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Molecular cloning of a novel phytochrome gene of the moss *Ceratodon purpureus* which encodes a putative light-regulated protein kinase

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Abstract

The phytochrome gene (*phyCer*) of the moss *Ceratodon purpureus* was isolated and characterized. *phyCer* is composed of three coding exons: exon I of 2035 bp, exon II of 300 bp and exon III of 1574 bp. The deduced polypeptide encoded by exon I and II exhibits substantial sequence homology to the conserved NH_2-terminal chromophore domain of known phytochromes. In contrast, the COOH-terminal polypeptide encoded by exon III shows no sequence homology to any phytochrome molecule. *phyCer* most likely represents a single-copy gene and is expressed in a light-independent manner. From the DNA sequence analysis it can be deduced that the PhyCer polypeptide is composed of 1303 amino acids (including the starting Met) which predicts a molecular mass for PhyCer of 145 kDa. The polypeptide encoded in exon III exhibits striking homology within the 300 carboxy-terminal amino acids to the catalytic domain of protein kinases. The carboxy terminus of PhyCer was found to be most homologous to protein-tyrosine kinases of *Dictyostelium discoideum* and to the products of retroviral oncogenes which belong to the Raf-Mos serine/threonine kinase family. From the hydropathy profile PhyCer appears to be a soluble protein. The predicted structure suggests that PhyCer represents a soluble light-sensor protein kinase which is linked with a cellular phosphorylating cascade.

Introduction

Phytochrome is one of the most important photoreceptors in plant cells. The red/far-red reversible chromoprotein controls a wide variety of photomorphogenetic responses including regulation of gene expression [24, 28, 44]. In angiosperms phytochrome is encoded by a small gene family of four to five divergent members. The primary structures of *phyA*, which code for labile phytochrome (PI) and of *phyB* and *phyC*, which are thought to code for stable phytochrome (PII) have been determined [39, 9]. A partial sequence of a phytochrome, designated *phyD*, was reported by Rüdiger and Thümmler [29].

Despite many efforts, the signal transduction

The *phyCer* sequence was submitted to the Martinsried Institute of Protein Sequences (MIPS; MPI f. Biochemie, FRG) under the accession number S20160.

chains that link the light dependent activation of phytochrome with the physiological plant responses are still unknown. So far the sequence analysis of phytochrome genes has not provided many clues about possible signal transduction mechanisms. Interestingly, Schneider-Poetsch *et al.* [36, 37] found that the COOH-terminal domains of phytochrome of the fern *Selaginella martensii* and the angiosperm phytochromes exhibit substantial homology to the cytosolic, COOH-terminal parts of bacterial sensor proteins, which are involved in the transduction of chemical stimuli within bacterial cells. These bacterial sensor proteins are generally composed of a NH_2-terminal sensor domain and a COOH-terminal catalytic regulator domain [42]. Thus, according to this model, phytochrome is composed of a NH_2-terminal domain functioning as a conserved light sensor and a catalytic, COOH-terminal regulator domain. However, up to now there exists no direct evidence supporting the existence of a catalytic activity of the COOH-terminal part of any phytochrome molecule.

The presence of several different phytochromes that respond to the same light stimulus complicate the analysis of phytochrome action in higher plants. For this reason, we looked for a more simple plant system to analyse the molecular action of phytochrome.

The development of mosses is in many ways under the control of phytochrome [20]. The best investigated example for phytochrome action in mosses is the red light-dependent positive phototropism of *Ceratodon purpureus* protonemata [19]. The low level of organization makes mosses a simple model system to study more complex cellular processes. Therefore we decided to investigate phytochrome action in the moss *C. purpureus*. Recently we were able to isolate phytochrome sequences from *C. purpureus* using the polymerase chain reaction [45]. We used these DNA-probes to screen a *C. purpureus* genomic library and were able to isolate and characterize a genomic clone coding for *C. purpureus* phytochrome.

Here we report the complete sequence of the phytochrome gene of *C. purpureus* (*phyCer*). *phyCer* represents an unusual, novel phytochrome gene. In contrast to all known phytochromes, the 3′ terminus of *phyCer* codes for a putative polypeptide with striking homology to protein kinases suggesting that phytochrome in mosses is a light-regulated protein kinase. The meaning of this finding for the understanding of phytochrome-dependent photomorphogenesis in plants will be discussed.

Materials and methods

Plant materials

Experiments were performed with sterile cultures of *Ceratodon purpureus* (Hedw.) Brid. protonemata which were originally derived from a single spore [18]. The cultures, a gift of Dr E. Hartmann, were subcultured by vegetative regeneration. The protonemata were grown in sterile liquid cultures or on solid medium containing 1.2% (w/v) agar at 20 °C under a light regime of 18 h light and 6 h dark. The medium used is a modified Kofler's medium described by Saxena and Rashid [35] containing: $Ca(NO_3)_2 \cdot 4H_2O$ (50 mg/l), K_2SO_4 (100 mg/l), KH_2PO_4 (220 mg/l), $FeSO_4 \cdot 7H_2O$ (10 mg/l), $ZnSO_4$ (1 mg/l), KNO_3 (770 mg/l), KCl (250 mg/l), $MgSO_4$ (250 mg/l), Na_2EDTA (10 mg/l), H_3BO_3 (1 mg/l), $MnSO_4$ (0.1 mg/l), KJ (0.01 mg/l), $CuSO_4$ (0.03 mg/l), 1% (w/v) glucose. The pH was adjusted to 5.8 using 0.1 M NaOH.

Oat seedlings (*Avena sativa* L. cv. Pirol; BayWa, Munich, FRG) were grown on Vermiculite at 25 °C in the dark for 3.5 days. *Arabidopsis thaliana* cv. Eil-O was grown for four weeks in a greenhouse in soil under short-day conditions (8 h light/16 h dark).

Genomic library construction and screening

C. purpureus genomic DNA was isolated according to a miniprep method as described by Davis *et al.* [6]. An additional precipitation step with LiCl to remove RNA and further interfering im-

purities from the DNA preparations was performed. The genomic library was constructed with *Sau*3AI partial digested, size fractionated DNA and the *LambdaGEM-11* phage vector (Promega) cut with *Xho* I according to the protocol provided by the manufacturer. The cloning strategy using partially filled-in *Xho* I lambda arms and partially filled-in *Sau*3AI-cut genomic DNA was followed. About 18000 recombinant phages were obtained in a typical cloning experiment. Without further amplification the phages were screened with the *phyCer*-specific probe, which was derived from a PCR fragment amplified with the primer combination L456/R740 [45]. Hybridization was carried out with a *DIG*-dUTP (Boehringer Mannheim) labelled, single-stranded probe. The labelling reaction was carried out with Amplitaq (Perkin Elmer Cetus) as described [45]. Plaque hybridization was carried out at low stringency in 30% formamide, 5 × SSC (0.75 M NaCl, 0.075 sodium citrate), 5% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, 0.02% SDS and denatured salmon sperm DNA (100 μg/ml) for 18 h at 42 °C. Filters were washed in 2 × SSC, 0.1% SDS at room temperature with final washes at 50 °C. Immunological detection of the hybrids was performed with the *DIG* DNA detection kit (Boehringer Mannheim) according to the kit instructions.

