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 (nonphototropic hypocotyl) 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 AvaIII, 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 32P. The library (1.2 × 109 plaque-forming units) was screened by plaque hybridization using the 32P-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.

This paper was submitted directly (Track II) to the Proceedings office. 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 2x SSPE/0.1% SDS twice at room temperature for 10 min and washed in 1x SSPE/0.1% SDS twice at 65°C for 15 min, and finally washed with 0.1x 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 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 A. phage clones were cloned into pBluescript II SK(+) and were subsequently sequenced. The 5.8-kb SalI/Xhol fragment that was hybridized with the probe was subcloned into XhoI 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 SEQUENCER version 3.1 (Gene Codes, Ann Arbor, MI) and aligned by using GENETYX.

**Rapid Amplification of the 3' cDNA Ends (3' RACE)**

Cloning of isolated total RNA is described in ref. 20. The procedure used for the 3' RACE followed the manufacturer's instruction (GIBCO/BRL). Total RNA (1 μg) 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 μl of LA Taq (Takara Shuzo, Kyoto, Japan) with its PCR buffer (+Mg2+) and dNTPs, 0.05 unit of cDNA, and 25 pmol of primers, a gene-specific primer (5'-GGAGAGGGCTGCAACTTGG-GAAAG-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'-GGATCTATAAAGGAGGACCTGGAGCTTC-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 Phytocrome Yeast Expression Vector.** To introduce the BgII site at the 5' end of Adiantum PHY3 coding region and the SalI 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, 2 μl of solution containing 1 μl of LA Taq buffer, dNTP, 10 ng of pAdi gPHY3-1, and 10 pmol of forward primer (5'-TCCCCGGGAGGATCTAATATGGGACTCCACG-3'; nucleotide positions 1-13 from the start codon), and 10 pmol of reverse primer (5'-GTCGACCGAATGTATCTTGAAA-GGAC-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, 72°C for 2 min, 72°C for 3 min, and 1 cycle at 72°C for 3 min. The resultant PCR products of all 20-μl reactions were electrophoresed in 0.8% 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), AF308864 (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 [32P]cDNA-labeled Adiantum PHY1 cDNA chromophore binding region as a probe. This probe 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

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**Text Content**

- **Adiantum PHY3**
- **Adiantum PHY4**
- **Plant Biology:** Nozue et al.

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**References**

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revealed that *PHY3* lacks introns and encodes a protein of 1,465 amino acids. The *PHY3* polypeptide consists of an NH₂-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. 1A and B).

The NH₂-terminal 564-aa region of the *PHY3* polypeptide is very similar to phytochrome chromophore-binding domains [52% identity to *Arabidopsis PHYA* (Fig. 1B) 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. 1B; 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. 2A) and spectrophotometric experiments (Fig. 2B) demonstrate that the full-length *Adiantum PHY3* polypeptide (160 kDa) covalently binds phycocyanobilin to yield a photochromic holo-
suggests that the NPH1-related domain of Adiantum light sensor. Molecular analysis of nph1 (Fig. 1) is a single-copy gene. RT-PCR experiments kinase activity will be light-regulated. That the Adiantum homolog, we hypothesize that this photoreceptor is a sensor of A

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dues are necessary for NPH1 function (7). Conservation of these two residues in young leaves) and in gametophytes (i.e., dark-imbibed spores in sporophytes (i.e., dark-grown young leaves and light-grown

PK (48.7%). Based on this compar-

ogy, we predict that the NPH1-related domain of Adiantum PHY3 contains the information that specifies this membrane 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 will play a role in light-induced phototropism in this organism.

The discovery of a phytochrome-NPH1 chimera in Adi-

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-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.

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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 chimera 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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