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A phytochrome from the fern *Adiantum* with features of the putative photoreceptor NPH1

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ABSTRACT **In plant photomorphogenesis, it is well accepted that the perception of red/far-red and blue light is mediated by distinct photoreceptor families, i.e., the phytochromes and blue-light photoreceptors, respectively. Here we describe the discovery of a photoreceptor gene from the fern *Adiantum* that encodes a protein with features of both phytochrome and NPH1, the putative blue-light receptor for second-positive phototropism in seed plants. The fusion of a functional photosensory domain of phytochrome with a nearly full-length NPH1 homolog suggests that this polypeptide could mediate both red/far-red and blue-light responses in *Adiantum* normally ascribed to distinct photoreceptors.**

Plants alter their growth and development in response to the light environment through a process known as photomorphogenesis. Several families of photoreceptors contribute to the whole-plant response to light from the UV-B to near-infrared region (1). Red/far-red light is perceived by phytochrome, a biliprotein of approximately 120 kDa encoded by a multigene family both in seed plants and in cryptogams (2, 3). Blue-light perception is primarily mediated by cryptochromes (CRYs; refs. 4 and 5) and probably by the product of *NPH1* locus (6). Based on the phenotypes of the *nph1* (*n*on*p*hototropic *h*ypocotyl) mutant (7) and *cry1cry2* double mutant (8), NPH1 has been implicated as the major photoreceptor responsible for blue light-dependent phototropic curvature in seed plants. Indeed, all of the strong *nph1* mutant alleles are defective in both first- and second-positive curvature (6), whereas *cry1cry2* double mutants are only impaired in first-positive phototropic curvature (8). Owing to the presence of putative flavin-binding sites on the NPH1 polypeptide (7) and epistasis analyses (9), NPH1 appears to be the primary photoreceptor mediating second-positive phototropism in plants.

The influence of phytochrome and blue-light photoreceptors on each other's activity is well documented (10). Genetic analyses have clearly demonstrated an interaction between phytochrome and CRY1 signaling pathways (11). Moreover, a direct interaction between phytochrome and the CRY photoreceptors was recently documented (12). In seed plants, blue light-mediated phototropism has been shown to be affected by red- and far red-light treatments (13). Based on these and other studies, it appears likely that the signal-transduction pathways for red- and blue-light photoreceptor families share at least one common component (see ref. 14 for review).

The co-action of phytochrome and blue-light photoreceptors has been examined at the cellular level in fern gametophytes, notably for the genus *Adiantum* (15, 16). In *Adiantum*, phytochrome-dependent spore germination is suppressed by blue-light irradiation (17). Phytochrome also prolongs blue light-induced cell-cycle progression in filamentous protonemal cells of *Adiantum* (18). By contrast, phytochrome and blue-light receptors act cooperatively to mediate phototropism of *Adiantum* protonemata (19) and to affect chloroplast photorelocation in the heart-shaped two-dimensional prothallus (16). Detailed analysis of both phenomena indicates that the photoreceptor co-action occurs within single cells (16, 19) and that the signals from photoreceptors do not pass from one cell to another (16).

While investigating the molecular nature of the blue-light photoreceptors in *Adiantum*, we recently identified several putative cryptochrome genes (20). To identify the photoreceptors responsible for the red/far-red responses in *Adiantum*, we screened a genomic library by hybridization with the previously isolated *Adiantum PHY1* cDNA clone (21). With this screen, we found an unknown phytochrome gene that encodes a polypeptide possessing features of both phytochrome and NPH1. In this report, we describe the features of this gene and examine its expression pattern and preliminary biochemical characteristics.

MATERIALS AND METHODS

Plant Materials. Sporophytes of *Adiantum capillus-veneris* used for DNA preparation were cultivated in a greenhouse under natural conditions. For RNA preparation, rhizomes whose leaves were cut off were kept under either natural conditions or complete darkness, and heads of resulting young leaves (crosiers) were collected and frozen with liquid nitrogen. Spores and protonemata were cultivated as described (22).

