PKS1, a Substrate Phosphorylated by Phytochrome That Modulates Light Signaling in *Arabidopsis*

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Plants constantly monitor their light environment in order to grow and develop optimally, in part through use of the phytochromes, which sense red/far-red light. A phytochrome binding protein, PKS1 (phytochrome kinase substrate 1), was identified that is a substrate for light-regulated phytochrome kinase activity in vitro. In vivo experiments suggest that PKS1 is phosphorylated in a phytochrome-dependent manner and negatively regulates phytochrome signaling. The data suggest that phytochromes signal by serine-threonine phosphorylation.

Phytochromes are red/far-red photoreceptors that regulate many aspects of plant development (*1*). The prototypical phytochrome is a homodimer consisting of 125-kD subunits. The protein is composed of two major domains: an NH_2-terminal chromophore-binding domain that is sufficient for normal photochemistry, and a COOH-terminal domain required for dimerization and thought to be a signal output domain (*2*). Phytochromes are localized in the cytoplasm or the nucleus, depending on the light conditions and the type of phytochrome (*3*). Phytochromes are synthesized in a red light–absorbing form (Pr) in the dark; upon absorption of red light, they convert to the far-red light–absorbing form (Pfr). Pfr is thought to be the active form because for many phytochrome-mediated responses, far-red light can dampen the response by converting Pfr back to Pr.

In *Arabidopsis*, each of the five phytochromes (phyA through phyE) has both unique and redundant functions (*4*). Analysis of mutants has demonstrated that the COOH-terminus is essential for phytochrome function in vivo, but the biochemical mechanism of phytochrome signaling remains unclear (*2*). Although cyanobacteria has a phytochrome with light-regulated histidine kinase activity (*5*), the COOH-terminal domain of plant phytochromes is only weakly related to histidine kinases (*6, 7*). Biochemical data point toward serine-threonine kinase activity associated with phyA, and autophosphorylation of recombinant oat phyA is light-regulated (*7*). Phytochromes might therefore belong to a growing class of eukaryotic serine-threonine kinases with histidine kinase ancestry (*7, 8*).

To investigate the mechanism of phytochrome signaling, we conducted a yeast two-hybrid screen to identify proteins that interact with the COOH-terminal domain of phyA (*9*). This screen yielded a clone encoding the COOH-terminus of PHYA (*10*), which confirmed the ability of this portion of phyA to dimerize. We also identified three other genes capable of specific interaction with phytochromes; one of these, *PKS1*, is the subject of this report. Yeast two-hybrid experiments showed that the PKS1 protein interacts with the COOH-termini of both PHYA and PHYB (Fig. 1A). These interactions were confirmed using full-length phyA or phyB purified from yeast and in vitro transcribed and translated PKS1 (*11*). Although Pr and Pfr, the two spectral forms of phytochrome, have different protein conformations, PKS1 appears to bind both forms (Fig. 1B).

PKS1 encodes a basic soluble protein of 439 amino acids with no recognizable sequence motifs or signals for subcellular targeting (*12*) (Fig. 2A). Fluorescence microscopy showed that PKS1-GFP (green fluorescent protein) fusions are cytoplasmic (*10*). *PKS1* mRNA is expressed in young seedlings in both darkness and light; later in development, *PKS1* mRNA amounts decrease, with moderate expression in rosette leaves and very low expression in roots and flowers (Fig. 2B). This distribution pattern overlaps with the mRNA distribution of *PHYA-PHYE* in *Arabidopsis* (*13*).

