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Isolation and characterization of homologues of plant blue-light photoreceptor (cryptochrome) genes from the fern *Adiantum capillus-veneris*

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Abstract Many blue-light mediated physiological responses have been studied in the fern *Adiantum capillus-veneris*. We have isolated genomic clones encoding sequences similar to those encoding blue-light photoreceptors (cryptochromes) in higher plants using the *Arabidopsis CRY1* cDNA as a probe, and these positive clones fall into five independent groups. Using RACE procedures, we obtained full-length cDNA sequences for three of these five groups. The deduced amino acid sequences include the photolyase-homologous domain in the N-terminal half, and they also contain a C-terminal extension of about 200 amino acids in length. These structural features indicate that the genes indeed encode *Adiantum* cryptochromes and represent a small gene family having at least three members.

Key words *Adiantum* · Cryptochrome · Fern · Plant blue-light photoreceptor · Plant gene family

Introduction

Numerous physiological responses mediated by blue light have been described in plants, including phototropic responses, hypocotyl elongation, cotyledon expansion, stomatal opening, chloroplast development, anthocyanin biosynthesis and gene expression. However, the molecular components of the signal transduction pathways are still unknown. In 1993, Ahmad and Cashmore isolated one of the blue-light photoreceptor genes from *Arabidopsis thaliana* (Ahmad and Cashmore 1993) and they referred to the protein encoded by this gene as CRY1 for the name of such photoreceptors, cryptochromes (Lin et al. 1995b). This gene was cloned from a T-DNA tagged *hy4* mutant line (*hy4–2*) that showed a deficiency in blue light-dependent inhibition of hypocotyl elongation. Using *hy4* mutant lines and transformed tobacco overexpressing CRY1, they demonstrated that this photoreceptor mediates not only hypocotyl elongation but also anthocyanin accumulation in germinating seedlings (Ahmad et al. 1995; Lin et al. 1995a). The significant structural features of CRY1 are similarity to microbial type I photolyases in the N-terminal 500 amino acids and an additional 180 amino acid extension in the C-terminus.

So far, only three additional, distinct, presumptive cryptochrome genes have been cloned: *Arabidopsis CRY2/PHH1* (Lin et al. 1996b; Hoffman et al. 1996), *Sinapis SA-PHR1* (Batschauer 1993) and *Chlamydomonas CPH1* (Small et al. 1995). The *CRY2* and *PHH1* sequences were isolated independently; however, the deduced amino acid sequences of these two genes are the same and thus we assume that these two sequences originated from the same locus in this work. The deduced amino acid sequences of the cryptochrome genes show quite high similarities to CRY1 in their photolyase-homologous regions. In addition, the CRY2 (Phh1) and CPH1 sequences also have C-terminal extensions like CRY1, but are less similar to CRY1 in this region than in the photolyase domain. Since SA-PHR1 lacks a C-terminal extension, it is debatable whether it should be referred to as a CRY1 homologue; however, since SA-PHR1 does not show any photolyase activity (Malhotra et al. 1995) we consider it to be a *Sinapis* cryptochrome. To date, cryptochrome genes have been identified in seed plants and a green alga; however, to study blue light signal perception and transduction mechanisms throughout the plant kingdom, more photoreceptor gene sequences from other plant species are required. Cryptogams, and especially mosses and ferns, have well characterized blue light-mediated responses (Wada and Kadota 1989). The identification and characterization of their cryptochromes is thus of particular interest.

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In the fern *Adiantum capillus-veneris*, photomorphogenic phenomena have been studied in detail (Wada and Sugai 1994). The simple organization of fern gametophytes has greatly facilitated physiological observations at the cellular level. Blue light mediates several responses in *Adiantum* gametophytes, such as inhibition of spore germination (Sugai and Furuya 1985), inhibition of protonemal cell elongation (Kadota et al. 1979), induction of apical swelling in tip-growing cells (Wada et al. 1978), induction of phototropic responses (Hayami et al. 1986), regulation of cell cycle and cell division (Wada and Furuya 1974; Miyata et al. 1979; Furuya et al. 1997), and promotion of chloroplast photo-orientation (Yatsuhashi et al. 1985; Kagawa and Wada 1994). Further physiological analysis has demonstrated the localization and dichroic orientation of the photoreceptors (Wada and Furuya 1978; Miyata et al. 1979; Kadota et al. 1986; Yatsuhashi et al. 1987; Hayami et al. 1992). In spite of the wealth of data, the nature of the blue-light photoreceptors is still obscure. Here, we report the isolation and characterization of cryptochrome-homologous genes from *Adiantum*. This is the first report to identify cryptochrome gene homologues in ferns, and indicates that cryptochromes in *Adiantum* are encoded by a small gene family of at least three, and perhaps five, members.

Materials and methods

Construction and screening of the genomic library

Adiantum genomic DNA was prepared from young sporophyte leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). For construction of the genomic library, DNA was partially digested with *Sau*3AI and size-fractionated on an agarose gel. Fragments of 15 to 20 kb were partially filled with dATP and dGTP using the Klenow fragment of DNA Polymerase I and ligated into the λ FIX II *Xho*I-partial fill-in vector (Stratagene). The ligated DNA was packaged using Gigapack Gold II (Stratagene) and transformed into *Escherichia coli* XL1-blue MRA(P2). Approximately 1.2×10^6 recombinant plaques were transferred to Hybond N+ (Amersham) membranes according to the manufacturer's procedures. Membranes were hybridized to a ^{32}P-labelled full-length cDNA fragment of *Arabidopsis CRY1* (a kind gift of Dr. A. R. Cashmore) at 60° C in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. These membranes were washed twice in 2 × SSPE, 0.1% SDS for 10 min at room temperature, then twice for 15 min with 1 × SSPE, 0.1% SDS at 42° C. The final wash step was 0.1 × SSPE, 0.1% SDS at 42° C for 30 min. After plaque purification, recombinant DNAs were prepared and analyzed by physical mapping and Southern hybridization. Genomic DNA fragments which hybridized to *Arabidopsis CRY1* cDNA were subcloned into the pBluescript II SK vector (Stratagene) and sequenced. DNA sequences were determined using a Cycle sequence core kit (Takara shuzo), synthetic internal primers and an ALF Express automated DNA sequencer (Pharmacia Biotech).

