PHYTOCHROME KINASE SUBSTRATE 1 is a phototropin 1 binding protein required for phototropism

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Phototropism, or plant growth in response to unidirectional light, is an adaptive response of crucial importance. Lateral differences in low fluence rates of blue light are detected by phototropin 1 (phot1) in *Arabidopsis*. Only NONPHOTOTROPIC HYPOCOTYL 3 (NPH3) and root phototropism 2, both belonging to the same family of proteins, have been previously identified as phototropin-interacting signal transducers involved in phototropism. PHYTOCHROME KINASE SUBSTRATE (PKS) 1 and PKS2 are two phytochrome signaling components belonging to a small gene family in *Arabidopsis* (*PKS1–PKS4*). The strong enhancement of *PKS1* expression by blue light and its light induction in the elongation zone of the hypocotyl prompted us to study the function of this gene family during phototropism. Photobiological experiments show that the PKS proteins are critical for hypocotyl phototropism. Furthermore, PKS1 interacts with phot1 and NPH3 *in vivo* at the plasma membrane and *in vitro*, indicating that the PKS proteins may function directly with phot1 and NPH3 to mediate phototropism. The phytochromes are known to influence phototropism but the mechanism involved is still unclear. We show that PKS1 induction by a pulse of blue light is phytochrome A-dependent, suggesting that the PKS proteins may provide a molecular link between these two photoreceptor families.

Arabidopsis thaliana | NONPHOTOTROPIC HYPOCOTYL 3 | photomorphogenesis photoreceptors

Plants' survival depends on their ability to orient growth appropriately at the very beginning of their development. Plants need to reach the light and start photosynthesis before the seed reserves have been exhausted. They determine their direction of growth by sensing and responding to the gravity vector and the direction of light. These processes are called gravitropism and phototropism (1–3). *Arabidopsis thaliana* hypocotyls use gravity in darkness to orient their growth in the soil. But as soon as the seedlings perceive a weak source of light, even under the soil, gravitropism is repressed and phototropism predominates (3–5). Under low fluence rates of blue light, phytochrome A (phyA) is the predominant photoreceptor that triggers repression of gravitropism (6, 7). Light direction is perceived by the phototropin family [phototropin 1 (phot1) and phototropin 2 (phot2) in *Arabidopsis*] of UV-A/blue light sensors (2, 8). Phot1 is necessary and sufficient under a weak source of blue light, whereas phot1 and phot2 act redundantly to mediate phototropism under high blue light (9). Phot1 and phot2 are not only required for phototropism but also for chloroplast movement, stomatal opening, and leaf flattening. Together, these responses all are believed to maximize photosynthetic light capture while minimizing photodamage (8, 10). Phototropin-mediated responses are thus particularly important for normal plant growth under extreme (very low or very high) light conditions (11–13).

Despite the obvious importance of phototropism, the signaling mechanisms operating downstream of light perception are poorly understood. Light triggers a conformational change in the photoreceptor that activates its protein kinase activity, but very few specific phototropism signaling components have been identified (2, 14, 15). NONPHOTOTROPIC HYPOCOTYL 3 (NPH3) and ROOT PHOTOTROPISM 2 (RPT2) function as signal transducers in phototropism signaling (16–19). They belong to a plant-specific family of proteins possessing a BTB/POZ (broad complex, tramtrack, bric à brac/pox virus, and zinc finger) and a coiled-coil domain, both thought to be involved in protein–protein interaction (16). *nph3* null mutants show no phototropic curvature at any blue light fluence rates, whereas the *rpt2* mutant is impaired in phototropism only at high fluence rates (17, 18). Phot1, NPH3, and RPT2 all are associated with the plasma membrane, particularly in elongating cells (16, 20). NPH3 and RPT2 can physically interact with phot1 and each other (16, 18). Moreover, COLEOPTILE PHOTOTROPISM 1, a rice homologue of NPH3, acts upstream of the redistribution of auxin induced by unilateral illumination of the seedling, further indicating that these proteins function early in this signaling pathway (19). In addition to these components specifically acting in phototropism signaling, establishment of a gradient of auxin responsiveness is required to initiate asymmetric growth associated with not only phototropism, but also gravitropism (21).

The phytochromes modulate phototropism through mechanisms that remain to be molecularly elucidated (4, 5). Here we show that the phyA signaling components PHYTOCHROME KINASE SUBSTRATE (PKS) 1 and PKS2 (22) and PKS4, another member of this gene family in *Arabidopsis* (23), are required for phototropism. PKS1 is localized at the plasma membrane and can form a complex with phot1 and NPH3. Physiological analysis of *pks1*, *pks2*, and *pks4* loss-of-function mutants demonstrates that the PKS proteins are necessary for normal phototropism under weak intensities of blue light. Hence our findings define the PKS proteins as components of phot1 signaling and suggest that the PKS proteins may represent a link between phytochrome and phototropin signaling.

