSETTE B19 (ABCB19), reviewed e.g. in Sakai & Haga, 2012; Christie & Murphy, 2013; Hohm *et al.*, 2013; Briggs, 2014; Liscum *et al.*, 2014) are located at the plasma membrane, a pivotal role for membrane-localised phot1 in mediating phototropism is consistent with this. The findings that other important components of phot signalling (e.g. NPH3 and PHYTOCHROME KINASE SUBSTRATE 1 (PKS1), reviewed e.g. in Sakai & Haga, 2012; Hohm *et al.*, 2013; Briggs, 2014; Liscum *et al.*, 2014), as well as a direct substrate of phot kinase activity (PKS4, (Demarsy *et al.*, 2012)), are also located at the plasma membrane further supports this conclusion. In light of our data, a role for internalisation in phot1 signalling itself seems highly unlikely although we

519 cannot fully exclude the possibility that cytosolic phot1 plays a role in responses
520 other than those tested here, e.g. stomatal opening that involves the cytosolic
521 protein kinase BLUE LIGHT SIGNALING1 (BLUS1). BLUS1 is a direct
522 phosphorylation target of phot1 and acts as a primary regulator to connect light
523 perception and activation of the H^+-ATPase necessary for the opening of stomata
524 (Takemiya *et al.*, 2013).
525
526 **Internalisation of phot1 does not play a role in receptor desensitisation or**
527 **the modulation of phototropic sensitivity**
528 Most hypotheses regarding the importance of phot1 internalisation somehow
529 centre on receptor desensitisation and an attenuation of receptor signalling by
530 removing it from its site of action, the plasma membrane. The observation that
531 red light pre-treatment retains phot1 at the membrane and enhances
532 phototropism provided strong support for a role of receptor internalisation in
533 attenuating the response (Han *et al.*, 2008). Consistent with this idea, oryzalin
534 treatment mediates phot1 internalisation and impairs phototropism (Sullivan *et*
535 *al.*, 2010). However, as shown here, membrane-anchoring phot1 has no major
536 impact on phototropic responsiveness in etiolated or de-etiolated seedlings (Figs
537 3 and S2). These findings suggest little, if any role for phot1 internalisation in
538 modulating phototropic sensitivity. Moreover, red light pre-treatment still
539 enhanced phototropic sensitivity in transgenic lines expressing lipid-modified
540 versions of phot1 (Fig. 6), indicating that this phenomenon is not driven by
541 retention of phot1 at the plasma membrane. Lindeboom *et al*. (2013) have
542 recently shown that phot1-stimulated microtubule reorientation through
543 katanin is required for hypocotyl curvature. Hence, phototropic impairment

following oryzalin treatment (Sullivan *et al.*, 2010) likely results from microtubule depolymerisation (Hugdahl & Morejohn, 1993) rather than an increase in phot1 internalisation.

Red light enhancement of hypocotyl phototropism is phyA-dependent (reviewed e.g. in Briggs, 2014). Our results are consistent with the view that enhancement is mediated by gene expression changes involving nuclear phyA, rather than by direct or indirect interaction of cytosolic phyA with phot1. Kami *et al*. (2012) reported that phyA was still able to enhance phototropism when localised in the nucleus but not in the cytosol. Indeed, nuclear phyA activates the expression of genes that are involved in the regulation of phototropism (e.g. *PKS1* and *ROOT PHOTOTROPISM 2* (*RPT2*); Kami *et al.*, 2012, 2014; Zhao *et al.*, 2013; Haga *et al.*, 2014). Despite these findings, a minor role for cytosolic phyA in the process cannot be excluded (Jaedicke *et al.*, 2012; Hughes, 2013).

Since etiolated seedlings combined with low intensity blue light were used for our phototropic analyses, the ratio of internalised to membrane-bound phot1 would be expected to be low during these experiments (Wan *et al.*, 2008). The phototropic response of de-etiolated seedlings was also examined to assess any effects of potentially higher ratios of internalised phot1 (Fig. S2). However, no difference in phototropic responsiveness was observed under these conditions between controls and lines expressing membrane-anchored phot1 (Fig. S2). In addition, our analysis of leaf flattening, petiole positioning and chloroplast movement provided no evidence for an enhanced phot1 activity in lines where phot1 was constitutively plasma membrane-associated (Figs 3 and 4). We therefore conclude from our membrane-anchoring studies that it is

unlikely for phot1 internalisation to play a major role in receptor desensitisation as well as signalling.

Plasma membrane-bound phot1 is turned over in response to high intensity blue light

Roberts *et al*. (2011) recently proposed a model in which NPH3, a phot1-interacting protein, which is essential for phototropism (Inada *et al.*, 2004; Lariguet *et al.*, 2006), acts as an adaptor in a CULLIN3-based E3 ubiquitin ligase ($CRL3^{NPH3}$) that mediates ubiquitination of phot1. Phot1 is mono/multi-ubiquitinated in response to low intensity blue light, a process that is proposed to stimulate receptor internalisation, which is required to mediate phototropism. Under higher intensities, phot1 is additionally poly-ubiquitinated, a modification that is linked to targeted degradation by the 26S proteasome, thereby representing a means of receptor desensitisation (Roberts *et al.*, 2011).

Blue light-dependent phot1 ubiquitination at Lys526 has been confirmed recently (Deng *et al.*, 2014). Knieb *et al*. (2004) also showed in mustard seedlings, that approximately 20 % of phot1 is internalised after high intensity blue light illumination. Interestingly, only the amount of this soluble phot1 fraction diminished over time while the membrane-bound fraction did not. This led the authors to speculate that internalised, soluble phot1 is targeted for degradation. By contrast, the membrane-attached phot1 is somehow protected from turnover (Knieb *et al.*, 2004).

The above conclusions therefore imply that phot1 internalisation is a prerequisite for its turnover. Intrinsic membrane-localised proteins are most commonly degraded through endocytosis (Murphy *et al.*, 2005; Luschnig & Vert,

2014). Phot1 internalisation has been shown to depend on clathrin-mediated endocytic recruitment that is triggered by receptor autophosphorylation (Kaiserli *et al.*, 2009). However, connecting phot1 internalisation with its degradation contrasts with the finding that the kinase-inactive mutant *phot1-7* is no longer internalised (Kaiserli *et al.*, 2009), but is still degraded upon exposure to high intensity blue light (Sullivan *et al.*, 2010). In accordance with the latter, we show that membrane anchoring does not affect the rate of phot1 turnover, as in *phot1-7* (Fig. 7). Thus, phot1 degradation can occur independently of internalisation. Hence, there must be another mechanism that mediates turnover of plasma membrane associated phot1 in response to light. It is conceivable that light-induced conformational changes of phot1 expose phosphorylation sites for other kinases that could initiate the degradation process, as has been shown for cryptochrome 2 (Shalitin *et al.*, 2002; Zuo *et al.*, 2012).