DNA subcloning, PCR experiments, nucleotide sequencing and sequence analysis

All manipulations were carried out according to standard protocols [2]. Conditions for PCR experiments, cloning of PCR fragments and labelling of PCR fragments were essentially as described [45]. Phage insert fragments and derivative subfragments were cloned into the *pBluescript-KS* + (Stratagene) plasmid vector. Nucleotide sequence determination was carried out by the dideoxy chain termination method [32] with double-stranded DNA templates using the T7 DNA polymerase (Pharmacia). Subclones for further sequence analysis were obtained by sequential deletions. The data were analysed on VAX-computers using GCG sequence analysis programs [10] at Martinsried Institute for Protein Sequences (MIPS), MPI für Biochemie, FRG. Multiple sequence alignments were made with the PILEUP program using standard parameters; the resembling phylogenetical distances of the aligned sequences were calculated with the DISTANCES program. Homology plots were drawn with the PLOTSIMILARITY program.

Mapping of intron-exon boundaries (reverse PCR)

Poly(A)$^+$ RNA was prepared by chromatography of total RNA isolated from *C. purpureus* protonemata (see below) on oligo-dT-cellulose (Type 7, Pharmacia). About 1 μg of poly(A)$^+$ RNA was used to synthesize oligo-dT-primed cDNA with the M-MLV reverse transcriptase (BRL), in a 20 μl reaction volume. The reaction mix was brought to 100 μl with H_2O and 1 μl was used directly in a subsequent PCR experiment. The oligonucleotide primers used were L2480 (5′-TGAGCAGAGAATGTTCATGGACAG-3′) and primer R3553 (5′-GCTGAAGAACCACT-TCCCAAGCTC-3′) (L = sense, R = antisense primer; the numbers indicate the positions of the 5′-terminal nucleotides within *phyCer* relative to the initiation ATG). The PCR products were purified on agarose gels, extracted from the gels with GEANCLEAN (BIO 101, USA) and subcloned into pBluescript. Nucleotide sequence determination was performed as described above.

Southern blot analysis

For Southern blot analysis of the *phyCer* gene, 10 μg genomic DNA was digested with *Eco* RI or *Hin*d III. The resulting fragments were separated by electrophoresis through a 1% agarose gel and blotted onto Biodyne A (PALL) nylon membrane. The DNA was fixed to the membrane by UV treatment. Synthesis of the single-stranded, *DIG*-dUTP-labelled probes, hybridization and immunological detection of the hybrids was essentially as described above for screening except that hy-

bridization solutions contained 50% formamide instead of 30% formamide. The subcloned *Eco* RI/*Pst* I and 1.2 kb *Hin*d III fragments were amplified prior to labelling with PCR using the T3 and T7 sequencing primers.

Northern blot analysis

Total RNA was isolated with a hot phenol method as described by de Vries *et al.* [11]. RNAs were prepared from light-grown *C. purpureus* protonemata, from protonemata kept in the dark for 3 days prior to RNA extraction, from 3.5-day-old etiolated oat seedlings and from leaves of 4-week-old *A. thaliana* plants, kept 3 days in the dark prior to RNA extraction. The RNAs (10 μg per lane) were separated in a formaldehyde containing, 1% agarose gel and blotted to Biodyne A (PALL) nylon membrane. The RNA was fixed to the matrix by UV-treatment. The blots were hybridized with single-stranded ^{32}P-dCTP-labelled probes at high stringency as described above for Southern analysis. *C. purpureus* blots were hybridized with the *phyCer*-specific PCR probe used for screening. The oat *phyA* probe was derived from a PCR fragment obtained with the primer combination L721/R1676 [45] and the full-length cDNA clone HM41 coding for oat phytochrome [47]. The *A. thaliana phyA* probe was derived from a PCR fragment obtained with the primer combination L1318/R1676 [45] and the genomic clone d4 containing the *phyA* gene of *A. thaliana* (Rollfinke and Thümmler, unpublished). Synthesis of the single stranded, ^{32}P-dCTP-labelled probes was performed as described previously [45]. Hybrids were detected by autoradiography. Transcript sizes were estimated from mobility relative to RNA molecular standards (Boehringer Mannheim).

Results

Isolation and characterization of phyCer

The *phyCer*-specific probe which was amplified in a PCR experiment with the primer combination L456/R740 [45] was used to search a *C. purpureus* genomic library (PCR probe; Fig. 1A). Of ca. 18000 plaques, four reacted positively with the *phyCer* probe. The positives were plaque purified; one of the initial clones turned out to be a false-positive. The remaining three clones exhibited similar restriction maps implicating that they are carrying the identical, partly overlapping gene. The clone designated A2 was used for further analysis. The physical map of A2 is given in Fig. 1A. The 5.8 kb *Xho* I fragment of A2 which hybridizes to the PCR probe and most likely contained *phyCer* completely was subjected to DNA sequence analysis. The nucleotide and derived amino acid sequences are shown in Fig. 2. The sequence analysis revealed that *phyCer* is composed of 3 major open reading frames (ORF): ORF I of about 2000 bp, ORF II of about 300 bp and ORF III of about 1500 bp.

The putative polypeptides encoded by ORF I and II exhibit striking sequence homologies to the NH_2 terminal, chromophore binding domain of the known phytochrome polypeptides (see below; Table 2). Because of the high degree of homology of *phyCer* to the *phyA* genes it was possible to localize the precise boundaries of exons I and II of *phyCer* based on the presence of conserved splice junctions. Exon I codes for a polypeptide of 678 amino acids calculated from the first in frame Met; the exon II-encoded polypeptide is 100 amino acids long. Exon I and II are separated by a 296 bp intron. As mentioned above, the gene structure of the *phyA* genes and the *phyCer* gene is conserved in this region (Fig. 1B). A large exon (exon I in *phyCer*) of about 2000 bp is coding for the chromophore-binding domain followed by a short intron (intron 1 in *phyCer*). The exon-intron junctions of this intron are conserved between *phyCer* and all known *phyA* genes and are most likely conserved in all *phy* genes [30]. Interestingly, exon II, which is about 820 bp long in all known phytochrome genes [5, 21, 23, 33] is only partly present in the *phyCer* gene. The corresponding exon II stops 300 nucleotides downstream from the intron 1/exon II junction.