Results

The PKS Proteins Are Crucial for Hypocotyl Phototropism Under Low Fluence Rates of Blue Light. PKS1 and PKS2 are phytochrome-binding proteins acting as components of the very low fluence response (VLFR) branch of phyA signaling (22, 24). PKS1 expression is transiently induced by light precisely in the elongation zone of the root and hypocotyl (22). Elongation zones contain cells that elongate in response to tropic stimulations to

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Abbreviations: phot1, phototropin 1; NPH3, nonphototropic hypocotyl 3; PKS, phytochrome kinase substrate; phyA, phytochrome A; VLFR, very low fluence response.

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induce organ curvature (1, 2). Light induction of *PKS1* in the hypocotyl elongation zone (22) and strong up-regulation of *PKS1* by blue light (Fig. 4, which is published as supporting information on the PNAS web site) prompted us to test the involvement of PKS1 in phototropism. *PKS1* belongs to a small gene family that appeared at the emergence of angiosperms and consists of four members in *Arabidopsis* (*PKS1–PKS4*) (23). To test for possible functional redundancy among members of this gene family we isolated T-DNA insertion lines disrupting the coding sequence of *PKS4* (Fig. 5, which is published as supporting information on the PNAS web site) and constructed all possible mutant combinations among *pks1*, *pks2*, and *pks4*. There is currently no insertional mutant available in the *PKS3* gene.

The hypocotyls of *phot1* seedlings are randomly oriented when irradiated with a low fluence rate of blue light from above, because phyA represses gravitropism and phototropism is completely impaired in the absence of phot1 (6). We took advantage of this clear phenotype to test whether the PKS proteins play a role in phototropism. The growth orientation profile was determined for all of the available loss-of-function *pks1*, *pks2*, and *pks4* single, double, and triple mutants by using WT Col-O, *phyA*, *phot1*, and *nph3* as controls (Fig. 1*A*). Seedlings were classified into groups according to the angle of their hypocotyl relative to vertical (0–20°, 20–40°, 40–60°, and >60°). As reported (6), *phyA* hypocotyls were even more vertically oriented than the WT, and *phot1* hypocotyls were randomly oriented (Fig. 1*A*). The *pks1*, *pks2*, and *pks4* single mutants were subtly less vertically oriented than the WT. This tendency of random growth behavior was more pronounced in *pks1pks2*, *pks2pks4*, and particularly in the *pks1pks4* double mutants. Interestingly, the growth orientation profile of *pks1pks2pks4* hypocotyls was as random as in *phot1*, suggesting that, as with *phot1* mutants, the *pks1pks2pks4* mutants responded neither to light direction nor to gravity (Fig. 1*A*). This genetic analysis showed that PKS1, PKS2, and PKS4 had a function in determining the growth direction of hypocotyls. They seem to act in a redundant way, with PKS4 playing the major role. The similarity between the *phot1* and *pks* double- and triple-mutant phenotype suggested that the PKS proteins act positively in phot1 signaling.

To examine further whether the PKS proteins were implicated in phototropism, WT, *phot1*, *nph3*, and *pks1pks2pks4* seedlings were treated with unilateral light. Seedlings were illuminated for 3 days with a lateral source of low intensity blue light, and the final growth orientations were measured (Fig. 1*B*). As observed (6), WT hypocotyls were phototropic, whereas *phot1* hypocotyls no longer responded to the directional blue light and had an inhibited gravitropic response (Fig. 1*B*). As expected from previous studies the phenotype of *nph3* mutants was very similar to that of *phot1* mutants (16). As with the *phot1* and *nph3* mutants, hypocotyls of the *pks1pks2pks4* triple mutant did not direct their growth toward blue light and had an inhibited gravitropic response. This result indicates that PKS1, PKS2, and PKS4 were essential for phototropism but not for inhibition of gravitropism under long-term blue-light irradiation. When the different *pks* single, double, and triple mutants were grown in darkness, hypocotyls grew against the gravity vector as did the WT, *phot1*, and *nph3* mutants, whereas the agravitropic *arg1* mutant (25) was more randomly oriented (Fig. 6, which is published as supporting information on the PNAS web site). Taken together our results indicate that the *pks* mutants have a normal gravitropic response in darkness but are deficient for phototropism during long-term irradiation.