3′-, 5′-RACE for cDNA cloning

For RNA preparation, imbibed *Adiantum* spores were kept in darkness for 4 days, then cultured for 3 days under red light to obtain protonemal cells. RNA was selectively precipitated by adding LiCl, a final concentration of 2 M, to total nucleic acids prepared by the CTAB method described above. RNA was further purified using a CsCl step gradient procedure (Ausubel et al. 1987). This total RNA fraction was used in first-strand cDNA synthesis for 3′- and 5′-RACE. Both 3′- and 5′-RACE procedures were performed using the 3′ RACE system for rapid amplification of cDNA ends (GibcoBRL) and 5′ RACE system for rapid amplification of cDNA ends, Version 2 (GibcoBRL) according to the manufacturer's protocols. In 3′-RACE, the gene-specific primer PHL3RA1 (5′-AAYTTYCCMTTHAGVCATGA-3′) was used for first-round amplification of the target DNA and PHL3RA2 (5′-CCYYTDGTKGATGCHGGVATG-3′) was used for nested PCR (Fig. 2A). The annealing temperature for first-round PCR was 55° C and for nested PCR was 60° C. Prior to the PCR, preamplification denaturation was performed for 3 min at 94° C. The parameters for PCR amplification were 1 min denaturation at 94° C, 2 min at annealing temperature, and 3 min elongation at 72° C for 30 cycles. To obtain full-length amplified products, a final extension at 72° C was performed for 7 min. After agarose gel electrophoresis, the amplified fragments were recovered from the gel, cloned into the pGEM-T easy vector (Promega) and sequenced as described above. In 5′-RACE, a gene-specific primer, 5-GSP1 (5′-AGCTCAAYWCCAGCKGCACG-3′), was used for first-strand cDNA synthesis. The other gene-specific primers for first-round PCR were 411-GSP1 (5′-CAGAGGGCAAGCGAGAGAG-3′), 9-GSP1 (5′-ATCCTATCCAATTCATGACC-3′) and 68-GSP1 (5′-GATTATCCATTCGGTCCAG-3′), corresponding to *Adiantum CRY1*, *CRY2*, *CRY3*, respectively. For nested PCR, 411-GSP2 (5-GGTTGTCAATGCGATAGAGC-3′), 9-GSP2 (5′-ACATCACTCTCCAAGTCTGC-3′) and 68-GSP2 (5′-ATATGTACTGCCAACCAAGG-3′) were synthesized for *CRY1*, *CRY2*, *CRY3*, respectively (Fig. 2B). The parameters for PCR and procedures for cloning and sequencing were the same as above.

DNA blot analysis

Five μg of genomic DNA was digested with *Eco*RV or *Sac*I, fractionated on a 0.5% agarose gel, and transferred to Hybond N+ (Amersham) membrane by the conventional capillary method (Ausubel et al. 1987). Transcript-specific probes were prepared using PCR products amplified with primers located on both sides of the fourth intron (Fig. 3). The primer pairs used were: for *Adiantum CRY1*, PHL4–3 (5′-GCAAGAGGCTCATGACAATGGGAG-3′) and PHL4–11(5′-ACCTGAATGGCTGCTAAGTC-3′), for *CRY2*, PHL9–1 (5′-GCACATGCGGCAA-GACGATTCTCAAG-3′) and PHL9–3 (5′-GTTACAACAACCCTACAGTG-3′), and for *CRY3*, PHL6–4 (5′-AGCAACGAATGGAGACATGG-3′) and PHL6–8 (5′-TCATGAACTCAAGTCT-CAGC-3′). The PCR products were electrophoresed on an agarose gel, excised and purified from the gel, and labelled with ^{32}P[dCTP] using a random primer labelling kit (Pharmacia Biotech). The membranes were hybridized with transcript-specific probes at 65° C in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. These membranes were washed twice in 2 × SSPE, 0.1% SDS for 10 min at room temperature, then once for 15 min with 1 × SSPE, 0.1% SDS at 65° C. The final wash step was with 0.1 × SSPE, 0.1% SDS at 65° C for 30 min.

RT-PCR

Total RNA from light-grown young leaves of *Adiantum* sporophytes was prepared by the procedure used for preparation of RNA from protonemal cells described above. Two μg of total RNA from gametophytes and sporophytes were reverse-transcribed using Superscript II reverse transcriptase and AP primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′) (GibcoBRL). An aliquot of the first-strand cDNA was used for PCR amplification with transcript-specific primers, i.e. PHL4–3 and PHL4–11 for *CRY1*, PHL9–1 and PHL9–3 for *CRY2*, and

PHL6–4 and PHL6–8 for *CRY3* as described in DNA blot analysis. As a control, genomic DNA was also amplified using the same specific primers. The PCR parameters were: denaturation for 1 min at 94° C, followed by 30 cycles of 10 s denaturation at 94° C, 1 min annealing at 55° C, and 1 min extension at 72° C, with a final extension for 5 min at 72° C. Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Computer analysis

Multiple sequence alignments of nucleotide and amino acid sequences were performed using the Clustal W version 1.6 program (Thompson et al. 1994). Hydropathy analysis was performed according to Kyte and Doolittle (1982) using a window of 9 amino acids. The DDBJ/EMBL/GenBank nucleotide sequence database accession number for *Escherichia coli phr* is K01299, *Arabidopsis thaliana CRY1* is S66907, *Arabidopsis thaliana CRY2* (*PHH1*) is U43397 (U62549), *Sinapis alba SA-PHR1* is X72019, and *Chlamydomonas reinhardtii CPH1* is L07561.

