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Interaction specificity of *Arabidopsis* 14-3-3 proteins with phototropin receptor kinases

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ABSTRACT

Phototropin receptor kinases play an important role in optimising plant growth in response to blue light. Much is known regarding their photochemical reactivity, yet little progress has been made to identify downstream signalling components. Here, we isolated several interacting proteins for *Arabidopsis* phototropin 1 (phot1) by yeast two-hybrid screening. These include members of the NPH3/RPT2 (NRL) protein family, proteins associated with vesicle trafficking, and the 14-3-3 lambda (λ) isoform from *Arabidopsis*. 14-3-3λ and phot1 were found to colocalise and interact in vivo. Moreover, 14-3-3 binding to phot1 was limited to non-epsilon 14-3-3 isoforms and was dependent on key sites of receptor autophosphorylation. No 14-3-3 binding was detected for *Arabidopsis* phot2, suggesting that 14-3-3 proteins are specific to phot1 signalling.

Structured summary:
MINT-7146953: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0915) with *ARF7* (uniprotkb:Q9LFJ7) by *two hybrid* (MI:0018)
MINT-7147335: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0914) with *14-3-3 phi* (uniprotkb:P46077) by *far Western blotting* (MI:0047)
MINT-7146854: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0915) with RPT2 (uniprotkb:Q682S0) by *two hybrid* (MI:0018)
MINT-7147215: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0914) with *14-3-3 lambda* (uniprotkb:P48349) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7147044, MINT-7147185, MINT-7147200, MINT-7147413: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0914) with *14-3-3 lambda* (uniprotkb:P48349) by *far Western blotting* (MI:0047)
MINT-7146983: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0915) with *14-3-3 lambda* (uniprotkb:P48349) by *two hybrid* (MI:0018)
MINT-7146871: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0915) with *NPH3-like* (uniprotkb:Q9S9Q9) by *two hybrid* (MI:0018)
MINT-7146905: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0915) with *ARF2* (uniprotkb:Q9M1P5) by *two hybrid* (MI:0018)
MINT-7147364: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0914) with *14-3-3 upsilon* (uniprotkb:P42645) by *far Western blotting* (MI:0047)
MINT-7147234: *PHOT1* (uniprotkb:O48963) *physically interacts* (MI:0914) with *14-3-3 kappa* (uniprotkb:P48348) by *far Western blotting* (MI:0047)

1. Introduction

Phototropins (phot1 and phot2) are plasma membrane-associated receptor kinases that respond specifically to UV/blue wavelengths and regulate processes that serve to optimise photosynthetic efficiency and promote plant growth [1]. These include phototropism, stomatal opening [2], chloroplast relocation movement [3], as well as leaf expansion [4] and positioning [5].

Phototropins comprise an N-terminal photosensory region coupled to a C-terminal serine/threonine kinase domain that belongs to the AGC family of protein kinases [1]. UV/blue light is detected by a repeated motif within the N-terminal region known as LOV1 and LOV2 [6]. The LOV domains associated with plant phototropins function as blue light sensors by binding the chromophore flavin

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mononucleotide (FMN) [7]. Phototropin activity is regulated predominantly by LOV2 [8,9], whereas LOV1 appears to mediate receptor dimerisation [10] and modulate receptor activity [11]. Consequently, kinase activation by LOV2 results in receptor autophosphorylation [12,13], a prerequisite for phototropin signalling [12].

While much effort has focussed on elucidating the primary mechanisms underlying phototropin receptor activation by light, the downstream signalling processes remain largely elusive. Several phototropin-interacting proteins have been identified. Phot1 from *Vicia faba* and *Arabidopsis thaliana* exhibit 14-3-3 binding [12,14]. However, the functional significance of this interaction is still unknown. Recent proteomic analysis has shown that interacting targets for 14-3-3 family members in barley [15] include NPH3, a phot1-interacting protein that is essential for phototropism [16], as well as PIN1, an auxin efflux carrier reported to play a role in the development of phototropic curvatures [17]. RPT2 and PKS1 represent additional signalling components that interact directly with phot1. RPT2 is closely related to NPH3 and mediates both phototropism and stomatal opening [18], whereas PKS1 has been shown to influence phototropic curvature [19].

Here, we adopted a yeast two-hybrid approach to identify signalling components that interact with phot1 from *Arabidopsis*. Among the candidates isolated was 14-3-3 lambda (λ). Detailed biochemical analysis revealed that 14-3-3 binding is specific to members of the non-epsilon group of *Arabidopsis* 14-3-3 proteins and is limited to phot1, suggesting that 14-3-3 binding is unique to phot1 signalling.

2. Materials and methods

2.1. Plant material

Wild-type (*gl-1*, ecotype Columbia) and the *phot1-5 phot2-1* mutant have been described previously [3]. *Arabidopsis* expressing phot1-GFP [20], phot2-GFP [21] and GFP-Lti6b [22] are as described. The *14-3-3λ* mutant was obtained from the SALK T-DNA mutant collection (SALK_075219). For the detection of transcripts total RNA was extracted from leaf tissue by the RNeasy plant mini kit (Qiagen) according to the manufacture's instructions. cDNA synthesis and RT-PCR were performed as described [23]. Primers used to amplify *14-3-3λ* transcripts were 5′-AGGCGCTACTCCAGCGGA-3′ and 5′-CAAAGGTTATGGGGATTTTGAGA-3′. Seeds were surface sterilised and planted on filter paper on half-strength Murashige and Skoog medium with 0.8% agar (w/v). Red and blue light was provided as described [24]. Plants were grown on soil in a controlled environment room (Fitotron, Weiss-Gallenkamp, Loughborough, UK) under 16/8 h 22/18 °C light-dark cycle (70 μmol m^{-2} s^{-1}).

