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Phytochrome in lower plants

Detection and partial sequence of a phytochrome gene in the moss *Ceratodon purpureus* using the polymerase chain reaction

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The polymerase chain reaction was carried out with primers hybridizing to conserved regions of the phytochrome genes. With DNA from the moss *Ceratodon purpureus* 5 overlapping fragments were obtained resulting in a continuous nucleotide sequence of 1474 bp. The deduced amino acid sequence showed homology of around 60% with all known phytochrome sequences. The sequences contained a conserved chromophore attachment site. In light-grown *Ceratodon* protonemata the phytochrome mRNA with the size of about 4.5 kb was detected.

Polymerase chain reaction; Phytochrome; Moss; DNA sequence; Amino acid sequence

1. INTRODUCTION

The red/far-red reversible sensor pigment phytochrome controls plant growth and development throughout the whole plant kingdom [1]. Many effects of light are initiated by the photochemical conversion of Pr, the red light absorbing form, to Pfr, the far-red light absorbing, physiologically active form of phytochrome. Red/far-red reversible reactions reach from chloroplast movement in the green alga *Mougeotia* [2] or phototropism of fern and moss protonemata [3] to regulation of gene expression in higher plants [4]. For none of these reactions, the signal transduction chain has been elucidated. Recent investigations on phytochrome genes in *Arabidopsis thaliana* [5] point to 3 (phyA, phyB and phyC) or more different phytochromes and probably as many different transduction chains in higher plants.

The protonemata of the moss *Ceratodon purpureus* exhibit a well characterized red/far-red reversible phototropism [6] implicating the existence of phytochrome in this moss. Western blot analysis revealed the presence of a phytochrome-related protein in several other mosses [7,8]. Here we present the first direct evidence for the existence of phytochrome in *Ceratodon purpureus* by determination of the partial sequence of a phytochrome gene. The sequence was derived from PCR-products amplified by using oligonucleotide primers directed to conserved regions of phytochrome genes. The phylogenetical relationship of this sequence to known phytochrome sequences is discussed.

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2. MATERIALS AND METHODS

2.1. *Plant materials, DNA and RNA extractions*

C. purpureus (Hedw.) Brid. cultures were a gift of Dr. E. Hartmann. For DNA and RNA extractions protonemata were grown in sterile liquid cultures [9] at 20°C under a light regime of 18 h light - 6 h dark. Genomic DNA was isolated according to a miniprep method [10] including an additional precipitation step of RNA with 2 M LiCl, which also removed further interfering impurities from the DNA preparations. RNA was isolated with a hot phenol method as described by De Vries et al. [11].

2.2. *Polymerase chain reaction and DNA labeling*

PCR experiments were performed using AmplitaqTM (Perkin Elmer Cetus) according to the protocol of the manufacturer in a reduced volume of 50 μl. Standard reaction conditions were as follows: 30 cycles of 1 min denaturing at 94°C, 2 min annealing at 50°C and 3 min elongation at 72°C. Primary PCR experiments with *Ceratodon* DNA (1 μg) were performed with the annealing temperature reduced to 40°C. For re-PCR the products of the primary PCR were separated by electrophoresis in 2% agarose gels. The products of interest were cut out and eluted by a squeeze-freeze method [12]. Re-PCR was performed under standard conditions using 1 μl of the squeeze-freeze eluates. Purified PCR fragments were cloned into the *Sma*I site of pBluescript KS + (Stratagene) as described below.

Single stranded DNA-probes were also synthesized with the AmplitaqTM. Starting material for the labeling reactions were PCR fragments (0.1 μg) amplified under standard conditions using cloned phytochrome DNA (0.1 μg) as template. The initial PCR for the synthesis of *Avena* phytochrome probes was performed with primers L456/R740 and L721/R1676 using a full length cDNA-clone coding for oat phytochrome previously isolated by J. Bonenberger [13].

For synthesis of DIG-dUTP labeled single stranded *Avena* phytochrome probes instead of the dNTP nucleotide mix provided with the AmplitaqTM kit, 5 μl of the DIG-dUTP labeling mix includ-

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ed in the DIG DNA labeling kit (Boehringer Mannheim) were used in a 50 μl reaction volume. The probes were extracted with phenol/chloroform. Free nucleotides were separated by a spin column [12] equilibrated with T-E buffer containing 0.1% SDS. For hybridization 5 μl of the eluates were used.

For the Northern experiment ^{32}P-labeled single stranded *Ceratodon* phytochrome probe was used. The probe was synthesized using 0.1 μg of PCR-template obtained with the primer combination L456/R740 in a 10 μl reaction mix containing 16.5 pmol [α-^{32}P]dCTP (3000 Ci/mmol; Amersham), 33.5 pmol unlabeled dCTP and dATP, dGTP and dTTP, 50 pmol each. Not incorporated nucleotides were separated by a spin column [12]. For hybridization 10^6 counts/ml hybridization solution of labeled probe was used.