In conclusion, this study provides evidence that plasma membrane-bound phot1 represents the active form of the receptor for all tested responses and internalisation is not essential for signalling. We also show that phot1 re-localisation is unlikely to represent an effective desensitisation mechanism as none of the tested responses were positively affected by constitutive membrane-association of phot1. Likewise, retention of phot1 at the plasma membrane does not appear to be the mechanism underlying the phyA-mediated red light enhancement of phototropism. Finally, we can convincingly show that phot1 degradation does not require release of the photoreceptor into the cytosol and occurs independently of autophosphorylation. The role of phot1 ubiquitination by CRL^{NPH3} in this context remains elusive, as does the mechanism that

617 eventually triggers phot1 for degradation. These aspects continue to be
618 fascinating topics for future research.
619

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Figure Legends

Figure 1. Myristoylated or farnesylated phot1 is no longer internalised upon blue light irradiation

(**a**) and (**b**) Localization of phot1 fusion protein in cortex cells of the hypocotyl elongation zone of three-day-old, etiolated seedlings. Fluorescence was detected with a 2-photon microscope. White arrows indicate phot1-mCitrine (**a**) and phot1-GFP (**b**) signal in the cytoplasm of control seedlings. (Note that there is already some cytosolic signal in the dark image of pPHOT1::*PHOT1*-GFPfarn$^{C\text{-}A}$ which is probably due to very fast internalisation during sample setup under transmitted light.) Schematic representations of the constructs used to make transgenic *Arabidopsis* plants expressing modified phot1 proteins are depicted below the images for (**a**) and (**b**).

BL, blue light; CDS, coding sequence; pPHOT1, endogenous *PHOT1* promoter

Figure 2. Lipid-modified phot1 is autophosphorylated and mediates NPH3-dephosphorylation

Three-day-old, etiolated seedlings grown on plates with half-strength MS agar medium were irradiated with blue light from the top at a fluence rate of 15 µmol m^{-2} s^{-1}. Samples were harvested at the indicated times after the start of irradiation. NPH3 and phot1 proteins were detected by Western blotting using anti-NPH3 and anti-phot1 antibodies, respectively. Note the reduction in electrophoretic mobility of phot1 after 10 and 120 minutes of blue light irradiation in all lines except *phot1-7*. (The shift is less visible in the case of pPHOT1 due to erratic running of the gel.)

916 phos., phosphorylated isoform of NPH3; dephos., dephosphorylated isoform of
917 NPH3; tagged, phot1 fusion protein with GFP- or mCitrine tag; WT, size of wild
918 type phot1; BL, blue light
919
920 **Figure 3. Plasma membrane-attached phot1 rescues phototropic bending**
921 **in a *phot1phot2* double mutant background**
922 Three-day-old, etiolated seedlings grown on vertical plates with half-strength MS
923 agar medium were irradiated with unilateral blue light at the described fluence
924 rates. Curvature was determined at the indicated times after the start of
925 irradiation. Bending was compared to the respective control lines (described in
926 the text; black triangles).
927 (**a**) and (**b**) Phototropic bending of transgenic seedlings expressing
928 myristoylated phot1-mCitrine (myri; red circles) at 1 and 0.1 µmol m^{-2} s^{-1},
929 respectively.
930 (**c**) and (**d**) Phototropic bending of transgenic seedlings expressing farnesylated
931 phot1-GFP (farn; green circles) at 1 and 0.1 µmol m^{-2} s^{-1}, respectively.
932 Data shown are means of two (**b**) or three (**a**) independent lines +/- two
933 standard errors. Asterisks indicate significant differences between lipidated lines
934 and controls (Student's t-test; ** $p < 0.01$; * $p < 0.05$).
935
936 **Figure 4. Petiole positioning and leaf flattening are rescued in**
937 **myristoylated or farnesylated phot1 transgenic lines**
938 (**a**) Petiole positioning of representative myristoylated (myri) or farnesylated
939 (farn) phot1 seedlings grown on soil for one week under 70 µmol m^{-2} s^{-1} white
940 light 16/8 hour light-dark cycle then transferred to 10 µmol m^{-2} s^{-1} 16/8 hour

941 light-dark cycle for a further week. Mutated farnesyl (mut farn) represents a
942 control for farn whilst pPHOT1 is a control for myri.
943 (**b**) Quantification of petiole angles from horizontal. Data shown are means of
944 three independent lines for each genotype. Error bars indicate standard error (n
945 > 10).
946 (**c**) The leaf flattening index of the leaves shown in (**a**). The leaf flattening index
947 is expressed as the ratio before and after artificial flattening. Data shown are
948 means of or three independent lines for each genotype. Error bars indicate
949 standard error (n = 10).
950
951 **Figure 5. Chloroplast accumulation movement is rescued in lipid-modified**
952 **phot1 lines**
953 (**a**) Myristoylated (myri) or farnesylated (farn) phot1 plants were grown on soil
954 for three weeks under 70 μmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light.
955 Rosette leaves were detached and treated with low intensity blue light (1.5 μmol
956 m^{-2} s^{-1}) for three hours or kept in the dark for three hours before observation of
957 chloroplast autofluorescence by confocal microscopy. Scale bar represents 20
958 μm. Mutated farnesyl (mut farn) represents a control for farn whilst pPHOT1 is a
959 control for myri.
960 (**b**) Quantification of the number of chloroplasts at the upper face of palisade
961 mesophyll cells of the plants described in (**a**). Data shown are means of three
962 independent lines for each genotype. Error bars indicate standard error of > 12
963 cells per transgenic line.
964

Figure 6. Enhancement of phototropism by red light is independent of the subcellular localisation of phot1

Seedlings were treated as described in Figure 3. Prior to exposure to lateral blue light (0.1 µmol m^{-2} s^{-1}), they were given a red-light pulse (1800 µmol m^{-2}) and incubated in darkness for one hour. Curvature was determined at the indicated times after the start of blue light irradiation. Bending was compared to control lines (described in the text; black triangles).

(**a**) and (**b**) Phototropic bending of transgenic seedlings expressing myristoylated phot1-mCitrine (myri; red circles).

(**c**) and (**d**) Phototropic bending of transgenic seedlings expressing farnesylated phot1-GFP (farn; green circles).

Data shown are means +/- two standard errors of 166 to 230 seedlings of three biological replicates. Asterisks indicate significant differences between red light pre-treated (solid lines) and control seedlings (dashed lines) (Student's t-test; $p < 0.005$).