To test whether the PKS proteins are required for phototropism in etiolated seedlings stimulated by a short blue-light treatment, dark-grown seedlings were exposed to blue-light pulses (Fig. 1*C*). Under these conditions phot1 functions as the essential blue-light receptor mediating perception of directional light (26, 27). *phyA* mutants show a reduced phototropic response possibly because phyA is required to inhibit gravitropism and/or because the phytochromes are required to modulate the level or activity of phototropism signaling components (3, 6). Interestingly PKS1 induction by a pulse of blue light was phyA-dependent (Fig. 4*C*). In accordance with these expectations *phot1* mutants exhibited no detectable phototropic response to pulsed irradiation, whereas *phyA* mutants were ≈50%

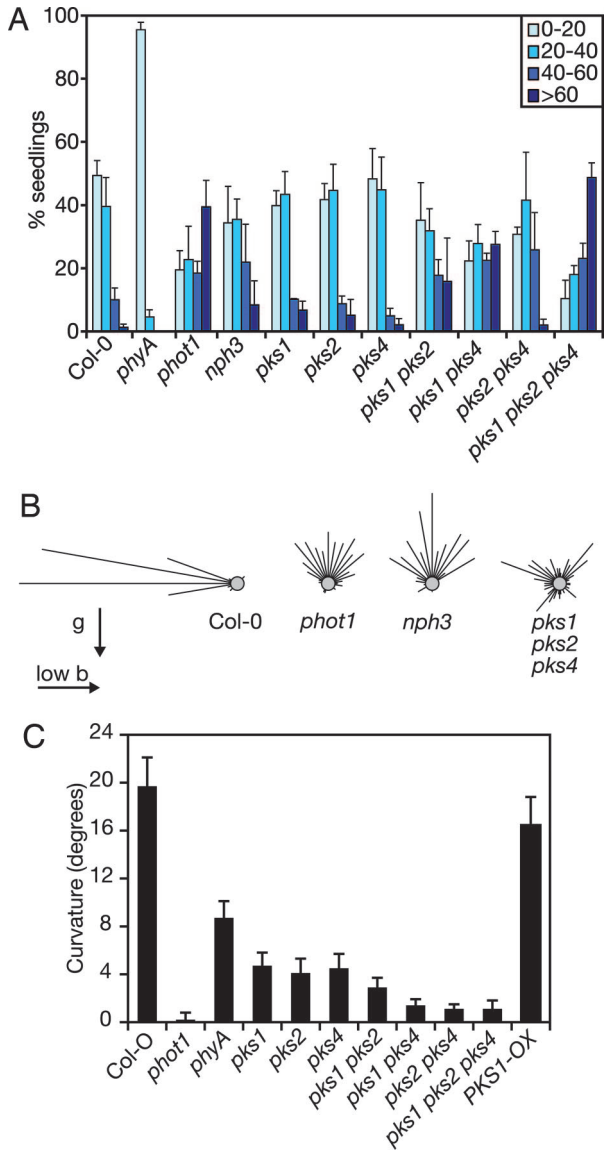

Fig. 1. PKS1, PKS2, and PKS4 are required for hypocotyl phototropism. (*A*) Hypocotyl growth orientation of Col-O, *phyA*, *phot1*, *nph3*, *pks1*, *pks2*, *pks4*, *pks1pks2*, *pks1pks4*, *pks2pks4*, and *pks1pks2pks4* grown on vertical plates for 3 days at 0.1 μmol·m^{-2}·s^{-1} blue light. The percentage of seedlings with hypocotyl angles relative to vertical (0°) is represented in four classes: 0–20°, 20–40°, 40–60°, and >60°. Data are average ± SD from three experiments with ≈50 seedlings for each experiment. (*B*) Hypocotyl growth orientation of Col-O, *phot1*, *nph3*, and *pks1pks2pks4* seedlings grown on vertical plates for 3 days subjected to continuous unilateral blue light (0.7 μmol·m^{-2}·s^{-1} coming from the left as indicated by the arrow). The repartition of the hypocotyl growth orientation is shown as circular histograms with 10° angle categories. The number of seedlings for each genotype is between 93 and 134. (*C*) Seedlings of the indicated genotypes were grown in darkness for 72 h and treated with six pulses of blue light (0.1 μmol·m^{-2} each) separated by 20 min of darkness. The hypocotyl phototropic curvature was determined 20 min after the last pulse. Data are average curvature angles ± SE with a minimum of 30 seedlings per genotype.

