Regulation of Phototropic Signaling in Arabidopsis via Phosphorylation State Changes in the Phototropin 1-interacting Protein NPH3*5

Ullas V. Pedmale and Emmanuel Liscum

From the Division of Biological Sciences and the Christopher S. Bond Life Sciences Center, University of Missouri, Columbia, Missouri 65211

Phototropism, or the directional growth (curvature) of various organs toward or away from incident light, represents a ubiquitous adaptive response within the plant kingdom. This response is initiated through the sensing of directional blue light (BL) by a small family of photoreceptors known as the phototropins. Of the two phototropins present in the model plant Arabidopsis thaliana, phot1 (phototropin 1) is the dominant receptor controlling phototropism. Absorption of BL by the sensory portion of phot1 leads, as in other plant phototropins, to activation of a C-terminal serine/threonine protein kinase domain, which is tightly coupled with phototropic responsiveness. Of the five phot1-interacting proteins identified to date, only one, NPH3 (non-phototropic hypocotyl 3), is essential for all phot1-dependent phototropic responses, yet little is known about how phot1 signals through NPH3. Here, we show that, in dark-grown seedlings, NPH3 exists as a phosphorylated protein and that BL stimulates its dephosphorylation. phot1 is necessary for this response and appears to regulate the activity of a type 1 protein phosphatase that catalyzes the reaction. The abrogation of both BL-dependent dephosphorylation of NPH3 and development of phototropic curvatures by protein phosphatase inhibitors further suggests that this post-translational modification represents a crucial event in phot1-dependent phototropism. Given that NPH3 may represent a core component of a CUL3-based ubiquitin-protein ligase (E3), we hypothesize that the phosphorylation state of NPH3 determines the functional status of such an E3 and that differential regulation of this E3 is required for normal phototropic responsiveness.

Although sessile, plants can respond adaptively to various environmental stimuli by altering their growth and development. Phototropism, or directional growth of a given organ (e.g. seedling stem) in response to a change in incident light direction, represents one such response (1–3). Plants utilize a specific class of photoreceptors, the phototropins, to sense directional blue light (BL) cues that induce phototropic responses (4–6). Two phototropins, phot1 (phototropin 1) and phot2, are present in the model plant Arabidopsis thaliana, and both are functional serine/threonine protein kinase photoreceptors (7, 8). Although much is known about the structural means by which phototropins absorb BL and thus activate the kinase domain, at present, no native substrate other than the phototropins themselves has been identified (4, 5). Moreover, although it is clear that perception of directional BL leads to phototropic curvature through differential accumulation of and response to the plant growth regulator auxin (9), the mechanism by which phototropin activation initiates signaling leading to this signal output remains elusive.

The identification of phototropin-interacting proteins represents a potentially powerful approach to elucidate how these photoreceptors transduce directional BL signals. Five phototropin-interacting proteins have been identified in recent years: NPH3 (non-phototropic hypocotyl 3) (10), RPT2 (root phototropism 2) (11), PKS1 (phytochrome kinase substrate 1) (12), Vicia faba phot1A-interacting protein (13), and a 14-3-3 protein (14). Of these proteins, NPH3 represents the most likely candidate to yield insights into how phot1 transduces directional BL cues to induce phototropic responses because mutants lacking this protein are completely aphototropic under phot1-dependent conditions (10, 11, 15–17) while retaining other major phot1 functions such as chloroplast movements (11) and stomatal aperture control (11, 18). The remaining four proteins either are involved in other phot1-dependent processes (V. faba phot1A-interacting protein and the 14-3-3 protein) (13, 14) or are not absolutely necessary for progression of phot1-dependent phototropic signaling (RPT2 and PKS1) (11, 12).

NPH3 is a novel 745-amino acid plasma membrane-associated protein and is a member of a large family of highly related plant-specific proteins that includes RPT2 (5, 10, 15). Three characteristics define members of the NPH3/RPT2 family of proteins: 1) an N-terminal BTB (Broad-Complex/Tramtrack/Bric-a-brac) domain (19, 20), 2) a 14-3-3 protein (Pfam accession number PF03000) in the middle of each protein, and 3) a C-terminal coiled coil in most members of the family (5). Although the functional role(s) of these domains are not fully

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1 To whom correspondence should be addressed. Tel.: 573-882-2672; Fax: 573-882-0123; E-mail: liscume@missouri.edu.