Genomic Library Screening. Preparation of total DNA and genomic library construction is described in ref. 20. For a probe, previously isolated *Adiantum PHY1* cDNA (21) was digested with *Ava*III, and a 1,059-bp fragment (positions 805–1,863) containing the putative chromophore-binding region was excised from an agarose gel and labeled with ^{32}P. The library (1.2×10^6 plaque-forming units) was screened by plaque hybridization using the ^{32}P-labeled probe. Hybridizations were done in 5× Denhardt's solution, 0.5% SDS, 5× SSPE [standard saline phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA], and 100 μg/ml denatured salmon sperm DNA with the probe at 65°C for overnight.

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Abbreviations: RACE, rapid amplification of cDNA ends; PK, protein kinase; RT-PCR, reverse transcription-PCR; CRY, cryptochrome; AP, poly(A)-complementary primer; LOV, light, oxygen, or voltage sensor.
Data deposition: The nucleotide and predicted amino acid sequence of the *Adiantum PHY3* has been deposited with GenBank, DNA Database Japan, and European Molecular Biology Laboratory databases (accession no. AB012082).
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The plaque lifts were rinsed in 2× SSPE/0.1% SDS twice at room temperature for 10 min and washed in 1× SSPE/0.1% SDS twice at 65°C for 15 min, and finally washed with 0.1× SSPE/0.1% SDS at 42°C for 30 min. The filters were dried and autoradiographed with an intensifying screen overnight at −70°C. Fragments of bacteriophage inserts that hybridized with the probe were cloned into Stratagene pBluescript II SK(+) and sequenced with an ALFexpress DNA sequencer (Pharmacia). Based on this sequence, appropriate other regions of λ phage clones were cloned into pBluescript II SK(+) and were subsequently sequenced. The 5.8-kb *Sal*I/*Xho*I fragment that was hybridized with the probe was subcloned into *Xho*I linearized pBluescript II SK(+) and named pAdi gPHY3-1. The DNA and derived protein sequences were analyzed by using GENETYX version 8.0 (Software Development, Tokyo) and SEQUENCHER version 3.1 (Gene Codes, Ann Arbor, MI) and aligned by using GENETYX.

Rapid Amplification of the 3′ cDNA Ends (3′ RACE) Cloning. Isolation of total RNA is described in ref. 20. The procedure used for the 3′ RACE followed the manufactures' instruction (GIBCO/BRL). Total RNA (1 ug) was prepared from dark-grown young leaves and reverse-transcribed with poly(A)-complementary primer (AP) in a 20-μl reaction volume. PCR was performed by using a Takara thermal cycler TP480 in a 25-μl reaction volume containing 1 unit of LA *Taq* (Takara Shuzo, Kyoto, Japan) with its PCR buffer ($+Mg^{2+}$) and dNTPs, 0.05 unit of cDNA, and 25 pmol of primers, a gene-specific primer (5′-CGGAGGAGGCTGCAACTTG-GAAGG-3′; nucleotides −69 to −46 from start codon), and a primer (AUAP) complementary to the AP for 1 cycle at 94°C for 1 min, 30 cycles at 98°C for 20 sec, 60°C for 2 min, 68°C for 10 min, and 1 cycle at 72°C for 10 min. After purification of ≈6-kb fragments of the first PCR product by excision of agarose gel, 1% of the solution containing the fragments was subjected to the second PCR by using a nested primer (5′-GGATCCATA*ATGGCGACTCCAGGGGGGC*-3′; nucleotides 1–19 from the start codon; gene-specific sequence is in italics) and AUAP with the same PCR conditions. The resultant products were cloned into pGEM T-Easy vector (Promega), and the clones were sequenced.