2.2. Yeast two-hybrid screening

The CLONTECH Matchmaker GAL4 Two-Hybrid System 3 was used for yeast two-hybrid analysis. *Arabidopsis PHOT1* was cloned into the pGBK-T7 bait vector and used to screen a cDNA library derived from 3-day-old etiolated *Arabidopsis* seedlings (*Arabidopsis* Biological Resource Centre, #CD4-22). Yeast strain AH109 was cotransformed with bait and prey plasmids and selected on synthetic dropout medium (SD) lacking leucine (Leu) and tryptophan (Trp). Contransformants were assayed for interaction on SD medium lacking adenine, histidine, leucine, tryptophan, and containing 5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside (X-α-gal). Yeast transformation, α-galactosidase assays and plasmid rescue were carried out in accordance with the manufacturer's instructions. Interaction tests with *14-3-3λ* cDNA in the pACT2 prey vector were performed with pGBK-T7 constructs encoding regions of phot1 (amino acids 1-202, 1-307, 1-423 and 1-627).

2.3. Heterologous protein expression

Coding sequences of *Arabidopsis* 14-3-3 isoforms 14-3-3λ (At5g10450), 14-3-3κ (At5g65430), 14-3-3φ (At1g35160), 14-3-3υ (At5g16050), 14-3-3ο (At1g34760) and 14-3-3ε (At1g223300) were PCR-cloned into the pGEX-4T1 vector (Amersham Biosciences) as a translational fusion with glutathione-*S*-transferase (GST). Recombinant 14-3-3 proteins were expressed and purified from the *Escherichia coli* host strain BL21(DE3) (Novagen). Protein expression of GST-fusion proteins and GST was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were lysed with a French press and purified with GST-Bind Resin (Novagen). Proteins were eluted with 50 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. The purity and integrity of purified proteins was confirmed by 12.5% SDS–PAGE and staining with coomassie brilliant blue R250 (Bio-Rad). The LOV1+2 regions of phot1 (amino acids 180–628) and phot2 (amino acids 116–542) were expressed and purified as calmodulin binding peptide fusions and [7]. Expression of phot1 and phot2 in insect cells was performed as described previously [25].

2.4. In vitro phosphorylation

Autophosphorylation assays were performed as described previously [8]. For far-Western blotting experiments, radiolabelled ATP was omitted from the reaction. Phosphorylation of the bacterially expressed LOV1+2 proteins was performed by incubation of 10 μg of purified protein with 1000 units of bovine protein kinase A catalytic subunit (Calbiochem) in accordance with the supplier's instructions.

2.5. Protein extraction and immunoprecipitation from Arabidopsis

Total protein and microsomal membrane protein was extracted as described previously [13]. For separation of soluble and membrane proteins, total protein extract was centrifuged at 100 000*g* at 4 °C for 75 min. The supernatant was used as the soluble fraction and the pellet resuspended in extraction buffer as the membrane fraction. Protein concentrations were determined by the Bradford colorimetric method (Bio-Rad). GFP immunoprecipitations were performed using the μMACS GFP isolation kit (Miltenyi Biotech) as described [13].

2.6. Western blot analysis

Proteins were detected with anti-phot1 and anti-phot2 polyclonal antibodies [9], anti-14-3-3λ purified polyclonal antibody raised against the peptide sequence VKDYRSKVESELSSVC (Eurogentec), anti-GFP monoclonal antibody (BD Biosciences), anti-GST monoclonal antibody (Novagen), anti-His antibody (Santa Cruz Biotechnology) and anti-UGPase antibody (AgriSera). Western blots were developed with either horseradish peroxidase (HRP)-linked secondary antibodies and Immobilon Western chemiluminescence HRP substrate (Millipore) or alkaline phosphatase-linked secondary antibodies and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) solution (Sigma). Far-Western blotting was carried out according to Kinoshita and Shimazaki [26]. Nitrocellulose membranes were incubated with purified GST-14-3-3 proteins or GST alone at a final concentration of 0.1 mM unless otherwise stated. Protein–protein interactions were detected using monoclonal anti-GST antibody (Novagen) at 1/10 000 dilution.

2.7. Sequence alignment and phylogenetic analysis

Alignment of the 14-3-3 amino acid sequences and phylogenetic trees were produced using ClustalX [27]. Trees were viewed using TreeView [28].