2 The abbreviations used are: BL, blue light; E3, ubiquitin-protein ligase; PPase or PP, protein phosphatase; OKA, okadaic acid; CN, cantharidin; ET, eto-dithall; MOPS, 4-morpholinepropanesulfonic acid; TBS, Tris-buffered saline; NPH3LS, light-state NPH3; NPH3DS, dark-state NPH3.
understood at present, the coiled-coil region of NPH3 is known to be necessary and sufficient for interaction with phot1 (10), whereas the BTB domain has been shown recently to interact with CULLIN3 (CUL3) in a heterologous insect cell expression system. Based on paradigms established in fungal and animal systems, in which BTB domain-containing proteins function as substrate adapters in CUL3-based ubiquitin-protein ligase (E3) (21–25), it has been proposed that NPH3 may function as a core component of a CUL3-based E3 complex that is necessary for phototropic signal progression (5). Although this hypothesis is currently under investigation, the physical and genetic interactions between phot1 and NPH3 suggest that, whatever the biochemical function of NPH3, it will somehow be regulated by phot1. Given that phot1 is a light-activated protein kinase (26), it seems reasonable to predict that such regulation of NPH3 function could occur through reversible phosphorylation.

NPH3 has been shown previously to exhibit different electrophoretic mobility states that are dependent upon in vivo irradiation conditions; in particular, BL specifically induces a state change to higher mobility relative to mock-irradiated samples (10). It was proposed that differing phosphorylation states of NPH3 could account for the observed alterations in electrophoretic mobility (10). However, no direct supporting evidence for this conclusion has been reported. In this study, we use a combination of pharmacological treatments and immunoblot assays to conclusively demonstrate that the higher mobility state of NPH3 does result from dephosphorylation and that this dephosphorylation is dark-reversible. In particular, we show that the BL-dependent increase in the electrophoretic mobility of NPH3 is sensitive to in vivo treatment with protein phosphatase (PPase) inhibitors and that in vitro treatment of samples never exposed to light with λ-PPase can phenocopy the effects of in vivo BL exposure. We further show that exposure of Arabidopsis seedlings to PPase inhibitors results in significantly reduced BL-dependent phototropism, consistent with the action of PPase(s) in phototropic signal transduction. Finally, we also provide evidence that the BL-dependent change in the electrophoretic mobility of NPH3 is dependent upon the phot1 photoreceptor and does not require functional phot2, the cryptochrome cry1 or cry2, or the phytochrome phyA or phyB. These results suggest that absorption of BL by phot1 leads to the activation of a PPase(s) that dephosphorylates an inactive phosphorylated form of NPH3 to allow further progression of phototropic signaling.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions and Light Treatments—Seedlings of A. thaliana L. accession Columbia (Col-0) and various mutant genotypes in this background were used as sources of microsomal membranes and for the phototropic assays described in this study. The mutants used in this study have been described previously: phot1-5 (7), nph3-6 (10), and phot2-1 and phot1-5 phot2-1 (27). Seedlings were grown either on agar-based medium or in liquid culture (depending upon the experiment) as described below. First, seeds were surface-sterilized using 30% (v/v) commercial bleach (20 min) and then washed five times with sterile water. Unless noted otherwise, seeds were next placed either on filter paper mounted on 1% agar-solidified half-strength Murashige and Skoog medium (28) in Petri plates or in 15 ml of half-strength Murashige and Skoog liquid medium in 125–250-ml Erlenmeyer flasks. The seeds were then cold-treated for 3 days in darkness before treatment with red light for 1 h to induce uniform germination (29). The plates or flasks were next transferred to complete darkness, and seedlings were allowed to grow for 3 days prior to any treatment. For growth of seedlings in liquid medium, the flasks were shaken at 50–60 rpm throughout their growth and subsequent treatments up to tissue collection. For BL treatments, seedlings growing on agar medium were either mock-irradiated or exposed to unilateral BL (29) of various intensities (0.01, 0.1, 1.0, and 10 μmol m⁻² s⁻¹) for different time intervals (1, 5, 30, and 240 min), whereas seedlings grown in liquid medium were irradiated with BL (1.0 μmol m⁻² s⁻¹) from underneath the flasks with constant shaking.

PPase Inhibitor Treatments—The following PPase inhibitors (Sigma) were used in this study: 9,10-deepethio-9,10-didehydroacanthofilicin (okadaic acid (OKA)); 2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-carboxylic acid anhydride (cantharidin (CN)); and a dicarboxylic acid derivative of CN, 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid (endothall (ET)). OKA and CN were dissolved in Me₂SO, whereas ET was dissolved in water. To test the influence of these compounds on the electrophoretic mobility of NPH3, a given compound was added to liquid cultures of 3-day-old etiolated (dark-grown) seedlings at the indicated concentrations with or without cycloheximide (Sigma) dissolved in 100% Me₂SO. Controls were treated with an equal volume of Me₂SO alone.

The influence of the PPase inhibitors on phototropism was assayed as follows. First, 3-day-old etiolated seedlings grown vertically on microscopic glass slides coated with half-strength Murashige and Skoog agar medium were completely immersed in half-strength Murashige and Skoog liquid medium containing a given inhibitor (or Me₂SO as a control) for 2 h. Next, seedlings on the glass slides were exposed to unilateral BL (2.0 μmol m⁻² s⁻¹) for 12 h. After collection of seedlings, hypocotyl lengths and phototropic curvatures were measured using Scion Image software (Scion Corp., Frederick, MD). Student’s t tests were performed to compare the mean responses of seedlings under different treatments.