PKS1 interacts with the COOH-termini of phyA and phyB in the region of highest homology with histidine kinases. Because oat phyA autophosphorylates on Ser/Thr (*7*) and because yeast two-hybrid screens have identified substrates for known protein kinases (*14*), we tested whether PKS1 is a substrate for the kinase activity of phytochrome in vitro. Recombinant oat phyA phosphorylated a glutathione S-transferase (GST)–PKS1 fusion protein (*15*). These experiments show that the extent of GST-PKS1 phosphorylation by phyA was light-regulated, with GST-PKS1 being a better substrate for Pfr's kinase activity than for Pr's kinase activity (Fig. 3A). Phytochrome autophosphorylation and PKS1 phosphorylation were stimulated by red light by a factor of 2 to 2.5. The residues phosphorylated on PKS1 were Ser and to a low extent Thr (*10*). Ser^{599} of oat phyA is phosphorylated preferentially as Pfr in vivo (*16*). When a mutant form of phyA (Ser^{599} → Lys, or S599K) was used for in vitro kinase assays, both autophosphorylation and phosphotransfer to PKS1 were no longer light-stimulated (Fig. 3A).

Thus, phyA exhibits both light-regulated autophosphorylation and PKS1 phosphotransferase activities. In vitro, phytochrome phosphorylates substrates such as histone H1, cry1, and cry2 (*17*), but not in a light-regulated manner. Our observation of light-modulated phosphoryl-

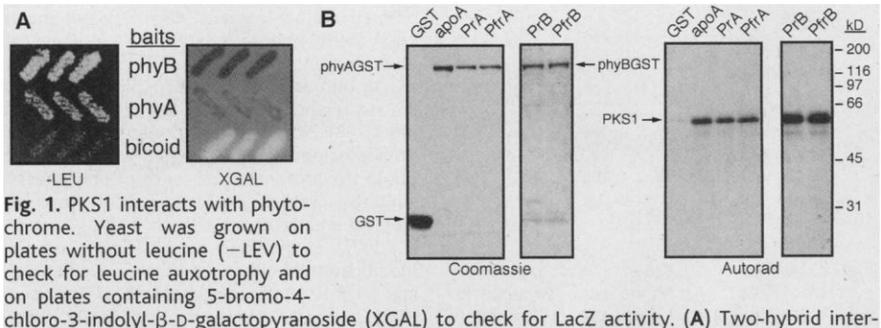

Fig. 1. PKS1 interacts with phytochrome. Yeast was grown on plates without leucine (−LEV) to check for leucine auxotrophy and on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGAL) to check for LacZ activity. (**A**) Two-hybrid interaction of PKS1 with the COOH-termini of PHYA and PHYB. (**B**) In vitro interaction of PKS1 with full-length phyA and phyB in Pr form (PrA, PrB) or in Pfr form (PfrA, PfrB). apoA refers to PHYA protein without chromophore. phyA and phyB were expressed as GST fusion proteins (phyAGST, phyBGST). Coomassie blue staining and autoradiography of the same gel are shown.

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ation in vitro suggests that PKS1 is a substrate of phytochrome's kinase activity in vivo. Analysis of PKS1 immunoprecipitates of light-grown seedlings with or without phosphatase treatment indicated that PKS1 is a phosphoprotein in vivo (*18*) (Fig. 3B). We have been unable to detect phyA or phyB coimmunoprecipitation under our assay conditions (*10*).

To assess whether PKS1 is phosphorylated in a light-dependent fashion, we performed PKS1 protein immunoblot analysis on dark-grown versus red light–grown seedlings (*19*). In the dark, PKS1 is mainly present as a faster migrating form, whereas in red light both slow- and fast-migrating forms of PKS1 can be seen. In red light–grown *Arabidopsis* plants that are overexpressing phyB, PKS1 is mainly present as a slower migrating form. Thus, PKS1 is phosphorylated in vivo in a phytochrome-dependent manner (Fig. 3C).

PKS1 maps to the top of chromosome II on bacterial artificial chromosome T17M13. No known photomorphogenic mutant maps to this region of the genome, and screening various T-DNA insertion collections did not yield any mutants disrupting the gene (*10*). Of 50 *PKS1* antisense lines analyzed by protein immunoblotting, nine had reduced amounts of PKS1 protein without affecting the plants' phenotypes (*10*), perhaps as a result of redundancy with *PKS1*-related sequences such as EST (T13904), which codes for an open reading frame 55% identical to PKS1 over the entire protein (*10*).

