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BIOTIC AND ABIOTIC STRESS

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Abiotic stress response in the moss *Physcomitrella patens*: evidence for an evolutionary alteration in signaling pathways in land plants

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Abstract The mechanisms plants use to adapt to abiotic stress have been widely studied in a number of seed plants. Major research has been focused on the isolation of stress-responsive genes as a means to understand the molecular events underlying the adaptation process. To study stress-related gene regulation in the moss *Physcomitrella patens* we have isolated two cDNAs showing homology to highly conserved small hydrophobic proteins from different seed plants. The corresponding genes are up-regulated by dehydration, salt, sorbitol, cold and the hormone abscisic acid, indicating overlapping pathways are involved in the control of these genes. Based on the molecular characterization of the moss homologs we propose that signaling pathways in response to abiotic stress may have been altered during the evolution of land plants.

Keywords *Physcomitrella patens* · Abiotic stress · Gene regulation

Abbreviation *ABA:* Abscisic acid · *EST:* Expressed sequence tag

Introduction

As sessile organisms, plants are to an extremely high degree affected by changes in the environmental conditions. To counteract the damaging effects of stress situations plants have developed specific molecular and biochemical mechanisms, the investigation of which has been a major research topic for several years (Blumwald 2000; Shinozaki and Yamaguchi-Shinozaki 2000; Knight and Knight 2001; Thomashow 2001; Xiong and Zhu 2001; Seki et al. 2003; Shinozaki and Dennis 2003; Shinozaki et al. 2003; Zhu 2003). Various plant species have been studied for adaptive responses to abiotic stress; these include the genetic model system *Arabidopsis thaliana* and major crop species like wheat, barley and rice. In addition, researchers have also focused on plant species that show an extremely high tolerance to particular stress situations; for example, the ice plant *Mesembryanthemum crystallineum* (Bohnert and Cushman 2000) is able to tolerate high concentrations of salt and therefore has been studied with respect to the mechanisms underlying this resistance. Two examples of model species showing resistance to severe water loss are *Craterostigma plantagineum* (Scrophulariacea) (Bartels et al. 1997; Bartels and Salamini 2001) and the moss *Tortula ruralis* (Oliver et al. 2000; Wood et al. 2000). These so-called resurrection plants are able to survive the desiccation of their vegetative tissues and return to normal growth upon rehydration.

The genetic preconditions that confer a high degree of tolerance to a particular stress situation evolved in different plant families, but they are limited to a small number of individual species. It would be interesting to identify stress response pathways that were conserved throughout land plant evolution. One approach to address this question is the identification of stress-responsive genes and subsequent analysis of their expression pattern following different stress treatments. It would be of particular interest to perform such an analysis using genes that are induced not only by one but multiple stress treatments.

The induction of these genes is achieved by cross-talks between different signaling pathways. A class of genes that meets these requirements encodes highly hydrophobic small proteins and has been identified in different plant species. In Arabidopsis, two well-characterized genes were isolated by screening a cDNA library using a subtracted cDNA probe enriched in cold-induced transcripts (Capel et al. 1997). As expected, the transcripts of the isolated genes—*RCI2A* and *RCI2B*—were found to be up-regulated by cold. As many cold-inducible genes are