Construction of the Strep-Tagged Phytochrome Yeast Expression Vector. To introduce the *Bgl*II site at the 5′ end of *Adiantum PHY3* coding region the and *Sal*I site at the 3′end of the coding region, PCR was performed by using a Takara thermal cycler TP240 in 50 μl of solution containing 1 unit of LA *Taq*, LA *Taq* buffer, dNTP, 10 ng of pAdi gPHY3–1, and 10 pmol of forward primer (5′-TCCCCCGGGAGATCTATA-*ATGGCGACTCCAG*-3′; nucleotide positions 1–13 from the start codon), and 10 pmol of reverse primer (5′-GTCGAC-C*GAATGTATCTTGAAAGGAC*-3′; nucleotide positions 4,377–4,395 from the start codon) with PCR conditions of 1 cycle at 94°C for 1 min, 30 cycles at 98°C for 10 sec, 50°C for 2 min, 72°C for 3 min, and 1 cycle at 72°C for 10 min. The resultant PCR products were cloned into pGEM T-Easy vector, and the insert was exchanged with the *Adiantum* PHY3 gene by the *Stu*I and *Bam*HI sites to avoid sequence mistakes derived from the PCR reaction. The nucleotide sequence of the resultant product (pPHY3BS) was sequenced to confirm that the construct was correct. A Strep-tagged (Biometra, Tampa, FL) yeast expression vector was derived from digestion by *Bam*HI and *Sal*I of a construct in which cyanobacterial phytochrome (Cph1) Strep-tagged at the COOH terminus was cloned into the yeast expression vector pMAC106 (23, 24). The insert of *Bgl*II and *Sal*I pPHY3BS fragment was ligated to *Bam*HI- and *Sal*I-digested Strep-tagged yeast expression vector, resulting in full-length *Adiantum* PHY3::Strep-tagged protein construct.

Expression of Full-Length *Adiantum* PHY3::Strep-Tagged Protein. *Adiantum* PHY3 protein Strep-tagged at the COOH terminus was expressed in *Saccharomyces cerevisiae* 29A (MAT*α*, *leu2–3*, *leu2–112*, *his3–1*, *ade1–101*, *trp1–289*), and 40% ammonium sulfate-fractionated *Adiantum* PHY3-containing extract was used for zinc-blot, Strep-tagged Western blot, and spectrophotometric analysis as described (23, 25).

Genomic Southern Analysis. Five-microgram aliquots of genomic DNA from light-grown sporophyte leaves were digested with *Eco*RV, *Dra*I, or *Hinc*II and fractionated on a 0.8% agarose gel and blotted to a nylon membrane (Hybond N^+; Amersham). Chemiluminescent analysis was performed by using the GeneImages labeling and detection system (Amersham). Three different regions of the *PHY3* genomic clone (Fig. 1*A*) were amplified by using PCR in a Takara thermal cycler TP240; primers used were 5′-AGATCTATAATGGC-GACTCCAGGGGGGC-3′ (forward) and 5′-ACGCGTC-GACCCCTCAGAATGAGCTGAAGAGACC-3′ (reverse) for the phytochrome chromophore-binding region, 5′-GAA-GATCTATAATGAGGGAGGACCTGGAGCAGTTC-3′ (forward) and 5′-ACGCGTCGACCATGCTTCAAC-CCCAGCCTCTC-3′ (reverse) for the central hinge region, and 5′-GAAGATCTATAATGTTCCGGCCCATCAAGCC-A-3′ (forward) and 5′-GTCGACCGAATGTATCTTGAAA-GGAC-3′ (reverse) for the protein kinase (PK) region. PCR containing 0.5 unit of EX *Taq* (Takara) with its buffer and dNTP, 20 pmol of primer, and 10 ng of pAdi gPHY3–1 were performed in 20 μl under the following conditions: 1 cycle at 94°C for 2 min, 30 cycles at 98°C for 20 sec, 55°C for 90 sec, 72°C for 2 min, and 1 cycle at 72°C for 7 min. These PCR fragments were labeled with fluorescein and used as probes. Prehybridization and hybridization were carried out according to the manufacturer's instructions. The membranes were washed in 1× SSC/0.1%SDS and 0.1× SSC/0.1%SDS at 60°C for 15 min, respectively.