To assess whether PKS1 might be sufficient (if not essential) for an altered light response, we generated lines overexpressing PKS1 (*20*) (Fig. 4C). These lines had an elongated hypocotyl when grown in white light (Fig. 4, A and B); this phenotype is reminiscent of phytochrome-deficient mutants and is indicative of reduced sensitivity to light. Growth in different monochromatic lights indicated that PKS1-overexpressing lines were less sensitive to red light and retained normal sensitivity to blue and far-red light (Table 1). Because phyB is the major photoreceptor for red light, these results are consistent with PKS1 acting as an inhibitor of phyB signaling. In accordance with this interpretation, the PKS1 overexpression phenotype is only 28% as effective in a *phyB* mutant background, relative to the effect in the wild type (*10*). Interestingly, when PKS1 is overexpressed in *phyA* mutants, the phenotype is more pronounced than in a wild-type background (Fig. 4, A and B). In white light, the role of phyA in hypocotyl elongation is only uncovered in *phyB* mutants; thus, the PKS1 overexpression phenotype in *phyA* mutants is consistent with PKS1 being an inhibitor of phyB signaling.

Our results suggest that plant phytochromes signal through light-regulated protein kinase activity. Although the amino acid sequence of PKS1 does not suggest a biochemical function, these results confirm that oat phyA is an atypical serine-threonine kinase (*7*). Other prokaryotic-like histidine kinases found in eukaryotes phosphorylate Ser and Thr rather than His or Asp (*8, 21*). However, phytochrome phosphorylation may pass through a less stable phospho-His or phospho-Asp intermediate, as seen with mitochondrial α-ketoacid dehydrogenase kinase phosphorylation (*8*).

PKS1 appears not to associate differently with the two spectral forms of phytochrome; however, phosphorylation might be an im-

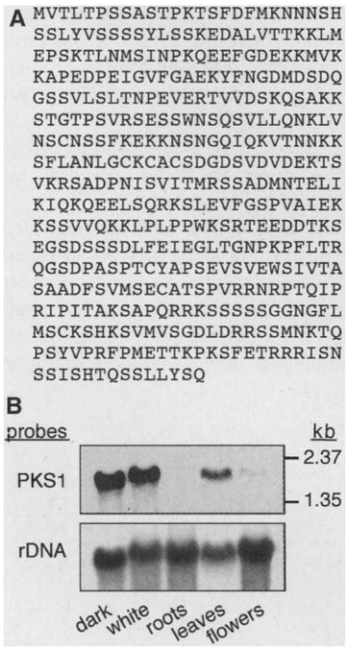

Fig. 2. Amino acid sequence and expression pattern of PKS1. (**A**) Amino acid sequence of the *PKS1* gene (*12*). This sequence has been deposited in GenBank (accession number AF149053). (**B**) Northern blot analysis of *PKS1* expression with 10 μg of total RNA for each lane.

