Plant Molecular Biology **23**: 1187–1198, 1993.

A novel blue light- and abscisic acid-inducible gene of *Arabidopsis thaliana* encoding an intrinsic membrane protein

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Received 25 May 1993; accepted in revised form 24 September 1993

Key words: Arabidopsis thaliana, blue light, floral induction, gene expression, membrane protein, DNA sequence

Abstract

Continuous irradiation with blue light (400–500 nm) induces flower formation in plantlets of *Arabidopsis thaliana* (C24) while red light (600–700 nm) is ineffective. This observation started a search for genes that are activated by blue light and initiate the morphogenic programme leading to flower formation. Several genes were identified via their cDNAs. From these clone AthH2, with an open reading frame for a hydrophobic 30.5 kDa polypeptide, was selected for further characterization of the corresponding gene. From a genomic library a DNA fragment of about 6.4 kb was isolated, comprising the coding region as well as 5′-upstream and 3′-downstream flanking segments. The coding region is composed of four exons, which specify a polypeptide of 286 amino acids. Several potential regulatory elements were found between position −670 and −1140 including GA and ABA sequence motifs. The latter could account for the observed induction of the AthH2 gene by ABA. Southern blot analysis of *Arabidopsis* genomic DNA suggests that the AthH2 gene is encoded by a single-copy gene. Hydropathy plots and secondary structure analysis of the putative polypeptide predict six membrane-spanning domains implicating a function as transmembrane channel protein. It displays significant homology with the proteins TR7a of pea (82%) and RD 28 of *A. thaliana* (68%).

Introduction

Light, as an environmental factor, plays a crucial role in the development of plants. Accordingly, they have evolved photoreceptor systems which respond to different qualities and quantities of light and thus control developmental processes. While the red/far-red sensing phytochrome is well characterized, the nature of the blue and near-UV light mediating receptor (cryptochrome) is still unknown. Recent studies indicate that both are part of a multiple photosensory system which exerts control over a number of physiological processes by mediating different light qualities [12]. However, it is not known which of the various responses is controlled, by which photoreceptor

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Database under the accession numbers Z17399 (genomic) and Z17424 (cDNA).

or to what extent the different receptor molecules have overlapping functions. To resolve these uncertainties a system is required where light responses can be dissected by setting up conditions in which either blue light- or red light-dependent processes take place. *Arabidopsis thaliana* was found to be a plant particularly well-suited for these investigations. Mutation analysis of hypocotyl elongation inhibition observed in blue light-grown seedlings revealed that certain genes involved in signal transduction are defective [27]. They are distinct from those found in the *hy* mutants of *Arabidopsis thaliana* [25], in which the phytochrome response is altered.

Floral induction as a typical light response of *Arabidopsis* plantlets could be accomplished by continuous exposure to blue light (485 nm) while red light (660 nm) administered under the same experimental conditions was completely ineffective [3]. The obvious blue light dependence of morphogenesis under these conditions offered a good opportunity to search for genes which are likewise preferentially activated by blue light because their products serve as structural, housekeeping or regulatory proteins in the process of flowering.

In a recent investigation, we found evidence for the existence of such genes in *A. thaliana*. Several of these are rapidly expressed upon blue light irradiation of young plants. The features of one gene, which encodes an intrinsic membrane protein, suggest a transporter function. Remarkably, for the same gene, induction by the plant hormone abscisic acid was observed. In this paper we describe its genomic structure and the mode of expression as well as features of the encoded polypeptide.

Materials and methods

Plant material and growth conditions

Dry seeds of *Arabidopsis thaliana* L. (strain C24) were surface-sterilized by immersion in ethanol + 0.02% Triton X-100 for 5 min followed by incubation with 5% hypochlorite + 0.02% Triton X-100 for 15 min. Germination and growth were carried out at 24 °C in darkness on a medium described by Estelle and Sommerville [7] supplemented with 2% sucrose and solidified with 0.8% agar. Seedlings after 2 days and plantlets after 14 days of culturing were exposed to either blue, red or white light. The blue light source consisted of Philips fluorescent tubes TL 36/W 18 providing 4–6 W/m^2 within the spectral range 400–550 nm. Red light of equal energy fluence rate (spectral range 600–700 nm) was produced by Philips fluorescent tubes TL 36/W 15. Osram incandescent tubes supplied white light of equal energy fluence rate. The set-up for irradiation with far-red light was as reported [26].