Reverse Transcription–PCR (RT-PCR) Analysis. Total RNA (1 ug) was reverse-transcribed as in 3′ RACE and PCR was performed in a Takara thermal cycler TP240. One-twentieth of the reaction product (20 μl) was subjected to the first PCR (containing 0.5 unit of EX *Taq* with its buffer and dNTP, and 20 pmol of 5′-GCAGGAAAAGGCAGAGGTG-GTTAG-3′ forward primer positioned within the PK domain and AUAP) under the following conditions: 1 cycle at 95°C for 1 min, 40 cycles at 95°C for 20 sec, 68°C for 30 sec, 72°C for 3 min, and 1 cycle at 72°C for 3 min. An aliquot (0.0025%) of the original PCR product was reamplified with nested PCR [5′-TTCGACCTTTCCTTCCTCACCTCC-3′ (forward primer) and AUAP] under the following conditions: 1 cycle at 95°C for 1 min, 40 cycles at 95°C for 20 sec, 72°C for 90 sec, and 1 cycle at 72°C for 3 min. The resultant PCR products of all 20-μl reactions were electrophoresed in 2.0% agarose gel and visualized with ethidium bromide staining. The PCR products were cloned and sequenced.

Amino Acid Sequence. The GenBank accession numbers of cDNAs used in this paper are AB016168 (*Adiantum* PHY1), X17341 (*Arabidopsis* PHYA), AF030864 (*Arabidopsis* NPH1), Z30332 (*Spinacia* PK), Z30333 (*Mesembryanthemum* PK), and J04555 (*Phaseolus* PVPK).

RESULTS AND DISCUSSION

By using methods and conditions described in *Materials And Methods*, a genomic library of *A. capillus-veneris* was constructed and subsequently screened with the [^{32}P]dCTP-labeled *Adiantum* PHY1 cDNA chromophore binding region as a probe. This region is conserved in all phytochromes previously isolated and therefore is a useful probe to screen for phytochrome genes and/or cDNAs (26, 27). With this screen, we identified two typical phytochrome genes (*Adiantum PHY1* and *PHY2*; ref. 28), a phytochrome-related sequence (*Adiantum PHY4*; ref. 29), and a photoreceptor gene (*Adiantum PHY3*) that is the subject of this report. Sequence comparison of *Adiantum PHY3* gene and its corresponding cDNA clones