Preparation of Microsomal Membrane Proteins—Microsomal membranes were isolated from seedlings after BL or inhibitor treatments as described by Liscum and Briggs (16) with noted exceptions. All manipulations described below were performed under dim red light in a 4 °C temperature-controlled room. Seedlings were ground in ice-cold homogenization buffer (25 mM MOPS-NaOH (pH 7.8), 250 mM sucrose, 0.1 mM MgCl₂, 8 mM cysteine, 10 mM NaF, 5 mM ε-aminocapric acid, 1 mM benzamidine, and Complete protease inhibitor mixture (Roche Applied Science)), followed by ultracentrifugation at 100,000 × g for 1 h, 15 min to pellet microsomes. Microsomal membranes were then resuspended in resuspension buffer (5 mM potassium phosphate (pH 7.8), 250 mM sucrose, 4 mM KNO₃, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydro-

³ N. Zheng and E. Liscum, unpublished data.
chloride, 5 mM e-aminocaproic acid, 1 mM benzamidine, and Complete protease inhibitor mixture) using a Dounce homogenizer. The resuspended microsomal membranes were aliquoted and stored in −70 °C until used. The concentration of proteins in the microsomal preparations was determined by the Bradford colorimetric method.

In Vitro Dephosphorylation Using λ-PPase—All steps of in vitro dephosphorylation reactions were performed under dim red light. Briefly, 20 µg of total microsomal proteins from etiolated seedlings were mixed with 400 units of λ-PPase (New England Biolabs) in manufacturer-supplied buffer supplemented with 2 mM MnCl2, 0.5 mM 4-(2-aminoethyl)benzene-sulfonate hydrochloride, and 0.5% Triton X-100 and incubated on ice for 5 min and then at 30 °C for 30 min. Reactions were terminated by the addition of 0.25 volumes of 4× electrophoresis sample buffer (180 mM Tris-HCl (pH 6.8), 40% glycerol, 4% SDS, 0.04% bromphenol blue, and 200 mM dithiothreitol). The samples were then heated at 90 °C for 5 min and subjected to SDS-PAGE as described below.

SDS-PAGE and Immunoblot Analysis—Total microsomal proteins (12 µg/sample) were separated on 9% SDS-polyacrylamide gels (30) until the 72 kDa-specific band of the prestained molecular mass marker (Fermentas, Hanover, MD) reached the end of the gel. SDS-PAGE-separated proteins were then transferred to a nitrocellulose membrane by electroblotting in 192 mM glycine, 25 mM Tris, and 20% methanol.

For immunodetection, the membrane was first blocked using 5% (w/v) dry milk in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween 20 (Tris-buffered saline (TBS)/Tween) for 3 h. This was followed by incubation with primary antibody raised against the C-terminal region of NPH3 (1:5000 dilution) (10) for 2 h in TBS/Tween plus 1% dry milk. The membrane was then washed three times with TBS/Tween, followed by incubation with secondary antibody. The secondary antibody used in this study was alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega Corp., Madison, WI) diluted to 1:15,000 in TBS/Tween plus 1% dry milk. Finally, the membrane was washed four times with TBS/Tween (without dry milk), and NPH3-specific bands were detected by chemiluminescence using Immobilon AP substrate (Millipore Corp. Billerica, MA).

RESULTS

Rapid, Reversible, and Light-dependent Post-translational Modification of NPH3—A previous study has shown that Arabidopsis NPH3 can exist in different modified states depending upon whether seedlings are grown and kept in darkness or exposed to light (10). In particular, NPH3 present in etiolated seedlings treated with BL exhibits increased mobility upon SDS-PAGE relative to NPH3 in seedlings kept in darkness or exposed to red light (10). In this study, we examined in detail several properties of this BL-dependent change in NPH3 mobility.

We first examined the fluence rate and time dependences of this response by performing immunoblot analyses on total microsomal proteins isolated from 3-day-old etiolated seedlings exposed to various fluence rates of BL for different periods of time. NPH3 exhibited a clear shift in electrophoretic mobility in membranes from seedlings exposed to very low (0.01 µmol m−2 s−1), low (0.1 µmol m−2 s−1), intermediate (1 µmol m−2 s−1), and high (10 µmol m−2 s−1) fluence rates of BL for as little as 5 min (Fig. 1A and supplemental Fig. S1). In fact, we observed little, if any, fluence rate dependence for the formation of the higher mobility state of NPH3 (hereafter referred to as light-state of NPH3 (NPH3LS)) over the complete range of light intensities examined. Additional time course studies over a narrower range of irradiation times from 5 to 30 min indicated that the maximal levels of NPH3LS accumulated sometime between 5 and 10 min after the start of BL exposure (Fig. 1B), although a shift in mobility could be detected after as little as 1 min of irradiation (Fig. 1, A and C).