For hormone treatment plantlets were grown for 2 h on the standard agar medium containing either 1 mM abscisic acid (ABA-*cis*, *trans*, Sigma) or gibberellic acid (GA_3, Sigma) in various amounts: 10 μM, 100 μM or 1 mM. For testing the effect of calcium ions, the concentration of $CaCl_2$ in the agar medium was raised to 10 mM.

Preparation and analysis of RNA

Plantlets (2 g fresh weight) were quickly chilled in liquid nitrogen and ground in a mortar. The resulting powder was suspended in 15 ml extraction buffer (0.6 M NaCl, 10 mM EDTA, 100 mM Tris-HCl pH 8.0, 4% SDS) plus 15 ml of a 1:1 mixture of phenol (80%) and chloroform. After shaking for 10 min and centrifugation at 20000 × *g* for 10 min the aqueous phase was recovered. 0.75 vol LiCl solution (8 M) was added and the mixture incubated for 1 h on ice, then centrifuged at 20000 × *g* and 4 °C for 10 min. The pellet was resuspended in 5 ml water treated previously with diethyl pyrocarbonate (DEPC). 1/10 vol sodium acetate (3 M) and 2.5 vol ethanol were added and left for 2 h at −20 °C. The precipitated RNA was collected by centrifugation, resuspended in a small volume of DEPC-treated water, and size-fractionated on 1% denaturing agarose-formaldehyde gel [5]. Total RNA from different preparations was standardized spectrophotometrically, by using the ethidium

bromide fluorescence of ribosomal RNA species and by hybridizing with ^{32}P-labelled oligo(dT) [19]. For northern hybridization the gel was transferred by vacuum blotting to nylon membrane, irradiated with UV for 2 min and incubated in Quickhyb solution (Stratagene). Hybridization to ^{32}P-labelled cDNA was performed at 68 °C for 1 h. The washing procedure was carried out following the suppliers' protocol (Stratagene). The air-dried membrane was exposed to Kodak XAR-5 film with intensifying screen at −80 °C.

Isolation of DNA and Southern blot analysis

Plant nuclei were isolated from 4-week old plantlets following the protocol of Jofuku and Goldberg [21]. Extraction by lysis of their DNA was performed according to Richards [32]. The crude DNA preparation thus obtained was subsequently digested with a mixture of the RNases T1 and A (1500 units T1 + 500 μg A). Further purification was achieved by precipitation with cetyltrimethylethylammonium bromide (CTAB) as described by Murray and Thomson [30]. For genomic Southern blot analysis standard procedures were used [33]; 1 μg of restriction fragments was applied per lane. Hybridization was with Quickhyb solution (see above). ^{32}P-labelling of genomic DNA was performed by following the protocol of either Feinberg and Vogelstein [8] or Sturzl and Roth [36], the latter for establishing strand specific probes by the use of *Taq* DNA polymerase and the primer ExH2-2: 5′-TTAGATCTGTCGACCTCAATCAGCTTTAGCTTCTGG-3′.

Cloning of complementary DNA (cDNA)

Polyadenylated RNAs (poly[A]RNA) for cDNA synthesis were isolated with oligo(dT) coupled to magnetic beads following the protocol of the manufacturer (Dynal). Samples of 1–2 μg were heat-denatured and transformed to cDNA by following the procedure of Gubler and Hoffman [15]. Pre-selection of relevant sequences was achieved by library subtraction as described by Schweinfest *et al.* [34]. In separate assays, the double-stranded cDNAs obtained from poly(A)RNAs of plantlets grown either in continuous blue light or red light (see above) were cloned into the vector λZAP II (Stratagene), after adding *Eco* RI adapters to both ends. Amplified libraries with about 10^6 primary plaques were established in the presence of helper phage R408 with *E. coli* XL-1 Blue as host. The single-stranded DNA, which had its origin in the poly(A)RNAs of red light-treated plantlets and was produced by the f1 phage, was biotinylated according to Duguid *et al.* (6) using a photobiotin labelling system (Gibco-BRL). This biotinylated DNA was hybridized in a 10-fold surplus to the single-stranded DNA of the phage library which had its origin in the poly(A) RNA of the blue light-treated plantlets. The biotin-containing hybrids which had formed were precipitated by adding streptavidin and 1 vol of 5 M ammonium acetate and removed by centrifugation. The remaining single-stranded DNA in the supernatant was subjected twice to the same subtraction procedure, then recovered by precipitation with ethanol and provided with the complementary strand using polymerase K and M13rev sequencing primer. Highly competent cells of *E. coli* XL-1 Blue were transformed with the plasmids obtained and the resulting ampicillin-resistant bacteria were grown to yield single-stranded f1 phage.