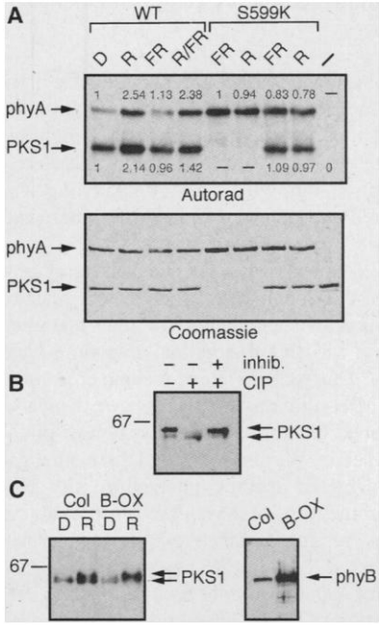

Fig. 3. Light-regulated PKS1 phosphorylation by phyA in vitro. Each kinase assay used 1 μg of oat phyA and 1 μg of GST-PKS1 or PKS1 alone (*15*). (**A**) GST-PKS1 is phosphorylated in a light-dependent fashion by oat phyA in vitro. Amounts of phyA phosphorylation are expressed relative to lane 1 [dark (D)]; amounts of phyA S599K phosphorylation are expressed relative to lane 5 [far-red (FR) light, without PKS1]; and amounts of PKS1 phosphorylation are expressed relative to lane 1 (D, wild-type phyA). (**B**) PKS1 is phosphorylated in vivo. Immunoprecipitated PKS1 treated with or without CIP in the presence or absence of phosphatase inhibitors was subjected to protein immunoblotting and detected with antibodies to PKS1. (**C**) PKS1 is modified by red light, and this modification is enhanced in lines overexpressing phyB. Protein immunoblots of wild-type (Col) and phyB-overexpressing (B-OX) lines grown in the dark (D) or in red light (R) were probed with antibodies to PKS1. The same lines grown in the dark were also probed with an antibody to phyB.

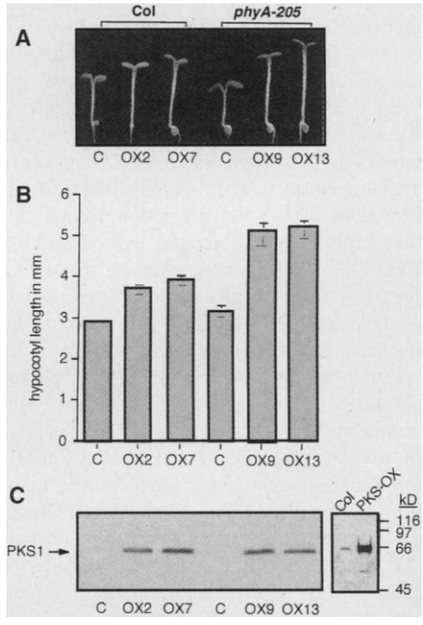

Fig. 4. Seedlings overexpressing PKS1 have reduced sensitivity to white light. This phenotype is more pronounced in a *phyA-205* background. We obtained 12 lines with increased amounts of PKS1 in a wild-type Col background and 4 lines in *phyA-205*. All lines have a longer hypocotyl in white light. Two representative lines in each background are shown. C, control lines transformed with an empty vector. (**A**) Hypocotyl elongation phenotype. (**B**) Quantification of hypocotyl length; average length (n = 16) and SE are indicated. (**C**) Protein immunoblot analysis of seedlings overexpressing PKS1 probed with a PKS1 antibody.

Table 1. *Arabidopsis* seedlings overexpressing PKS1 have a reduced sensitivity to red light. Seedlings were grown and measured as described (*21*). Mean hypocotyl lengths ± SE are indicated.

Genotype	Light conditions and hypocotyl lengths (mm)				
	Dark	Blue	Red	Far-red	White
Col vector	12.58 ± 0.35	3.26 ± 0.12	6.42 ± 0.19	2.03 ± 0.1	2.91 ± 0.07
Col PKS-OX7	12.9 ± 0.34	3.05 ± 0.14	7.37 ± 0.18	1.88 ± 0.05	3.93 ± 0.11
phyA-205 vector	12.6 ± 0.33	3.61 ± 0.1	6.88 ± 0.17	10.24 ± 0.39	3.21 ± 0.11
phyA-205 PKS-OX13	12.86 ± 0.36	3.49 ± 0.17	7.88 ± 0.15	10.84 ± 0.52	5.25 ± 0.13

portant regulatory step of this interaction. Although the exact function of PKS1 remains unknown, the phenotype of PKS1-overexpressing lines suggests that PKS1 is a negative regulator of phyB signaling. PKS1 might modulate phyB kinase activity or subcellular localization. Alternatively, it may coordinate phyA and phyB function or inhibit phytochrome signaling by titrating out a positively acting factor.