In contrast to the *phyA* genes, the COOH terminus of *phyCer* is encoded by a long continuous

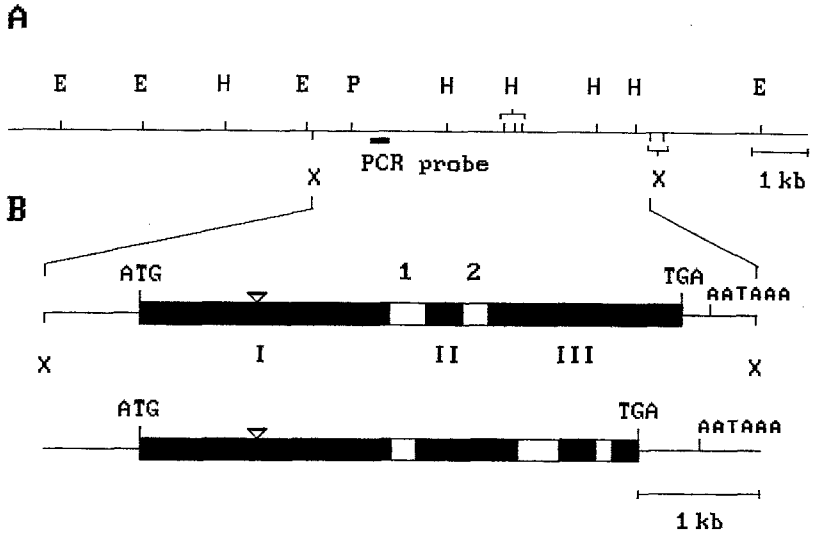
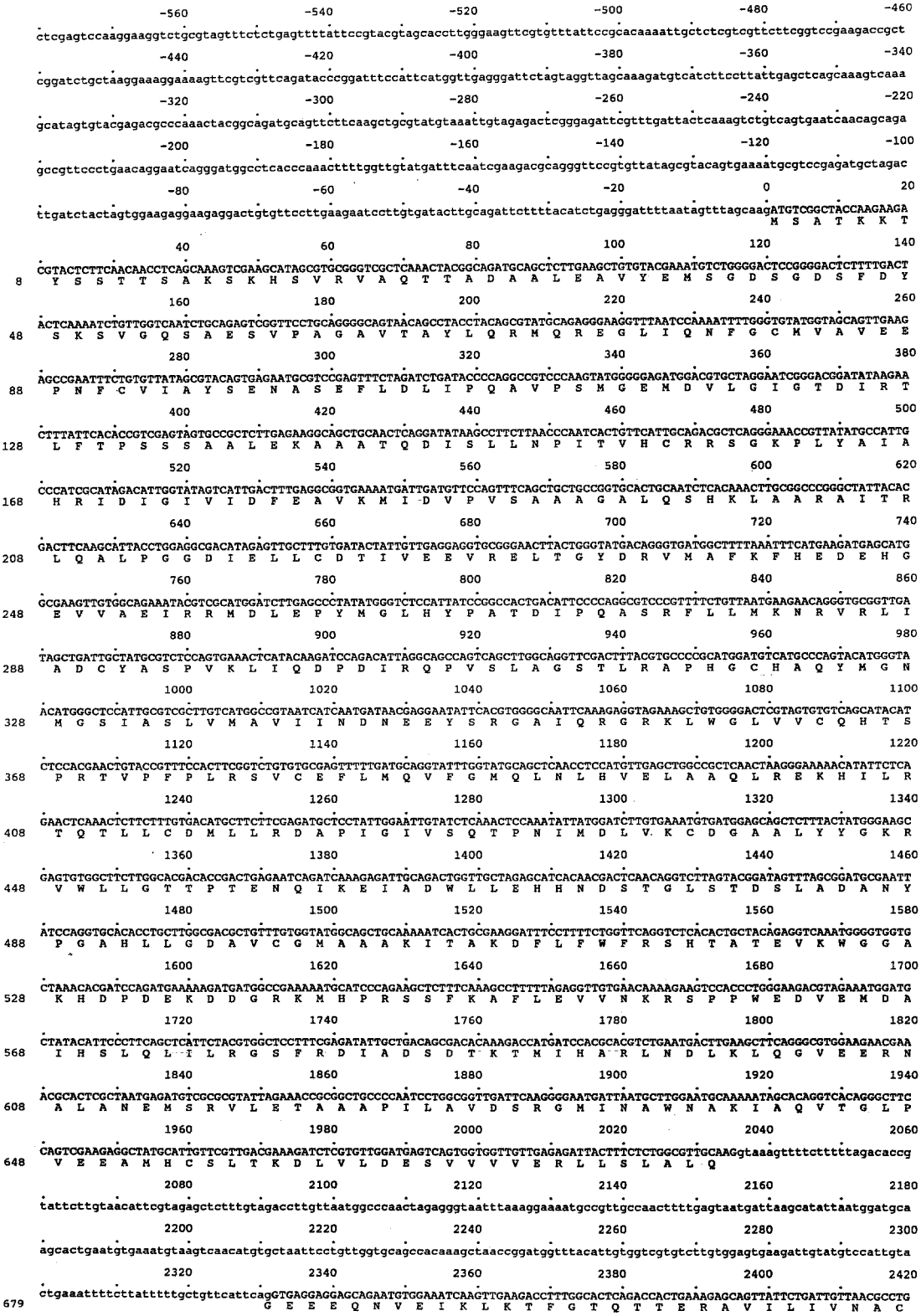


Fig. 1. Analysis of the *phyCer* gene. A. Restriction map of the genomic clone A2. E, H, P and X represent restriction sites of *Eco* RI, *Hind* III, *Pst* I and *Xho* I, respectively. The thick bar indicates the site of hybridization of the 289 bp DNA fragment (PCR probe) used for screening. B. Comparison of the structures of the *phyCer* gene and the *phyA* gene from oat [21]. The 5.8 *Xho* I fragment of A2 containing the *phyCer* gene is shown at the top. X represents the restriction sites of *Xho* I. The black boxes indicate the coding sequences. The open boxes indicate intron sequences and the lines indicate 5′ and 3′ flanking sequences. I, II and III = exon I, II and III of *phyCer*; 1 and 2 = intron 1 and 2 of *phyCer*. The arrowheads indicate the positions of the chromophore attachment sites.

ORF (see Fig. 1B). The predicted polypeptide encoded in ORF III has no sequence homology to the known phytochrome polypeptides. 236 bp downstream of the termination codon (TGA) of ORF III a polyadenylation signal (AATAAA) is found, implicating that ORF III is coding for the COOH terminus of the *phyCer* polypeptide. Downstream of the polyadenylation signal other consensus sequences are found, which are considered to be also essential for the formation of active mRNA; 23 bp downstream of AATAAA the sequence TGTGTTCC is found which fits the consensus sequence YGTGTTYY present in many mammalian mRNAs [25]; 10 bp downstream of this sequence the motif CAYTG is present which is usually found close to the site of poly(A) addition [3]. It is interesting to note that all consensus sequences involved in precise polyadenylation in mammals are present in the 3′ terminus of *phyCer*; genes of higher plants are usually much more diverse in this region [7].