It is interesting to note that, after 240 min of BL exposure, some portion of immunoreactive NPH3 appeared to exhibit slightly retarded mobility compared with that of NPH3LS observed at 30 min (Fig. 1A). Such a change in mobility could result either from a reduction/reversal in whatever modification is causing the dark-state NPH3 (NPH3DS)-to-NPH3LS change or from a simple increase in protein abundance. To test the latter possibility, the time course experiment at 1.0 µmol m−2 s−1 BL was repeated in the presence of cycloheximide to prevent de novo synthesis of NPH3. Because the mobility of NPH3 was indistinguishable between 30 and 240 min in cycloheximide-treated samples (Fig. 1C and supplemental Fig. S1), we concluded that the “thicker” band observed at 240 min in the samples without cycloheximide (Fig. 1A) resulted from a de novo increase in NPH3 protein abundance rather than an alteration in post-translational modification.

We next investigated whether NPH3DS could be recovered from NPH3LS by placing in vivo BL-treated seedlings back into darkness. Etiolated seedlings were irradiated for 2 h with 1.0 µmol m−2 s−1 BL and then returned to darkness for various times (Fig. 1D). Indeed, we did observe nearly complete recovery of NPH3DS from NPH3LS in the absence or presence of cycloheximide (Fig. 1, E and F). The recovery of NPH3DS from NPH3LS in darkness was visible within 5 min independent of cycloheximide treatment. Interestingly, the recovery of NPH3DS from NPH3LS in darkness followed the same kinetics as that observed for the conversion of NPH3DS to NPH3LS in BL (Fig. 1, compare B and F). Together, these results clearly illustrate that the observed modification of NPH3 is post-translational, dependent upon light, and reversible.

Upon long-term (e.g. 12–24 h) exposure to BL, phot1 is known to move from plasma membrane to cytoplasmic locations (31), likely endomembrane compartments (32). Such prolonged light treatment also renders phot1 less immunodetectable in immunoblot analyses (32). In contrast, NPH3 was detectable as NPH3LS after prolonged exposure to BL for 24 h at 2.0 µmol m−2 s−1 at levels comparable to those observed in dark-grown samples (data not shown). Moreover, treatment of seedlings for 12 h with BL followed by transfer to darkness for 12 h allowed reversion of NPH3LS back to NPH3DS, similar to what we observed upon short-term light/dark treatments (data not shown).

Dark and Light States of NPH3 Represent Different Phosphorylated Forms of the Protein—It has been proposed previously that the post-translational modification(s) leading to altered mobility of NPH3 upon SDS-PAGE might reflect differing
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Phosphorylation states of the protein (10). We therefore examined the influences of PPase inhibitors on the state change of NPH3 in response to BL. If the conversion of NPH3DS to NPH3LS indeed reflects a dephosphorylation event, we should be able to prevent this conversion by pharmacologically blocking the activity of PPases (Fig. 2A).

As shown in Fig. 2B, we found that OKA failed to block the BL-dependent conversion of NPH3DS to NPH3LS in 3-day-old etiolated seedlings at concentrations below 1 μM, whereas at 1 μM, this state change was almost entirely prevented. These results indicate that NPH3 in dark-grown seedlings (NPH3DS) exists as a phosphorylated species. Moreover, given that OKA is a cell-permeable toxin that preferentially inhibits PP2A (IC50 = 0.1–1.0 nM) compared with PP1 (IC50 = 10–100 nM) (33, 34) and has no effect on PP2C (35), these results also suggest that the conversion of NPH3DS to NPH3LS in response to BL treatment occurs through dephosphorylation, likely mediated by either PP1 or PP2A.

To further investigate the role of PP1 and PP2A in the dephosphorylation of NPH3DS, we employed two additional selective PP1/PP2A inhibitors: CN and ET. Both of these are potent membrane-permeable inhibitors with higher specificity for PP2A (CN, IC50 = 40 nM; and ET, IC50 = 970 nM) than for PP1 (CN, IC50 = 473 nM; and ET, IC50 = 5 μM) (36–38). As shown in Fig. 2 (C and D), treatment of seedlings with either CN or ET at concentrations >1 μM inhibited the BL-dependent conversion of NPH3DS to NPH3LS, with complete blockage of the state change occurring at ~50 μM. These pharmacological studies are consistent with the interpretation that the BL-dependent formation of NPH3LS results from the dephosphorylation of NPH3DS and that the dephosphorylation response is mediated by either PP1 or PP2A.