There are precedents for negative regulators of a protein kinase being substrates of that same protein kinase. For example, Far1 and Sic1 are inhibitors of Cdc28 protein kinase activity that are degraded after phosphorylation by Cdc28 (*22*). Alternatively, PKS1 may promote segregation of PfrB to the nucleus, much as I-κB retains NF-κB in the cytoplasm until appropriate signals dissociate this interaction and expose NF-κB's nuclear localization signals (*23*). Light-regulated cytoplasmic sequestration of phytochromes by PKS1 represents an attractive model because of phytochrome's newly postulated role in the nucleus (*3, 24*).

The mechanism by which phytochromes affect numerous developmental responses has long eluded us. Phytochromes could phosphorylate multiple substrates present in various subcellular locations, in tissues, and during different developmental stages. Alternatively, these different responses could result from branching later in the signaling pathways. Determining the number of substrates phosphorylated by phytochromes is one way of answering this important question.

References and Notes

1. H. Smith, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 289 (1995).
2. P. H. Quail, *Plant Cell Environ.* **20**, 657 (1997).
3. K. Sakamoto and A. Nagatani, *Plant J.* **10**, 859 (1996).
4. P. F. Devlin, S. R. Patel, G. C. Whitelam, *Plant Cell* **10**, 1479 (1998); G. C. Whitelam and P. F. Devlin, *Plant Cell Environ.* **20**, 752 (1997); M. J. Aukerman *et al.*, *Plant Cell* **9**, 1317 (1997).
5. K. C. Yeh, S. H. Wu, J. T. Murphy, J. C. Lagarias, *Science* **277**, 1505 (1997).
6. H. A. W. Schneider-Poetsch, *Photochem. Photobiol.* **56**, 839 (1992); T. D. Elich and J. Chory, *Cell* **91**, 713 (1997).
7. K. C. Yeh and J. C. Lagarias, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13976 (1998).
8. R. A. Harris *et al.*, *Adv. Enzyme Regul.* **37**, 271 (1997).
9. A bait coding for the last 160 amino acids of *Arabidopsis* PHYA was cloned into pEG202 and used to screen an *Arabidopsis* cDNA library [H. Zhang, J. Wang, H. M. Goodman, *Biochim. Biophys. Acta* **1353**, 199 (1997)]. Positive clones were tested against a bicoid bait for specificity [J. Gyuris, E. Golemis, H. Chertkov, R. Brent, Cell **75**, 791 (1993)] and against a bait corresponding to the last 175 amino acids of *Arabidopsis* PHYB.
10. C. Fankhauser, K. C. Yeh, J. C. Lagarias, J. Chory, data not shown.
11. A full-length *PKS1* clone was obtained from an *Arabidopsis* cDNA library [J. Kieber, M. Rothenberg, G. Roman, K. Feldmann, J. Ecker, *Cell* **72**, 427 (1993)]. This cDNA was polymerase chain reaction–amplified with Bam HI adapters and cloned into pCMX-PL1. In vitro transcription/translation was performed with a Promega TnT kit. A PHYA-GST fusion protein was created by cloning the *Arabidopsis PHYA* cDNA with a Bam HI site at the 5′ end and a Not I site after the last amino acid into pDS472a [S. L. Forsburg and D. A. Sherman, *Gene* **191**, 191 (1997)] and expressed in *Schizosaccharomyces pombe* as described [S. L. Forsburg, *Nucleic Acids Res.* **21**, 2955 (1993)]. A PHYB-GST fusion protein was created by adding a Not I site after the last amino acid of PHYB in clone p41 and adding an in-frame GST cDNA with Not I and Xba I adapters. GST, PHYA-GST, or PHYB-GST (1 μg) were bound onto glutathione-agarose beads (G4510, Sigma). The beads were washed three times with extraction buffer (EB). [T. D. Elich and J. Chory, *Plant Cell* **9**, 2271 (1997)]; in vitro transcribed and translated PKS1 (diluted 1:10 in EB) was added to the beads and incubated for 60 min at 4°C with gentle shaking. The beads were washed five times with EB; the bound proteins were eluted with EB containing 10 mM glutathione, then separated by SDS–polyacrylamide gel electrophoresis (PAGE) [U. K. Laemmli, *Nature* **227**, 680 (1970)], stained, and exposed.