Extension and amplification of the 5′ cDNA end

The materials for this procedure designed by Frohmann [11] came from a 5′ RACE kit (Gibco/BRL), and the manufacturer's protocol was followed, apart from minor modifications. Gene-specific primers were used, viz. GSP1H2 (CACTATGTAGTACACAGCTC) and GSP-2H2 (CGAAACCTTCCTAGCTAAGAACAAACCG). The product was subsequently introduced into the Bluescript plasmid SK II (Stratagene) using *Spe* I and *Hin*d III restriction sites. The full-length clone was obtained by ligating the truncated original isolate to the cloned RACE product at the *Hin*d III site.

DNA sequence analysis

Both strands of cDNA insertions which were previously subcloned in the vector Bluescript SK II (see above) as well as genomic DNAs were analysed by the dideoxy chain-termination technique with T7 polymerase [37] by using materials from a commercial T7 sequencing kit (Pharmacia, USB). The cDNA insertions and the genomic DNAs were successively deleted with reagents from a kit designed for nested deletion of double-stranded DNA (Pharmacia). Sequence data analyses and derived protein sequences were performed using the PC Gene Program (Intelligenetics). Searches for protein sequences similar to those derived from the various cDNA and genomic clones were conducted with the FASTA program (EMBL).

Results

Initial studies were performed to characterize the light-dependent process of floral induction in plantlets of the *Arabidopsis thaliana* strain C24. The same spectral dependency was observed as that described for seedlings of the Estland strain of *A. thaliana* by Brown and Klein ([3]; see Introduction). Continuous irradiation with blue light (400–550 nm; 5 W/m^2) brought about a high percentage of flower formation within three weeks. However, continuous red light of identical energy fluence rate completely suppressed flowering and enhanced vegetative growth. On the other hand, with plantlets grown in continuous white light, flower formation was delayed for an average of two weeks.

Selection of transcripts coding for blue light-induced proteins

In order to identify genes which are related to blue light-induced photomorphogenesis in *Arabidopsis* under the growth conditions described, cDNA libraries were constructed from poly(A)RNAs which were isolated from plantlets irradiated for two weeks with either continuous blue or red light of equal energy fluence rate. We chose this period of time because the plantlets grown under both light regimes were morphologically quite alike. They had reached a developmental stage most favourable for selecting blue light-induced genes and eliminating those representative of red and white light-treated plantlets. By differential hybridization and selection (cDNA library subtraction; see Materials and methods) of the 21 independent clones obtained, 14 were found to encode transcripts which accumulate preferentially in blue light-irradiated plantlets. We focused on AthH2 because the corresponding gene exhibited maximum expression in blue light as compared to red light or darkness (see below). The molecular analysis of AthH2, which is representative of a new class of light-induced species, and the deduced structure of the encoded protein are reported here.

Expression pattern of AthH2

The level of the AthH2 mRNA was studied by northern blot analysis in plantlets grown under the different light regimes for 14 days (Fig. 1A). Hybridization signals of the highest intensity were monitored for the blue light-treated plantlets while those for the other samples tested were moderate or, like the dark-grown control, very low. As depicted by Fig. 1B, abscisic acid (ABA) could adequately replace blue light in activating the AthH2 gene when plantlets were treated with the hormone for 2 h in darkness. Additionally, conditions were tested which induce expression in other species of genes coding for proteins homologous to the AthH2 protein: heat shock (hs; Fig. 1B) and gibberellic acid (GA, not shown) were as ineffective in this respect as tissue wounding and enhanced extracellular Ca^{2+} supply (data not shown). These findings suggest that blue light and ABA share common elements in the signal transduction pathway.

Alternatively, plantlets were pre-treated with red light for 14 days, then exposed to blue light of identical energy fluence rate, and the steady-state level of the AthH2 transcript determined up to 6 h