To prove that this unusual phytochrome gene is indeed an actively transcribed gene and to localize the precise exon/intron boundaries between exon II and ORF III we investigated the corresponding cDNA of *phyCer*. cDNA clones of interest were isolated with the reverse PCR method [13] using primer combinations flanking the putative exon/exon boundary. With poly(A)$^+$ mRNA isolated from *C. purpureus* protonemata oligo(dT)-primed single-stranded cDNA was synthesized. With the primers L2480/R3553 and the single stranded cDNA as template a PCR product of about 900 bp was obtained. Sequence analysis of this PCR product revealed that exon II and ORF III are indeed spliced together into an actively transcribed mRNA. From this clone the precise exon/intron boundaries between exon II and exon III could be localized; the size of intron 2 turned out to be 210 bp. Figure 3 shows the sequence data around the junction site between exon II and III. The *phyA* genes contain introns within the 5′ non-coding region; in the case of rice this intron is 2582 bp long [23]. It is not known if *phyCer* contains a corresponding intron in this region. This is possible since up-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M S A T K K T
40 60 80 100 120 140
CGTACTCTTCAACAACCTCAGCAAAGTCGAAGCATAGCGTGCGGGTCGCTCAAACTACGGCAGATGCAGCTCTTGAAGCTGTGTACGAAATGTCTGGGGACTCCGGGGACTCTTTTGACT
8 Y S S T T S A K S K H S V R V A Q T T A D A A L E A V Y E M S G D S G D S F D Y
160 180 200 220 240 260
ACTCAAAATCTGTTGGTCAATCTGCAGAGTCGGTTCCTGCAGGGGCAGTAACAGCCTACCTACAGCGTATGCAGAGGGAAGGTTTAATCCAAAATTTTGGGTGTATGGTAGCAGTTGAAG
48 S K S V G Q S A E S V P A G A V T A Y L Q R M Q R E G L I Q N F G C M V A V E E
280 300 320 340 360 380
AGCCGAATTTCTGTGTTATAGCGTACAGTGAGAATGCGTCCGAGTTTCTAGATCTGATACCCCAGGCCGTCCCAAGTATGGGGGAGATGGACGTGCTAGGAATCGGGACGGATATAAGAA
88 P N F C V I A Y S E N A S E F L D L I P Q A V P S M G E M D V L G I G T D I R T
400 420 440 460 480 500
CTTTATTCACACCGTCGAGTAGTGCCGCTCTTGAGAAGGCAGCTGCAACTCAGGATATAAGCCTTCTTAACCCAATCACTGTTCATTGCAGACGCTCAGGGAAACCGTTATATGCCATTG
128 L F T P S S S A A L E K A A A T Q D I S L L N P I T V H C R R S G K P L Y A I A
520 540 560 580 600 620
CCCATCGCATAGACATTGGTATAGTCATTGACTTTGAGGCGGTGAAAATGATTGATGTTCCAGTTTCAGCTGCTGCCGGTGCACTGCAATCTCACAAACTTGCGGCCCGGGCTATTACAC
168 H R I D I G I V I D F E A V K M I D V P V S A A A G A L Q S H K L A A R A I T R
640 660 680 700 720 740
GACTTCAAGCATTACCTGGAGGCGACATAGAGTTGCTTTGTGATACTATTGTTGAGGAGGTGCGGGAACTTACTGGGTATGACAGGGTGATGGCTTTTAAATTTCATGAAGATGAGCATG
208 L Q A L P G G D I E L L C D T I V E E V R E L T G Y D R V M A F K F H E D E H G
760 780 800 820 840 860
GCGAAGTTGTGGCAGAAATACGTCGCATGGATCTTGAGCCCTATATGGGTCTCCATTATCCGGCCACTGACATTCCCCAGGCGTCCGTTTTCTGTTAATGAAGAACAGGGTGCGGTTGA
248 E V V A E I R R M D L E P Y M G L H Y P A T D I P Q A S R F L L M K N R V R L I
880 900 920 940 960 980
TAGCTGATTGCTATGCGTCTCCAGTGAAACTCATACAAGATCCAGACATTAGGCAGCCAGTCAGCTTGGCAGGTTCGACTTTACGTGCCCCGCATGGATGTCATGCCCAGTACATGGGTA
288 A D C Y A S P V K L I Q D P D I R Q P V S L A G S T L R A P H G C H A Q Y M G N
1000 1020 1040 1060 1080 1100
ACATGGGCTCCATTGCGTCGCTTGTCATGGCCGTAATCATCAATGATAACGAGGAATATTCACGTGGGGCAATTCAAAGAGGTAGAAAGCTGTGGGGACTCGTAGTGTGTCAGCATACAT
328 M G S I A S L V M A V I I N D N E E Y S R G A I Q R G R K L W G L V V C Q H T S
1120 1140 1160 1180 1200 1220
CTCCACGAACTGTACCGTTTCCACTTCGGTCTGTGTGCGAGTTTTGATGCAGGTATTTGGTATGCAGCTCAACCTCCATGTTGAGCTGGCCGCTCAACTAAGGGAAAAACATATTCTCA
368 P R T V P F P L R S V C E F L M Q V F G M Q L N L H V E L A A Q L R E K H I L R
1240 1260 1280 1300 1320 1340
GAACTCAAACTCTTCTTTGTGACATGCTTCTTCGAGATGCTCCTATTGGAATTGTATCTCAAACTCCAAATATTATGGATCTTGTGAAATGTGATGGAGCAGCTCTTTACTATGGGAAGC
408 T Q T L L C D M L L R D A P I G I V S Q T P N I M D L V K C D G A A L Y Y G K R
1360 1380 1400 1420 1440 1460
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1480 1500 1520 1540 1560 1580
ATCCAGGTGCACACCTGCTTGGCGACGCTGTTTGTGGTATGGCAGCTGCAAAAATCACTGCCAAGGATTTCCTTTTCTGGTTCAGGTCTCACACTGCTACAGAGGTCAAATGGGGTGGTG
488 P G A H L L G D A V C G M A A A K I T A K D F L F W F R S H T A T E V K W G G A
1600 1620 1640 1660 1680 1700
CTAAACACGATCCAGATGAAAAAGATGATGGCCGAAAAATGCATCCCAGAAGCTCTTTCAAAGCCTTTTTAGAGGTTGTGAACAAAAGAAGTCCACCCTGGGAAGACGTAGAAATGGATG
528 K H D P D E K D D G R K M H P R S S F K A F L E V V N K R S P P W E D V E M D A
1720 1740 1760 1780 1800 1820
CTATACATTCCCTTCAGCTCATTCTACGTGGCTCCTTTCGAGATATTGCTGACAGCGACACAAAGACCATGATCCACGCACGTCTGAATGACTTGAAGCTTCAGGGCGTGGAAGAACGAA
568 I H S L Q L I L R G S F R D I A D S D T K T M I H A R L N D L K L Q G V E E R N
1840 1860 1880 1900 1920 1940
ACGCACTCGCTAATGAGATGTCGCGCGTATTAGAAACCGCGGCTGCCCCAATCCTGGCGGTTGATTCAAGGGGAATGATTAATGCTTGGAATGCAAAAATAGCACAGGTCACAGGGCTTC
608 A L A N E M S R V L E T A A A P I L A V D S R G M I N A W N A K I A Q V T G L P
1960 1980 2000 2020 2040 2060
CAGTCGAAGAGGCTATGCATTGTTCGTTGACGAAAGATCTCGTGTTGGATGAGTCAGTGGTGGTTGTTGAGAGATTACTTTCTCTGGCGTTGCAAGgtaaagttttcttttagacaccg
648 V E E A M H C S L T K D L V L D E S V V V V E R L L S L A L Q
2080 2100 2120 2140 2160 2180
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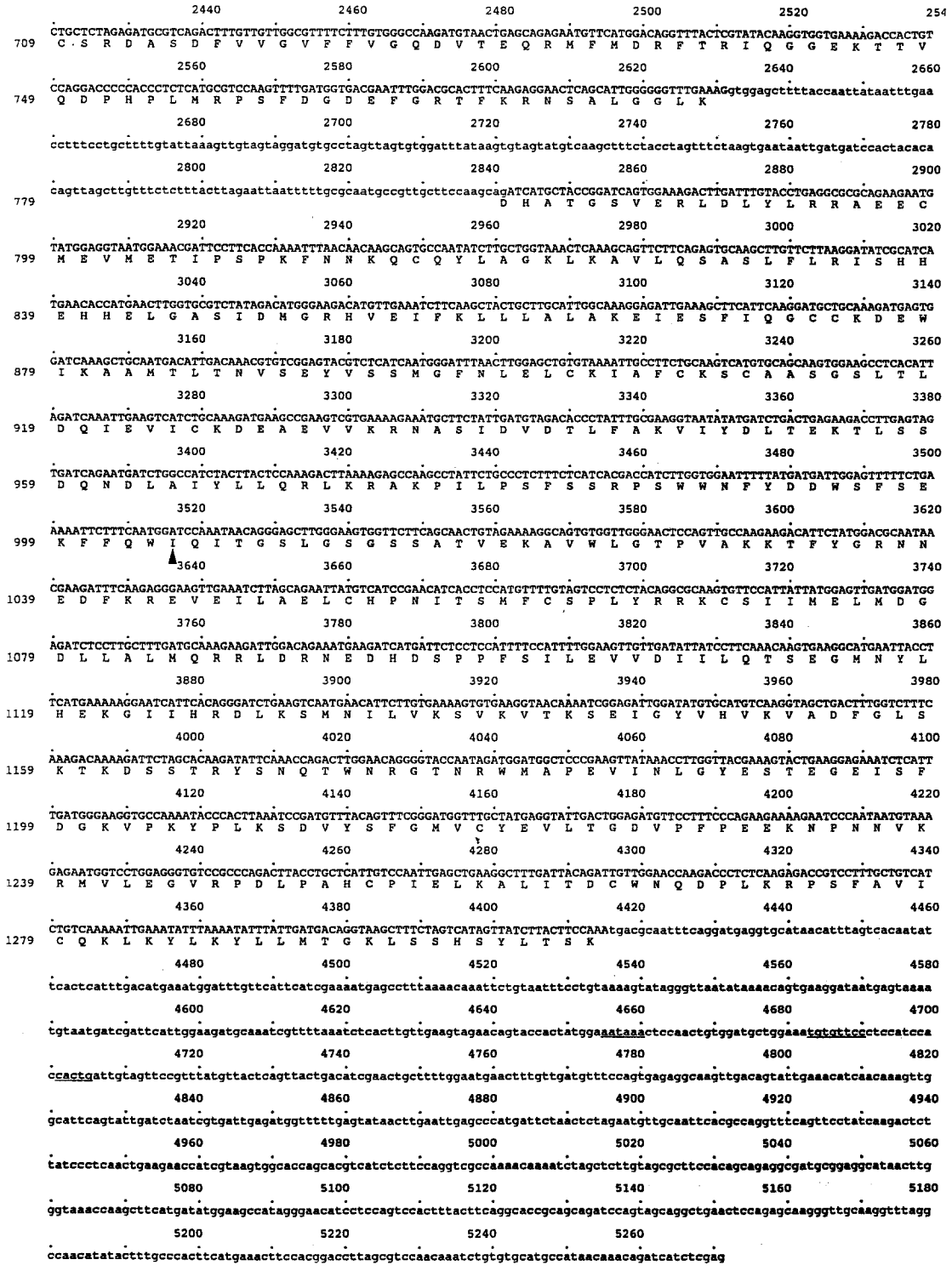