Results

Genomic library screening and isolation of cryptochrome cDNAs from *Adiantum*

To isolate cryptochrome-related sequences from *Adiantum*, we first screened a genomic library of 1.2×10^6 recombinant phages. We used a full-length cDNA from *Arabidopsis CRY1* (Ahmad and Cashmore 1993) as a probe and, after three rounds of plaque isolation and purification, nine positive genomic clones were obtained. Analysis by restriction enzyme digestion showed that these clones could be divided into five independent groups (Fig. 1). Southern analysis of these clones identified restriction fragments that hybridized with *Arabidopsis CRY1* cDNA (Fig. 1). We then subcloned these restriction fragments into a phagemid vector and sequenced them. The fragments contain CRY1-related sequences and all the homologous regions showed similarity to type I photolyases (data not shown).

Based on these genomic sequences, we designed degenerate primers for 3′-RACE to isolate cDNAs corresponding to each independent group (Fig. 2A). Since we have observed many blue light-dependent photoresponses in *Adiantum* gametophytes, we extracted total RNA from protonemal cells grown under red light and used this as a template for first-strand cDNA synthesis. After nested PCR with the primer PHL3RA2, we obtained three groups of amplified fragments. The nucleotide sequences of these fragments corresponded to three of the five genomic clone groups, λ-#4 and #11, λ-#9, and λ-#6 and #8. To isolate the 5′ ends of these fragments, we performed the 5′-RACE procedure using gene-specific primers (Fig. 2B) and obtained amplified fragments corresponding to all the 3′-RACE products. As the result of the 3′- and 5′-RACE, three independent cDNAs were identified and these cDNAs encode polypeptides comprising 637 amino acids (*Adiantum CRY1*), 679 amino acids (*Adiantum CRY2*) and 718 amino acids (*Adiantum CRY3*) (Figs. 3A, 4). *Adiantum CRY1* corresponds to the genomic group λ-#4 and #11, *CRY2* corresponds to λ-#9, and *CRY3* corresponds to λ-#6 and #8. Genomic Southern blot analysis using each gene-specific probe (probes a, b, c) indicated that each gene is present as a single copy in the haploid genome (Fig. 3B). We determined the nucleotide sequences of the genomic clones encoding *CRY1*, *2*, and *3* in order to determine the structure of these genes. Comparison of the cDNA and genomic sequences revealed that the genes all contain four introns (Figs. 3A, 4). While the length of the intron at each position is variable, the intron positions are similar. Furthermore, the position of the first intron of *Adiantum CRY*s is exactly conserved in both *Arabidopsis CRY1* and *CRY2* (*PHH1*), and the third intron insertion position is identical to the fourth intron site of *Arabidopsis CRY2* (*PHH1*) (Figs. 3A, 4).

Comparison of the *Adiantum* CRYs with blue-light photoreceptors from higher plants and with microbial photolyases

The deduced amino acid sequences of *Adiantum CRY1*, *2* and *3* were aligned with those of the *Arabidopsis* cryptochromes *CRY1* and *CRY2* (*PHH1*), and the

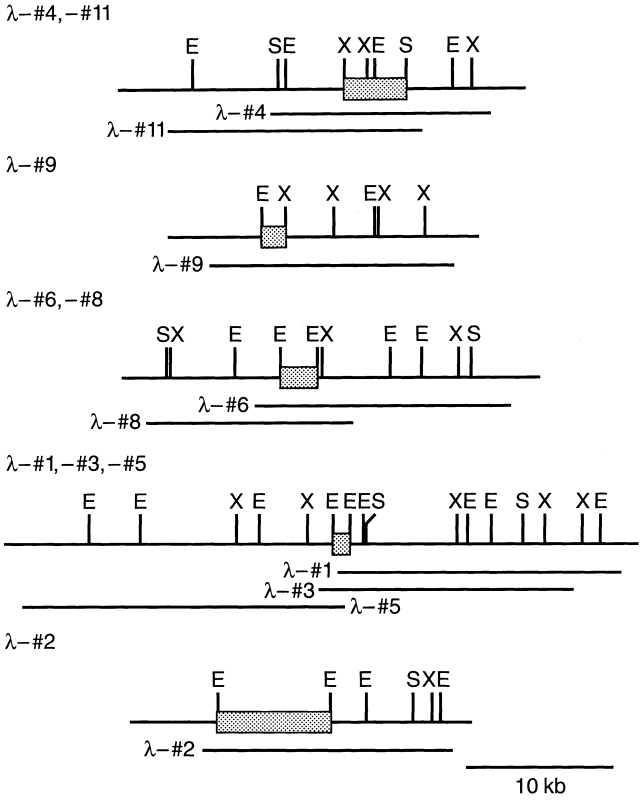

Fig. 1 Physical maps of nine *Adiantum* genomic clones hybridized with *Arabidopsis CRY1* cDNA. Isolated genomic clones are shown by *solid bars*. *Solid boxes* indicate the restriction endonuclease fragments which hybridize the *Arabidopsis CRY1* cDNA probe. Recognition sites for *Eco*RI (E), *Sal*I (S) and *Xho*I (X) cleavage are indicated