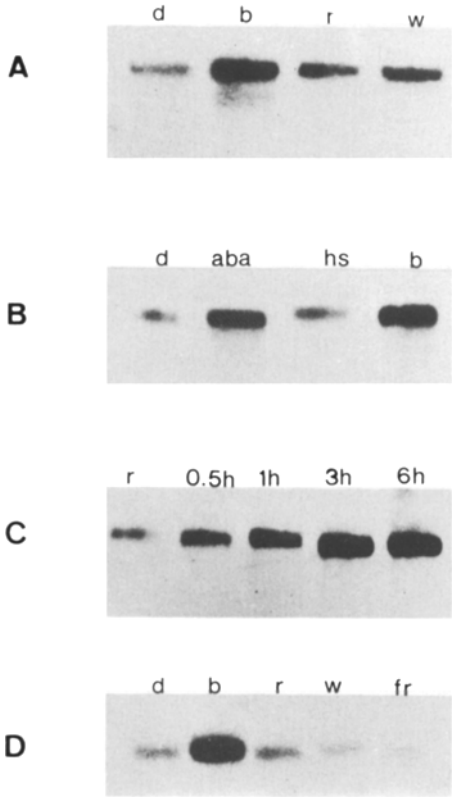


Fig. 1. Northern blot analysis of the AthH2 transcript. Total RNA (10 μg per lane) from plantlets subjected to various experimental conditions prior to RNA extraction was separated in a formaldehyde-containing 1.5% agarose gel, blotted onto nylon membrane and hybridized with the ^{32}P-labelled cDNA of clone AthH2. A. 14-day-old plantlets grown in darkness (d), continuous blue (b), red (r) or white light (w). B. Dark-grown seedlings (d) treated with abscisic acid (aba), heat-shock (40 °C for 2 h; hs) or irradiated with blue light for 2 h (b). C. Plantlets kept in red light for 14 days (r) were exposed to blue light for various lengths of time (0.5 to 6 h). D. Plantlets kept in darkness for 14 days (d) were exposed for 2 h to blue (b), red (r), white (w) or far-red light (fr).

from the onset of blue light irradiation (Fig. 1C). The hybridization signals intensified as the specific transcript accumulated with increasing exposure time to blue light. This tendency is observed as early as 30 min after changing to blue light. In contrast, pre-illumination with red light failed to bring about a significant accumulation of the specific mRNA as indicated by the weak hybridization signal (lane r).

To verify that light induction of the gene solely depends on blue light, even if administered for relatively short periods, plantlets grown for 14 days in darkness were exposed to red, far-red or white light for 2 h. While the blue light brought about a significant accumulation of the specific transcript, the other light qualities as well as darkness failed to evoke such an effect (Fig. 1D).

Genomic organization

To assess the structural organization and gene copy number of the AthH2 gene, gel blot analyses of the genomic DNA of *A. thaliana* were performed, applying stringent hybridization conditions. Equal amounts of total DNA were digested to completion with selected restriction enzymes, *Hin*d III, *Sty* I and *Eco* RI and analysed by Southern blot hybridization employing the cDNA insertion of clone AthH2 as probe (Fig. 2). Each enzyme tested gave one single strongly hybridiz-

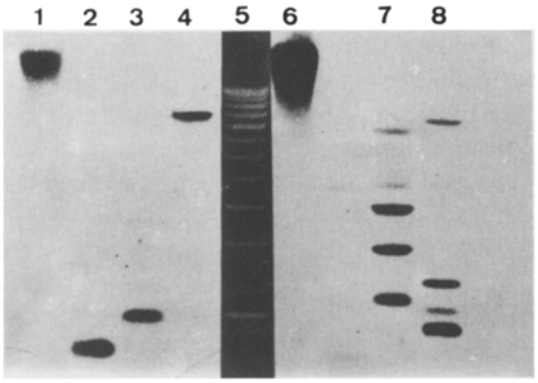


Fig. 2. Southern blot analysis of the AthH2 gene. Genomic DNA treated with the restriction enzymes *Hin*d III (lane 2), *Sty* I (lanes 3, 7) *Eco* RI (lanes 4, 8) or untreated (lanes 1, 6) was size-fractionated by electrophoresis through a 1% agarose gel (1 μg per lane), blotted onto nylon membrane, and probed with the radiolabelled cDNA insertion of AthH2 (lanes 1–4). For comparison, a hybridization in parallel was carried out with a cDNA probe of about equal length and specific activity, corresponding to exon III and common to the four *rbcS* genes of *Arabidopsis thaliana* (lanes 7, 8). The intensities of the hybridization signal in lane 6 correspond to 4 copies of *rbcS* per genome; thus the three main hybrid bands in lane 7 as well as the two in lane 8 represent a single-copy gene each. In contrast, the single hybrid band in lane 1 comprised only about 25% of the radioactivity bound in that of lane 6. Lane 5: DNA size marker (1 kb ladder, Gibco/BRL).

ing fragment. These findings when compared with the behaviour of another single-copy gene, indicate that the gene represented by clone AthH2 is present in the genome as a single-copy gene.