Fig. 2. Nucleotide and derived amino acid sequences of *phyCer*. The coding regions are represented by capital letters; intron and flanking regions are shown in lower-case letters. Nucleotides are numbered above the sequences; amino acids are numbered at the left side starting with the first in-frame methionine of the *phyCer*-encoded polypeptide. Consensus sequences presumably involved in polyadenylation of the *phyCer* transcript are underlined. The arrow indicates the approximate position of the beginning of the protein kinase catalytic domain (see Fig. 4).

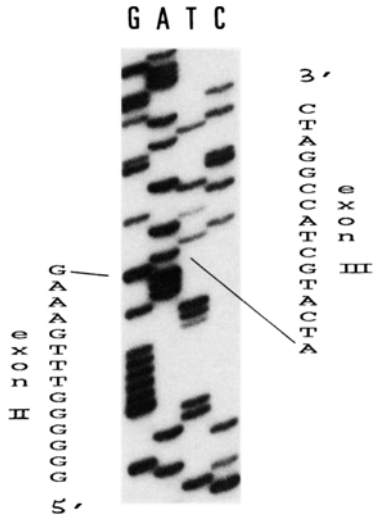

Fig. 3. Mapping of intron 2; nucleotide sequence analysis of the cDNA clone obtained by the reverse PCR experiment. The sequence around the junction of exon II and exon III is shown. GATC are the respective lines of the dideoxy sequencing ladder.

stream of the putative initiation ATG we were not able to detect conserved sequence motives which are usually found in typical eucaryotic plant promoters.