as responsive as the WT (Fig. 1*C*). The *pks* single and multiple mutants exhibited phototropic responses that fell between those of the *phot1* and *phyA* mutants (Fig. 1*C*). The role of the different PKS proteins appeared to be partially redundant with the double mutants having a more pronounced phenotype than the single mutants (Fig. 1*C*). If the PKS proteins were influencing phototropism solely through a phyA-mediated mechanism we would have expected the *pks* mutants to exhibit phototropic responses at least as robust as those of the *phyA* mutant. Our results thus suggest that under pulsed conditions the PKS proteins function mainly in the phot1-dependent pathway. This interpretation is also consistent with the growth orientation of *pks1pks2pks4* triple mutants under long-term irradiation that is similar to that of *phot1* but distinct from the one of *phyA* (Fig. 1 *A* and *B*) (6). It is important to point out that etiolated *pks* mutants do not have a hypocotyl growth phenotype, indicating that their phototropism phenotype is not the result of a growth defect (22) (data not shown). Finally, overexpression of PKS1 did not lead to an increase in phototropic curvature, indicating that a higher level of PKS1 was not sufficient to enhance this physiological response (Fig. 1*C*).

PKS1 Is a Plasma Membrane-Associated Protein. Our physiological analysis demonstrated that PKS1, PKS2, and PKS4 are required for phot1-mediated phototropism under low fluence rates of blue light (Fig. 1). Primary sequence analysis of the PKS proteins indicates that they are basic soluble proteins devoid of a domain with a known function (24). In an attempt to address the molecular function of the PKS proteins, we examined the subcellular localization of PKS1 by using transgenic lines expressing PKS1-GFP. *PKS1* mRNA is transiently light-induced in the elongation zone of hypocotyls and roots of etiolated seedlings (22). Microscopic examination of transgenic seedlings expressing *PKS1*:*PKS1-GFP* confirmed this observation at the protein level (Fig. 7, which is published as supporting information on the PNAS web site, and data not shown). By using confocal microscopy we observed that the PKS1-GFP signal was mainly at the periphery of the cells, distinct from the subcellular localization of soluble GFP but very similar to the subcellular localization of plasma membrane-localized GFP (Fig. 2 *A*–*C*). Interestingly, this tissue-level and subcellular localization of PKS1-GFP is very similar to that of phot1 and NPH3, which is associated with the plasma membrane (16), and strongly expressed in elongating cells of etiolated hypocotyls (20) (Fig. 8, which is published as supporting information on the PNAS web site).

To examine whether, like phot1 and NPH3, PKS1 was membrane-associated we prepared microsomal and cytoplasmic fractions from 3-day-old WT seedlings. Cell fractionations were performed either from etiolated seedlings or after an additional 4 h of white-light treatment to allow induction of PKS1 protein expression (22). Phot1 was used as a positive control for microsomal proteins (20). As described (20), phot1 was membrane-associated in dark-grown seedlings, and a small fraction of phot1 was released into the cytosol upon light exposure. PKS1 protein accumulation was induced in seedlings exposed to white light compared with seedlings grown in darkness (22) (Fig. 2*D*). In both conditions PKS1 was detected mainly in the microsomal fraction, suggesting that PKS1 was associated with membranes (Fig. 2*D*).

PKS1, like all of the other PKS proteins, lacks any obvious membrane attachment sequence (24). To determine whether PKS1 is a peripheral membrane protein, pellets of microsomal fractions were treated with high salt, alkali buffer, or the detergent Triton X-100. PKS1 could only be released from microsomes by solubilization with 1% Triton X-100 (Fig. 2*E*), suggesting that PKS1 is actually membrane-anchored rather than a peripheral membrane protein. This possibility was confirmed by using a Triton X-114 partitioning experiment that allows the separation of hydrophilic proteins in the aqueous phase from lipophilic proteins in the detergent phase (28) (data not shown).