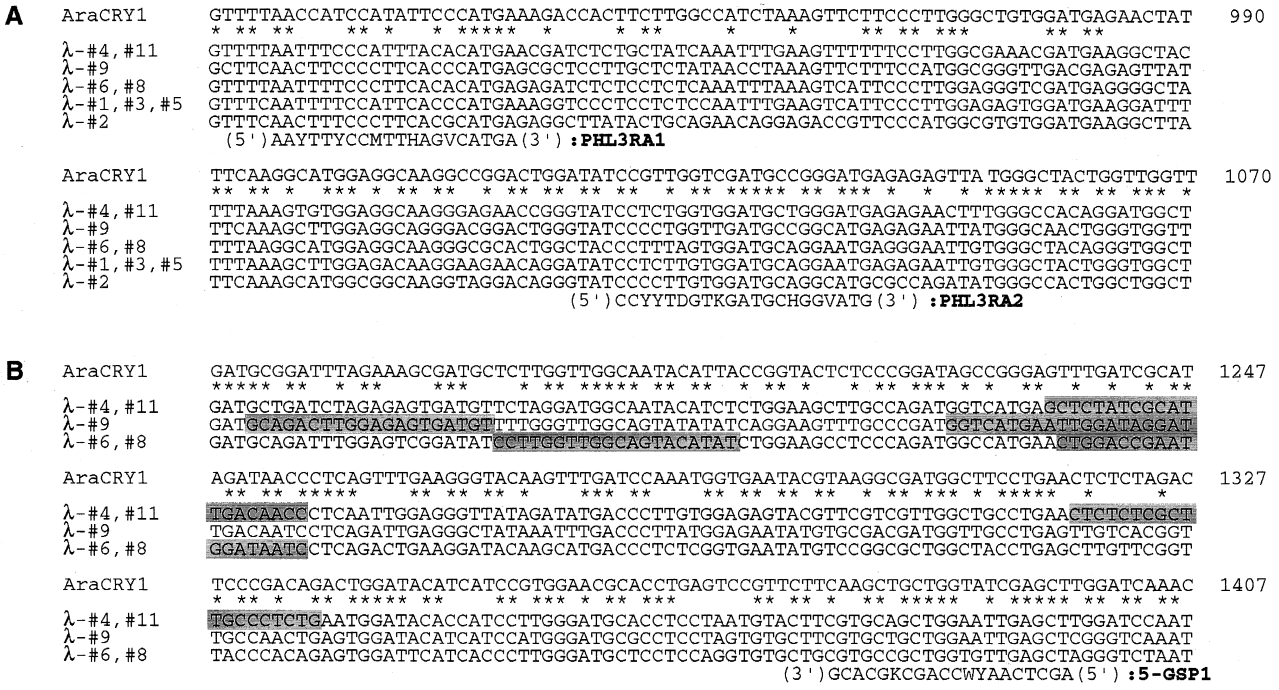

Fig. 2A, B Alignment of nucleotide sequences of *Arabidopsis CRY1* and *Adiantum* genomic clones. Nucleotides conserved in all the sequences are indicated by *asterisks*. AraCRY1 indicates the nucleotide sequence of the *Arabidopsis CRY1* cDNA. **A** Degenerate primers used for 3′-RACE procedure (PHL3RA1, PHL3RA2) are shown *below* the alignment. **B** A degenerate primer for first-strand cDNA synthesis on 5′-RACE (5-GSP1) is indicated *below* the alignment as a complementary sequence. Gene-specific primers for nested PCR are indicated by the *shaded boxes* (see Materials and methods)

sequences of an approximately 500-amino acid domain extending from the N-termini were found to be remarkably similar to each other. In contrast, the C-terminal portions of these sequences showed fewer similarities (Fig. 4). Hydropathy profiles of the N-terminal 500 amino acids of *Adiantum* CRYs were also found to be quite similar to those of *Arabidopsis* CRY1, CRY2 (Phh1) and the *E. coli* photolyase (Fig. 5). The N-terminal region of cryptochromes is known to be similar to that of microbial type I photolyases (Ahmad and Cashmore 1993; Lin et al. 1996b; Hoffman et al. 1996). Thus, *Adiantum* CRYs contain an N-terminal photolyase-homologous domain and a C-terminal extension, like other plant cryptochromes. Based on these observations, we consider that each *Adiantum CRY* sequence encodes a blue-light photoreceptor, or cryptochrome, and refer to them as *Adiantum* cryptochrome (*CRY*) genes *1*, *2* and *3*. Similarity searches against sequence databases detected no sequences that were highly homologous to any of the C-terminal extensions of the *Adiantum* CRYs. Although the C-terminal extensions of cryptochromes show little similarity, these extensions all contain the sequence motif DQMVP [DQQVP in *Arabidopsis* CRY2 (Phh1)]. In addition, *Adiantum* CRY3 contains the serine-rich region noted by Hoffman et al. (1996) in Arabidopsis CRY1 and CRY2 (Phh1) in its C-terminal extension.

Microbial type I photolyases contain two co-factors: one is a light harvesting co-factor – either methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF) – and the other is a catalytic co-factor, flavin adenine dinucleotide (FAD) (Sancar 1994). Like type I photolyases, the *Arabidopsis* CRY1 and *Sinapis* SA-PHR1 associate with FAD (Lin et al. 1995b; Malhotra et al. 1995). Since several amino acid residues of the photolyase have been predicted to bind co-factors based on the crystal structure of the DNA photolyase from *E. coli* (Park et al. 1995), we compared the amino acid sequences of cryptochromes to that of the *E. coli* photolyase helical domain which contains the FAD binding site (Fig. 6). Out of 12 amino acid residues that interact with FAD in *E. coli* photolyase, 11 are conserved in all cryptochromes; the exceptions are phenylalanine 233 of *Chlamydomonas* CPH1 (*E. coli* Tyr222) and asparagine 359 of *Arabidopsis* CRY1 (*E. coli* Asn341). The non-conserved residue, asparagine 378 of *E. coli*, is replaced by aspartate in all the cryptochromes, although aspartate is also present in the *Halobacterium halobium* photolyase at this position (Takao et al. 1989). The TGYP motif is also found in all cryptochromes (Fig. 6). This motif is conserved in all type I photolyases and lies in the flavin-binding domain (Malhotra et al. 1992; Raibekas and Jorns 1994). On the other hand, DNA-binding amino acid residues are also predicted in the *E. coli* photolyase based on the crystallographycal studies. These residues are not well conserved between the *E. coli* photolyase and cryptochromes; thus, tryptophan 277 of the *E. coli* photolyase, which is presumed to be involved in binding to DNA (Li and Sancar 1990) (Fig. 6) is not found in the cryptochromes.

Gene expression

The isolation of three CRY sequences as cDNAs means that at least three CRY genes are expressed in protonemal cells of *Adiantum*. This might be expected since many blue light-mediated responses have been observed in *Adiantum* gametophytes. In the diploid phase of *Adiantum*, few blue light-dependent phenomena have been observed, the best characterized being the blue light-induced phototropic response in *Adiantum cuneatum* sporophytes (Wada and Sei 1994). To investigate the expression of *Adiantum CRY* mRNAs in diploid tissues, we performed RT-PCR using total RNA prepared from young sporophyte leaves grown under natural light. Figure 7 shows that transcripts corresponding to *CRY1*, *2*, *3* accumulated, not only in gametophytes but also in sporophytes. In addition, transcripts of *CRY1*, *2*, *3* were similarly processed at the fourth intron in both haploid and diploid tissues.