3. Results

3.1. Identification of phot1-interacting proteins

Arabidopsis phot1 was used as bait in a yeast two-hybrid screen using a random cDNA library from *Arabidopsis* [29]. A total of 8×10^4 colonies were screened, from which 57 grew on full selection medium. Further characterisation led to the isolation of five cDNA clones encoding proteins of interest that are summarised in Table 1. Proteins known to interact with phot1, such as RPT2 [18] were identified demonstrating the veracity of the approach. Specifically, the N-terminal region of RPT2 (amino acid residues 1–187) was isolated (Supplementary Fig. S1) including the BTB/POZ domain (amino acid residues 32–128) which is known to be important for mediating the interaction between RPT2 and phot1 [18]. A novel member of the NPH3/RPT2-like (NRL) family was also identified. This protein, designated NPH3-like (NPH3-L), comprised a truncated region of the protein (amino acid residues 243–433) downstream of the BTB/POZ domain which resides within the NPH3 signature region spanning amino acids 213 to 484 in NPH3-L (Supplementary Fig. S1). In addition, we identified two ADP-ribosylation factors (ARF2 and ARF7), members of the Ras superfamily of GTP-binding proteins that play important roles in the assembly and disassembly of coat proteins associated with driving vesicle budding and fusion [30]. In each case, full-length cDNAs were isolated encoding the entire region of the protein (Supplementary Fig. S1). Similarly, a full-length cDNA was obtained encoding 14-3-3λ (Supplementary Fig. S1), that interacts specifically with phot1 in yeast, although weaker in comparison to the interaction between positive controls expressing murine p53 and the SV40 large T-antigen (Fig. 1A). Similarly, NPH3-L, ARF2 and ARF7 were not found to autoactivate the yeast two-hybrid system in the absence of phot1 (data not shown), and thus represent bona fide interacting proteins.

3.2. Effect of blue light on phot1 interactions

Yeast growth was screened in the absence of continuous illumination to identify the afore-mentioned proteins. To establish whether light had any effect, yeast were grown in darkness or in continuous white light (20 μmol m^{-2} s^{-1}). The interaction between ARF2 and phot1 was severely attenuated in the presence of light (Fig. 1B) and was specific to blue light (Fig. 1C). A similar light sensitivity was observed for the interaction between ARF7 and phot1 (data not shown) suggesting that photoactivation of phot1 by blue light disrupts its binding to ARF proteins. By contrast, light had no effect on phot1 interactions with RPT2, NPH3-L and 14-3-3λ (Fig. 1B).

14-3-3 proteins are key regulators of protein function in eukaryotes and preferentially bind to phospho-serine/threonine-containing motifs [31]. Since phot1 undergoes blue light-dependent autophosphorylation [25], yeast growth in darkness (Fig. 1B) would indicate that 14-3-3λ binding to phot1 is not dependent on the phosphorylation status of the receptor. However, previous reports have shown by far-Western blotting that a different member of the *Arabidopsis* 14-3-3 family, 14-3-3 phi (ϕ), binds

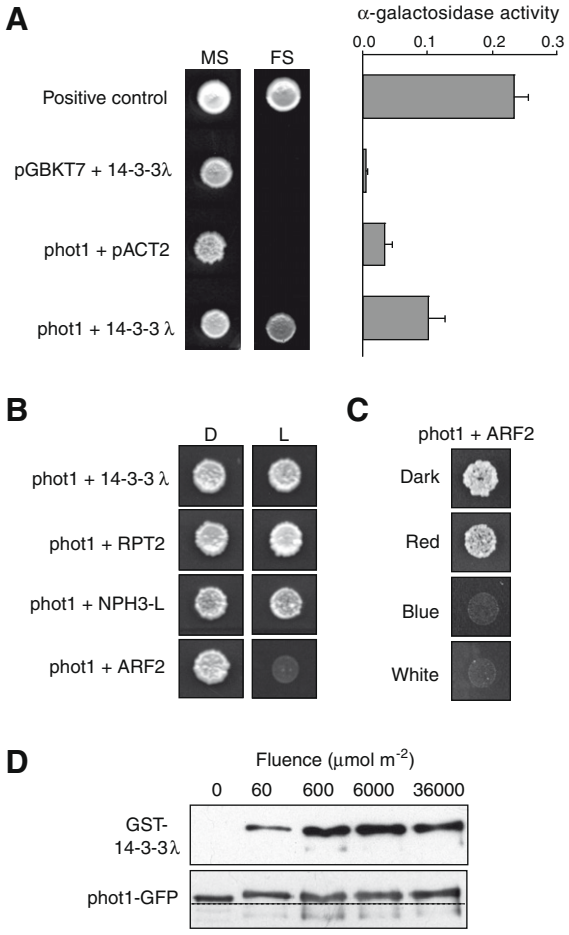

Fig. 1. Yeast two-hybrid analysis of phot1-interacting proteins. (A) Yeast two-hybrid analysis of phot1 and 14-3-3λ. Yeast growth on minimal selection (MS) medium selects for cotransformants while growth on full selection (FS) medium selects for interacting proteins. No interaction was observed with empty vector transformations. Vectors encoding murine p53 and the SV40 large T-antigen were included as a positive control. Interactions were quantified using the α-galactosidase assay. Error bars indicate standard error ($n = 3$). (B) Effect of white-light irradiation on phot1 interactions. Yeast growth on FS medium grown in darkness (D) or continuous white light (L) at 20 μmol m^{-2} s^{-1}. (C) Effect of blue-light irradiation on phot1 interaction with ARF2. Yeast growth on FS medium grown in white (20 μmol m^{-2} s^{-1}), red or blue light (10 μmol m^{-2} s^{-1}, respectively). (D) Far-Western blotting of immunoprecipitated phot1-GFP with 14-3-3λ. Three-day-old etiolated seedlings expressing phot1-GFP were treated with the indicated fluence of blue light. Phot1-GFP was immunoprecipitated from microsomal membrane fractions and analysed by far-Western blotting with GST-14-3-3λ. As a control for protein loading blots were probed with anti-GFP antibody. Dashed line indicates lowest mobility edge of phot1-GFP.