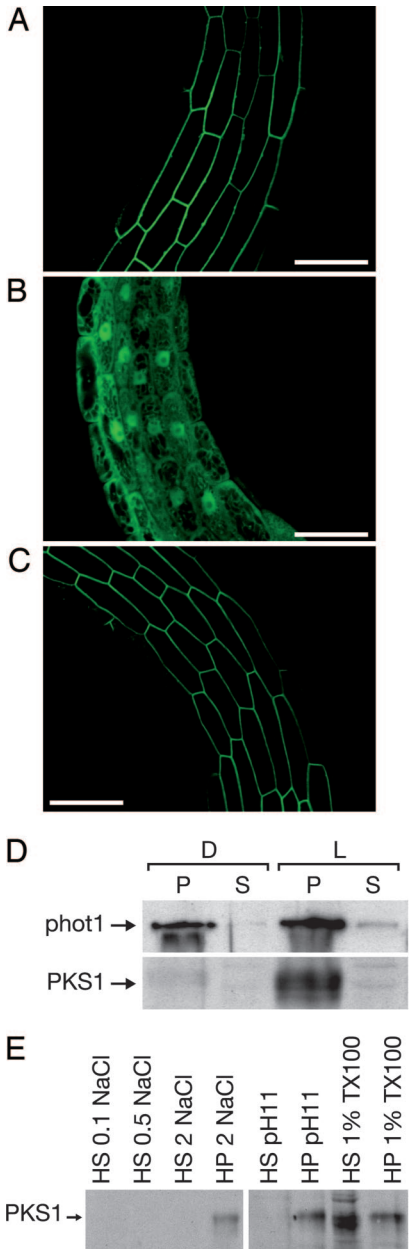

Fig. 2. PKS1 protein is associated with the plasma membrane. (*A*) Localization of PKS1-GFP in 2-day-old etiolated *35S:PKS1-GFP* seedlings. The seedling was imaged with a confocal microscope with a ×40 objective with a 488-nm excitation line. (*B*) Localization of GFP in 2-day-old etiolated *35S:GFP* seedlings. The seedling was imaged as above. (*C*) Localization of plasma membrane-targeted GFP in 2-day-old etiolated *35S:GFP-LTI6b* seedlings. The seedling was imaged as above. (Scale bars: 50 μm.) (*D*) Immunoblot analysis of PKS1 localization after cell fractionation of etiolated seedlings (D) or etiolated seedlings treated with 4 h of white light (L). Microsomal (P) or cytosolic (S) fractions were separated by SDS/PAGE, transferred onto nitrocellulose, and subjected to Western blot analysis by using phot1- or PKS1-specific antibodies. (*E*) Immunoblot analysis of PKS1 localization after solubilization of microsomal fractions from etiolated seedlings treated for 4 h with white light. Microsomal pellets were treated with 0.1 M NaCl, 0.5 M NaCl, 2 M NaCl, carbonate buffer (pH 11), or 1% Triton X-100. HS (high speed supernatant) corresponds to the solubilized proteins and HP (high speed pellet) corresponds to the proteins still associated with the microsomes. Those fractions were separated by SDS/PAGE, transferred onto nitrocellulose, and subjected to Western blot analysis using a PKS1-specific antibody.