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also induced by other abiotic stress treatments, the expression pattern of *RCI2A* and *RCI2B* were analyzed in response to dehydration, salt and the plant hormone abscisic acid (ABA), the latter being a mediator of the stress response. Both genes were found to be up-regulated by dehydration and ABA, but not by salt. The regulation of these genes upon various different stress treatments suggests overlapping signal transduction pathways that are initiated upon exposure to different stresses. The encoded proteins are also believed to act in the adaptation to different abiotic stress situations. Protein sequence analyses revealed two putative membrane-spanning regions, which led to the hypothesis that these proteins may be involved in the maintenance of membrane structure and function during water-stressed situations caused by environmental conditions resulting in reduced water availability (Capel et al. 1997). A functional characterization of the Arabidopsis *RCI2A* gene was carried out in a yeast mutant defective in the *RCI2* homologous yeast gene *SNA1*. This mutant showed retarded growth in the presence of 1 *M* NaCl compared to that shown by the wild type. Expression of the Arabidopsis *RCI2A* gene was able to complement the salt-sensitive phenotype of the *SNA1* deletion mutant, suggesting a role in sodium tolerance (Nylander et al. 2001). As a functional characterization of the two Arabidopsis genes by over-expression in both sense and antisense orientation is still unavailable, the properties of these genes remain hypothetical. However, when searching the nucleotide and protein databases homologs of the *RCI2* genes can be found in different other species, including plants, animals, fungi and bacteria, suggesting a common ancestor and a high degree of conservation throughout evolution. In addition to the *RCI2* genes in Arabidopsis, homologous genes identified in other plant species have also been associated with the abiotic stress response. Interestingly, those genes that were analyzed with respect to their expression pattern upon different stress treatments show a deviating regulation pattern with respect to gene expression. For instance, the barley genes *blt101.1* and *blt101.2*, which were identified by a differential screen of a barley cDNA library, were shown to be up-regulated by cold, but not by dehydration, ABA or salinity (Goddard et al. 1993; Brown 1998). Another member of this class of genes encoding small hydrophobic proteins was identified in the salt-tolerant wheatgrass *Lophopyrum elongatum*. Enhanced mRNA levels for the gene *ESI3* have been shown in response to NaCl, KCl, ABA and mannitol (Gulick et al. 1994), whereas an up-regulation of this gene by cold has not been reported.

The presence of this class of small proteins throughout groups of divergent organisms suggests that they play an important role in the molecular response to different stress factors. Based on their different expression patterns in plants these proteins may be either involved in the response to changes in a single factor, like the barley *blt101* gene family, which is exclusively induced by cold, or they may play a role in overlapping mechanisms induced by several stress factors. The different regulation patterns of these genes that have been observed in various plant species imply that the regulatory mechanisms in response to abiotic stress may have been altered throughout evolution. To provide evidence for this hypothesis we isolated two *RCI2* homologs from the moss *Physcomitrella patens*, which is representative of a land plant that diverged from seed plants more than 450 million years ago (Theissen et al. 2001). Analysis of the expression pattern of the moss homologs could provide information on the regulatory pathways that were developed during an early stage of land plant evolution.

Materials and methods

Plant material and growth conditions

The cultivation of *Physcomitrella patens* plants has been described by Reski et al. (1994). Plants were grown axenically in liquid Knop medium containing 250 mg l^{-1} KH_2PO_4, 250 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 250 mg l^{-1} KCl, 1,000 mg l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 12.5 mg l^{-1} $FeSO_4 \cdot 7H_2O$, pH 5.8, in Erlenmeyer flasks at 25±1°C under a 16/8-h (light/dark) photoperiod with light supplied at an intensity of 55 μmol m^{-2} s^{-1}. The plants were subcultured at 7-day intervals. For salt and sorbitol treatments, plants were transferred to Knop medium supplemented with either 250 m*M* NaCl or 600 m*M* sorbitol. ABA treatment was carried out by adding 50 μM (±)-*cis-trans* ABA to the liquid cultures. Cold treatments were performed by placing the cultures on ice. For the dehydration experiments, the medium was removed using a Buchner funnel and the plants allowed to become dessicated at room temperature.

Isolation of cDNAs

The cDNA clones for *PpSHP1* and *PpSHP2* were obtained following a protein-protein BLAST search (Altschul et al. 1990) of the *P. patens* expressed sequence tag (EST) library, which was generated during a joint project of the University of Freiburg and BASF Plant Science GmbH (Rensing et al. 2002) with the *Arabidopsis thaliana* protein sequence RCI2A (Swiss-Prot accession no. Q9ZNQ7) as query. Two identified cDNA clones were retrieved from the bacterial stock depository, and the full-length cDNA clones were sequenced on both strands.