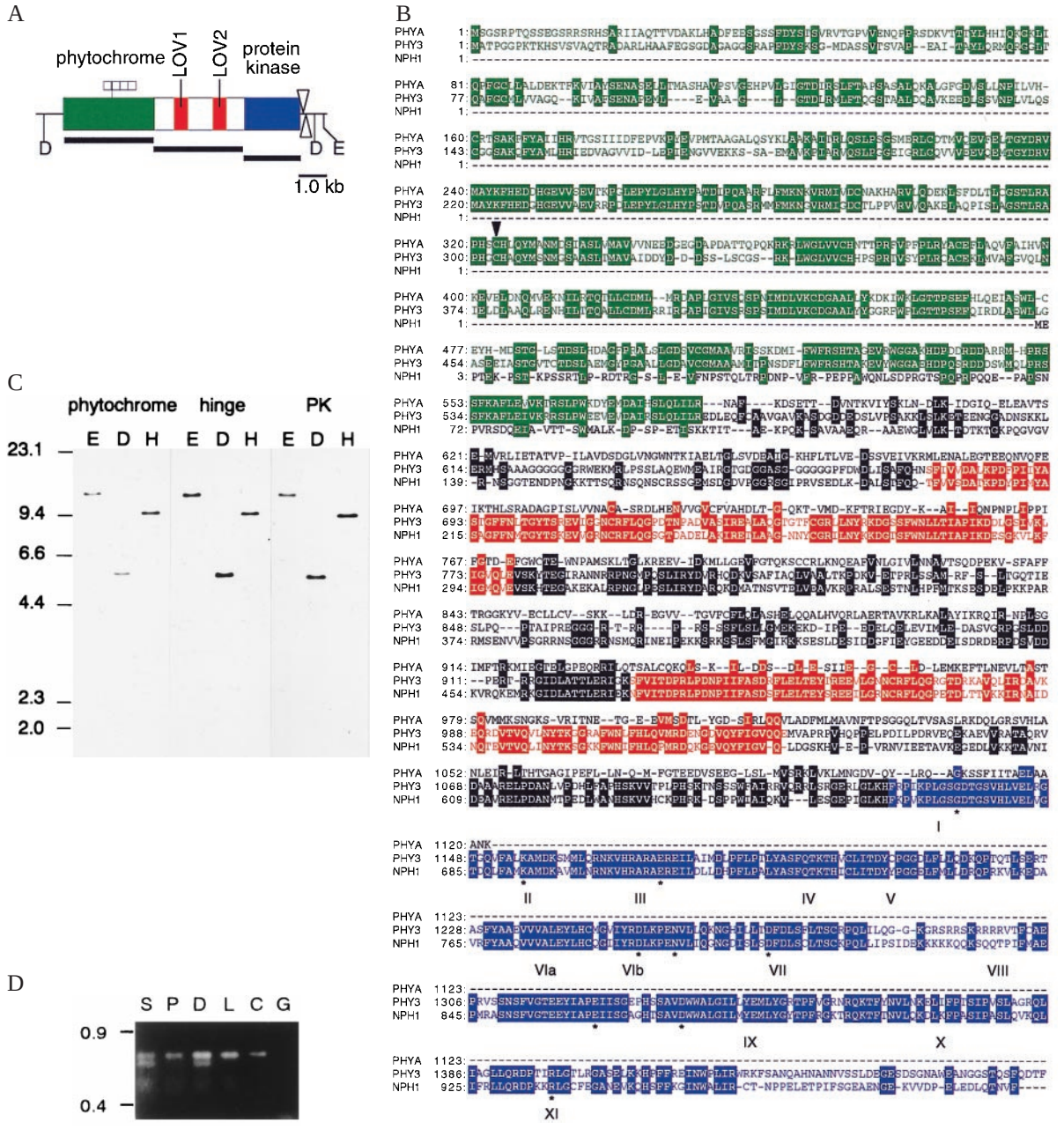

FIG. 1. Isolation and molecular characterization of the *Adiantum PHY3*. (*A*) Schematic representation of domains in the predicted *Adiantum* PHY3 protein. Three different domains are indicated by green, red, and blue, respectively. The positions of Poly(A) addition sites are indicated by arrowheads. The positions of the probes used in *C* are indicated by solid lines. E, *Eco*RV; D, *Dra*I. (*B*) The predicted amino acid sequence of *Adiantum* PHY3 aligned with *Arabidopsis* PHYA and *Arabidopsis* NPH1. The alignment was constructed with GENETYX version 8. The putative phytochrome chromophore binding site (Cys) is indicated by an arrowhead. The 12 conserved subdomains of the PK domain (shown in blue) are indicated by roman numerals. All amino acids conserved among Ser/Thr PKs are indicated by ∗. Residues identical to those of the *Adiantum* PHY3 are highlighted. Color indication is the same as for *A*. (*C*) Genomic DNA blot analysis. Blots were hybridized with phytochrome, the central hinge, or PK domain probes. Marker sizes are shown on the left in kb. E, *Eco*RV; D, *Dra*I; H, *Hinc*II. (*D*) RT-PCR analysis. S; spores imbibed for 1 day under darkness; P, spores imbibed for 4 days under darkness and cultured for 3 days under unilateral red light (0.6 W/m^2); D, newly emerged sporophytic crosier grown under darkness; L, newly emerged sporophytic crosier grown under natural conditions; C, cDNA clone; G, genomic clone. Marker sizes are shown on the left in kb.

revealed that *PHY3* lacks introns and encodes a protein of 1,465 amino acids. The PHY3 polypeptide consists of an NH_2-terminal domain quite similar to known phytochrome chromophore-binding domains that is fused to a large polypeptide structurally related to the NPH1 protein from *Arabidopsis thaliana* (Fig. 1*A* and *B*).