The 3' terminus of phyCer *exhibits homology to protein kinases*

A database comparison of the amino acid sequence of the polypeptide encoded in exon III revealed striking homology within the 300 carboxy-terminal amino acids to the catalytic domains of protein kinases. Each residue thought to be essential to phosphotransferase activity [17] is present at an analogous position in PhyCer (see Fig. 4). PhyCer was found to be most homologous to previously described protein-tyrosine kinases of *Dictyostelium discoideum* [43] (DPYK1 and DPYK2) and to the products of retroviral oncogenes and their cellular counterparts which belong to the Raf-Mos serine/threonine kinase family [16] (see Table 1).

The catalytic region of the kinase domain of PhyCer was compared with over 120 protein kinase catalytic domains included in the SALK protein-kinase database [16] using GCG multiple alignment programs [10]. The PILEUP program [12] clustered the PhyCer kinase between Mos and Raf protein serine/threonine kinases together with DPYK1 and DPYK2 (see Fig. 4 in Hanks and Quinn [16]. The phylogenetic distances and the percentage of identical residues between the different kinases are given in Table 1. The multiple alignment is shown in Fig. 4. The homology of PhyCer to DPYK1, DPYK2 and Raf and Mos is interesting: Raf and Mos represent serine/threonine kinases which are phylogenetically closest to the protein-tyrosine kinases [17]; DPYK1 and DPYK2 are protein-tyrosine kinases which possess elements of both protein-tyrosine and serine/threonine kinases [43]. In fact, PhyCer also exhibits this 'structural mosaic' (see Fig. 4).

Table 1. Phylogenetical distances and percentage of identical residues between the kinase catalytical domain of protein kinases which are closest to PhyCer.

	DPYK1	DPYK2	A-Raf	C-Src	PhyCer	C-Mos
DPYK1	–	59(42)	49(32)	47(32)	46(31)	43(25)
DPYK2		–	53(32)	52(33)	49(33)	46(29)
A-Raf			–	54(34)	47(31)	45(26)
C-Src				–	46(30)	46(31)
PhyCer					–	38(22)

The distance value is the number of matches between each sequence pair divided by the length of the shorter sequence of the pair without gaps multiplied by 100. Percentage of identical residues (in parenthesis) were calculated by dividing the number of identical residues between each sequence pair by the length of the shorter sequence of the pair without gaps and multiplying the result by 100. The region of the kinase domains compared to each other is shown in Fig. 4. References for A-Raf, C-Mos and C-Src are found in Hanks and Quinn [16], DPYK1 and DPYK2 in Tan and Spudich [43].

Fig. 4. Multiple amino acid sequence alignment of protein kinase catalytic domains and the kinase domain of PhyCer. Numbering is according to Fig. 2. The PhyCer sequence is compared with those of protein-serine/threonine kinases C-Mos and A-Raf, the authentic protein-tyrosine kinase C-Src and recently described *C. discoideum* protein-tyrosine kinases DPYK1 and DPYK2. References for the kinases are given in Table 1. The amino acid residues highly conserved in typical protein-tyrosine kinases (ConsTyr) and serine/threonine kinases (ConsSer) [16] are indicated below the sequences. The conserved residues also present in PhyCer are given in capitals, conserved residues not found in PhyCer are given in lower-case letters. The arrow indicates the lysine (K^{1129}) found in almost all serine/threonine kinases. Roman numerals above the sequences indicate conserved kinase subdomains designated by Hanks and Quinn [16].

The residue strictly conserved in typical protein-serine/threonine kinases which is also present in PhyCer is Lys^{1129} found in region VIb defined by Hanks and Quinn [16]. In contrast, only 4 of 34 residues specifically conserved in well characterized tyrosine kinases are not present in PhyCer. From these homologies we tentatively conclude that the *phyCer* gene product is a protein-tyrosine kinase likely to be regulated in a red/far-red reversible manner.

Southern blot analysis of phyCer

Genomic Southern blots of *C. purpureus* DNA were hybridized with DNA probes directed to different regions of *phyCer*. The 808 bp *Eco* RI-*Pst* I fragment hybridizes to the 5′ untranslated end including 160 bp of exon I. The 289 bp PCR fragment used for screening hybridizes to exon I about 220 bp upstream of the chromophore attachment site. The 1267 bp *Hin*d III fragment almost completely covers exon III. These experiments revealed the fragments expected from the restriction map of A2, but in addition larger fragments were detected with the PCR probe hybridizing to exon I (Fig. 5, lane B). Since the hybridization conditions were stringent, all bands shown in Fig. 5 represent DNA fragments homologous to the *phyCer* gene. The gel blots implicate that *phyCer* is present in a single copy within the *C. purpureus* genome. In addition, a stretch of

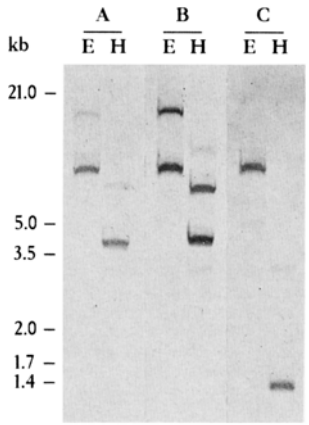

Fig. 5. Southern blot analysis of the *phyCer* gene. Genomic DNA prepared from *C. purpureus* protonemata was digested (10 μg per line) with *Eco* RI (E) or *Hin*d III (H), separated by electrophoresis through a 1% agarose gel and blotted onto Biodyne A (PALL). Hybridization was performed at high stringency with the following probes: the 808 bp *Eco* RI/*Pst* I fragment containing the 5′ end of A2 (A), the PCR probe hybridizing to exon I (B) and the 1.2 kb *Hin*d III fragment containing exon III nearly complete (C). The probes were labelled with DIG-dUTP and the hybrids were detected with immunological methods (see Materials and methods).

phyCer containing the chromophore attachment site is present twice. If this fragment represents a pseudogene or another active phytochrome gene in *C. purpureus* is not known yet. The presence of phytochrome pseudogenes has also been described for pea and maize [5, 33, 34].

Structural features of the phyCer-*encoded polypeptide*

From the predicted polypeptide encoded by *phyCer*, it can be deduced that PhyCer is composed of three major domains, the conserved chromophore domain encoded by exon I and II, the COOH-terminal kinase domain and a hinge region of about 220 amino acids which links the chromophore and the kinase domain. The hinge region of PhyCer does not show significant homology to any protein found in the databases. From the DNA sequence analysis the deduced PhyCer polypeptide is composed of 1303 amino acids (aa) which predicts a molecular mass of the *C. purpureus* phytochrome polypeptide of about 145 kDa and is thus larger than other *phy*-coded polypeptides (e.g. PhyA oat 1129 aa, 125 kDa; *A. thaliana* PhyA 1122 aa, 124 kDa; PhyB 1172 aa, 129 kDa and PhyC 1111 aa, 124 kDa).