Sequence of the AthH2 cDNA

We determined the nucleotide sequence of the cDNA insertion of clone AthH2. It was analysed for coding regions by the methods of Shepherd [35] and Fickett [9]. A plausible continuous reading frame was determined. The putative polypeptide encompases 286 amino acids with a computed molecular mass of 30563 Da.

Isolation of a genomic clone corresponding to AthH2

A genomic library of *A. thaliana* (Columbia) was screened with the ^{32}P-labelled cDNA insertion of AthH2 as probe. One specific clone was identified containing an insertion of about 6.4 kb. From the results of restriction analyses (not shown) it was established that the genomic DNA fragment is comprised of the coding region and a 5′-upstream flanking sequence of about 4.4 kb as well as a 3′-downstream portion of about 0.6 kb. A 3.5 kb fragment of the original insertion including the promoter and the complete coding region as well as the 3′-downstream region was sequenced (Fig. 3).

DNA organization of the AthH2 gene

To obtain a full-length cDNA of the AthH2 and to determine the transcription start site, respectively, the method of rapid amplification of cDNA ends (RACE; see Materials and methods) was applied. Sequence comparison with the truncated fragment of the specific genomic clone (see above) revealed the following features: the coding region is interrupted by three introns, the transcription starts 75 bp 5′-upstream from the initiation codon; 137 bp form the untranslated 3′-end. Moreover, sequence motifs with resemblance to the TATA and CAAT box occur at positions −32 to −28 and −79 to −76, respectively. Additionally, sequence elements with relation to the action of GA and ABA are present in the AthH2 promoter between positions −1140 to −1027 and −712 to −670, respectively. In the distal segment a putative enhancer consensus sequence (PuCACGPy) resides, which was originally identified in the promoter of the ABA-inducible gene EM of wheat [29]. Also, two pyrimidine boxes (PyCTTTTPy) typical for the promoter of α-amylase genes from rice, wheat and barley which are inducible by GA and Ca^{2+} [16, 20], and an interrupted GCAACG tandem repeat identified in the promoter of the GA-responsive cathepsin-like gene in wheat [4] were detected. An α-amylase consensus sequence box (TAACAA/GA) and the affiliated pyrimidine box [16] were found in the proximal region −712 to −670. The occurrence of these sequence elements is in agreement with the observed inducibility of AthH2 by ABA. GA and Ca^{2+} were ineffective on AthH2-mRNA steady-state concentrations under the experimental conditions applied. The sequence homologies to the related boxes may not be relevant, however, the fact that ABA and GA are often acting as antagonists implies that the expression of the AthH2 gene might be regulated by both substances.

Structural homologies of the AthH2 protein

As already mentioned, the cDNA of clone AthH2 encodes a predicted polypeptide of 286 amino acid residues. Computer analyses of the secondary structures revealed the presence of 6 transmembrane helices in an arrangement that fits the requirements for an intrinsic membrane protein and is characteristic of a specific class of channel proteins (Fig. 4). The N-terminus is hydrophilic and hence lacks a cleavable signal sequence. The amino acid residues 173 to 180 form an ATP/GTP-binding site. Moreover, three computer-predicted phosphorylation sites exist at the positions 27, 128 and 194 with preference to protein kinase C, cAMP/cGMP-dependent kinase and