Figure 7. Blue light induced receptor turnover targets membrane-localised phot1

Three-day-old, etiolated seedlings grown on plates with half-strength MS agar medium were irradiated with blue light from above at a fluence rate of 120 µmol m^{-2} s^{-1}. Samples were harvested at the indicated times after the start of irradiation. phot1 protein was detected by Western blotting using an anti-phot1 antibody. Expression of DET3 was used as an internal control and lane intensities were quantified using the Image Quant LAS 4000 software.

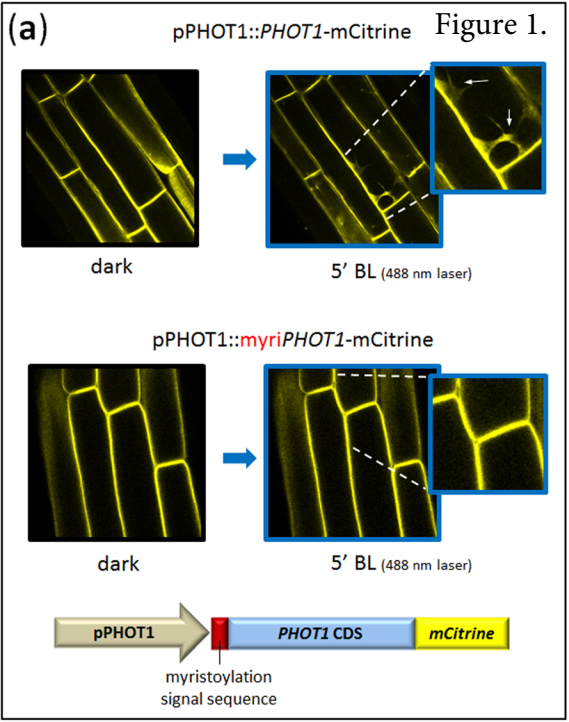
(**a**) Relative amount of myristoylated phot1-mCitrine (myri; red bars) as compared to the control line (pPHOT1; dark grey bars).

(**b**) Relative amount of farnesylated phot1-GFP (farn; green bars) as compared to the control line (mutated farn; light grey bars).

(**c**) Relative amount of kinase-inactivated phot1 (*phot1-7*; light grey bars) as compared to the wild type (Col-0; dark grey bars).

Data shown are means +/- two standard errors of at least two biological replicates with at least two technical replicates each.

Figure 1.


(a)
pPHOT1::PHOT1-mCitrine
dark
5′ BL (488 nm laser)
pPHOT1::myriPHOT1-mCitrine
dark
5′ BL (488 nm laser)
pPHOT1
PHOT1 CDS
mCitrine
myristoylation signal sequence

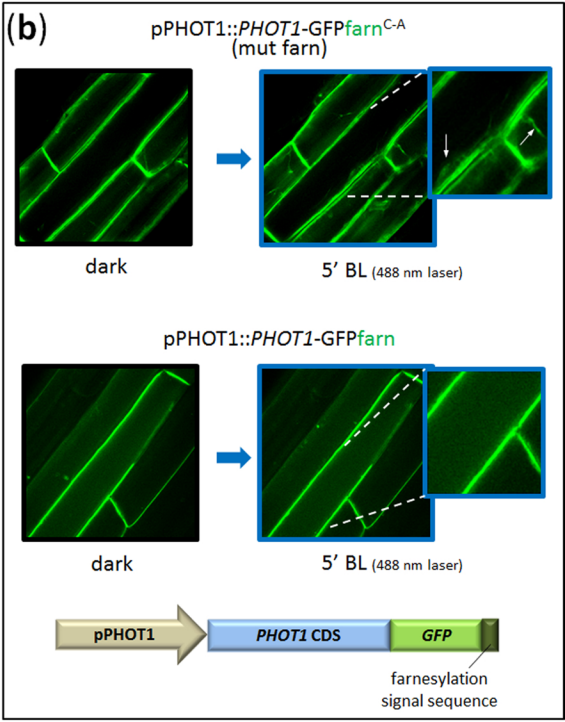

(b)
pPHOT1::PHOT1-GFPfarnC-A
(mut farn)
dark
5′ BL (488 nm laser)
pPHOT1::PHOT1-GFPfarn
dark
5′ BL (488 nm laser)
pPHOT1
PHOT1 CDS
GFP
farnesylation signal sequence