Discussion

Despite the fact that there are many blue light-mediated responses in plants, to date, *Arabidopsis* is the only the plant in which cryptochromes have been shown to be encoded by a gene family. The *Arabidopsis* cryptochrome gene family comprises two members: *CRY1* and *CRY2* (*PHH1*); however, it is difficult to believe that these two genes could mediate all the divergent blue-light responses in *Arabidopsis*. In fact, *hy4* (*cry1*) mutants show normal blue light-dependent phototropic responses (Chory 1992; Liscum and Briggs 1995), and CRY2-overexpressing transformants do not demonstrate any alterations in phototropic responses (Cashmore 1997). Together, these observations imply the existence of additional blue-light photoreceptor(s). Recently, Huala et al. (1997) identified the *Arabidopsis NPH1* (*non*phototropic *h*ypocotyl 1) gene, which encodes an essential component involved in phototropism; however, which photoreceptor mediates the blue light-dependent phototropic response is still unclear. In this study, we have demonstrated that three cryptochrome genes are expressed in *Adiantum*. We have also isolated two additional independent genomic DNA fragments which contain cryptochrome-homologous regions. This is the first report of the isolation of cryptochrome-homologous genes from ferns, and suggests that blue-light signal perception through cryptochromes may be a widespread mechanism, at least in vascular plants. Our findings also indicate that cryptochromes in *Adiantum* are encoded by a small gene family containing at least three, and possibly as many as five, members, the largest CRY family reported to date. The range of divergent phenomena mediated by blue light in *Adiantum* is consistent with a family size of greater than three members. *Adiantum* CRYs are quite dissimilar to *Arabidopsis* CRYs in their C-terminal extensions. A blue light-induced phototropic response is also known to exist in

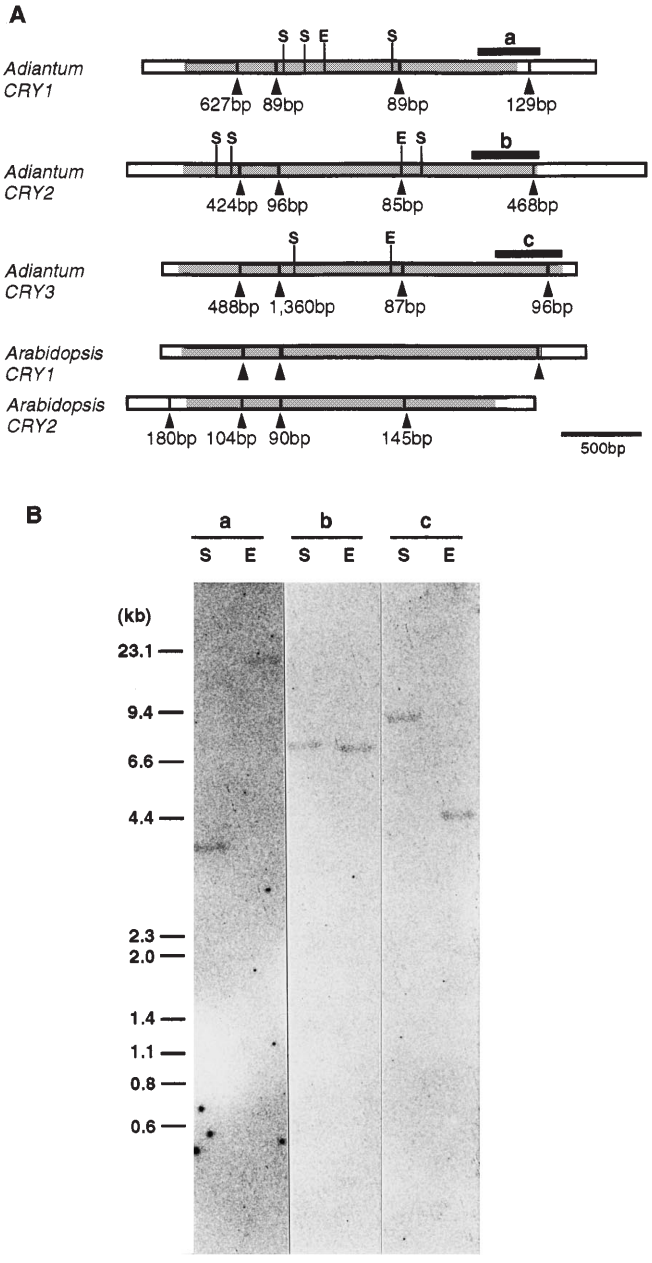

Fig. 3A, B Gene structure and Southern analysis. **A** Schematic representation of the gene structure. *Open boxes* and *shaded boxes* indicate untranslated and protein-coding regions, respectively. Intron insertion positions are indicated by *arrowheads* together with the intron lengths. The locations of gene-specific probes used for Southern analysis are shown by the *solid bars* (a, b, c). Restriction sites for *Sac*I (S) and *Eco*RV (E) are indicated. For comparison, the gene structures of *Arabidopsis CRY1* and *CRY2* (*PHH1*) are also presented. **B** Southern blot analysis of total *Adiantum* DNA hybridized with probes specific for *CRY1* (a), *CRY2* (b), and *CRY3* (c). DNA fragments (5 μg) treated with restriction enzymes [*Sac*I (S), *Eco*RV (E)] were fractionated on a 0.5% agarose gel and probed with radiolabelled specific probes described above. The positions of the molecular size marker are indicated on the left