Table 1
Identification of phot1-interacting proteins. Proteins of interest identified from yeast two-hybrid screening of an *Arabidopsis* cDNA library with full-length phot1.

Interacting protein	Accession number	Protein function	% Frequency
RPT2	At2g30520	Phototropism, stomatal opening	2
NPH3-like	At1g30440	Unknown	4
ARF2	At3g62290	GTP binding, vesicle trafficking	7
ARF7	At5g17060	GTP binding, vesicle trafficking	2
14-3-3λ	At5g10450	Phosphoprotein binding	2

to the phosphorylated form of phot1 immunoprecipitated from *V. faba* [14] and *Arabidopsis* [12]. A transgenic *Arabidopsis* line expressing full-length phot1 fused to green fluorescent protein (phot1-GFP) under the control of the native *PHOT1* promoter has been shown to complement the null *phot1-5* allele [20]. This line was therefore used to immunoprecipitate phot1-GFP from 3-day-old dark-grown seedlings to assess 14-3-3λ binding to phot1 by far-Western blotting. As shown in Fig. 1D, binding of recombinant 14-3-3λ fused to glutathione-*S*-transferase (GST) was not detected for phot1-GFP immunoprecipitated from dark-grown seedlings. However, a reduced electrophoretic mobility of phot1-GFP, indicative of receptor autophosphorylation, and concomitant 14-3-3λ binding was observed when seedlings were exposed to blue light. 14-3-3λ binding was also fluence-dependent, saturating at 600 μmol m^{-2}. These findings concur with studies using 14-3-3ϕ [12,14] demonstrating that, at least for plant-derived phot1, 14-3-3 binding is dependent on receptor autophosphorylation.

3.3. Ser^{410} in addition to Ser^{350} and Ser^{376} is required for binding of 14-3-3λ to phot1

Phospho-serine residues required for the 14-3-3ϕ binding to *Arabidopsis* phot1 have been mapped to the intervening linker region between LOV1 and LOV2 [12] and conform to protein kinase A (PKA)-like consensus sequences [13]. To determine whether this was also the case for 14-3-3λ, truncation analysis from the C-terminus of phot1 was performed to assess regions important for 14-3-3 binding in yeast. Interactions between phot1 and 14-3-3λ were only evident when the LOV-linker region was present (Fig. 2A), indicating the importance of this peptide sequence. *Arabidopsis* phot1 is phosphorylated on three serine residues within this region, namely Ser^{350}, Ser^{376} and Ser^{410} [12,13]. Phosphorylation of both Ser^{350} and Ser^{376} is required for binding of 14-3-3ϕ to *Arabidopsis* phot1 [12]. We therefore investigated whether these residues were also required for 14-3-3λ binding to phot1. Fragments of *Arabidopsis* phot1 containing both LOV domains, designated LOV1+2 were generated for this purpose. In vitro phosphorylation of LOV1+2 by the catalytic subunit of PKA resulted in strong 14-3-3λ binding as measured by far-Western blotting (Fig. 2B). Although mutation of Ser^{350} and Ser^{376} to alanine substantially reduced the interaction, residual 14-3-3λ binding to phot1 in response to PKA treatment could still be detected (Fig. 2B). Mutation of Ser^{410} to alanine (Fig. 2C) abolished this interaction demonstrating an accessory role for this residue in mediating 14-3-3λ binding to phot1.

3.4. 14-3-3λ-binding is limited to phot1

While an interaction between 14-3-3λ and phot1 was readily detectable by far-Western blotting, no interaction was observed for *Arabidopsis* phot2. In vitro phosphorylation of the LOV1+2 region of phot2 by PKA was unable to mediate binding of 14-3-3λ (Fig. 3A). For independent verification, we investigated 14-3-3λ binding to phot1 and phot2 expressed in insect cells as both proteins exhibit light-induced kinase activity in this system (Fig. 3B). 14-3-3λ binding was specific to phot1, but did not appear to be light dependent. This finding may coincide with the basal level of kinase activity detected for phot1 in vitro [25]. Indeed, treatment with λ-phosphatase abolished radiolabelled phosphate incorporation in addition to 14-3-3λ binding to phot1 under dark and light conditions (Supplementary Fig. S2). Despite showing light-dependent phosphorylation activity in insect cells, phot2 did not bind 14-3-3λ either in darkness or in response to irradiation (Fig. 3B). As further confirmation, we examined whether phot2 from