2.3. *DNA/RNA blotting and hybridization*

For Southern analysis, PCR-products were separated by electrophoresis in 2% agarose. The gels were denatured and neutralized as described [12] and the DNA fragments were then transferred to Biodyne A (PALL) membrane by drying the gel onto the membrane at room temperature using a gel dryer. The DNA was fixed to the membrane by UV treatment [12]. Hybridization was carried out with DIG-dUTP labeled probes. The filters were prehybridized for four hours at 42°C with the antiblocking reagent provided with the DIG DNA detection kit (Boehringer Mannheim) containing 30°C formamide according to the instructions of the manufacturer. Hybridization was performed over night under identical conditions. Filters were washed in 2×SSC, 0.1% SDS at room temperature with final washes at 50°C. The development of the filters was according to the kit instructions.

Northern blotting with 10 μg total *Ceratodon* RNA was performed as described [12] using Biodyne A membranes. RNA was fixed to the matrix by UV treatment. Hybridization conditions were the same as described for the Southern experiment.

2.4. *Cloning and Sequencing*

PCR fragments were made blunt ended by incubating the PCR products with 1 U of Klenow fragment of DNA polymerase I (Boehringer Mannheim) for 15 min at 37°C within the PCR-reaction mix. After phenol/chloroform extraction and purification on spin columns the fragments were ligated into the *Sma*I site of the pBluescript KS+ vector. Sequence determination was carried out by the dideoxy chain-termination method [14] using T7 DNA polymerase (Pharmacia). Subclones for further sequence analysis were obtained by sequential deletions. The data were analysed on IBM-AT computer with PCGENE programs compiled by Amos Broich (Genofit, Geneva) and homology searches were carried out with the FASTA program [15].

3. RESULTS

The polymerase chain reaction (PCR) was carried out with oligonucleotide primers directed to highly conserved regions around the chromophore binding site. Several products were obtained using genomic DNA from the moss *C. purpureus* (Fig. 1). The approach was as follows: we chose primer combinations which should result in PCR-products sized between 200 and 900 bp. After 30 PCR-cyles under low stringency conditions (40°C annealing temperature) the products were separated by gel electrophoresis. The expected PCR products were not visible on ethidium bromide stained agarose gels, but hybridization revealed the presence of a low amount of specific products. Therefore, in a duplicate gel these products were cut out blind and eluted by a 'squeeze-freeze' technique. The eluates were then used for re-PCR at more stringent conditions

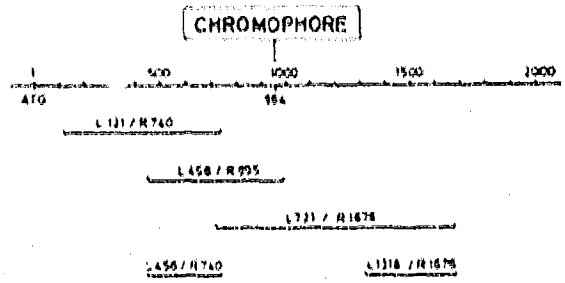

Fig. 1. Positions of the PCR-fragments obtained with genomic DNA from *C. purpureus* in relation to exon II of the *Avena* phytochrome gene [19]. The primer combinations used for amplification are shown above the bars representing the PCR-products. The nucleotide sequences of the primers are included in Fig. 2.

(50°C annealing temperature) with the respective primer combinations. After re-PCR single products were obtained which were subcloned and sequenced. The partial sequences were aligned to a continuous nucleotide sequence of 1474 bp. The sequence is given in Fig. 2, the derived amino acid sequence in Fig. 3. Sequence homology comparison on amino acid level revealed an average sequence identity with the known phytochromes of about 60% (Table I). This proves that we were indeed amplifying phytochrome sequences with our primers.

A Northern experiment (see Fig. 4) revealed, that in light grown *Ceratodon* protonemata the phytochrome transcript is easily detectable. The size of the mRNA is about 4.5 kb, about the size of a 'typical' phytochrome transcript (4.2 kb in the case of *Avena* [16]).

4. DISCUSSION

The present paper describes for the first time a (partial) gene sequence for phytochrome from a lower plant. Overlap of the fragments obtained by PCR (Fig. 1) was large enough to obtain a continuous sequence of 1474 bp. The molecular mass of oat phytochrome is 124 kDa corresponding to 3387 bp of coding nucleotides [17]. Assuming that *Ceratodon* phytochrome has about the same size (see Fig. 4), the elucidated sequence represents about half of the coding sequence of the *Ceratodon* phytochrome. It is remarkable that we did not amplify intron sequences in any of our PCR experiments. This implicates that the intron/exon structure of the *Ceratodon* gene corresponds to that of all other phytochrome genes so far investigated, at least around the chromophore binding site (see Fig. 1). This is of interest in evaluating the phylogeny of the phytochrome gene.