The NH_2-terminal 564-aa region of the PHY3 polypeptide is very similar to phytochrome chromophore-binding domains [52% identity to *Arabidopsis* PHYA (Fig. 1*B*) and 61% identity to *Adiantum* PHY1]. Indeed, this domain possesses all of the signature residues required for the covalent attachment of the linear tetrapyrrole prosthetic group, including the invariant Cys residue (indicated by the arrowhead in Fig. 1*B*; ref. 30). That *Adiantum* PHY3 contains a functional phytochrome chromophore-binding domain was addressed by expression of the apoprotein and its reconstitution with the chromophore precursor phycocyanobilin. Zinc-blot analysis (Fig. 2*A*) and spectrophotometric experiments (Fig. 2*B*) demonstrate that the full-length *Adiantum* PHY3 polypeptide (160 kDa) covalently binds phycocyanobilin to yield a photochromic holo-

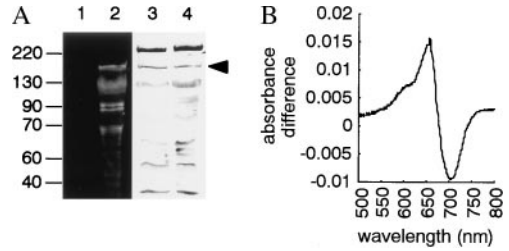

FIG. 2. Spectrophotometric and Western blot analysis of recombinant *Adiantum* PHY3. (*A*) Zinc blot (lanes 1 and 2) and Strep-tag Western blot (lanes 3 and 4) analyses. Each lane contained 300 ng of *Adiantum* PHY3 extract to which phycocyanobilin (PCB) was added (lanes 2 and 4) or omitted (lanes 1 and 3) and then resolved on 10% SDS/PAGE gels and transblotted to poly(vinylidene difluoride) membrane. Lanes 1 and 2 show zinc-treated membrane visualized by fluorescence using a Molecular Dynamics Storm imager. Lanes 3 and 4 are the same protein blot probed with streptavidin-alkaline phosphatase conjugate. Full-length of *Adiantum* PHY3 PCB adducts (indicated by arrowhead) show fluorescence on zinc blot only when PCB was added (lane 2). Marker sizes are shown on the left in kDa. (*B*) Phytochrome-difference spectrum of *Adiantum* PHY3 after incubation with PCB used in *A*.

phytochrome species. The absorption-difference spectrum of the phycocyanobilin adduct of *Adiantum* PHY3 (Fig. 2*B*) is quite similar to those of other recombinant phytochromes (24, 25, 31).

Alignment of *Adiantum* PHY3, *Arabidopsis* PHYA, and *Arabidopsis* NPH1 proteins shown in Fig. 1*B* also illustrate that the COOH-terminal region of *Adiantum* PHY3 is strikingly similar to NPH1 (i.e., 57% identity). In this regard, the COOH-terminal domain of PHY3 possesses two 103-aa LOV domain repeats (44% identity) found in *l*ight, *o*xygen, or *v*oltage sensors (labeled LOV1 and LOV2 in Fig. 1*A*), followed by a Ser/Thr PK domain—both hallmarks of the putative NPH1 blue-light photoreceptor (7). The LOV domain of NPH1 has been proposed to reflect a flavin-binding site, strongly implicating NPH1 as the photoreceptor for phototropism in plants (7). It is therefore conceivable that *Adiantum* PHY3 contains both bilin and flavin prosthetic groups.

The Ser/Thr PK domain of *Adiantum* PHY3 has all of the essential subdomains and conserved amino acids of Ser/Thr PKs (Fig. 1*B*; ref. 32). The Ser/Thr PK domain of *Adiantum* PHY3 exhibits strong homology to AGC group (i.e., A kinase, G kinase, C kinase), which includes a *Spinacia* PK (62.8%), an *Arabidopsis* NPH1 (61.5%), a *Mesembryanthemum* PK (60.1%), and a *Phaseolus* PK (48.7%). Based on this comparison, we predict that *Adiantum* PHY3 will be a PK. The presence of both LOV and phytochrome chromophore domains adjacent to the Ser/Thr PK domain suggests that this kinase activity will be light-regulated.

Genomic Southern blot analysis (Fig. 1*C*) indicated that *Adiantum PHY3* is a single-copy gene. RT-PCR experiments (Fig. 1*D*) demonstrated that *Adiantum PHY3* is expressed both in sporophytes (i.e., dark-grown young leaves and light-grown young leaves) and in gametophytes (i.e., dark-imbibed spores and protonemata). This distribution suggests multiple photoregulatory roles for *Adiantum PHY3* in this fern.