Figure 2

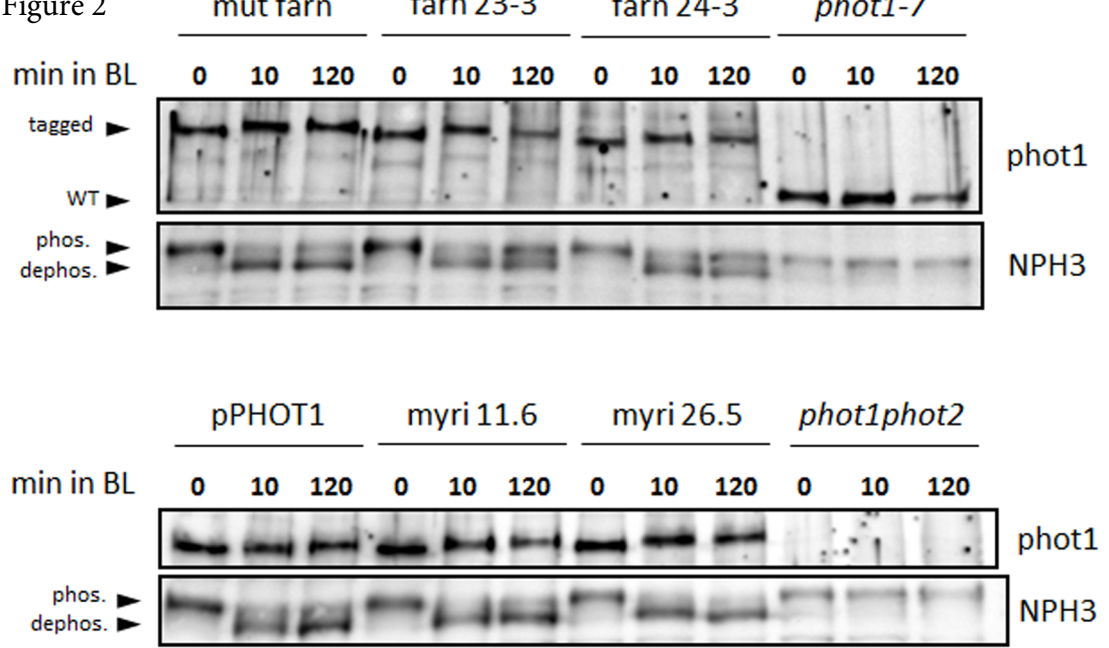
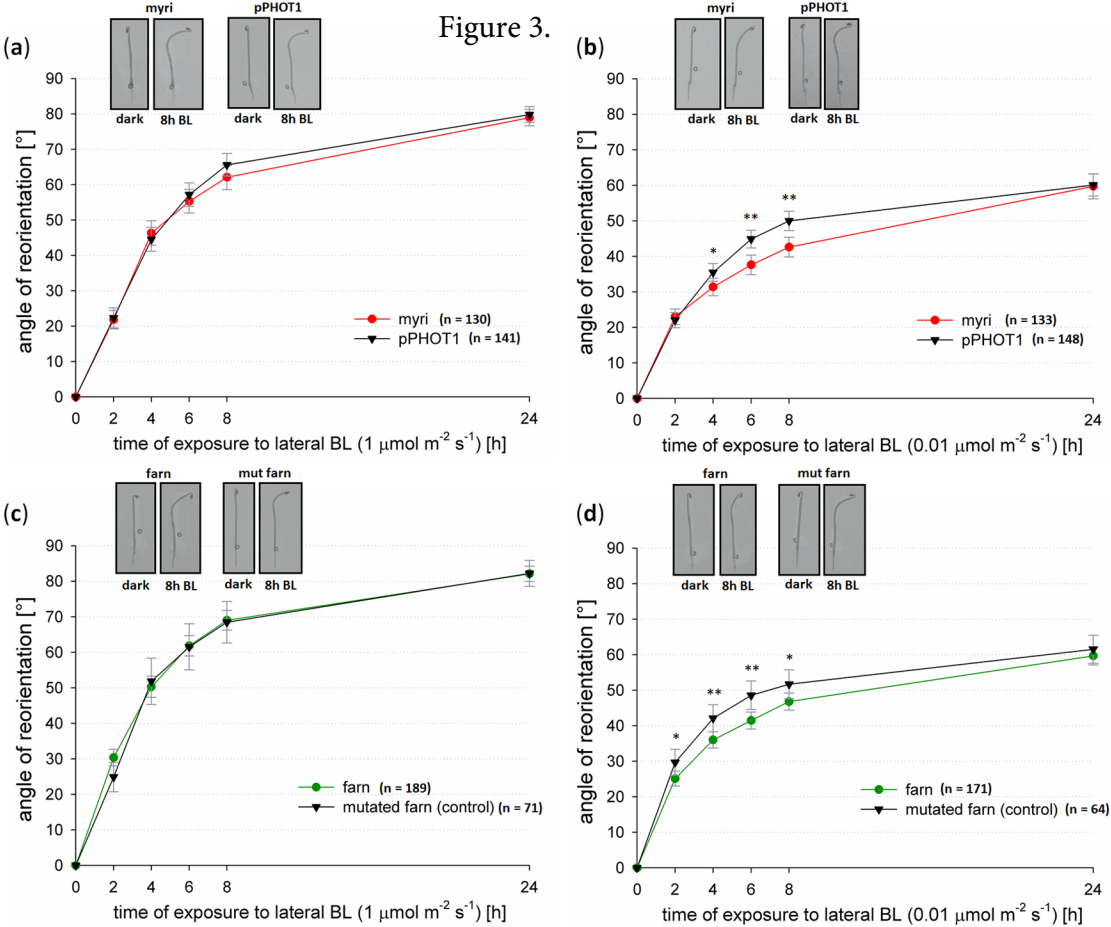

mut farn
farn 23-3
farn 24-3
phot1-7
min in BL
0 10 120 0 10 120 0 10 120 0 10 120
tagged
WT
phos.
dephos.
phot1
NPH3
pPHOT1
myri 11.6
myri 26.5
phot1phot2
min in BL
0 10 120 0 10 120 0 10 120 0 10 120
phos.
dephos.
phot1
NPH3

Figure 3.


(a)
myri
pPHOT1
dark
8h BL
dark
8h BL
angle of reorientation [°]
time of exposure to lateral BL (1 μmol m-2 s-1) [h]
myri (n = 130)
pPHOT1 (n = 141)
(b)
myri
pPHOT1
dark
8h BL
dark
8h BL
angle of reorientation [°]
time of exposure to lateral BL (0.01 μmol m-2 s-1) [h]
myri (n = 133)
pPHOT1 (n = 148)
(c)
farn
mut farn
dark
8h BL
dark
8h BL
angle of reorientation [°]
time of exposure to lateral BL (1 μmol m-2 s-1) [h]
farn (n = 189)
mutated farn (control) (n = 71)
(d)
farn
mut farn
dark
8h BL
dark
8h BL
angle of reorientation [°]
time of exposure to lateral BL (0.01 μmol m-2 s-1) [h]
farn (n = 171)
mutated farn (control) (n = 64)