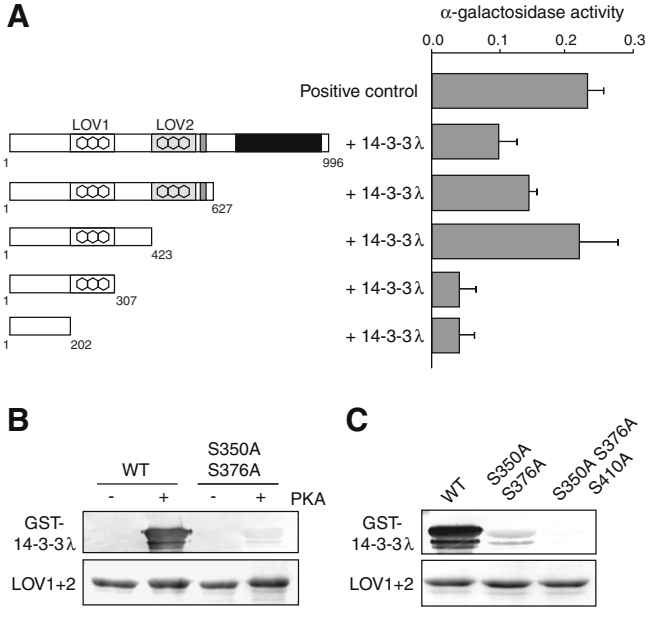

Fig. 2. 14-3-3λ binding to phot1 is dependent on its phosphorylation status. (A) Yeast two-hybrid analysis of a phot1 deletion series and 14-3-3λ. Interactions were quantified using the α-galactosidase assay. Error bars indicate standard error ($n = 3$). Positive control vectors are as described in Fig. 1A. (B) Far-Western blotting of wild-type (WT) and S350A/S376A mutant LOV1+2 protein fragments of phot1 with GST-14-3-3λ. LOV1+2 was subjected to in vitro phosphorylation in the presence or absence of PKA (+ or −). Ponceau S staining of the nitrocellulose membrane was used as a control for protein loading. (C) Far-Western blotting of wild-type (WT), S350A/S376A mutant and S350A/S376A/S410A mutant LOV1+2 protein fragments of phot1 with GST-14-3-3λ. LOV1+2 proteins were treated as in (B).

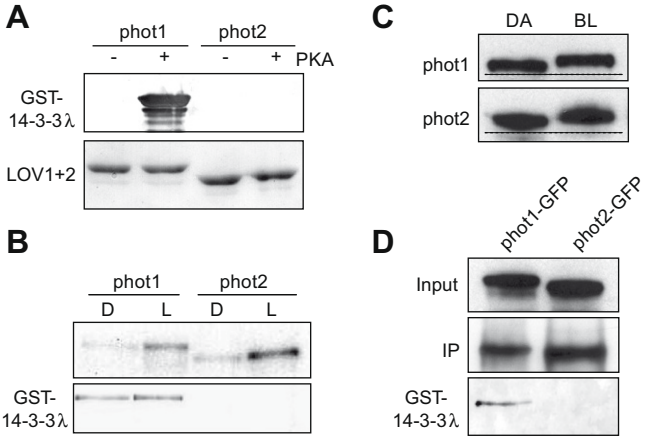

Fig. 3. Specificity of 14-3-3λ-phototropin interactions for phot1. (A) Far-Western blotting of phot1 and phot2 LOV1+2 proteins with GST-14-3-3λ. LOV1+2 proteins were treated as in Fig. 2B. (B) Far-Western blotting analysis of phot1 and phot2 with GST-14-3-3λ. Autoradiograph showing light-dependent autophosphorylation activity of phot1 and phot2 in protein extracts prepared from insect cells (upper panel). All manipulations were carried out under dim red light. Samples were given a mock irradiation (D) or irradiated with white light (L) at a total fluence of 10 000 μmol m^{-2} upon addition of radiolabelled ATP. Lower panel shows far-Western blotting of phot1 and phot2 with GST-14-3-3λ. (C) Western blot analysis of 5-day-old seedlings expressing phot1-GFP or phot2-GFP grown under white light (50 μmol m^{-2} s^{-1}, 16 h/8 h light-dark cycle) before being dark adapted (DA) for 12 h. Plants were either kept in darkness (DA) or irradiated with 100 μmol m^{-2} s^{-1} of blue light (BL) for 30 min. Blots were probed with anti-GFP antibody. Dashed line indicates lowest mobility edge. (D) Far-Western blotting of immunoprecipitated phot1-GFP and phot2-GFP with GST-14-3-3λ. Five-day-old seedlings expressing phot1-GFP or phot2-GFP were grown and treated with blue light as described in (C). Phot1-GFP and phot2-GFP were immunoprecipitated from microsomal membrane fractions and samples analysed by far-Western blotting with GST-14-3-3λ. Phot1- and phot2-GFP protein levels prior to (input) and following immunoprecipitation (IP) were assessed using anti-GFP antibody.

Arabidopsis could bind 14-3-3λ. Light-grown seedlings were used for our analysis since phot2 is poorly expressed in dark-grown *Arabidopsis* [8]. Seedlings were incubated in darkness overnight to restore receptor activity to its ground state. Subsequent irradiation of seedlings with blue light resulted in an electrophoretic mobility shift for phot1 and phot2 indicative of receptor autophosphorylation (Fig. 3C). Hence, identical illumination conditions were used to immunoprecipitate functional phot1-GFP and phot2-GFP from transgenic *Arabidopsis* (Fig. 3D). Consistent with our efforts to monitor 14-3-3λ binding to phot2 in vitro and insect cells, 14-3-3λ binding was only detected for phot1-GFP. Taken together, these data imply that 14-3-3λ binding is specific to phot1.