The distribution of amino acid sequence identity along aligned pairs of oat and *A. thaliana* PhyA and of *A. thaliana* PhyA and PhyCer are compared in Fig. 6. It is striking to note that the drop of homology between the PhyA sequences [9, 21, 38, 39] marks the fusion point between the chromophore and the kinase domain of PhyCer. From the hydropathy profile (not shown) PhyCer appears to be a soluble protein. It has no stretch of hydrophobic amino acids in the amino termi-

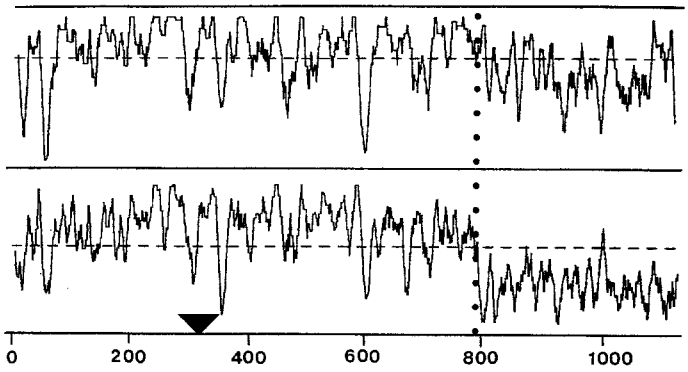

Fig. 6. Homology plots of oat and *A. thaliana* PhyA and PhyCer. Top panel: oat versus *A. thaliana* PhyA, bottom panel: *A. thaliana* PhyA versus PhyCer. Numbering of amino acid residues refers to residue positions in PhyCer. The arrowhead indicates the chromophore attachment site; the dashed line marks the average homology of the compared sequences and the dotted line indicates the junction of the chromophore and the kinase domain of PhyCer.

nus characteristic for signal peptide sequences nor a cluster of hydrophobic amino acids characteristic of a membrane-anchoring domain.

The predicted structure of PhyCer implicates that the NH_2 terminus of phytochrome undergoes light-dependent conformational changes, triggered by a reversible Z-E isomerization of the chromophore [31, 46], which are transmitted to the COOH-terminus which in turn is linked with a cellular phosphorylating cascade.

Phylogeny of phyCer

The 778 aa polypeptide encoded by exon I and II of *phyCer* representing the conserved chromophore binding domain was compared with all available phytochrome sequences using GCG multiple alignment programs [10]. The phylogenetic distances and the percentage of identical residues between the conserved chromophore domains are given in Table 2. The moss and fern phytochromes turned out to be related more closely to each other than the higher-plant PhyA, PhyB and PhyC sequences to each other. Furthermore, according to these data the chromophore domains of the PhyB phytochromes are related more closely to PhyCer and *S. martensii* phytochrome than to PhyA or PhyC.

PhyCer *is constitutively expressed in* C. purpureus *protonemata*

Northern blot analysis shows that in light-grown and dark-adapted *C. purpureus* protonemata *phyCer* mRNA is expressed at the same level (Fig. 7). The transcript hybridizing to the *phyCer*-specific probe is about 4.8 kb and is larger than the *phyA* transcript in oat or *Arabidopsis thaliana*. The *phyA* transcripts are reported to be about 4.2 kb [22, 39]. This finding correlates with the predicted molecular mass of the PhyCer subunit which is about 20 kDa larger than the PhyA subunits (see above).

Table 2. Phylogenetical distances and percentage of identical residues between the conserved chromophore domain of various phytochromes.

	PhyA						LP		PhyB		PhyC
	oat	rice	maize	Arab	pea	zucc	PhyCer	Sel	Arab	rice	Arab
Oat	–	95(92)	94(90)	82(70)	82(69)	83(71)	72(56)	74(60)	69(53)	69(53)	71(54)
Rice		–	95(90)	84(70)	83(70)	84(71)	73(56)	76(61)	69(54)	69(54)	72(54)
Maize			–	84(70)	83(70)	84(70)	73(56)	76(62)	69(53)	69(54)	71(54)
Arab				–	91(83)	90(83)	75(59)	78(62)	71(56)	71(55)	73(57)
Pea					–	90(83)	75(60)	78(63)	71(56)	72(56)	73(57)
Zucc						–	74(57)	77(62)	70(56)	71(56)	72(55)
PhyCer							–	85(75)	74(60)	74(60)	71(55)
Sel								–	78(65)	78(64)	74(58)
Arab									–	84(74)	71(55)
Rice										–	71(54)

Distance values and percentage of identical residues (in parenthesis) were calculated according to Table 1. The regions of the different phytochrome polypeptides compared to each other resemble the 778 aa conserved chromophore domain (encoded by exons I and II) of PhyCer. LP = lower plants. References for the different phytochrome sequences: oat [21], rice PhyA [23], maize [5], *Arab(idopsis)* PhyA, PhyB and PhyC [39], pea [33], rice PhyB [9], Zuc(chini) [38], Sel = *Selaginella:* S. Hanelt and H.J.A.W. Schneider-Poetsch (unpublished), EMBL database accession number X61458.

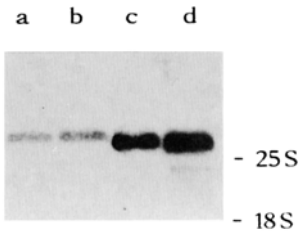

Fig. 7. Northern blot analysis of the *phyCer* transcript. Total RNAs (10 μg per lane) from light grown *C. purpureus* protonemata (lane a), from protonemata kept in the dark for 3 days prior to RNA extraction (lane b), from 3-day-old etiolated oat seedlings (lane c) and from 4-week-old *A. thaliana* leaves kept 3 days in the dark prior to RNA extraction (lane d) were separated in a formaldehyde containing 1% agarose gel and blotted to Biodyne A (PALL) nylon membrane. Hybridization was carried out at high stringency with ^{32}P-dCTP-labelled single-stranded probes and RNA-DNA hybrids were detected by autoradiography. *C. purpureus* blots were hybridized with the *phyCer*-specific PCR probe used for screening (lanes a and b). Filters with oat and *Arabidopsis* RNA were hybridized with a mixture of probes specific to *phyA* from oat and from *A. thaliana*. The probes detected specifically the oat *phyA* transcript (lane c) and the *A. thaliana phyA* transcript (lane d). Mobilities of the 25S and 18S rRNA are indicated.