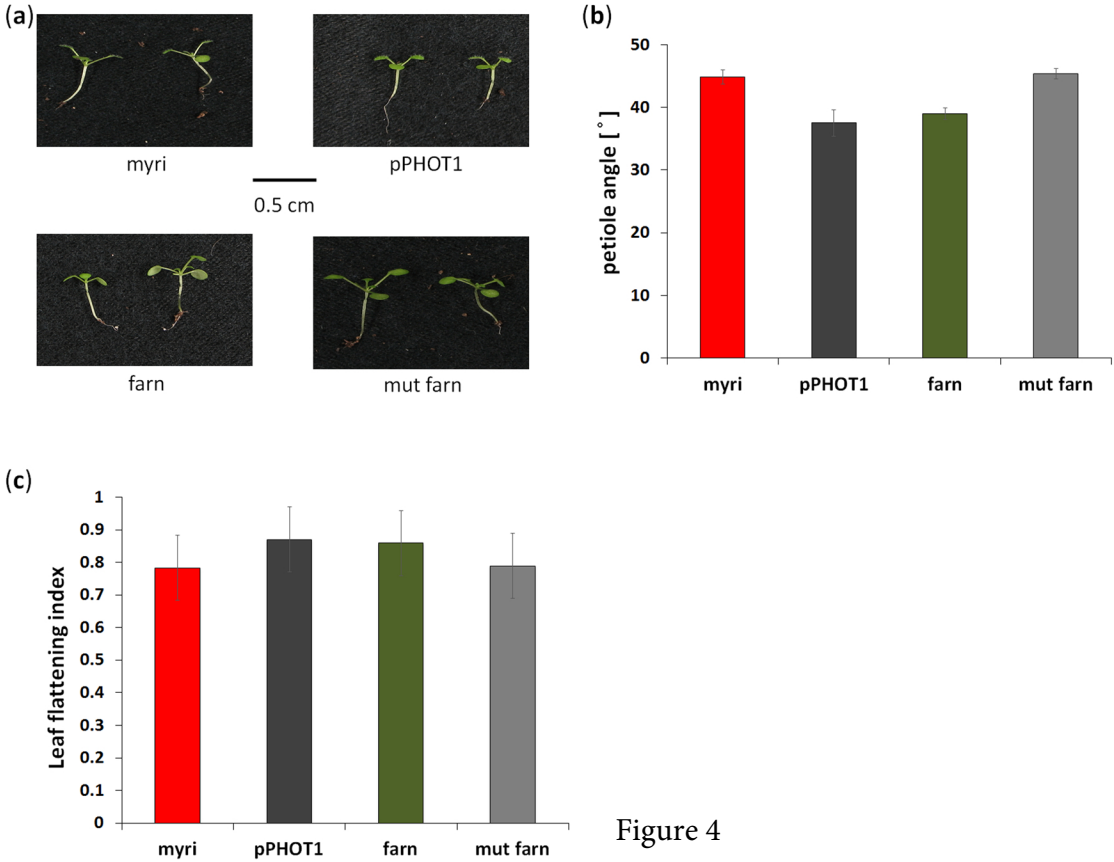

(a)
myri
pPHOT1
0.5 cm
farn
mut farn
(b)
petiole angle [°]
50
40
30
20
10
0
myri
pPHOT1
farn
mut farn
(c)
Leaf flattening index
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
myri
pPHOT1
farn
mut farn

Figure 4

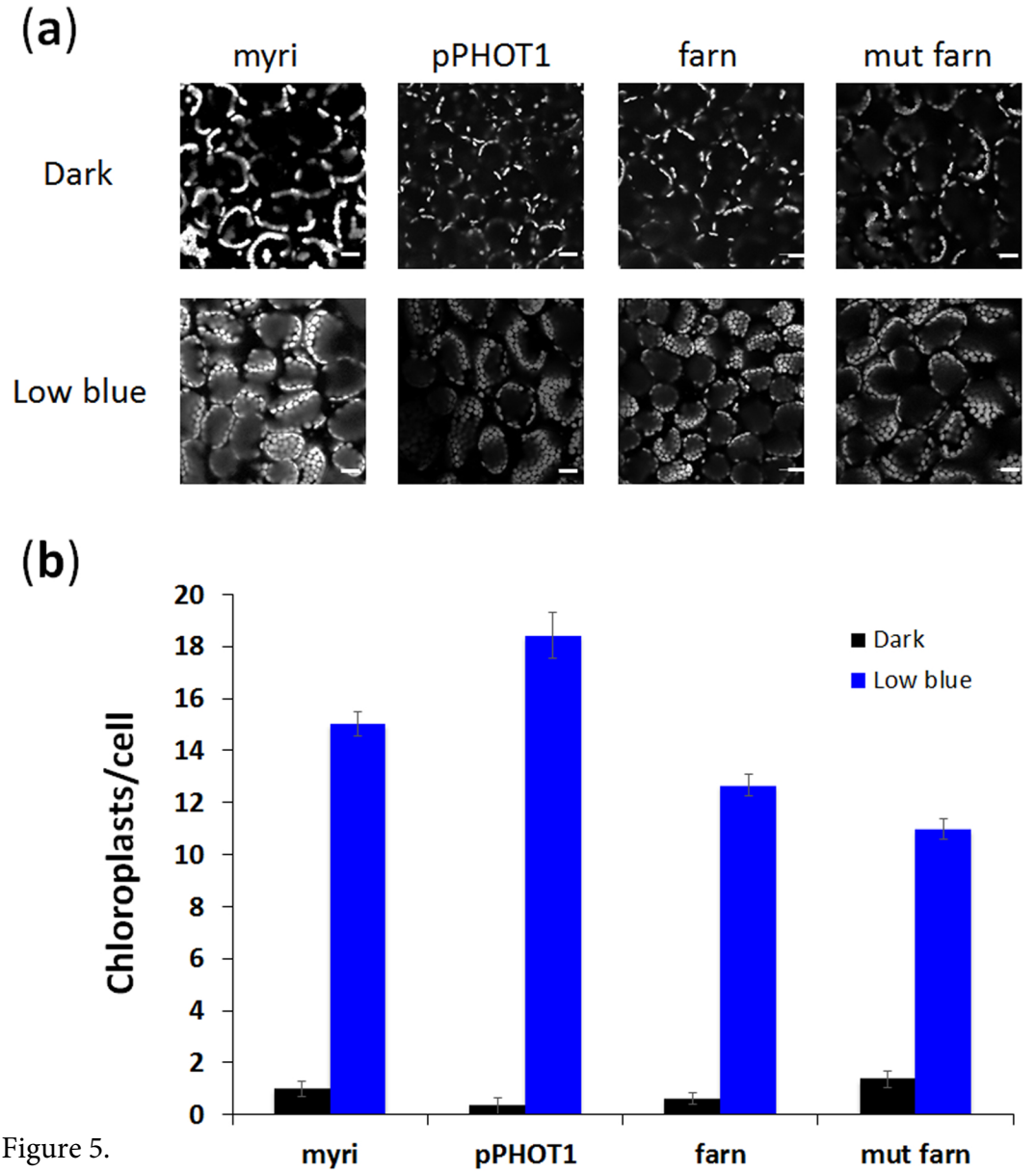

(a)
myri
pPHOT1
farn
mut farn
Dark
Low blue
(b)
Chloroplasts/cell
20
18
16
14
12
10
8
6
4
2
0
Dark
Low blue
myri
pPHOT1
farn
mut farn

Figure 5.

Figure 6.