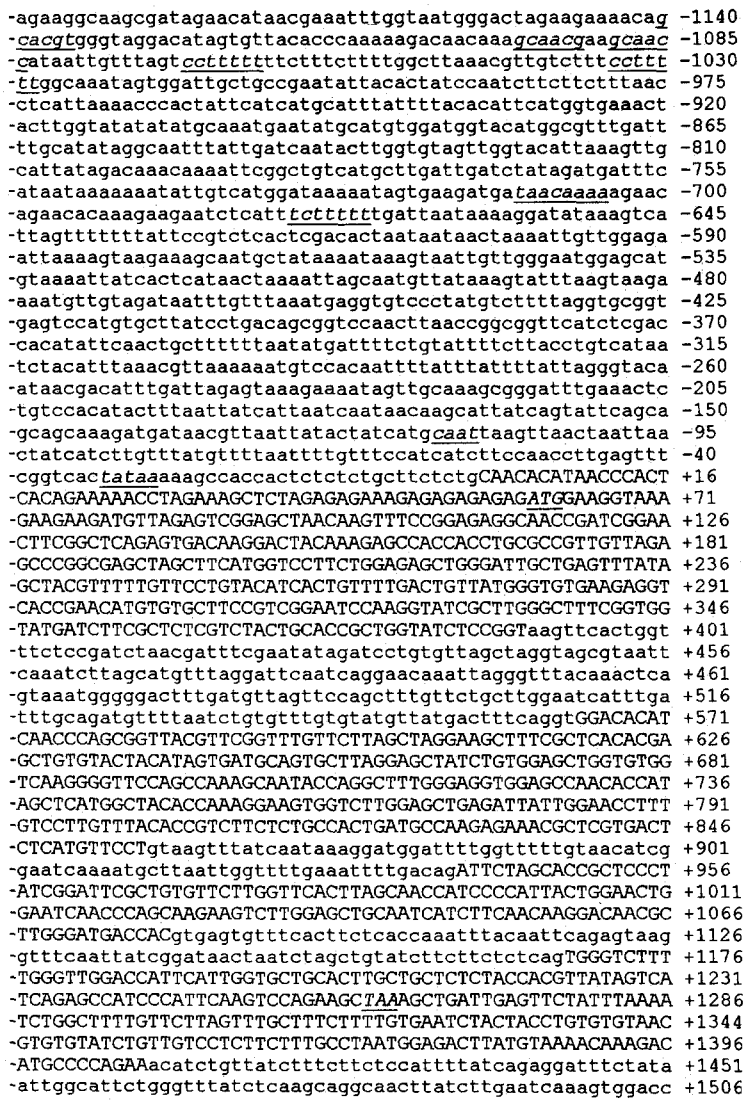


Fig. 3. Nucleotide sequence of the AthH2 gene. The coding regions corresponding to the cDNA are represented by capital letters, introns and flanking regions by lower-case letters. Nucleotide numbering refers to the transcription start (+ 1). The putative TATA box, the CAAT box, the first in-frame ATG initiation and the translation stop codon are underlined and written in italics as well as putative promoter sequence elements discussed in the text.

casein kinase II, respectively. The search for sequence homologies to the AthH2 protein using the entries of a data base of protein sequences (EMBL) revealed a substantial overall homology with the sequences of 6 proteins (Fig. 5). 82% of the amino acids are identical in the TR7a protein of pea with unknown function which is induced by water stress, heat shock and ABA [18]. 68% and 34%, respectively, of the residues match those of the RD28 protein of *A. thaliana* [38] and those of the major intrinsic protein of bovine lens fiber gap junctions (MIP; [15]). About 29% identity exists with a putative root membrane channel protein [39] and with the tonoplast intrinsic protein TIP of *A. thaliana* [18]. With the nodulin-26 protein from soybean [10] a 24% identity was found. These 7 proteins share a characteristic motif of 10 amino acid residues, SGGHXN-

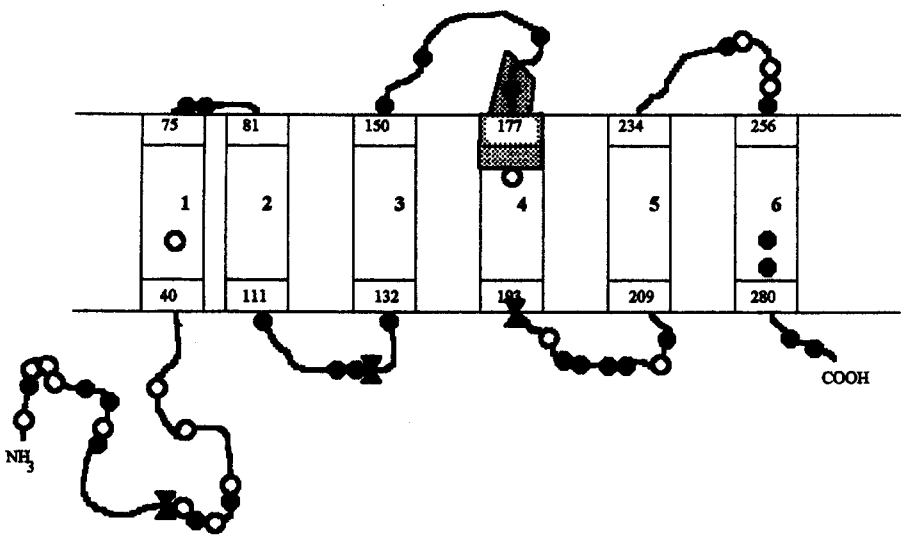


Fig. 4. Predicted secondary structures of the AthH2 protein. Small numbers depict the positions of the first and the last amino acid residue in each of the six transmembrane helices (rectangles with bold numbers). Open circles: acidic amino acids (Asp, Glu); full circles: basic amino acids (His, Lys, Arg). Putative phosphorylation sites are indicated by rhombic symbols. The region of the predicted ATP/GTP-binding site is stippled.