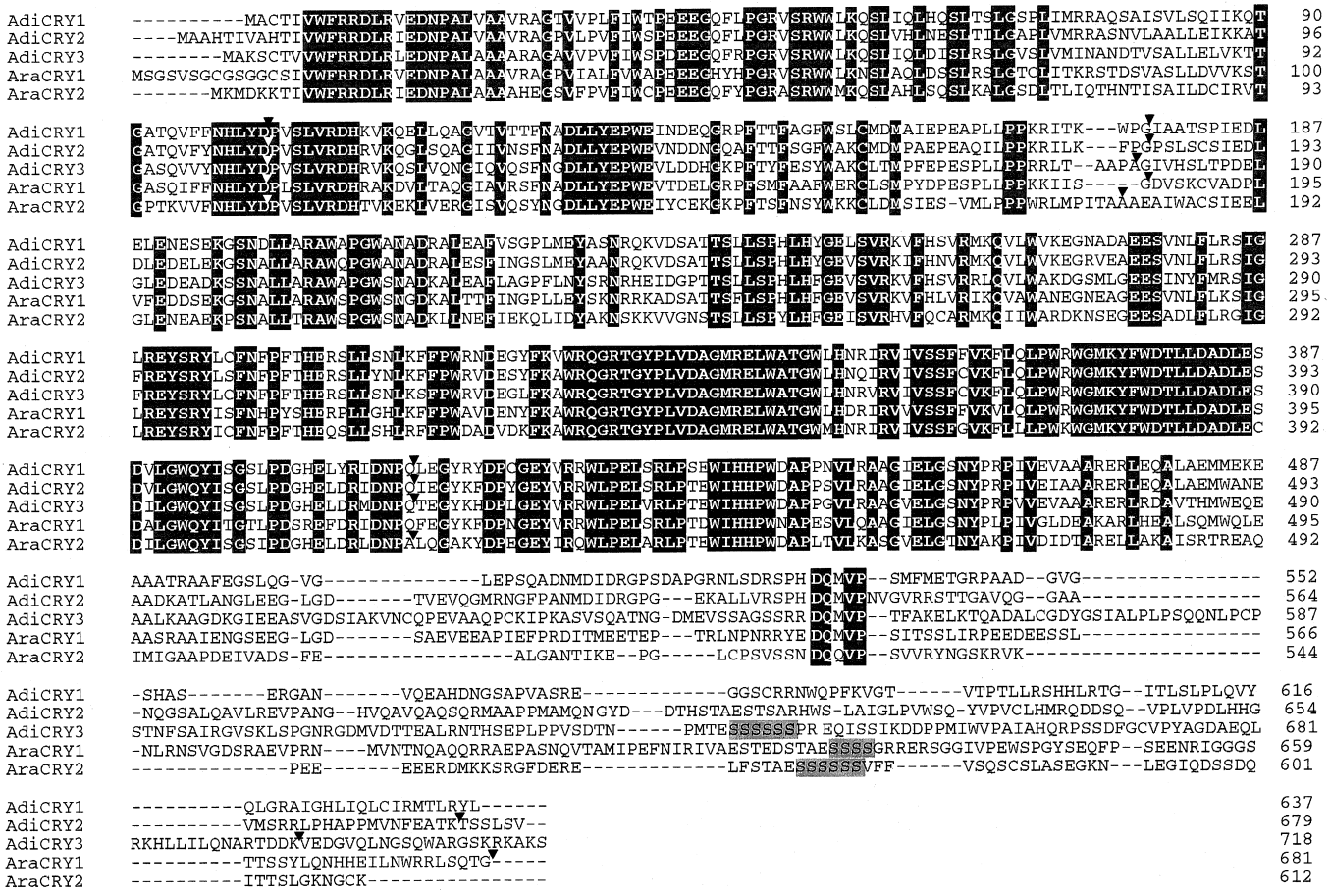

Fig. 4 Alignment of the deduced amino acid sequences of cryptochrome genes. Residues conserved in all five sequences are marked with *black boxes containing white lettering*. Intron insertion sites are indicated by *arrowheads*. AdiCRY1, AdiCRY2, AdiCRY3, AraCRY1, and AraCRY2 indicate the amino acid sequences predicted by *Adiantum CRY1*, *Adiantum CRY2*, *Adiantum CRY3*, *Arabidopsis CRY1*, and *Arabidopsis CRY2* (*PHH1*), respectively. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank databases under the accession numbers AB012626 (*Adiantum CRY1* cDNA), AB012627 (*Adiantum CRY2* cDNA), AB012628 (*Adiantum CRY3* cDNA), AB012629 (*Adiantum CRY1* gene), AB012630 (*Adiantum CRY2* gene), and AB012631 (*Adiantum CRY3* gene)

Adiantum, as in *Arabidopsis*. This evidence raises the possibility that a cryptochrome responsible for blue light-dependent phototropism in *Adiantum* may be among the *CRY*s we have isolated.

Our previous work using polarized light and/or microbeam irradiation demonstrated the intracellular localization of blue-light photoreceptors in *Adiantum*. Blue-light receptors involved in a shortening of the G1 phase, induction of cell division and inhibition of protonemal elongation are probably localized in or around the nucleus (Miyata et al. 1979; Kadota et al. 1986; Wada and Furuya 1978; Kadota et al. 1979). Receptors that confer apical cell swelling, polarotropism and chloroplast movement are localized on or close to the plasma membrane (Wada et al. 1978; Hayami et al. 1992; Yatsuhashi et al. 1987). The deduced amino acid sequences of the *Adiantum CRY*s do not contain characteristic nuclear localization signal (NLS); however, it is possible that *Adiantum* CRYs are imported into the nucleus by attachment to other nuclear proteins. Lin et al. showed that *Arabidopsis* CRY1 is a soluble protein and contains no predicted hydrophobic domains (Lin and Cashmore 1996; Lin et al. 1996a), but they did not exclude the possibility that *Arabidopsis* CRY1 transiently attaches to membranes. Computer analysis also predicted no hydrophobic membrane-spanning domains in *Adiantum* CRYs (data not shown), but the possibility still remains that they associate with the plasma membrane using some other anchoring mechanism. If the two additional cryptochrome-related sequences in the *Adiantum* genome are expressed and show structural similarity to cryptochrome, their gene products might also contain NLS or membrane-spanning domains.