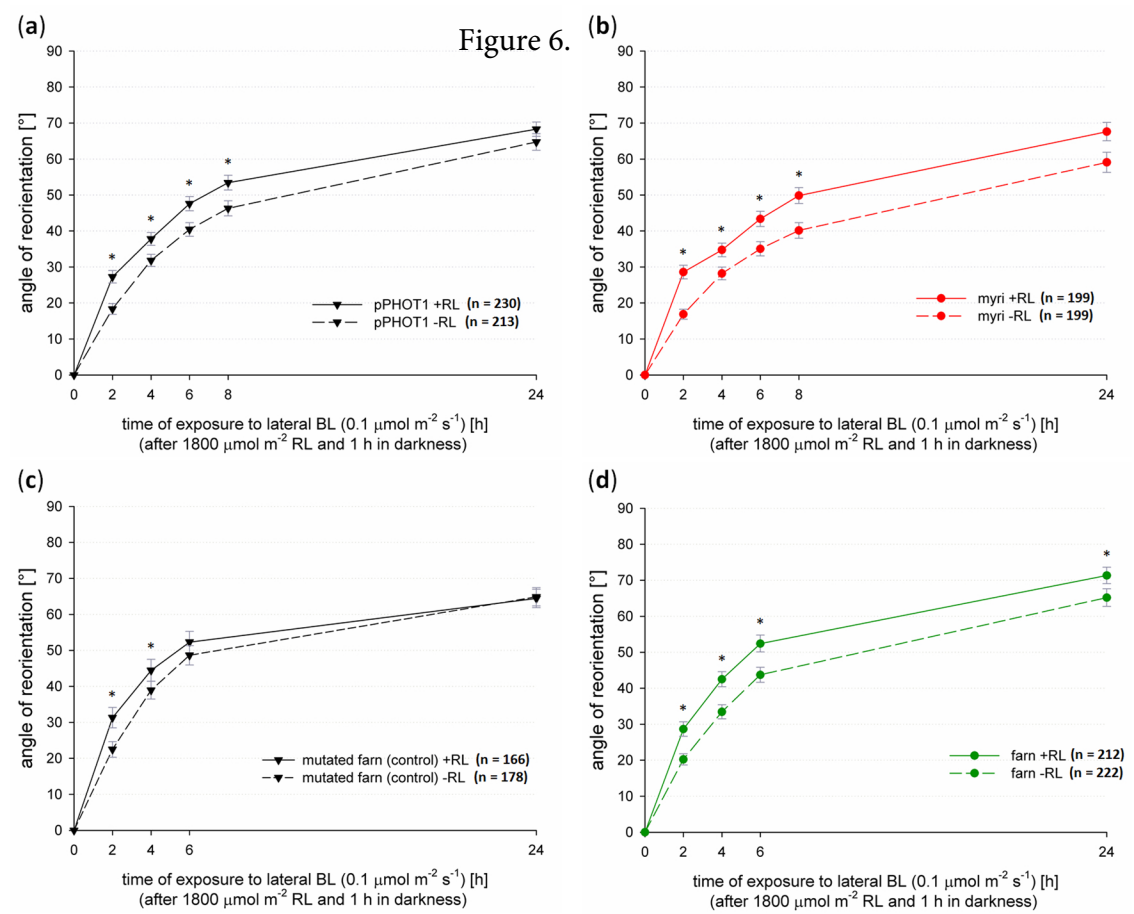
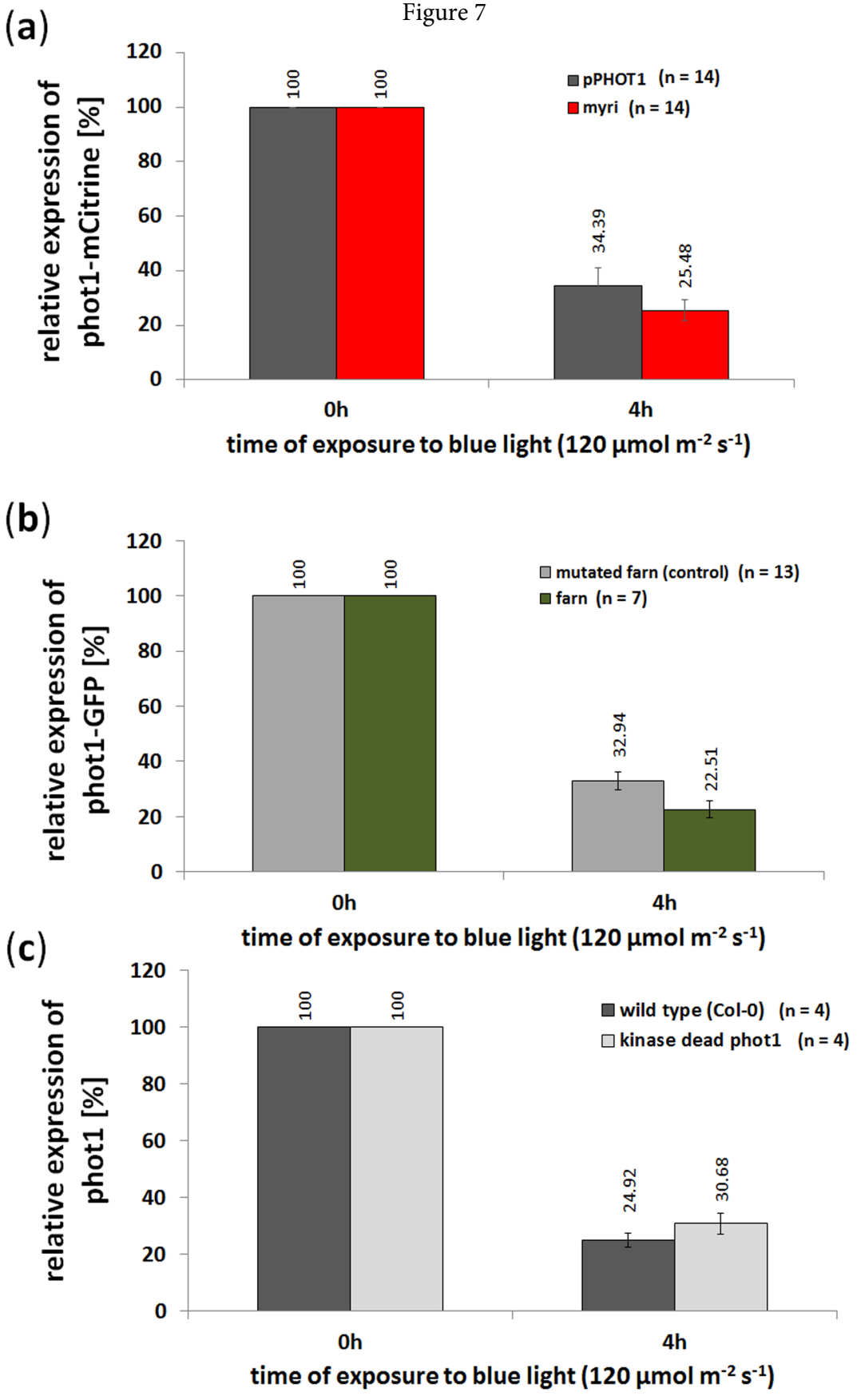

(a)
angle of reorientation [°]
pPHOT1 +RL (n = 230)
pPHOT1 -RL (n = 213)
time of exposure to lateral BL (0.1 μmol m-2 s-1) [h]
(after 1800 μmol m-2 RL and 1 h in darkness)
(b)
angle of reorientation [°]
myri +RL (n = 199)
myri -RL (n = 199)
time of exposure to lateral BL (0.1 μmol m-2 s-1) [h]
(after 1800 μmol m-2 RL and 1 h in darkness)
(c)
angle of reorientation [°]
mutated farn (control) +RL (n = 166)
mutated farn (control) -RL (n = 178)
time of exposure to lateral BL (0.1 μmol m-2 s-1) [h]
(after 1800 μmol m-2 RL and 1 h in darkness)
(d)
angle of reorientation [°]
farn +RL (n = 212)
farn -RL (n = 222)
time of exposure to lateral BL (0.1 μmol m-2 s-1) [h]
(after 1800 μmol m-2 RL and 1 h in darkness)