3.5. 14-3-3λ and phot1 colocalise and associate in vivo

The 14-3-3-protein family comprises 13 members in *Arabidopsis* [31]. Phylogenetic analysis shows that its members can be separated into two evolutionary branches: the epsilon and non-epsilon groups. 14-3-3λ is closely related to 14-3-3 kappa (κ) and in a subbranch of the non-epsilon group (Fig. 5A). Thus, a peptide region unique to 14-3-3λ was used to generate antibodies specific to 14-3-3λ (see Section 2). A T-DNA insertion mutant lacking *14-3-3λ* transcripts was used to test the antibody generated (Fig. 4A). No 14-3-3λ protein was detected in the T-DNA insertion mutant verifying the specificity of the 14-3-3λ antibody, as well as confirming that this allele is a null mutant (Fig. 4B). Western analysis revealed that 14-3-3λ is ubiquitously expressed in wild-type plants and particularly prevalent in roots, flowers, cauline and rosette leaves (Fig. 4C). Biochemical fractionation demonstrated that the majority of 14-3-3λ is soluble, however a portion of 14-3-3λ was found to associate with the membrane fraction purified from *Arabidopsis* (Fig. 4D) indicating that 14-3-3λ, at least in part, colocalises to the same subcellular compartment as phot1 [20]. Membrane localisation of 14-3-3λ was still observed in the *phot1 phot2* double mutant indicating that its association with the membrane is not dependent on phototropins (Fig. 4D). However, 14-3-3λ was found to co-purify with phot1-GFP immunoprecipitated from *Arabidopsis* and not with the plasma membrane marker GFP-Lti6b (Fig. 4E) suggesting that 14-3-3λ and phot1 interact in vivo.

3.6. 14-3-3 binding to phot1 is specific for non-epsilon 14-3-3 family members

We also tested the ability of phot1 to interact with other members of the *Arabidopsis* 14-3-3 family. Epsilon (ε) and omicron (ο) were chosen as representative candidates from the epsilon subbranches, whereas kappa (κ), upsilon (υ) and phi (φ) were chosen as further representatives of the non-epsilon group (Fig. 5A). Each of these proteins were expressed as GST fusions (Fig. 5B) and used for far-Western analysis with phot1-GFP immunoprecipitated from *Arabidopsis* alongside 14-3-3λ. Initially, binding of only 14-3-3λ and 14-3-3κ were observed (Fig. 5C). Upon longer exposure, binding of 14-3-3φ and, to a much lesser extent, 14-3-3υ was also visible. No binding of the 14-3-3ε and 14-3-3ο was detected under these conditions implying that 14-3-3 binding to phot1 is restricted to non-epsilon family members. While binding of 14-3-3φ to phot1 agrees with previous reports [12,14], our findings show that phot1 has a stronger affinity for 14-3-3λ followed by 14-3-3κ (Fig. 5C) consistent with their placement in the phylogenetic tree (Fig. 5A). The difference in binding affinity between 14-3-3λ and 14-3-3κ was also evident upon diluting phot1-GFP proteins levels (Fig. 6A). A similar specificity for 14-3-3 binding was observed when using phot1 expressed in insect cells (data not shown). Like 14-3-3λ, 14-3-3κ binding was abolished upon mutation of Ser^{350}, Ser^{376} and Ser^{410} to alanine in phot1 expressed in insect cells (Fig. 6B). No binding of 14-3-3κ could be detected for phot2 expressed in insect cells (Fig. 6C), as was the case for the other 14-3-3 candidates examined (data not shown) again indicating that 14-3-3 binding is a property unique to phot1.