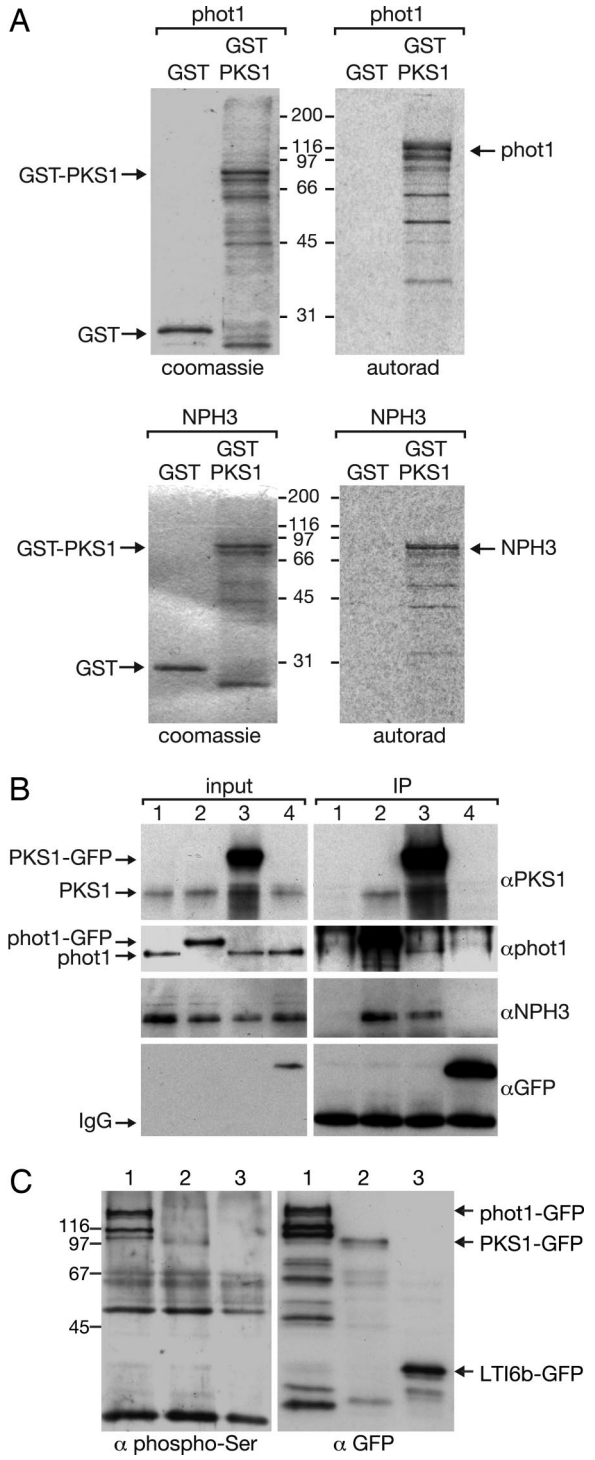

Fig. 3. PKS1 interacts with phot1 and NPH3 *in vitro* and *in vivo*. (*A*) PKS1 interacts with phot1 and NPH3 *in vitro*. Bacterially produced GST or GST-PKS1 were bound onto glutathione-agarose beads and incubated with *in vitro*-transcribed and -translated PHOT1 or NPH3. Beads were extensively washed, and proteins binding to the beads were eluted with reduced glutathione and separated by SDS/PAGE. (*Left*) The Coomassie blue-stained gels. (*Right*) Autoradiograms of the same gel. Note: GST-PKS1 is unstable in *Escherichia coli*, leading to a number of breakdown products in addition to the ≈80-kDa protein full-length fusion protein. *In vitro*-transcribed and -translated NPH3 and PHOT1 gave rise to a number of smaller proteins that are the result of either degradation or internal translation initiation. (*B*) PKS1 interacts with phot1 and NPH3 *in vivo*. Solubilized microsomal fractions were prepared from etiolated seedlings treated for 150 min with white light. The following genotypes were used in this assay: Col (lanes 1), *PHOT1*:*PHOT1-GFP phot1* (lanes 2), *35S*:*PKS1-GFP* (lanes 3), and *35S*:*LTI6b-GFP* (lanes 4). An aliquot was mixed 1:1 with 2XFSB (input). These extracts were immunoprecipitated with a covalently attached anti-GFP antibody coupled to agarose beads and specifically bound proteins eluted with 2XFSB (IP). Proteins were separated on SDS/PAGE, Western-blotted, and probed with various antibodies as described in *Materials and Methods*. (*C*) PKS1-GFP and phot1-GFP were phosphorylated *in vivo*. Immunoprecipitates of *PHOT1*:*PHOT1-GFP phot1* (lanes 1), *35S*:*PKS1-GFP* (lanes 2), and *35S*:*LTI6b-GFP* (lanes 3) were separated by SDS/PAGE and Western-blotted as above but probed with anti-GFP or anti-phospho-Ser/Thr antibodies.

Molecular Interactions Among PKS1, Phot1, and NPH3. Phot1, NPH3, and PKS1 all localize to the plasma membrane and are involved in phototropic responses (16, 20) (Figs. 1 and 2). We therefore decided to test whether those proteins interact with each other. We first examined whether PKS1 was able to bind phot1 and NPH3 *in vitro* by using a GST pull-down assay (Fig. 3*A*). Bacterially produced GST or GST-PKS1 fusions were bound to glutathione-agarose beads. The beads were incubated with ^{35}S-Met-labeled *in vitro*-transcribed and -translated PHOT1 or NPH3. The bound proteins were separated by SDS/PAGE, and the stained gel was subjected to autoradiography. This experiment showed that GST-PKS1 could interact with phot1 and NPH3, whereas GST did not interact with either of them (Fig. 3*A*).

To determine whether these interactions also occur *in vivo* we performed coimmunoprecipitation experiments with protein extracts from etiolated *Arabidopsis* seedlings treated for 150 min with white light to induce PKS1 expression and activate the phototropins (Fig. 3*B*). Microsomes were isolated from four different genotypes: Col-O, a transgenic line expressing the phot1-GFP fusion driven by the *PHOT1* promoter (*phot1*, *PHOT1*:*PHOT1-GFP*) (20), a line overexpressing PKS1-GFP (*35S*:*PKS1-GFP*) (24), and finally a line expressing a plasma membrane-localized GFP fusion protein (GFP fused to LTI6b) (29). Solubilized microsome extracts were incubated with an anti-GFP antibody linked to agarose beads. The beads were extensively washed, and the proteins specifically bound to the anti-GFP beads were analyzed by immunoblotting. Phot1-GFP, PKS1-GFP, and LTI6b-GFP all were present in the microsomal fraction and efficiently immunoprecipitated by the anti-GFP antibody (Fig. 3*B*). Given that the seedlings were exposed to white light before and during the immunoprecipitation experiment, we expected PKS1-GFP and phot1-GFP to be phosphorylated (2, 8). To test the phosphorylation status of phot1 and PKS1 we probed the immunoprecipitated proteins with an anti-phospho Ser/Thr antibody and an anti-GFP antibody as a loading control (Fig. 3*C*). PKS1-GFP and phot1-GFP were recognized by the anti-phospho Ser/Thr antibody, whereas LTI6b-GFP was not (Fig. 3*C*). This result indicates that phot1-GFP and PKS1-GFP were indeed phosphorylated during the *in vivo* immunoprecipitation and ruled out the possibility that the phosphorylation occurred on the GFP moiety.