12. Amino acid abbreviations are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
13. D. E. Somers and P. H. Quail, *Plant J.* **7**, 413 (1995); L. Goosey, L. Palecanda, R. A. Sharrock, *Plant Physiol.* **115**, 959 (1997).
14. X. Yang, E. J. Hubbard, M. Carlson, *Science* **257**, 680 (1992).
15. In vitro kinase assays were performed (7) with recombinant oat phyA and a GST-PKS1 fusion protein. The NH_2-terminal 215 amino acids of PKS1 were fused to GST in pGEX-4T1 (Pharmacia). The fusion protein was expressed and purified. Phospho-amino analysis was performed as described [K. Beemon and T. Hunter, *J. Virol.* **28**, 551 (1978)].
16. V. N. Lapko, X.-Y. Jiang, D. L. Smith, P.-S. Song, *Protein Sci.* **8**, 1 (1999); R. W. J. McMichael, thesis, University of California, Davis (1991). The S599K mutant oat PHYA was obtained by site-directed mutagenesis, expressed, and purified as described (*7*). Recombinant S599K had wild-type spectral properties (*10*).
17. Y.-S. Wong, R. W. J. McMichael, J. C. Lagarias, *Plant Physiol.* **91**, 709 (1989); M. Ahmad, J. A. Jarillo, O. Smirnova, A. R. Cashmore, *Mol. Cell* **1**, 939 (1998).
18. After germination, seedlings were grown for 5 days at 20°C in constant light on 1X MS media (Gibco-BRL). Light treatments were as described [M. M. Neff and J. Chory, *Plant Physiol.* **118**, 27 (1998)]. Red light–grown seedlings were extracted as described [D. M. Lagarias, M. W. Crepeau, M. D. Maines, J. C. Lagarias, *Plant Cell* **9**, 675 (1997)] except that 0.1% NP40 and 25 mM KF were added to the extraction buffer. An antibody to the GST-PKS1 protein (*15*) was produced and affinity-purified. PKS1 was immunoprecipitated with this antibody covalently attached to protein A–agarose (Pierce). The immunoprecipitates were treated with calf intestine phosphatase (CIP; Boehringer Mannheim) with or without phosphatase inhibitors [S. Lanker, M. H. Valdivieso, C. Wittenberg, *Science* **271**, 1597 (1996)]. Samples were separated by 9% SDS-PAGE, subjected to protein immunoblotting, and probed with affinity-purified antibody to PKS1. Binding of a horseradish peroxidase–coupled secondary antibody was revealed with SuperSignal (Pierce).
19. Proteins from 5-day-old seedlings grown in the dark or in red light were extracted, run on SDS-PAGE, subjected to protein immunoblotting, and probed with PKS1 antibody (*18*). PhyB was detected with the mBA2 antibody [T. Shinomura *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8129 (1996)].
20. Plants overexpressing PKS1 were obtained by in planta transformation of wild-type Col or *phyA-205* with a pCGN18 vector [T. Jack, G. L. Fox, E. M. Meyerowitz, *Cell* **76**, 703 (1994)] containing the *PKS1* cDNA in the Bam HI site. Homozygous lines with a single insertion were selected for analysis. Proteins were extracted by grinding 20 1-week-old seedlings in 50 μl of final sample buffer. Protein immunoblots were performed as described (*18*).
21. J. M. Brown and R. A. Firtel, *Curr. Biol.* **8**, R662 (1998); C. Chang and R. C. Stewart, *Plant Physiol.* **117**, 723 (1998).
22. W. Krek, *Curr. Opin. Genet. Dev.* **8**, 36 (1998).
23. I. Stancovski and D. Baltimore, *Cell* **91**, 299 (1997).
24. M. Ni, J. M. Tepperman, P. H. Quail, *ibid.* **95**, 657 (1998).

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