Figure 7


(a)
relative expression of phot1-mCitrine [%]
120
100
80
60
40
20
0
100
100
34.39
25.48
pPHOT1 (n = 14)
myri (n = 14)
0h
4h
time of exposure to blue light (120 µmol m-2 s-1)
(b)
relative expression of phot1-GFP [%]
120
100
80
60
40
20
0
100
100
32.94
22.51
mutated farn (control) (n = 13)
farn (n = 7)
0h
4h
time of exposure to blue light (120 µmol m-2 s-1)
(c)
relative expression of phot1 [%]
120
100
80
60
40
20
0
100
100
24.92
30.68
wild type (Col-0) (n = 4)
kinase dead phot1 (n = 4)
0h
4h
time of exposure to blue light (120 µmol m-2 s-1)

***New Phytologist* Supporting Information**

Article title: Should I stay or should I go? - Lipid anchoring of *Arabidopsis* phototropin 1 to assess the functional significance of receptor internalisation.
Authors: Tobias Preuten, Lisa Blackwood, John M. Christie, Christian Fankhauser
Article acceptance date: 29T

The following Supporting Information is available for this article:

Fig. S1. Lipid modified phot1 fusion protein is not internalised upon prolonged exposure to blue light

(**a**) and (**b**) Localization of phot1 fusion protein in the same cortex cells of the hypocotyl elongation zone of three-day-old, etiolated seedlings as shown in Figure 1 of the main article. Fluorescence was detected with a 2-photon microscope.
BL, blue light

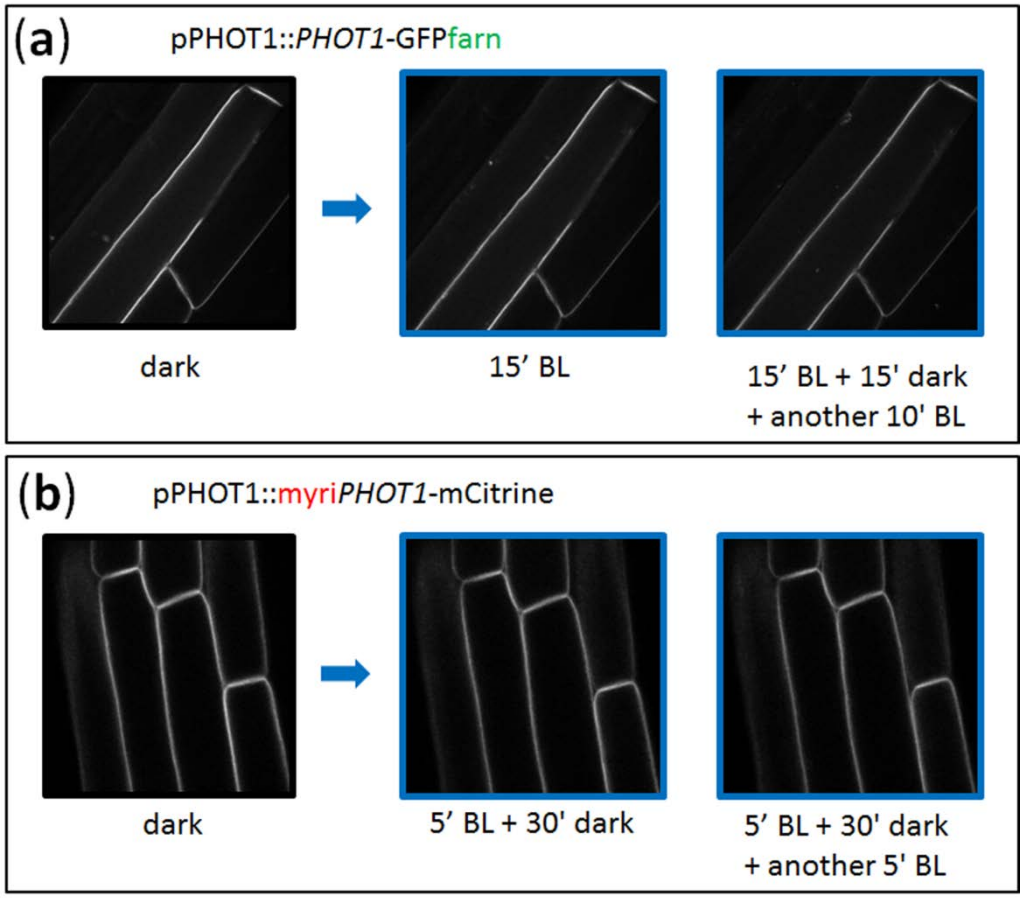


Fig. S2. De-etiolated seedlings expressing plasma membrane-anchored phot1 do not show altered phototropism

Three-day-old, light-grown seedlings grown on vertical plates with half-strength MS agar medium were irradiated with unilateral blue light at a fluence rate of 1 µmol m^{-2} s^{-1}. Curvature was determined at the indicated times after the start of irradiation. Bending was compared to control lines (described in the text; black triangles).
(**a**) Phototropic bending of transgenic seedlings expressing myristoylated phot1-mCitrine (myri; red circles).
(**b**) Phototropic bending of transgenic seedlings expressing farnesylated phot1-GFP (farn; green circles).
Data shown are means +/- two standard errors of 62 to 90 seedlings.