Interestingly, PKS1 coimmunoprecipitated with phot1-GFP and phot1 coimmunoprecipitated with PKS1-GFP, whereas neither of those proteins were present in the Col-O and LTI6b-GFP controls, showing that phot1 and PKS1 interact *in vivo* (Fig. 3*B*). Moreover, NPH3 coimmunoprecipitated with both phot1-GFP and PKS1-GFP, confirming the previously described interactions between NPH3 and phot1 (16) and the *in vitro* interaction we observed between NPH3 and PKS1 (Fig. 3). Both NPH3 and PKS1 coimmunoprecipitated with phot1-GFP (Fig. 3*B*). Conversely, NPH3 and phot1 both coimmunoprecipitated with PKS1-GFP, whereas NPH3 protein was not pulled down in control WT and LTI6b-GFP-expressing seedlings (Fig. 3*B*). The three proteins were thus present as a complex in solubilized microsomes. Finally, PKS1-GFP and

phot1-GFP did not coimmunoprecipitate the membrane-associated protein DET3 (30), highlighting the specificity of the interactions observed here (data not shown). It is noteworthy that the phot1–PKS1 interaction was observed in phot1–GFP-expressing plants where both proteins were present at WT levels (20) (Fig. 3*B*). The physical interaction thus occurred *in planta* at physiological concentrations of the two proteins. Our molecular data thus indicate that PKS1, NPH3, and phot1 are likely to form a complex at the plasma membrane. This observation is entirely consistent with and supports our physiological data showing that the PKS proteins are important for phototropism.

Discussion

Our photobiological experiments establish an important role for the PKS proteins during hypocotyl phototropism. The facts that PKS1 and PKS2 act in phyA signaling (22) and that *phyA* mutants are impaired in phototropism (3–5) suggest, at first glance, that the phototropism phenotype of *pks* mutants may be exclusively caused by alterations in phyA signaling. Two distinct sets of observation make this hypothesis unlikely: first, *pks1* and *pks2* mutants have an increased phyA VLFR when treated with pulses of far red light, whereas the *pks1pks2* double mutant shows a normal VLFR (22). In contrast, in response to a pulse of blue light the *pks1* and *pks2* mutants have a weaker phototropic response than *phyA*, a phenotype that is further enhanced in the *pks1pks2* double mutant (Fig. 1*C*). The phenotypes of *pks1* and *pks2* mutants are thus distinct when comparing far red and blue light. The PKS proteins appear to function as negative regulators of the phyA-VLFR (22), but positive regulators of phototropism in blue light (Fig. 1). Second, under long-term irradiation experiments the *pks* mutants behaved similarly to *phot1* and *nph3* mutants and very differently from the *phyA* mutant (Fig. 1 *A* and *B*). We have previously proposed that the reduced phototropic response of *phyA* mutants results from a reduced inhibition of gravitropism (6, 7). However, in contrast to *phyA* mutants, *phot1*, *nph3*, and *pks1pks2pks4* mutants clearly show an inhibition of the gravitropic response but exhibit no phototropic response under long-term low fluence rate blue-light illumination (6, 7) (Fig. 1*B*). The most parsimonious interpretation of these results is that the PKS proteins are positive regulators of phot1 signal transduction in blue light.

Given that phototropin signaling components are differentially required for the different phototropin responses (2, 8), it will be interesting to test whether the PKS proteins are also important for additional phototropin responses. PKS1, PKS2, and PKS4 do not appear to control leaf flattening by themselves, because *pks1pks2pks4* triple mutants have WT leaves that are very easy to distinguish from the curled leaves of *phot1phot2* double mutants (Fig. 9, which is published as supporting information on the PNAS web site). Future experiments should determine whether the PKS proteins regulate chloroplast movements and stomatal aperture.

The interpretation of our genetic results functionally coincides with the tissue distribution, subcellular localization, and protein–protein interaction data obtained for PKS1 (Figs. 2, 3, and 7). PKS1, phot1, and NPH3 are highly expressed in the hypocotyl elongation zone (20, 22) (Figs. 7 and 8). All three proteins are rather tightly associated with the plasma membrane through a mechanism that remains to be identified (16, 20) (Fig. 2). Finally, PKS1 strongly interacts with phot1 and NPH3 both *in vivo* and *in vitro* (Fig. 3). The fact that phot1-GFP can interact with PKS1 *in vivo* when both proteins are expressed at WT concentrations is a strong indication that this interaction is physiologically meaningful.