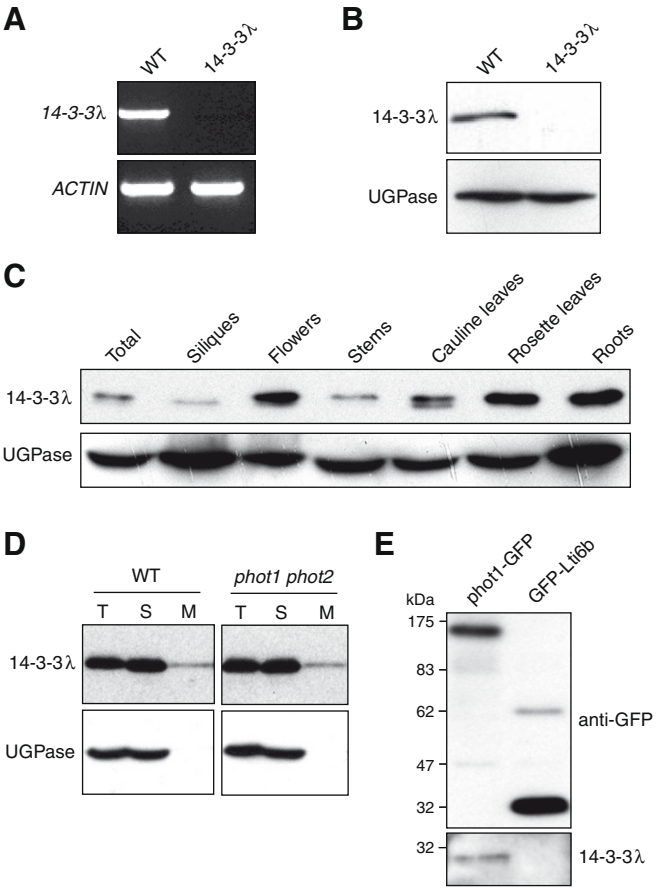

Fig. 4. Analysis of 14-3-3λ localisation in planta and its interaction with phot1. (A) RT-PCR analysis of *14-3-3λ* transcripts in wild-type (WT) and a 14-3-3λ null mutant. Actin was monitored as a control. (B) Western blot of total proteins extracted from wild-type (WT) and a 14-3-3λ null mutant. Total protein extracts (10 μg) were probed with an anti-14-3-3λ specific antibody. As a control for protein loading blots were probed with antibody raised against UDP-glucose pyrophosphorylase (UGPase). (C) Western blot analysis of 14-3-3λ protein levels in different tissues from wild-type plants. Total protein was extracted from tissues of 5-week-old *Arabidopsis*. Total protein extracts (10 μg) were probed with anti-14-3-3λ antibody and anti-UGPase as a loading control. (D) Western blot analysis of 14-3-3λ localisation in wild-type (WT) and *phot1 phot2* double mutant plants. Total protein extract (T) was separated into soluble (S) and membrane (M) fractions by ultracentrifugation. Equal volumes of each fraction were probed with anti-14-3-3λ antibody. Soluble UGPase protein levels were also monitored. (E) 14-3-3λ interacts with phot1 in vivo. Three-day-old etiolated seedlings expressing phot1-GFP were irradiated with 100 μmol m^{-2} s^{-1} of blue light for 30 min. Phot1-GFP was immunoprecipitated from microsomal membrane fractions and subjected to Western blot analysis with anti-GFP and anti-14-3-3λ antibodies. The plasma membrane marker line, GFP-Lti6b, was used as a negative control for non-specific interactions.

4. Discussion

The most prominent paradigms involving 14-3-3 proteins in plant regulatory events include regulation of plasma membrane H^+ATPase activity, nitrate reductase and sucrose phosphate synthase [31]. More recently, a role for 14-3-3 proteins in brassinosteroid signalling has been indentified [32]. In addition, phot1 from both *V. faba* and *Arabidopsis* has been shown to interact with 14-3-3φ [12,14], a member of the non-epsilon group. Previous studies have shown isoform-specific binding preferences between different 14-3-3-isoforms and their target proteins [33–35]. Yet,

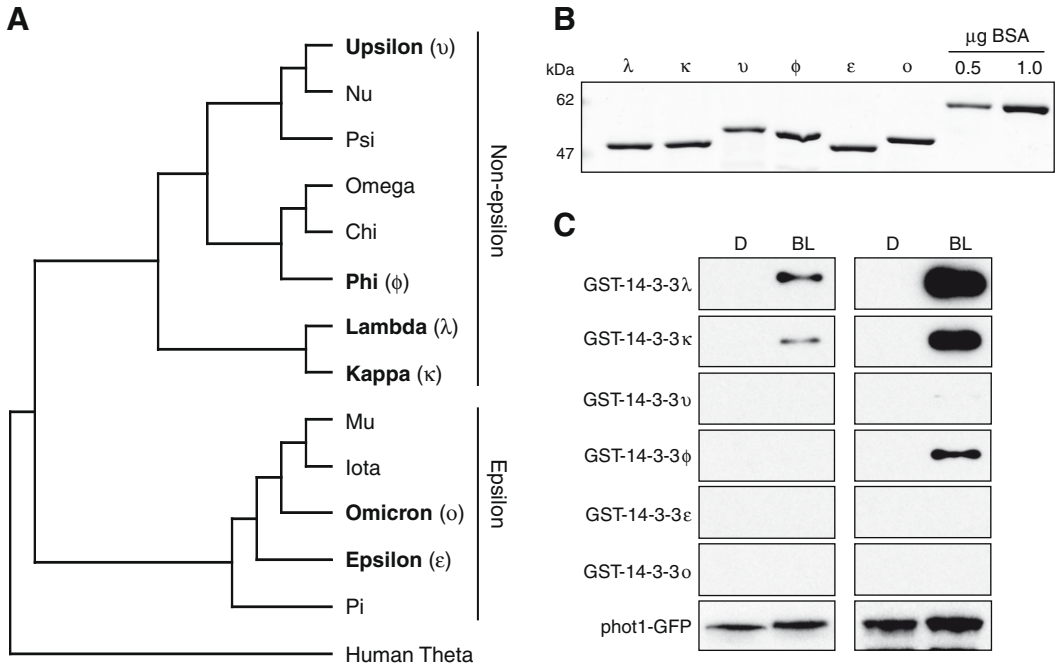

Fig. 5. Specificity of the 14-3-3-phot1 interaction. (A) Phylogentic tree of *Arabidopsis* 14-3-3 protein sequences. Non-epsilon and epsilon groups are indicated. Isoforms in bold were expressed in *E. coli* and used for far-Western blotting experiments. Human 14-3-3 isoform theta was designated as the outgroup. (B) Coomassie blue-stained SDS-PAGE gel of purified recombinant GST-14-3-3 proteins expressed in *E. coli*. Bovine serum albumin (BSA) was used as a loading control. (C) Far-Western blotting of immunoprecipitated phot1-GFP with different 14-3-3 isoforms. Three-day-old etiolated seedlings were either kept in the dark (D) or treated with blue light at a total fluence of 36 000 μmol m^{-2} (BL). Phot1-GFP was immunoprecipitated from microsomal membrane fractions and analysed by far-Western blotting. Left panel shows a short exposure whereas the right panel shows a longer exposure. Blots were probed with anti-GFP antibody as a loading control. Far-Western blotting was performed simultaneously to allow a direct comparison of 14-3-3 binding between the different isoforms.