NPH3LS Can Be Generated from NPH3DS by Treatment with λ-PPase in the Absence of BL—Because PP1 and PP2A inhibitors were able to prevent the in vivo BL-dependent conversion of NPH3DS to NPH3LS, we next investigated whether the state change could take place in the absence of light by simply exposing microsomal membranes to an active PPase in vitro. λ-PPase was chosen for these assays because it represents a highly active enzyme that has been shown to hydrolyze the phosphate group from seryl, threonyl, tyrosyl, and histidyl residues (39). Fig. 2E clearly demonstrates that the conversion of NPH3DS to NPH3LS can occur in the absence of BL in vitro, provided that...
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FIGURE 2. BL-induced conversion of NPH3DS to NPH3LS requires PPase activity. A–D, 3-day-old etiolated wild-type Arabidopsis seedlings (Col-0) grown in liquid culture were treated with the indicated concentrations of the PPase inhibitors OKA, CN, and ET for 2 h and then either mock-irradiated or irradiated with BL (1.0 μmol m⁻² s⁻¹) for 2 h. Treatments with equal volumes of solvent (Me₂SO (DMSO) or water) used to dissolve the inhibitors were also included with every inhibitor treatment as a control. Total microsomal proteins were incubated with the buffer alone (Fig. 2E). Moreover, when sodium fluoride and sodium orthovanadate, common chemical inhibitors of PPases (40), were used in the assay, λ-PPase failed to dephosphorylate NPH3 (Fig. 2E). These results are consistent with the conclusion that the interconversion of NPH3 between NPH3DS and NPH3LS results from the dephosphorylation/phosphorylation of the protein.

PP1 and PP2A Inhibitors Perturb Phototropic Curvature—A previous study has shown that BL-dependent proton pumping and stomatal opening in Vicia guard cells, processes since shown to be dependent upon phototropins (14), are suppressed by PPase inhibitor treatment (41). We therefore investigated whether the phot1-dependent phototropic curvature of Arabidopsis seedlings could be abrogated by PPase treatment (Fig. 3, A–D).

Intriguingly, we found that preincubation of seedlings in 50 μM CN, a concentration that completely abolished the dephosphorylation of NPH3DS (Fig. 2C), also abolished hypocotyl phototropism (Fig. 3, A and B). At lower CN concentrations, the inhibitory effects on both responses were reduced similarly (Figs. 2C and 3, A and B). The effects of ET were similar to those of CN in that concentrations inhibiting phototropism most effectively (Fig. 3C) were similar to those most efficient in inhibiting in vivo dephosphorylation of NPH3DS (Fig. 2D). In contrast to the effects of CN and ET, a concentration of OKA that maximally inhibited the conversion of NPH3LS to NPH3DS (1 μM) (Fig. 2B) only minimally affected BL-induced phototropism (Fig. 3D). It has been reported previously that exposure of OKA to UV light (300–400 nm) results in its degradation (42). As our BL source contains a trace amount of UVA light (364 nm peak, comprising <2% of the total incident quanta), the tempered effects of OKA on phototropic curvature compared with those on the dephosphorylation of NPH3DS observed here may reflect reduced levels of active OKA present during the longer BL exposures utilized in the former experiments (12 h versus 2 h). We have also observed that exposure to higher concentrations of OKA did not enhance the repressive effects of the inhibitor (data not shown), consistent with this conclusion.

Although the aforementioned results suggest that the phosphorylation state of NPH3 is linked with phototropic responsiveness, one could argue that exposure of intact seedlings to the high concentrations of PPase inhibitors simply results in repression of basal metabolism such that hypocotyl cell elongation is reduced to such an extent that differential growth for phototropic curvature is also impaired. However, this appears not to be the case relative to the present experiments because hypocotyl elongation was not dramatically affected by exposure and NaF, were added at 100 and 50 mm, respectively, to duplicate reactions to rule out nonspecific degradation in the sample. Reactions were terminated by the addition of SDS-PAGE loading buffer, followed by boiling. Total microsomal proteins from seedlings treated with BL (1.0 μmol m⁻² s⁻¹) 2 h are shown in the last lane as a control to represent the in planta converted NPH3DS state. The asterisk indicates the lane containing the input protein sample from etiolated seedlings containing NPH3DS used for in vitro dephosphorylation reactions. NPH3-specific bands were detected by immunoblotting using an anti-NPH3 antibody. Dashed lines in B–E depict the lowest mobility edge of NPH3DS.
to PPase inhibitors (Fig. 3, E–G). For example, 10 μM CN had a slightly significant effect on total hypocotyl elongation (Fig. 3E) and inhibited phototropism by ~50% (Fig. 3A), whereas exposure to 50 μM CN completely inhibited hypocotyl phototropism (Fig. 3A), but did not significantly influence total elongation (Fig. 3, B and E). Collectively, the pharmacological results are consistent with a model in which the BL-dependent dephosphorylation of NPH3DS and development of phototropism are linked and indicate that the former may be a prerequisite for the establishment of the latter.