PAVT, and have been classified accordingly as the MIP/NOD26/GlpF family [1, 31].

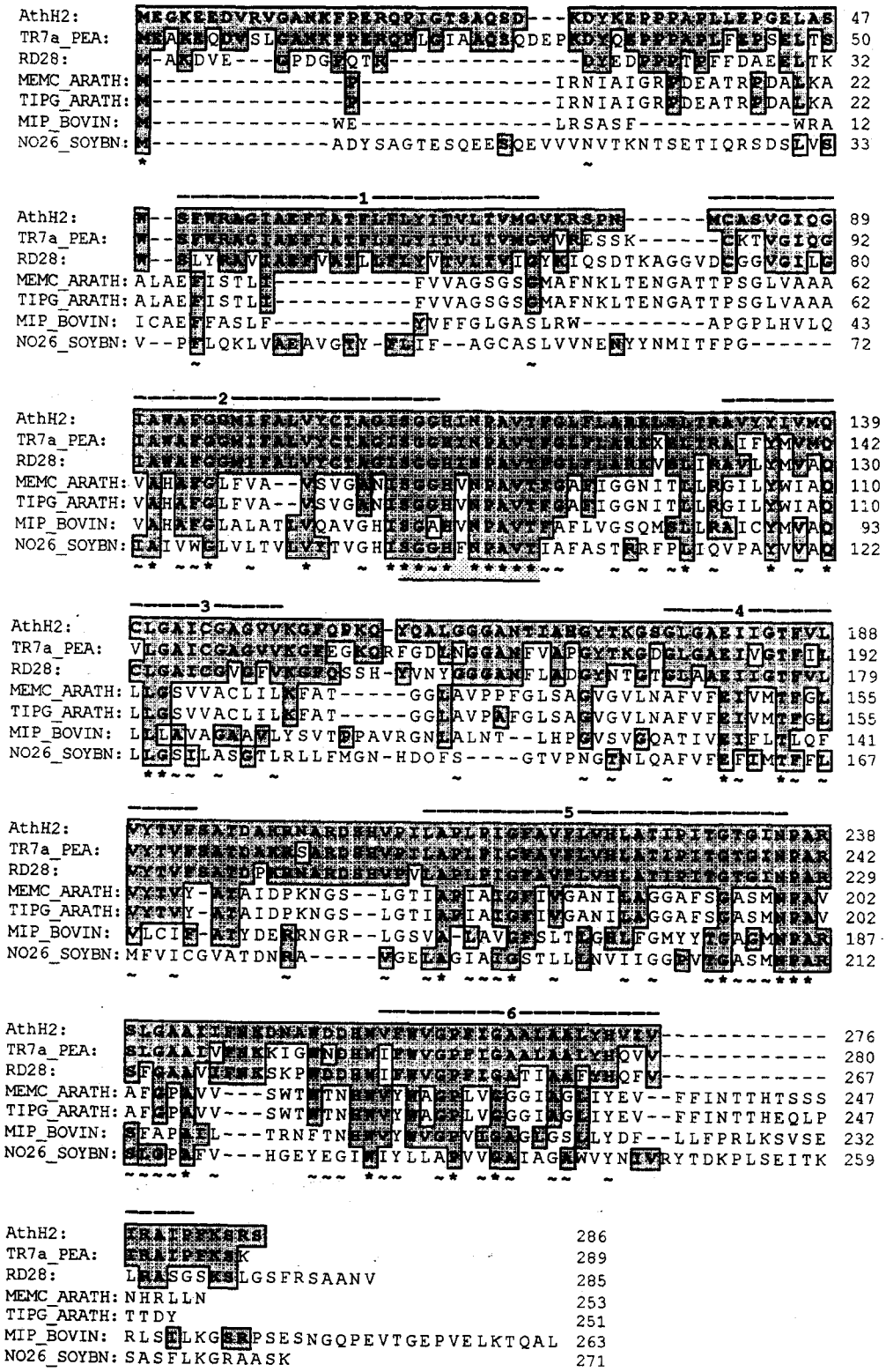
Putative function of the AthH2 protein

The predicted 6 membrane-spanning helices in the AthH2 encoded protein is in accordance with the proposed structural organisation of a class of plant channel proteins. Therefore, an equivalent function of the protein is being taken into consideration.