Included in Fig. 3 are the conserved sequences shared between phyA, phyB and phyC [5]; around the chromophore binding site they represent about 30% of the amino acids. Except for a few amino acids these 'consensus sequences' which are probably functionally

Fig. 2. Partial nucleotide sequences of *Ceratodon* phytochrome. The continuous sequence of *Ceratodon* phytochrome was derived by aligning the sequences of the PCR-fragments obtained with the primer combinations L121(X = C + T)/R740, L456/R995, L721 (X = A + G + C + T, Y = A + G) /R1676(X = A + G), L456/R740 and L1318 (X = A + G + C + T)/R1676(X = A + G) (see Fig. 1). Sense oligonucleotide primers are shown above, antisense primers below the *Ceratodon* sequence. The arrows indicate the direction of DNA-synthesis driven by the primers. The codon for the cystein (TGT) residue representing the presumable chromophore attachment site is indicated by an asterisk.

important are also present in *Ceratodon* phytochrome. In this region of the phytochrome gene, function should mean chromophore stabilization for photoreversibility [18]. The cysteine residue (marked with an asterisk in Fig. 3) representing the chromophore attachment site is present in *Ceratodon* phytochrome too, indicating that

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CERAT        KSVGQSAESVPAGAVTAYLQRMQREGLIQNFGCMVAVEEPNFCVIAYSENASEFLDLIPQAVPSMGEMDVLGIGTDIRTLFTPSSSAALEKAA
CONS                          YL IQ    IQPFGC    DE     I  SEN  E L      VP       L  GTD  SLF       L  A

CERAT  ATQDISLLNPITVHCRRSGKPLYAIAHRIDIGIVIDFEAVKMIDVPVSAAAGALQSHKLAARAITRLQALPGGDIELLCDTIVEEVRELTGYDRVMAFKF
CONS         LNP   H     KPFYAI HR       ID EP      P    AGA  S KLA   I  LQ LP G    LCD  V  V  LTGYDRVM YKF

CERAT  HEDEHGEVVAEIRRMDLEPYMGLHYPATDIPQASRFLLMKNRVRLIADCYASPVKLIQDPDIRQPVSLAGSTLRAPHGCHAQYMGNMGSIASLVMAVIIN
CONS   HED HGE V E      EPY GLHY ATDIPQA RFLF  N VRMI DC A    V QD  L     L GSTLRAPH CH QYM NM S ASL M V  N

CERAT  DNEEYSRGAIQRGRKLWGLVVCQHTSPRTVPFPLRSVCEFLMQVFGMQLNLHVELAAQLREKHILRTQTLLCDMLLRDAPIGIVSQTPNIMDLVKCDGAA
CONS                  LWGLVVCH    R  PFPLRYACEFL Q F    N E       EK  L TQ  LCDML R  P GIV QSP IMDLVKCDGAA

CERAT  LYYGKRVWLLGTTPTENQIKEIADWLLEHHNDSTGLSTDSLADANYPGAMLLGDAVCGMAAAKITAKDFLFWFRSHTATEVKWGGAKHDPDEKDDGRK
CONS     Y      LG  P E       W    H   TG  T SL   G P A  LG   CGMA   I   D  FWFRS TA    WGGA H P D D   R
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Fig. 3. Partial amino acid sequences of *Ceratodon* phytochrome deduced from the sequence given in Fig. 2. Residues conserved in phyA, phyB and phyC [5] are shown below the sequences. Residues specifically exchanged in *Ceratodon* phytochrome are marked with an arrowhead. The cysteine residue representing the presumable chromophore attachment site is indicated by an asterisk.

also here a tetrapyrrole chromophore is covalently bound to the protein. Hence we would expect to find very similar spectral properties of *Ceratodon* phytochrome like in *Avena* phytochrome.

The presence of phytochrome mRNA in light grown protonemata (see Fig. 4) suggests that *Ceratodon* phytochrome should be more closely related to type II (green, type B or C) than to type I (etiolated, type A) phytochrome of higher plants. But according to the data given in Table I, *Ceratodon* phytochrome cannot unequivocally be related to any of the known phytochrome types in higher plants. This is somewhat surprising, because phytochrome of lower plants was thought to be more related to type II phytochrome of higher plants [3].