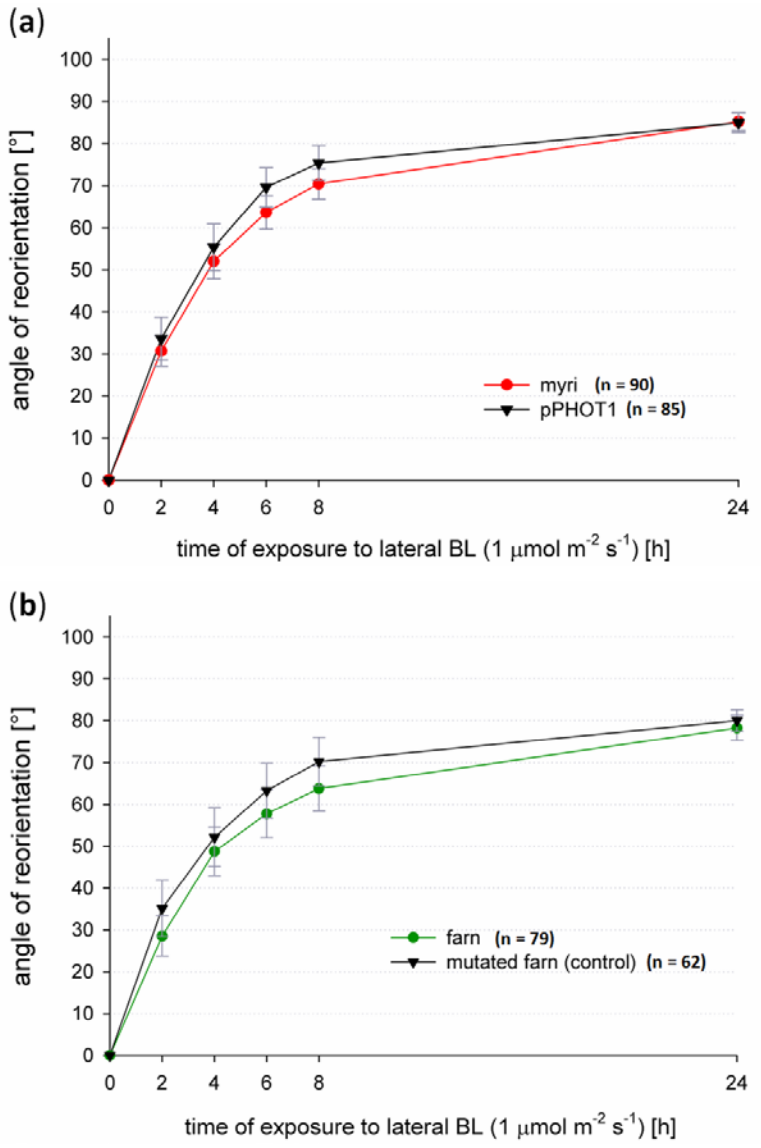


Fig. S3. Comparison of petiole positioning and leaf flattening measurements in wild type and control lines

(**a**) Petiole positioning of representative wild type, *phot1-5phot2-1* and control seedlings grown on soil for one week under 70 µmol m^{-2} s^{-1} white light 16/8 hour light-dark cycle then transferred to 10 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle for a further week. Mutated farnesyl (mut farn) represents a control for farn whilst pPHOT1 is a control for myri.
(**b**) Quantification of petiole angles from horizontal. Data shown are means of three independent lines for each genotype. Error bars indicate standard error (n > 10).
(**c**) The leaf flattening index of the leaves shown in (**a**). The leaf flattening index is expressed as the ratio before and after artificial flattening. Data shown are means of three independent lines for each genotype. Error bars indicate standard error (n = 10).

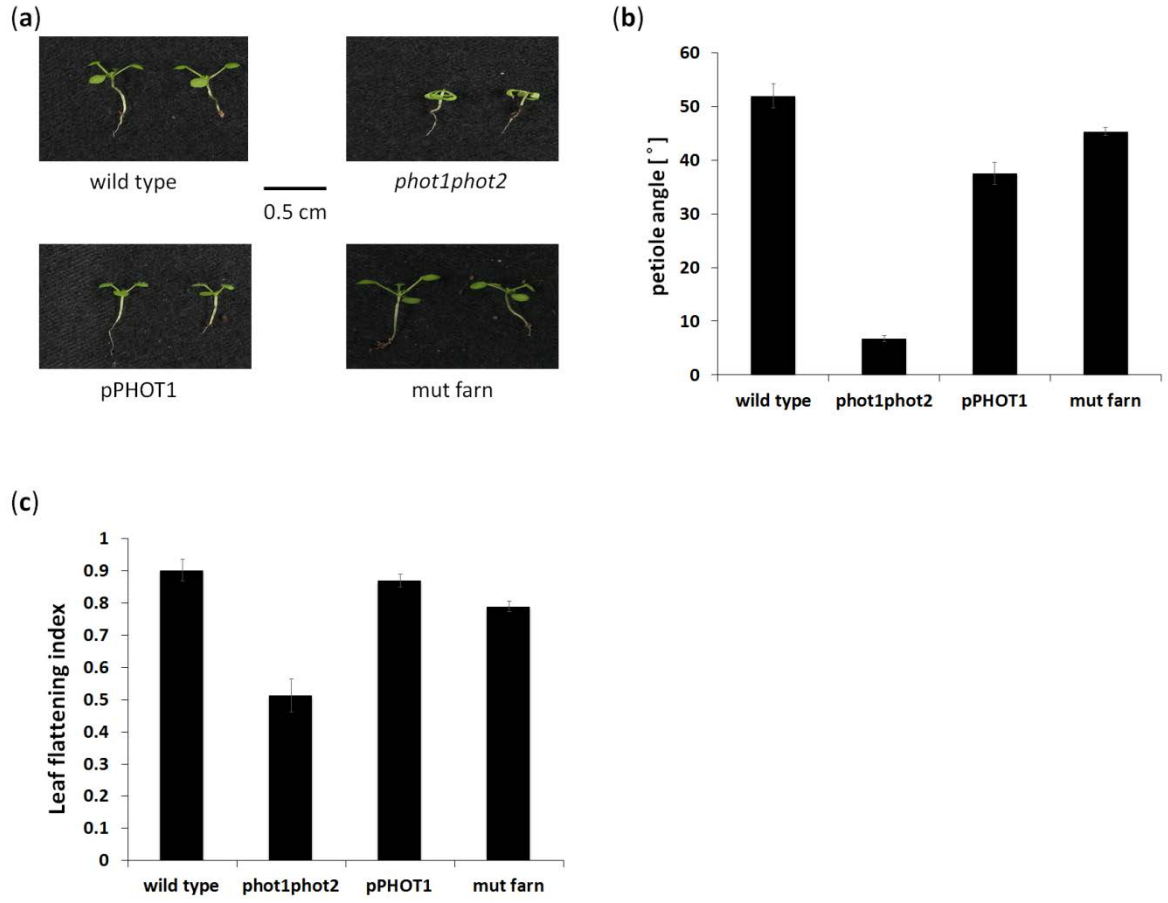


Fig. S4. Comparison of chloroplast movement in wild type and control lines

(**a**) Wild type, *phot1-5phot2-1* and control plants were grown on soil for three weeks under 70 µmol m^{-2} s^{-1} 16/8 hour light-dark cycle white light. Rosette leaves were detached and treated with low intensity blue light (1.5 µmol m^{-2} s^{-1}) for three hours or kept in the dark for three hours before observation of chloroplast autofluorescence by confocal microscopy. Scale bar represents 20 µm. Mutated farnesyl (mut farn) represents a control for farn whilst pPHOT1 is a control for myri.
(**b**) Quantification of the number of chloroplasts at the upper face of palisade mesophyll cells of the plants described in (**a**). Data shown are means of three independent lines for each genotype. Error bars indicate standard error of > 12 cells per transgenic line.