**BL-dependent Dephosphorylation of NPH3DS Requires Functional phot1**—Not only are NPH3 and phot1 localized in the same cellular compartment, but they also interact with each other physically (10–12). With deference to this knowledge and to the fact that phot1 mediates phototropic responsiveness under both low and high fluence rate BL conditions (8) that are capable of stimulating NPH3 dephosphorylation (Fig. 1), we wished to determine the role of phot1 and other photoreceptors in the BL-dependent conversion of NPH3DS to NPH3LS. As shown in Fig. 4A, unilateral BL exposure stimulated normal NPH3DS-to-NPH3LS conversion in etiolated seedlings containing functional phot1 (Col-0 and phot2-1 lanes). However, only lower mobility NPH3, indistinguishable in mobility from NPH3DS in mock-irradiated wild-type seedlings (Fig. 4A, lane 1), was observed in seedlings lacking phot1 (Fig. 4A, phot1-5 and phot1-5 phot2-1 lanes). The results presented in Fig. 4B demonstrate that NPH3 in seedlings lacking phot1 was in fact present as the phosphorylated form (NPH3DS) independent of in vivo BL exposure because in vitro λ-PPase treatment of microsomal membranes isolated from such seedlings resulted in the conversion of NPH3 to a higher mobility state (NPH3LS), as occurred with λ-PPase-treated wild-type microsomes (Fig. 2E). It thus appears
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In planta BL-induced dephosphorylation of NPH3DS requires phot1. A, wild-type Arabidopsis Col-0, phot1-5, phot2-1, and phot1-5 phot2-1 seedlings were grown in darkness for 3 days and then either mock-irradiated (0) or exposed to BL (0.1 or 2.0 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) for 2 h. Total microsomal proteins were extracted and separated by SDS-PAGE. NPH3 was detected by immunoblotting with an anti-NPH3 antibody (\( \alpha \)-NPH3). B, shown is the in vitro dephosphorylation of total microsomal protein samples from Col-0 and phot1-5 seedlings using \( \lambda \)-PPase. Phosphatase reactions on microsomal samples from mock- and BL-irradiated seedlings were terminated by the addition of SDS-PAGE loading buffer. Samples were then separated by SDS-PAGE and subjected to immunoblot analysis. NPH3 was detected with an anti-NPH3 antibody. C, etiolated 3-day-old Col-0, phot2-1, pks1 pks4, and phyA phyB cry1 cry2 seedlings were mock-irradiated or exposed to BL (2.0 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) for 2 h, followed by total microsomal protein extraction, SDS-PAGE, and immunoblot analysis with an anti-NPH3 antibody. Dashed lines depict the lowest mobility edge of NPH3DS.

That phot1 is dispensable with respect to NPH3 phosphorylation, but is necessary for the BL-dependent dephosphorylation of NPH3DS.

In addition to phot1, NPH3 has also been shown to physically interact with RPT2 (11) and PKS1 (12). To understand the role these NPH3-interacting proteins might play in the state change of NPH3 in response to BL, we examined the phosphorylation state of NPH3 in rpt2 and pks1 pks4 seedlings treated with BL at 2.0 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for 2 h. As shown in Fig. 4C, the NPH3DS-to-NPH3LS transition occurred normally in both rpt2 and pks1 pks4 backgrounds, indicating that neither RPT2 nor PKS1 is essential for this phot1-dependent response. Because the BL-absorbing cryptochromes cry1 and cry2 and the predominantly red-light-absorbing phytochromes phyA and phyB are known to function as secondary receptors in the modulation of phot1-dependent phototropism (44–47), we also examined the possibility that these photoreceptors could influence the phosphorylation state of NPH3. However, as shown in Fig. 4C, the BL-dependent conversion of NPH3DS to NPH3LS was completely unaffected in a phyA phyB cry1 cry2 quadruple mutant, indicating that the influence of cry1, cry2, phyA, and phyB on phot1-dependent phototropism does not occur through alterations in the phot1 regulation of NPH3 phosphorylation status. Taken together, the results presented in Fig. 4 demonstrate that the primary phototropic photoreceptor, phot1, is the only essential receptor regulating the phosphorylation state of NPH3.

**DISCUSSION**

Reversible protein phosphorylation is arguably one of the best understood and most common post-translational means of regulating cellular processes (48–50). Quite often, the phosphorylation state of a given protein alone determines the “on/off” state of a given signaling process (51–54). In this regard, it is intriguing to note that the three major morphogenic photoreceptor types in plants (cryptochrome, phytochrome, and phototropin) exhibit protein kinase activity (4, 55–61) and that protein phosphorylation/dephosphorylation events are associated with their signaling pathways (5, 62–66). In this study, we have demonstrated that the phot1-interacting protein NPH3 is a phosphoprotein in etiolated seedlings and that the BL-induced dephosphorylation of this protein may be necessary for phot1-dependent phototropic signaling.