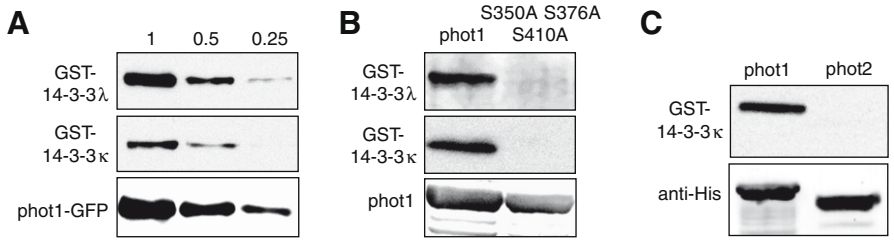

Fig. 6. Analysis of 14-3-3κ interactions with phot1 and phot2. (A) Comparison of 14-3-3κ and 14-3-3λ interaction with phot1-GFP by far-Western blotting. Phot1-GFP was immunoprecipitated as in Fig. 5C. Relative amounts of immunoprecipitate loaded are shown above each lane. Blots were probed with anti-GFP antibody as a loading control. Far-Western blotting was performed simultaneously to allow a direct comparison of 14-3-3 binding between the different isoforms. (B) Far-Western blotting of wild-type phot1 and the S350A/S376A/S410A mutant. Protein extracts from insect cells were irradiated with white light at a total fluence of 10 000 μmol m^{-2}. Far-Western blotting was performed with GST-14-3-3κ and 14-3-3λ. Phot1 protein levels are shown below. (C) Far-Western blotting of phot1 and phot2 expressed in insect cells. Soluble protein extracts were treated as described in (B). Far-Western blotting was performed with GST-14-3-3κ. Blots were probed with anti-His antibody as a loading control.

the specificity of phot1 for specific members of the *Arabidopsis* 14-3-3 family has not been investigated. Likewise, the ability of phot2 to interact with 14-3-3 proteins has not been examined. 14-3-3λ was identified as a phot1-interacting protein from our yeast two-hybrid screen indicating that phot1 also binds family members other than 14-3-3ϕ. In contrast to phot1 derived from *Arabidopsis*, binding of 14-3-3λ to phot1 in yeast was not light dependent (Fig. 1D). Phosphorylation of phot1 by a protein kinase endogenous to yeast may account for this discrepancy as has been reported for the *Arabidopsis* plasma membrane H^+ATPase AHA2 [36].

As well as interacting with 14-3-3λ, our findings demonstrate that phot1 exhibits different binding affinities for non-epsilon group members (Fig. 5). From the 14-3-3 representatives examined, 14-3-3λ appears to show the strongest affinity for phot1, followed by 14-3-3κ, 14-3-3ϕ and 14-3-3υ. The different binding affinities observed for non-epsilon isoforms matches well with their placement in the phylogenetic tree. The apparent difference in binding affinities between 14-3-3λ and 14-3-3κ is intriguing given these proteins are almost identical in protein sequence (93%). However, interactions between members of the epsilon group could not be detected implying that genetic analysis should now be restricted to non-epsilon members to evaluate the biological consequences of 14-3-3 binding to phot1. Our characterisation of *Arabidopsis* mutants lacking both 14-3-3λ and 14-3-3κ has been unsuccessful in identifying a physiological role for 14-3-3 binding to phot1 (data not shown) and maybe due to functional redundancy between other non-epsilon members such as 14-3-3ϕ. Overexpression of *Arabidopsis* 14-3-3λ in cotton results in an enhanced tolerance to drought stress [37] indicating that 14-3-3λ may, in conjunction with the phot1, regulate stomatal function. However, mutation of Ser^{350} and Ser^{376} does not impair phot1-induced stomatal opening in *Arabidopsis* [12]. Whether this is because Ser^{410} alone can promote residual 14-3-3 binding (Fig. 2D) is not known. Alternatively, 14-3-3 proteins may play a role in photoresponses that are specific to phot1 since we were unable to detect 14-3-3 binding to phot2 using a variety of approaches (Fig. 3). Indeed,

phospho-motifs present in the LOV linker region of phot1 are less well conserved in phot2 [13].

Phosphorylation status has been shown to be important for NPH3 function [38] and PIN-mediated auxin transport [39], both of which are influenced by phototropin activity. 14-3-3 binding may influence their interaction with phot1 given that these proteins also exhibit 14-3-3 binding [15]. Besides 14-3-3λ, we identified additional members of the NRL family in addition to ARFs as novel phot1-interacting proteins. The ARF Guanine Exchange Factor (GEF), GNOM is at least partially responsible for the polar localisation at the plasma membrane of the auxin efflux carrier PIN1 [40]. Since phot1 internalises from the plasma membrane in response to blue light [20], it will now be interesting to establish whether the ARFs identified here are involved in this process. Alternative but complementary proteomic-based strategies will also prove useful in validating these interactions as well as identifying additional phototropin signalling components.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.06.011.

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