RNA gel blot analyses

Isolation of total RNA was performed according to Pawlowski et al. (1994). Briefly, 400 mg of plant material were ground to fine powder under liquid nitrogen and transferred into an Eppendorf tube. A 500-μl aliquot of a 1:1 (v/v) mixture of phenol/extraction buffer [0.1 *M* LiCl, 0.1 *M* Tris-HCl, pH 9.0, 10 m*M* EDTA, 1% (w/v) sodium dodecyl sulfate (SDS)] was heated to 90°C and added to the plant material. The suspension was incubated for 5 min on a rotating platform, 250 μl $CHCl_3$ was added and the mixture was incubated for an additional 5 min. After centrifugation at 13,000 *g*, the aqueous phase was extracted once with phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) and once with chloroform. The aqueous phase was isolated, and the RNA was precipitated by the addition of one volume of LiCl overnight at 4°C. RNA was recovered by centrifugation for 20 min at 13,000 *g*, resuspended in 250 μl H_2O and again precipitated by the addition of 0.1 volume of 3 *M* NaOAc, pH 5.2 and two volumes of ethanol. The RNA was resuspended in 40 μl H_2O. Total RNA (20-μg aliquots) of each sample was separated in an 1% (w/v) denaturing agarose gel and transferred onto a nylon membrane as described by Sambrook and Russel (2001). cDNA hybridization probes of *PpSHP1* and *PpHSP2* were generated using gene-specific primers to amplify sections

of the 3′ untranslated regions. The primers used were 5′-GCAATGAGTCCCTCTTCCT-3′ and 5′-GGTAAGGAAGATGCTAAGG-3′ for *PpSHP1* and 5′-TCTGGAAGGTCTGCTCGAG-3′ and 5′-CATGATCATTTCTGGTGTG-3′ for *PpSHP2*. Hybridization was carried out under high-stringency conditions in modified Church Buffer (1 m*M* EDTA, 0.5 *M* sodium phosphate, pH 7.2, 7% SDS, 100 μg ml^{-1} denatured salmon sperm DNA) at 68°C. The membranes were washed for 10 min in 1× SSC, 0.1% SDS at 68°C followed by three washing steps of 10 min each in 0.5× SSC, 0.1% SDS at 68°C. Following hybridization, the membranes were exposed to an imaging plate. The plate was scanned using the Personal Molecular Imager FX System (Bio-Rad, Munich, Germany) imaging device.

Sequence analyses

For comparative sequence analysis, the following protein sequences were retrieved from the databases by means of protein-protein BLAST (BLASTP) searches (Altschul et al. 1990) using the *A. thaliana* RCI2A protein sequence as query (if possible, protein names are given in brackets): AAK50619 (RCI2A), AAF26091 (LTI6B), AAK50618 (RCI2B), AAM15078, Q9M095 (RC23), Q9SUI0 (RC24), Q9FE70 (RC21), O82232 (RC22), AAL31117 from *A. thaliana*; AAG46140, AAM46894, BAC16385, Q9LRI7 (OSR8), AAO16991 from rice; Q9ARD5 (BLT101.2), Q42509 (BLT101.1) from barley; AAN06944, BAB03288 from wheat; AAA21847 (ESI3) from the wheatgrass *Lophopyrum elongatum*; BAC23051 from potato; Q22700 from *Caenorhabtidis elegans*; EAA31469 from *Neurospora crassa*; AAK86229 from *Agrobacterium tumefaciens*; ZP_00016294 from *Rhodospirillum rubrum*; ZP_00086832 from *Pseudomonas fluorescens*; AAN66004 from *Pseudomonas putida*; AAO54050 from *Pseudomonas syringae*. Multiple sequence alignments were performed with the CLUSTALW algorithm (Thompson et al. 1994), and topology prediction of the proteins were carried out utilizing the TMAP algorithm (Persson and Argos 1994, 1996).