Sequence similarities between cryptochromes and microbial type I photolyases suggest that the basic mechanism for absorbing blue light photons and transferring electrons may be the same in both photoreceptors. It is particularly noteworthy that almost all the amino acid residues that interact with FAD in the *E. coli* photolyase are conserved in cryptochromes. The TGYP motif that is thought to be involved in formation of the chromophore-binding pocket in both photolyase and

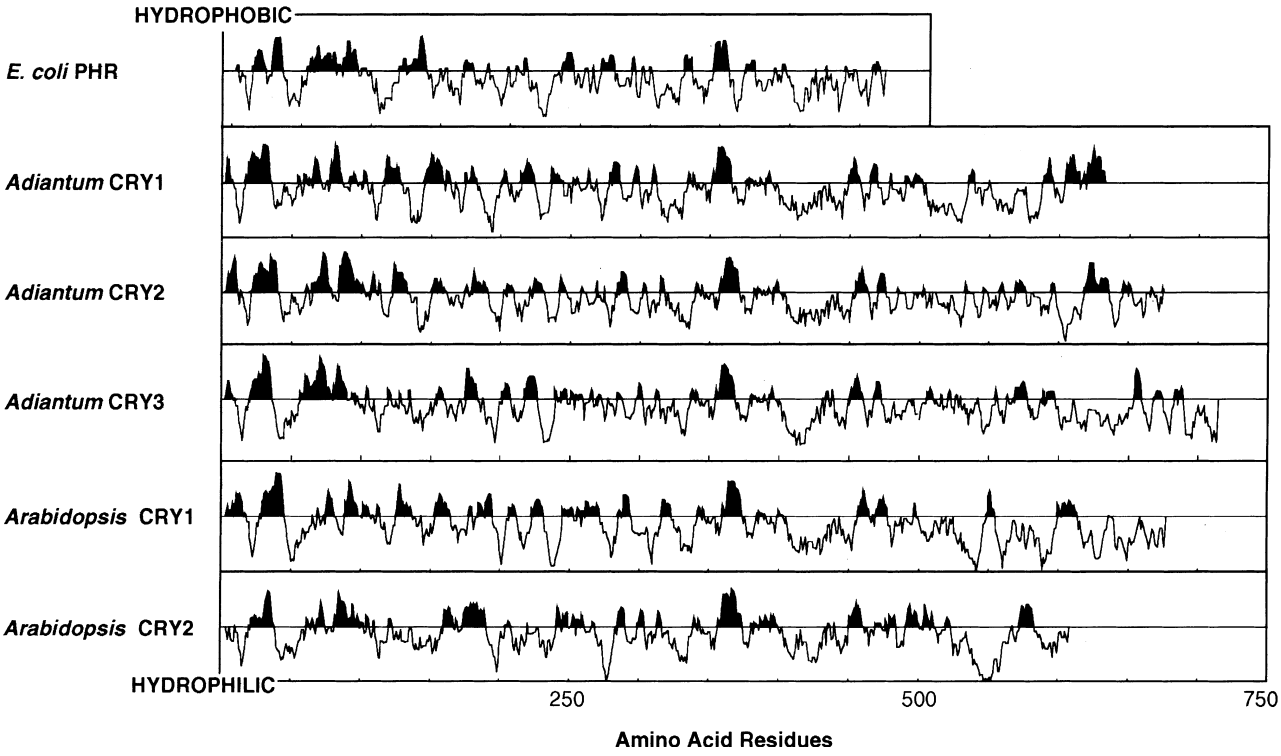

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Fig. 5 Hydropathy profiles of cryptochromes and microbial photolyase. Hydropathy analysis was performed according to Kyte and Doolittle (1982) with a window size of 9

Arabidopsis CRY1 (Ahmad et al. 1995) is also conserved in all cryptochromes. This evidence supports the hypothesis that the information from blue light signals perceived by cryptochromes is transmitted to their signal transduction pathways via electron donation from the FAD.

Ahmad et al. (1995) emphasized the critical importance of the C-terminal extension for *Arabidopsis* CRY1 function, because they found seven point mutations that mapped to this region. We found the DQMVP motif in this variable C-terminal region of cryptochromes. Two

Fig. 6 Alignment of amino acid sequences around the putative chromophore-binding site. Amino acids predicted to interact with FAD are marked by the *black boxes containing white lettering*. Residues presumed to interact with DNA are shown by *shaded boxes*. The TGYP motif is indicated by *asterisks*. E. coli, AdiCRY1, AdiCRY2, AdiCRY3, AraCRY1, AraCRY2, Sinapis and Chlamy indicate the amino acid sequences predicted by *E. coli phr*, *Adiantum CRY1*, *Adiantum CRY2*, *Adiantum CRY3*, *Arabidopsis CRY1*, *Arabidopsis CRY2* (*PHH1*), *Sinapis alba SA-PHR1* and *Chlamydomonas CPH1*, respectively

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E.coli      EKAAIAQLRQFCQNGAGEYEQQRDFPAVEGTSRLSASLATGGLSPRQCLHRLLAEQPQA-LDGGAGSV-----W   271

AdiCRY1     WANADRALEAFVSGPLMEYASNRQKVDSATTSLLSPHLHYGELSVRKVFHSVRMKQVLWVKEGNADAEESVNLF   282
AdiCRY2     WANADRALESFINGSLMEYAANRQKVDSATTSLLSPHLHYGEVSVRKIFHNVRMKQVLWVKEGRVEAEESVNLF   288
AdiCRY3     WSNADKALEAFLAGPFLNYSRNRHEIDGPTTSLLSPHLHFGEVSVRKVFHSVRRLQVLWAKDGSMLGEESINYF   285
AraCRY1     WSNGDKALTTFINGPLLEYSKNRRKADSATTSFLSPHLHFGEVSVRKVFHLVRIKQVAWANEGNEAGEESVNLF   290
AraCRY2     WSNADKLLNEFIEKQLIDYAKNSKKVVGNSTSLLSPYLHFGEISVRHVFQCARMKQIIWARDKNSEGEESADLF   287
Sinapis     WSNADKILNEFIEKQLIDYAKNSKKVVGNSTSLLSPYLHFGEISVRRVFQCARMKQIIWARDKNGEGEESADLF   286
Chlamy      VGGAISELEHFLAERLTEFEHDRAKVDRDSTSRLSPWIHIGSISVRYIFYRVRQCQAEW-LAAGTDRAQSCDDF   287