Discussion

The objective of this investigation was to identify genes which are rapidly activated upon blue light irradiation of *Arabidopsis* plantlets and to characterize their products. To this purpose the morphogenic programme was altered by adjusting the culture conditions in such a way that floral induction as a blue light-dependent response was favoured. A number of novel transcripts could be detected via their cDNA which failed to appear completely, or in comparable amounts, after an equivalent treatment with continuous red or far-red light. Apparently, blue light seems to mediate the activation of the corresponding genes. Differences among these were found with regard to the time course and the extent of transcript accumulation. Moreover, the cDNA clones so far examined differ in nucleotide sequence and deduced amino acid sequence. These observations are reminiscent of those made with cell culture lines of *Chenopodium rubrum* where we also discovered

Fig. 5. Comparison of the amino acid sequence of proteins structurally related to the AthH2 protein. Optimized alignment for the following species: TR7a of pea (TR7a–PEA), RD 28 (RD 28), membrane channel protein (MEMC–ARATH) and tonoplast intrinsic protein (TIPG–ARATH) of *Arabidopsis thaliana*, bovine membrane intrinsic protein (MIP–BOVIN), and nodulin 26 of soybean (NO26–SOYBN). Gaps were introduced to optimize matches. Boxes with bold letters: amino acids identical with those of AthH2. The asterisks indicate amino acids identical in all sequences; the squiggel symbols denote those with a conservative replacement. Bars with numbers on top are predicted transmembrane helices. The MIP/NOD26/GlpF-family motif is underlined.


AthH2:
TR7a_PEA:
RD28:
MEMC_ARATH:
TIPG_ARATH:
MIP_BOVIN:
NO26_SOYBN:
1
2
3
4
5
6

a set of rapidly blue light-activated nuclear and plastid genes [2, 24]. One of these genes encoding a glycine-rich protein of as yet unknown function [23] has counterparts in other plant systems where it is induced by ABA [28]. This leads us to assume that common signal transduction elements for both blue light and ABA are involved in the activation of this type of gene.

Since one of the novel *Arabidopsis* genes identified by clone AthH2 in this survey is likewise activated by either blue light or ABA we saw a good chance to investigate the *cis* elements known to be implicated in the response to light or a phytohormone. The results obtained establish the presence of such sequence elements in the extended promoter region that have been recognized as relevant for GA- and ABA-induced responses in several instances. Further studies, however, are necessary to substantiate the proposed functions of these elements *in vivo*. Surprisingly, we failed to detect any DNA segments within the 5′-upstream region that act as light-responsible *cis* elements in other plants systems [13]. Thus, the question arises whether the observed blue light regulation of the AthH2 gene is achieved by a separate transduction pathway relying perhaps on new, still unidentified, promoter elements.

The rapid response to blue light irradiation of the AthH2 gene could be due to an increase of the transcription rate rather than to transcript stabilization [2].

The six membrane-spanning helices deduced from the amino acid sequence of the AthH2-encoded protein seem to be a common structural entity of plant proteins active in transporting ions or small molecules through a membrane. The topological model depicted in Fig. 4 bears resemblance to that of the RD28 protein which has been identified in *A. thaliana* as product of a desiccation responsive (RD) gene [38]. Due to its structural similarity with several channel proteins like the bovine MIP, a gap junction protein [14], or the nodulin 26, a component of the peribacteroid membrane [10], a role as membrane transport system has been assigned to the RD28 protein. A similar model of a membrane-spanning protein derived from the data of hydropathy plot and secondary structures analysis has been proposed for TIP, an intrinsic tonoplast protein of storage vacuoles in seeds [22]. A novel feature is the inducibility of the AthH2 gene by either blue light or ABA in relation to a developmental process, i.e. flower formation.

The remarkable sequence homology and the similar conformation of the various intrinsic membrane proteins found in plants also apply to certain species of animal origin, such as MIP in the bovine lens fibre cell membrane junctions [14]. In this context we have emphasized the presence of the sequence motif SGGHXNPAVT shared by all these proteins. Thus the question arises whether they have a common evolutionary origin. We are far from understanding the importance of the AthH2 protein formed as a response to blue light irradiation in plantlets of *A. thaliana*. Further analysis of its native structure and *in vivo* function(s) with respect to signal transduction and/or photomorphogenesis will contribute to the understanding of the molecular mechanisms underlying these processes.

Acknowledgements

This research was supported by the Deutsche Forschungsgemeinschaft. We thank Ms I. Liebscher and Ms P. Trzebiatowski for competent assistance.

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