If we assume that *Ceratodon* phytochrome represents an ancient phytochrome form, each of the known phytochrome types from *Arabidopsis* (A, B, or C) is about equally distant from ancient phytochrome. They must be therefore specific forms of phytochrome in the Angiosperms. Sharrock and Quail [5] state that the gene duplication events leading to phytochrome types A, B and C occurred early in the evolution of vascular plants (before the branching of monocots and dicots). From our data (Table I) this would also hold true for *Ceratodon* phytochrome. We have to admit that this statement holds only true for a part of the phytochrome molecule (about half of *Ceratodon* phytochrome),

Table I

Percent amino acid identity among phytochromes from various plant species and the polypeptide derived from *Ceratodon* phytochrome DNA sequence

	Oat	Rice	Corn	Zucchini	Pea	*Arabidopsis* A	B	C
Ceratodon	59	60	61	61	63	63	64	63

Values are taken from pairs of alignments performed with the FASTA [15] program. References for previously published sequenced are oat [17], rice, corn, zucchini, pea [20–23] and *Arabidopsis* [5].

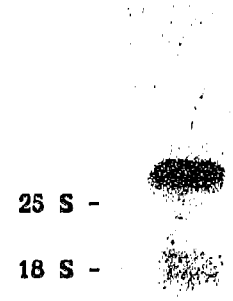

Fig. 4. Northern blot hybridization of phytochrome mRNA present in total RNA of *C. purpureus*. 10 μg of total RNA isolated from 3 week old light grown protonemata were electrophoresed in 1% agarose and blotted onto a nylon membrane. The blot was probed with *Ceratodon* PCR-product obtained with the primer combination L456/R740. Transcript size was estimated from the mobility relative to the rRNA molecules.

nevertheless the sequence is long enough to allow speculations about phylogenetic relationships.

Sites of phytochrome molecules which are linked to the various physiological reaction chains could be located at less conserved domains, representing the specific roles of the different phytochrome types in the plant cell. Thus it will be of great interest to see, if other parts of the *Ceratodon* phytochrome gene are more similar to one of the known phytochrome types in higher plants. This investigation is currently in progress.

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REFERENCES

[1] Shropshire, Jr. W. and Mohr, H. (1983) Photomorphogenesis, Encyclopedia of Plant Physiology, New Series, Vol 16A/B, Springer, Berlin.
[2] Wagner, G. and Rossbacher, R. (1980) Planta 149, 298–305.
[3] Wada, M. and Kadota, A. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 169–191.
[4] Kuhlemeier, C., Green, P.J. and Chua, N.-H. (1987) Annu. Rev. Plant Physiol. 38, 221–257.
[5] Sharrock, R.A. and Quail, P.H. (1989) Genes and Development 3, 1745–1757.
[6] Hartmann, E. and Weber, M. (1988) Planta 175, 39–49.
[7] Cordonnier, M.M., Greppin, H. and Pratt, L.H. (1986) Plant Physiol. 80, 982–987.
[8] Schneider-Poetsch, H.J.A.W., Schwarz, H., Grimm, R. and Rudiger, W. (1988) Planta 173, 61–72.
[9] Hartmann, E., Klingenberg, B. and Bauer, L. (1983) Photochem. Photobiol. 5, 599–603.
[10] Davis, R.W., Thomas, M., Cameron, J., St. John, T.P., Schere, S. and Padgett, R.A. (1980) Methods Enzymol 65, 404–411.
[11] De Vries, S., Hodge, H. and Bisseling, T. (1988) in: Plant Molecular Biology Manual (Gelvin, S.B. et al. eds) B6 pp. 1–13, Kluwer, Dordrecht.
[12] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman., J.G., Smith, J.A. and Struhl, K. (1988) Current Protocols in Molecular Biology, John Wiley, Media, Pennsylvania.
[13] Bonenberger, J. (1989) Diploma thesis, Botanisches Institut der Universität München.
[14] Biggins, M.D., Gibson, T.J. and Hong, G.F. (1983) Proc. Natl. Acad. Sci. USA 80, 3963–3965.
[15] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[16] Colbert, J.T., Hershey, H.P. and Quail, P.H. (1985) Plant Mol. Biol. 5, 91–101.
[17] Hershey, H.P., Barker, R.F., Idler, K.B., Lissemore, J.L. and Quail, P.H. (1985) Nucleic Acids Res. 13, 8543–8559.
[18] Rüdiger, W. (1987) in: Phytochrome and Photoregulation in Plants (Furuya, M. ed) pp. 127–138, Academic Press Inc., Tokyo.
[19] Hershey, H.P., Barker, R.F., Kenneth, B.I., Murray, G.M. and Quail, P.H. (1987) Gene 61, 339–348.
[20] Kay, S.A., Keith, B., Shinozaki, K., Chye, M.L. and Chua, N.-H. (1989) Plant Cell 1, 351–360.
[21] Lissemore, J.L., Colbert, J.T. and Quail, P.H. (1987) Plant Mol. Biol. 8, 485–496.
[22] Sato, N. (1988) Plant Mol. Biol. 11, 697–710.
[23] Christensen, A.H. and Quail, P.H. (1989) Gene 85, 381–390.