E.coli      LNELIWREFYRHLITYHPSLCKHRPFIAWTDRVQWQSNPAHLQAWQEGKTGYPIVDAAMRQLNSTGWMHNRLRM   345
                                                          ****
AdiCRY1     LRSIGLREYSRYL-CFNFPFTHERSLLSNLKFFPWRNDEGYFKVWRQGRTGYPLVDAGMRELWATGWLHNRIRV   355
AdiCRY2     LRSIGFREYSRYL-SFNFPFTHERSLLYNLKFFPWRVDESYFKAWRQGRTGYPLVDAGMRELWATGWLHNQIRV   361
AdiCRY3     MRSIGFREYSRYL-CFNFPFTHERSLLSNLKSFPWRVDEGLFKAWRQGRTGYPLVDAGMRELWATGWLHNRVRV   358
AraCRY1     LKSIGLREYSRYI-SFNHPYSHERPLLGHLKFFPWAVDENYFKAWRQGRTGYPLVDAGMRELWATGWLHDRIRV   363
AraCRY2     LRGIGLREYSRYI-CFNFPFTHEQSLLSHLRFFPWDADVDKFKAWRQGRTGYPLVDAGMRELWATGWMHNRIRV   360
Sinapis     LRGIGLRDYSRII-CFNFPFTHEQSLLSHLRFFPWDADVDKFKAWRQGRTGYPLVDAGMRELWATGWMHNRIRV   359
Chlamy      LQQMGYREYSRYL-AFHFPFIHERSLLGHLRACPWRIDQHAFKAWRQGQTGYPIVDAAMRQLWSSGWCHNRGRV   360

E.coli      ITASFLVKDLLIDWREGERYFMSQLIDGDLAANNGGWQWAASTGTDAAPYFRIFNPTTQGEKFDHEGEFIRQWL   419

AdiCRY1     IVSSFFVKFLQLPWRWGMKYFWDTLLDADLESDVLGWQYISGSLPDGHELYRIDNPQLEGYRYDPCGEYVRRWL   429
AdiCRY2     IVSSFCVKFLQLPWRWGMKYFWDTLLDADLESDVLGWQYISGSLPDGHELDRIDNPQIEGYKFDPYGEYVRRWL   435
AdiCRY3     IVSSFCVKFLQLPWRWGMKYFWDTLLDADLESDILGWQYISGSLPDGHELDRMDNPQTEGYKHDPLGEYVRRWL   432
AraCRY1     VVSSFFVKVLQLPWRWGMKYFWDTLLDADLESDALGWQYITGTLPDSREFDRIDNPQFEGYKFDPNGEYVRRWL   437
AraCRY2     IVSSFAVKFLLLPWKWGMKYFWDTLLDADLECDILGWQYISGSIPDGHELDRLDNPALQGAKYDPEGEYIRQWL   434
Sinapis     IVSSFAVKFLLLPWKWGMKYFWDTLLDADLECDIIGWQYISGSLPDGHELDRLDNPAIQGAKYDPEGEYIRQWL   432
Chlamy      VAASFLVKDLLLPWQWGLKHYWDAQIDADLECDALGWQYVSGGMSDAHPFSYMMDLEKEARRFDPDGEYVRRWL   434
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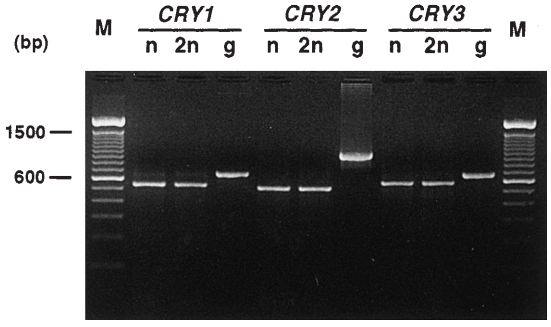

Fig. 7 RT-PCR analysis for *Adiantum CRY*s transcripts. Total RNA was isolated from protonemal cells (n) and young leaves of sporophytes (2n). RT-PCR was performed with the gene-specific primers on both side of the fourth intron (see Materials and methods). As a control, genomic DNA was also amplified with the same specific primers (g). The products were fractionated on a 2% agarose gel and stained with ethidium bromide. A 100-bp ladder marker was electrophoresed alongside the products (M) yielding the scale shown on the left

HY4 alleles (*hy4–3*, *hy4–9*) in *Arabidopsis* contain this motif (Ahmad and Cashmore 1993; Ahmad et al. 1995). In *hy4–9*, the proline residue of this motif is replaced by leucine although the CRY1 protein expression level is the same as that of wild type. These results indicate that this amino acid substitution has an effect on the signal transduction pathway via CRY1 and causes a decrease in the inhibition of hypocotyl elongation and anthocyanin biosynthesis. Therefore, it is of great interest to analyze the intramolecular function of this motif in cryptochromes.

The domain structure of cryptochrome is quite similar to that of the red/far-red light photoreceptor phytochrome. They both have a highly conserved domain involved in chromophore binding in their N-terminal regions, while the C-terminal domains are presumed to be concerned with substrate interactions (Furuya 1993; Clack et al. 1994). A question of great interest for the phylogenetic relationships of cryptochromes is whether diversification of cryptochrome genes in *Adiantum* occurred after the origin of seed plants, as in the case of phytochromes (Mathews and Sharrock 1997), and thus, whether there is any relationship between the cryptochromes from cryptogams and cryptochrome subfamilies in *Arabidopsis* (or seed plants). Answers to these interesting questions await the identification of more cryptochrome gene sequences from other plant species.

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