Discussion

The structure of phyCer *expression*

The structure of the *phy* genes, including *phyCer* is highly conserved in the region surrounding the chromophore attachment site (see Fig. 1B). This implicates that all phytochromes have a common ancestor gene with an identical gene structure within this 5′-terminal region. In contrast to the conserved chromophore domain which is involved in highly specific and complex interactions with the tetrapyrrol chromophore, the less conserved COOH-terminal domains obviously reflect the different roles of the different phytochrome types in the plant cells responding to the same light stimulus. All angiosperm phytochromes and the fern phytochrome have a conserved overall structure in the C-terminal domains [9, 36]. In contrast, in mosses a major rearrangement of the phytochrome gene must have occurred bringing a different catalytic domain under the control of the conserved light regulator domain. As mentioned above, the fusion point of the chromophore and kinase domain in PhyCer (at Lys^{778}) also marks a significant drop in the homology between different PhyA proteins (see Fig. 6). Therefore this point probably marks the link between the chromophore and the regulatory domains in all phytochromes. According to Deforce *et al.* [8], a NH_2-terminal polypeptide of only 549 aa of pea phytochrome is already sufficient to assemble *in vitro* with the phytochromobilin chromophore and to exhibit red/far-red photoreversibility.

That *phyCer* indeed represents a novel, active phytochrome gene and not a cloning artefact is supported by the facts that (1) the structure of *phyCer* could be confirmed by the genomic Southern experiments (2) *phyCer* contains very large open reading frames which are not found within pseudogenes and (3) a cDNA clone could be isolated containing the in frame splicing product of the chromophore- and the kinase-domain coding open reading frames. This cDNA clone also demonstrates that *phyCer* is expressed in the protonemata cells.

The steady-state concentration of *phyCer* mRNA is not affected by light (Fig. 7). Therefore, with respect to the expression *phyCer* is also more closely related to type II (light-stable) phytochrome [39]. Whether PhyCer is a light-stable protein is not known since we were not able to detect PhyCer in protein extracts of *C. purpureus* protonemata with antibodies raised against oat and maize PhyA; antibodies specific to PhyCer are presently not available.

Homology and phylogeny

In Table 2 a comparison of the phylogenetical distances and the percent identical residues between the conserved chromophore domains of various phytochromes is given. The values are somewhat higher and less divergent compared with the values obtained with the complete phytochrome sequences [9] reflecting the less pronounced sequence conservation within the COOH-terminus of the different phytochromes. Nevertheless, with respect to the fern and angiosperm phytochromes, basically the same con-

clusions drawn from the complete phytochrome sequences can be drawn alone from the conserved chromophore domain sequence, namely: (a) the three angiosperm phytochromes (PhyA, PhyB and PhyC) are equally divergent from each other reflecting an early three way divergence of the different phytochrome types preceding the divergence of monocots and dicots (b) PhyB diverged more slowly in evolution [9]. The observation that PhyB diverged more slowly in evolution is supported by the now available data from lower-plant phytochromes because, as expected, PhyB is closer to the (ancient) lower plant phytochromes than to PhyA and PhyC. Additional conclusions can be made with the lower-plant data: (c) dicot PhyA is closer to lower-plant phytochrome (as well to PhyB and PhyC sequences) than monocot PhyA; (d) the moss and fern phytochromes are related more closely to each other than the higher-plant PhyA, PhyB and PhyC sequences to each other; (e) PhyC represents an isolated phytochrome type which is most divergent from all known phytochromes. The observation that PhyB is less divergent from the lower-plant phytochromes could implicate that PhyB in higher plants is functionally related to lower-plant phytochrome. In lower plants phytochrome mainly triggers rapid reversible modulations within plant development [48]. In our previous study [45] we stated that phyCer is equally distant to the different angiosperm phytochromes. In this study only a partial sequence of phyCer around the highly conserved chromophore attachment site was available which did not reflect the phylogenetical relationship of phyCer to the higher phytochrome types so clearly. We do not know yet if in *C. purpureus* in addition to PhyCer further phytochromes corresponding to PhyA, PhyB or PhyC do exist or not. In our screening experiment which was performed at low stringency we isolated only one type of phytochrome.

Kinase activity and phytochrome action

The proposal that the COOH-terminus of PhyCer has a protein kinase activity has implications on the mechanism of transduction of light stimuli in plant cells. Protein kinases play a key role in signal transduction; the involvement of protein kinases within the phytochrome-dependent reaction pathways has been speculated and red/far-red-dependent changes in phosphorylation patterns have been reported [1, 27, 41]. Interestingly, preparations of oat type A phytochrome can be phosphorylated in a red/far-red light-dependent manner [26, 49]. This phosphorylating activity, however, is most likely not due to autophosphorylation of the PhyA polypeptide but is rather associated with a copurified protein [14]. Although angiosperm and fern phytochrome do not exhibit the typical consensus sequences of eucaryotic protein kinases, the close association of PhyA and kinases in higher plants suggests a functional connection also in angiosperms. Schneider-Poetsch *et al.* [36, 37] found homologies of angiosperm and fern phytochrome to the cytosolic region of bacterial sensor proteins. All these bacterial sensor proteins possess a conserved cytosolic histidine kinase domain [42] and probably the higher-plant and fern phytochromes use signal transduction ways similar to those found in bacteria.

In contrast to fern and higher plant phytochromes, PhyCer exhibits the clear features of an eucaryotic light receptor protein kinase. Receptor protein kinases are usually tyrosine-specific transmembrane protein kinases [4]. In fact, PhyCer exhibits strong homologies to tyrosine kinases (see Fig. 4), but the hydropathy profile (not shown) does not indicate that PhyCer is an integral membrane protein. This implies that PhyCer is not involved in trans-membrane signalling; PhyCer appears rather to be involved in intracellular signal transduction. This is very likely because the light stimulus is present in all protonemata cells at the same time. Nevertheless, from experiments with polarized light, PhyCer appears to be located in a fixed position close to the plasma membrane in *C. purpureus* protonemata [20].

Concluding remarks

Signalling via protein kinases is a fundamental regulatory mechanism. Surprisingly, homology of eukaryotic protein kinases to phytochrome is only observed in primitive mosses and not in higher plants. Phytochrome in higher plants is encoded by a multigene family. Since not all members of this family have been characterized yet [39] there is still the option that the protein kinase feature of PhyCer may also be associated with higher-plant phytochrome.

The function of the *D. discoideum* protein-tyrosine kinases which show strong homologies with PhyCer are not known. Blue-light effects on the development of *D. discoideum* have been described [15]; it will be interesting to see if DPYK1 or DPYK2 are involved in these processes, this time representing the catalytic domain of a blue-light receptor. Blue light modifies the phosphorylation of a single plasma membrane protein in pea [40]. This protein itself is probably a protein kinase and may be the blue light receptor.

The identification of PhyCer provides a unique opportunity to gain fresh insight into the mechanism of transduction of light stimuli in plant cells.

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