To date, just one genetic locus that is apparently indispensable for BL-induced phototropism has been identified: NPH3 (10, 11, 15–17). This, together with the knowledge that the NPH3 protein interacts physically with phot1 (10, 11, 12) and at least two other presumed signaling components, RPT2 (11) and PKS1 (12), makes NPH3 a likely target for regulation to modulate phototropic signal output. Previous analysis had shown that the electrophoretic mobility of NPH3 from etiolated seedlings irradiated in vivo with BL was enhanced compared with that of NPH3 from mock-irradiated siblings when resolved by SDS-PAGE, consistent with some type of post-translational modification, possibly phosphorylation (10). The results presented here clearly demonstrate that differing mobility states are in fact a result of different phosphorylation states of NPH3, the dark state (NPH3DS) being more phosphorylated than the light state (NPH3LS) (Figs. 1 and 2).

Although we have yet to identify the specific residues exhibiting differences in phosphorylation state between NPH3DS and NPH3LS, it is likely that seryl/threonyl groups are targets because the BL-dependent dephosphorylation of NPH3DS is sensitive to three different compounds (OKA, CN, and ET) (Fig. 2, B–D) that inhibit PPases with specificity for phosphoserine and phosphothreonine (67). The compounds tested here inhibit both type 1 (PP1) and type 2A (PP2A) PPases, with each inhibitor having ∼5–10-fold higher specificity for PP2A than for PP1 (34, 37, 38). On the basis of the observed concentration dependences for the inhibitor effects on the BL-induced dephosphorylation of NPH3DS (Fig. 2, B–D), we conclude that this process is likely mediated by PP1. The finding that exposure of seedlings to micromolar levels of CN and ET also inhibited phot1-dependent phototropism (Fig. 3, A–C) without dramatically impinging upon hypocotyl elongation (Fig. 3, E and F) is consistent with a model in which the PPase-dependent dephosphorylation of NPH3DS is a necessary component of the phototropic response (Fig. 5).

The involvement of a PPase in phototropin signaling may not be that surprising when one considers that PPases appear to play a role in a variety of photoresponses. For example, PP2A,
it appears that PP1 represents a common element in what otherwise appear to be distinct phot1-dependent signaling pathways (4, 5, 65).

The finding that the BL-induced conversion of NPH3\textsuperscript{DS} to NPH3\textsuperscript{LS} does not occur in seedlings lacking phot1 (Fig. 4A) but can occur in phot1 mutant extracts treated with λ-PPase (Fig. 4B) is interesting for two reasons. First, the latter result indicates that NPH3\textsuperscript{DS} present in the phot1 mutant background in fact exists as a phosphorylated protein and that phot1 activity is not required for its phosphorylation in darkness. The observation that the immunoreactive NPH3 protein present in etiolated phot1 phot2 double mutants exhibits an electrophoretic mobility indistinguishable from that of NPH3\textsuperscript{DS} in the wild type and phot1 single mutants (Fig. 4A) suggests that phot2 is also dispensable with respect to the phosphorylation of NPH3 in darkness. These results imply that a protein kinase(s) other than a phototropin must also interact with NPH3 at some time to mediate phosphorylation of the NPH3 protein (Fig. 5). The observed rapid dark recovery of NPH3\textsuperscript{DS} from NPH3\textsuperscript{LS} in wild-type seedlings (Fig. 1, E and F) suggests that the protein kinase acting on NPH3 may in fact be "constitutively" present (Fig. 5). Thus, the phosphorylation state of NPH3 at any given point in time is likely to reflect the balance between constitutive phosphorylation and BL-induced, phot1-dependent dephosphorylation, where the PPase activity is apparently dominant to the kinase activity (Fig. 5).

Second, the failure of NPH3\textsuperscript{DS} to become dephosphorylated in response to BL in phot1 mutant backgrounds indicates that phot1 is required, however, for the presumed PP1 to act on NPH3\textsuperscript{DS} (Fig. 5). On the basis of the observed electrophoretic mobilities upon one-dimensional SDS-PAGE, we estimate that the fluence threshold for the dephosphorylation of NPH3\textsuperscript{DS} is between 1 and 3 μmol m\textsuperscript{-2} (Fig. 1A). Although this fluence threshold is at least an order of magnitude lower than that necessary for BL induction of bulk autophosphorylation of phot1 in etiolated Arabidopsis seedlings as estimated from SDS-PAGE mobility (73), partial autophosphorylation of phot1 does occur at lower fluences (e.g. 1 μmol m\textsuperscript{-2}) (supplemental Fig. S1C) (74). There is also similarity in the temporal requirements for BL-induced phot1 autophosphorylation and NPH3\textsuperscript{DS} dephosphorylation, as well as dark recovery of the light sensitivity of the two responses (Fig. 1) (73). These overlapping photobiological properties of phot1 protein kinase activation and presumed PP1-dependent dephosphorylation of NPH3\textsuperscript{DS} suggest that activation of the protein kinase domain of phot1 may be a prerequisite for activation of the PPase (Fig. 5). This conclusion is certainly in harmony with the findings that NPH3\textsuperscript{DS} remains phosphorylated in phot1 mutant backgrounds (Fig. 4A) and that phot1 kinase activity is required for phototropic responsiveness (26).\textsuperscript{4}