Because *Adiantum* PHY3 possesses a phytochrome photosensory domain fused to an essentially full-length NPH1 homolog, we hypothesize that this photoreceptor is a sensor of both red/far-red and blue light. Our results clearly establish that the *Adiantum* PHY3 protein is a bona fide red/far-red light sensor. Molecular analysis of *nph1* mutant alleles also suggests that the NPH1-related domain of *Adiantum* PHY3 is functional. In *nph1–1* and *nph1–2* mutants, Val-774 and Arg-936 are impaired, respectively, indicating that these two residues are necessary for NPH1 function (7). Conservation of these two residues in *Adiantum* PHY3 (Val-1,237 and Arg-1,398) is consistent with the hypothesis that *Adiantum* PHY3 is a functional NPH1 homolog. The LOV domains of NPH1 and *Adiantum* PHY3 also encompass the direct repeat found in PAS domains, a motif which has been implicated in protein–protein interactions in many prokaryotic and eukaryotic regulatory proteins (33, 34). Because phytochromes also possess a PAS domain in the region adjacent to the chromophore-binding domain (35), a domain that appears critical for the transduction of the light signal perceived by phytochrome (36), it is tempting to speculate that the PAS domain represents the site for direct protein–protein interaction between the two photoreceptors. Such an interaction could account for the observed co-action or cross-talk between these distinct photoreceptor families in higher plants.

In *Adiantum*, molecular evolution appears to have created a chimeric photoreceptor that likely functions to integrate red/far red- and blue-light signaling information—a function that typically may be accomplished via direct interaction between members of the two photoreceptor families. The simplest model for *Adiantum* PHY3 action is that of a dual red/far-red- and blue-light photoreceptor. Alternatively, *Adiantum* PHY3 may be a red/far red photoreceptor that modulates the activity of a covalently joined component of one or more blue-light signaling pathways in this organism. In either case, we hypothesize that *Adiantum* PHY3 will relay light information to interacting proteins by a change in its PK activity. The development of improved expression and purification systems will be necessary to address this hypothesis adequately.

It is of considerable interest to determine which physiological responses are mediated by *Adiantum* PHY3. Because *Adiantum* PHY3 possesses a domain homologous to NPH1—a key component in seed-plant phototropism—we expect that it will play a role in light-induced phototropism in *Adiantum*. Unlike in other plants, where phototropic response requires a blue-light stimulus and red light only alters the action of blue light (13), phototropism in *Adiantum* gametophytes and sporophytes can be induced by both red and blue light (19, 37, 38). Physiological studies suggest that phytochrome and the blue-light photoreceptor for phototropism of fern protonema are both associated with the plasma membrane and/or ectoplasm (39, 40). This is consistent with the plasma membrane localization of a 120-kDa phosphoprotein, the putative product of the *NPH1* gene (7). Because hydropathy profiles indicate that *Adiantum* PHY3 has no transmembrane domains (data not shown), as is also the case for NPH1 (7), the nature of its (putative) association with the membrane is unclear. By analogy, we predict that the NPH1-related domain of *Adiantum* PHY3 contains the information that specifies this membrane association. The possibility that *Adiantum* PHY3 regulates the cell cycle and cell growth in protonemata is unlikely, because the phytochrome responsible for these responses is localized very near the plasma membrane, whereas the antagonistic effect of blue light is found only in the nuclear region (41, 42). These results suggest *Adiantum* PHY3 to be the photoreceptor for both red and blue light-induced phototropism in this organism.

The discovery of a phytochrome-NPH1 chimera in *Adiantum* raises interesting questions about the evolution of these two photoreceptor families. For example, does this type of molecule exist in other species? If not, why didn't it occur in other species? The answer to the first question (so far) is no; however, another phytochrome–PK chimeric gene was reported in the moss *Ceratodon* (*phyCer*; ref. 43). The PK domain of this moss phytochrome is quite dissimilar to the *Adiantum* PHY3 photoreceptor, suggesting that the two proteins have distinct evolutionary ancestries. The answer to the second question may reflect the difficulty of creating a functional fusion protein between phytochrome and NPH1 by random genetic events or the lack of sufficient selection pressure to retain such a gene product.

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