The existence of phototropin signaling elements that would be induced by the phytochromes has been postulated (3). Phytochrome-mediated induction of *PKS1* and *PKS2* expression (22) may thus partly explain the reduced phototropism in *phyA* mutants in response to blue-light pulses (Fig. 1*C*) (27). Indeed, PKS1 protein and mRNA are light-induced by a phyA-dependent VLFR (22). Moreover, we have shown that a pulse of blue light induces PKS1 protein levels and that this induction is lost in *phyA* mutants (Fig. 4*C*). Thus a pulse of blue light suffices to trigger both phototropism and phyA-dependent induction of PKS1. There may be additional levels of regulation whereby the PKS proteins could enable a coordination of phytochrome and phototropin action. Our coimmunoprecipitation results indicate that, under our assay conditions, both phot1 and PKS1 were phosphorylated (Fig. 3*C*). Phosphorylation may thus represent another level of regulation of this interaction *in planta*. We would, however, like to point out that *in vitro*-transcribed and -translated phot1 can interact with bacterially produced PKS1 (Fig. 3*A*), suggesting that plant-specific phosphorylation is not a prerequisite for this interaction. Finally, given that PKS1 and PKS2 are capable of interaction with the phytochromes *in vitro* (22) and that our data show that PKS1 interacts with phot1 *in vivo* (Fig. 3*B*), the PKS proteins may represent a link between these two photoreceptor families that have long been known to cooperate during the early steps of phototropism (3, 4). Such a cooperation between the phytochromes and phototropins is not incompatible with the independent effects we have observed for phyA and phot1 in the control of hypocotyl growth orientation in long-term experiments (6).

Materials and Methods

Plant Material and Growth Conditions. The Columbia (Col-O) ecotype of *A. thaliana* was used as the WT. All of the mutant alleles were in the Col-O background. The mutants were the following: *phot1-5* (31), *phyA-211* (32), *nph3-6* (16), *arg1-42* (Salk T-DNA insertion allele in *ARG1* from the laboratory of P. Masson, University of Wisconsin, Madison), *pks1-1*, *pks2-1* (22), and *pks4-1* (this study). Seeds were surface-sterilized and plated as described (6). With the exception of pulse-light experiments (see below), experiments were performed with continuous blue light at 22°C as described (6).

Generation of Mutants. The *pks4-1* mutant was identified by PCR-screening 40,000 T-DNA insertion lines using the *PKS4* (At5g04190)-specific primer CF259 (5′-GGAATCATCTC-CCAAGTTCCCAACTCGTGA-3′) and the T-DNA-specific primer JMLB1 (5′-GGCAATCAGCTGTTGCCCGTCTCACT-GGTG-3′). The PCR conditions were as described (33). The exact insertion site, determined by sequencing the PCR product, was after the 114th codon. The kan^R/kan^S ratio indicated the presence of a single T-DNA in the line, and the line was backcrossed to Col-O before future analysis. *pks4-1* was genotyped by PCR using a primer pair that detects the presence of the T-DNA (JMLB1, 5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′ and CF329, 5′-CTTGGGACTCGTAGGATTCA-3′) and a primer pair to test for homozygocity (CF329 and CF262, 5′-CAATGGCG-CAAACTACTGTC-3′). The phenotypes observed for *pks4-1* were confirmed with *pks4*-2, a second allele obtained from the GABI collection (line 312E01) (I.S. and C.F., unpublished work) (34). *pks* double and triple mutants were obtained by crossing. Genotyping of *pks1* and *pks2* was performed as described (22), and *pks4-1* genotyping was performed as described above.

Hypocotyl Growth Orientation. For long-term irradiation experiments seedlings were grown on vertically oriented half-strength Murashige and Skoog plates treated and measured as described (6). Phototropism in response to pulses of blue light was performed as described (27).

Transgenic Plants. Transgenic lines expressing PKS1-GFP under the control of the *PKS1* promoter were obtained by cloning a

4.3-kb sequence 5′ of the PKS1 initiator ATG upstream of the PKS1-cDNA fused to GFP5-S65T and the rbcs terminator into pPZP212 (35) in the EcoRI and HindIII cloning sites, to give rise to pCF334. pCF202 is the same construct but with a 35S promoter. These constructs were transformed into Col-O by *Agrobacterium*-mediated transformation by using the spray method (36). Single insertion lines were selected based on the ratio of kan^R/kan^S and homozygous lines were used for the study. The *phot1-5*, *PHOT1:PHOT1-GFP* line was provided by Winslow Briggs (Carnegie Institution of Washington, Stanford, CA) (20). Seedlings overexpressing the GFP-LTI6b fusion and PKS1 were as described (29).

Microscopy and Biochemical Techniques. Detailed procedures are provided in *Supporting Text*, which is published as supporting information on the PNAS web site.

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