But how does phot1 activation stimulate the PPase action on NPH3\textsuperscript{DS}? It seems appropriate to consider this question within the context of two broad alternative regulatory means: direct versus indirect. Direct regulation would require photoactive phot1 to physically interact with a PPase to regulate its ability to

\textsuperscript{4} E. Liscum, unpublished data.
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dephosphorylate NPH3\textsuperscript{DS}. Although there is currently no evidence for an interaction between a PPase and phot1, such an interaction cannot be formally discounted. So how could an interaction between phot1 and a PPase modulate the activity of the latter? Given the protein activity of phot1, one can envision a mechanism by which a PPase is converted from an inactive to active state via direct phosphorylation by photoactive phot1. Although certainly not unheard of, PPases are not typically regulated through phosphorylation by a protein kinase (75–78). Alternatively, phot1 could exhibit an allosteric inhibition on an associated PPase in darkness that is relieved in response to conformational changes in phot1 as a result of light absorption (79–82). Although allosteric regulation of PPase activity is very common (83, 84), the observation that NPH3\textsuperscript{DS} is not dephosphorylated in the phot1 mutant background (Fig. 4A) indicates that simple removal of phot1 is not sufficient for derepression of the PPase. Thus, if phot1 is functioning as a direct allosteric regulator of a PPase that acts on NPH3\textsuperscript{DS}, it must do so as both an inhibitor and an obligate facilitator of the PPase. Although we cannot ignore this possible mode of regulation, it seems unnecessarily complex.

As opposed to direct regulation, phot1 could influence the activity of the presumed PP1 that dephosphorylates NPH3\textsuperscript{DS} through an indirect means that does not require physical interaction between phot1 and the PPase, yet such indirect regulation would be expected to involve similar phot1-dependent mechanisms as described above for direct regulation, the major difference being that at least one additional factor would be required for the transfer of signal from phot1 to the PPase.

Relative to an allosteric mode regulation, light-induced conformational changes in phot1 could alter the interaction with and activity of an allosteric regulator of a PPase to allow activation of the latter. phot1 would have just one required role in an indirect allosteric mode of regulation, viz. alteration of the allosteric regulator (either an inhibitor or activator). This is in stark contrast to a direct mode of regulation, where phot1 must act as both an inhibitor and activator. In the absence of a non-phot1 substrate for the phot1 protein kinase (4), we propose that phot1 regulates the activity of a PPase that targets NPH3\textsuperscript{DS} for dephosphorylation through an indirect influence on an allosteric regulator of the PPase (Fig. 5).

Perhaps the most intriguing question to arise from this study is not how phot1 mediates the dephosphorylation of NPH3\textsuperscript{DS}, but rather how the conversion of NPH3\textsuperscript{DS} to NPH3\textsuperscript{LS} promotes phototropic signaling and response. The genetic requirement for NPH3 in phototropic responsiveness is well known (10, 11, 15–17, 85), yet data presented here suggest that it is not just the mere presence of NPH3 that is required for signal progression, but that the proper post-translational state (NPH3\textsuperscript{LS}) must exist (Fig. 3). To recap, NPH3\textsuperscript{LS} is generated through a BL- and phot1-dependent dephosphorylation of NPH3\textsuperscript{DS} (Figs. 1 and 4). So what could the dephosphorylation of NPH3\textsuperscript{DS} do to trigger phototropic signaling?

As introduced earlier, although novel and plant-specific, NPH3 is a B boxes domain-containing protein (10, 15, 21) that has been proposed to function as a core component of a CUL3-based E3 complex (5). It is intriguing to note that the activity of E3 ligases can be influenced by phosphorylation (86). For example, it is well documented that the phosphorylation state of many cell cycle control proteins determines their recognition and subsequent ubiquitination by E3 ligases (87, 88). More directly, the phosphorylation state of E3 components themselves can determine ligase activity (43, 89–92). It therefore seems plausible that the light/dark interconversion of NPH3 between its phosphorylated and dephosphorylated forms acts as a switch to regulate the activity of a CUL3-based E3 complex of which NPH3 is part (Fig. 5). The finding that the dephosphorylation of NPH3\textsuperscript{DS} appears to be correlated with phototropism (Fig. 3, A–D) suggests that such an E3 will contain NPH3\textsuperscript{LS} when its function promotes phototropic signaling (Fig. 5).

To conclude, the results presented here indicate that the phototropic signaling protein NPH3 exists as a phosphorylated protein in darkness and is rapidly dephosphorylated, likely by PP1, in response to phot1 photoactivation by BL. It appears that the dephosphorylated form of NPH3 (NPH3\textsuperscript{LS}) is required for phototropic signal progression, possibly through alterations in the activity of a CUL3-based E3 containing NPH3 (Fig. 5). Future studies will be aimed at identifying the kinase and PPase involved in this regulatory process, the mechanism by which phot1 modulates the activity of that PPase, and the exact nature of the signal output from a NPH3–CUL3 protein complex.

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