Results

Isolation and sequence analyses of *PpSHP1* and *PpSHP2*

To identify *P. patens* homologs of the class of small hydrophobic proteins described in various plant species we used the *A. thaliana* protein RCI2A for BLAST searches of a *P. patens* EST database (Rensing et al. 2002). As a source for the preparation of cDNA libraries from *Physcomitrella* we used material collected from all stages of the life cycle and plant material which had undergone treatments with different plant hormones. The searches revealed two significant hits in the EST database, one single EST sequence (singleton S_PP030008084; *E*-value 6×10^{-20}) and a second one representing a contig sequence (C_PP015060185; *E*-value 2×10^{-17}) consisting of three EST sequences (PP015026310, PP015030196, PP015060185). The four corresponding cDNA clones were retrieved from the bacterial stock depository for plasmid isolation and sequencing. In the case of the contig sequence, the three cDNA inserts were amplified using standard M13-20 and M13 reverse primers to select the clone harboring the longest cDNA. The clone PP015026310 contained the largest cDNA insert and was chosen for sequencing. Sequence analyses revealed that PP015026310 comprises a full-length cDNA of 875 bp, denoted *PpSHP1* (*P. patens* small hydrophobic protein 1; GenBank accession no. AY496071) encoding a protein of 59 amino acids. The clone PP030008084 also harbors a full-length cDNA comprising 698 bp; it has been designated *PpSHP2* (*P. patens* small hydrophobic protein 2; GenBank accession no. AY496072) and codes for a protein of 58 amino acids. The deduced amino acid sequences of both cDNAs share 67% of sequence identity (Fig. 1a). Based on the protein sequence of this class of proteins, we hypothesized that the proteins contain two membrane-spanning regions. Using the algorithm developed by Kyte and Doolittle (1982) we analyzed both protein sequences to identify putative membrane-spanning regions. The existence of two membrane-spanning regions was predicted for both proteins (Fig. 1b). To determine the degree of conservation between members of this protein family from different organisms, we performed a multiple sequence alignment (Thompson et al. 1994) with 29 protein sequences showing significant homology [*E*-value$<4\times10^{-4}$) to the Arabidopsis protein RCI2A (Fig. 1c)]. The multiple sequence alignment was used to predict membrane-spanning regions with the TMAP algorithm (Persson and Argos 1994, 1996). All sequences that were included in this analysis exhibit two putative membrane-spanning regions. Moreover, the sequence alignment shows that identical protein sequences can be found in different organisms because the amino acid sequences of proteins from barley (BLT101.1, accession no. Q42509), wheat (accession no. BAB03288) and the wheatgrass *L. elongatum* (ESI3, accession no. AAA21847) are completely consistent. The high degree of sequence conservation as well as the high consistency of protein topology suggest a common function of these proteins in different organisms.

PpSHP1 and *PpSHP2* are induced by cold, ABA, salt, osmotic stress and dehydration

The genes encoding the class of small hydrophobic proteins were initially identified during screening approaches designed to isolate abiotic stress-responsive genes. To demonstrate the role of these genes in the abiotic stress response their expression patterns following different abiotic stress treatments were analyzed. The genes that have been studied to date have been shown to be responsive to abiotic stress treatments, but their induction has been limited to a selection of stresses. For example, barley genes *blt101.1* and *blt101.2* are inducible by cold, but not by ABA, salt or dehydration (Goddard et al. 1993; Brown 1998). The Arabidopsis genes *RCI2A* and *RCI2B* are up-regulated by cold, dehydration and ABA, but not by salt (Capel et al. 1997). We performed expression analysis of the two *Physcomitrella* genes *PpSHP1* and *PpSHP2* in order to elucidate the regulation of these genes by different abiotic stresses. RNA gel blot analyses were carried out on samples derived from plants treated with cold, salt, dehydration and osmotic stresses and ABA (Fig. 2). Unlike the expression of homologous

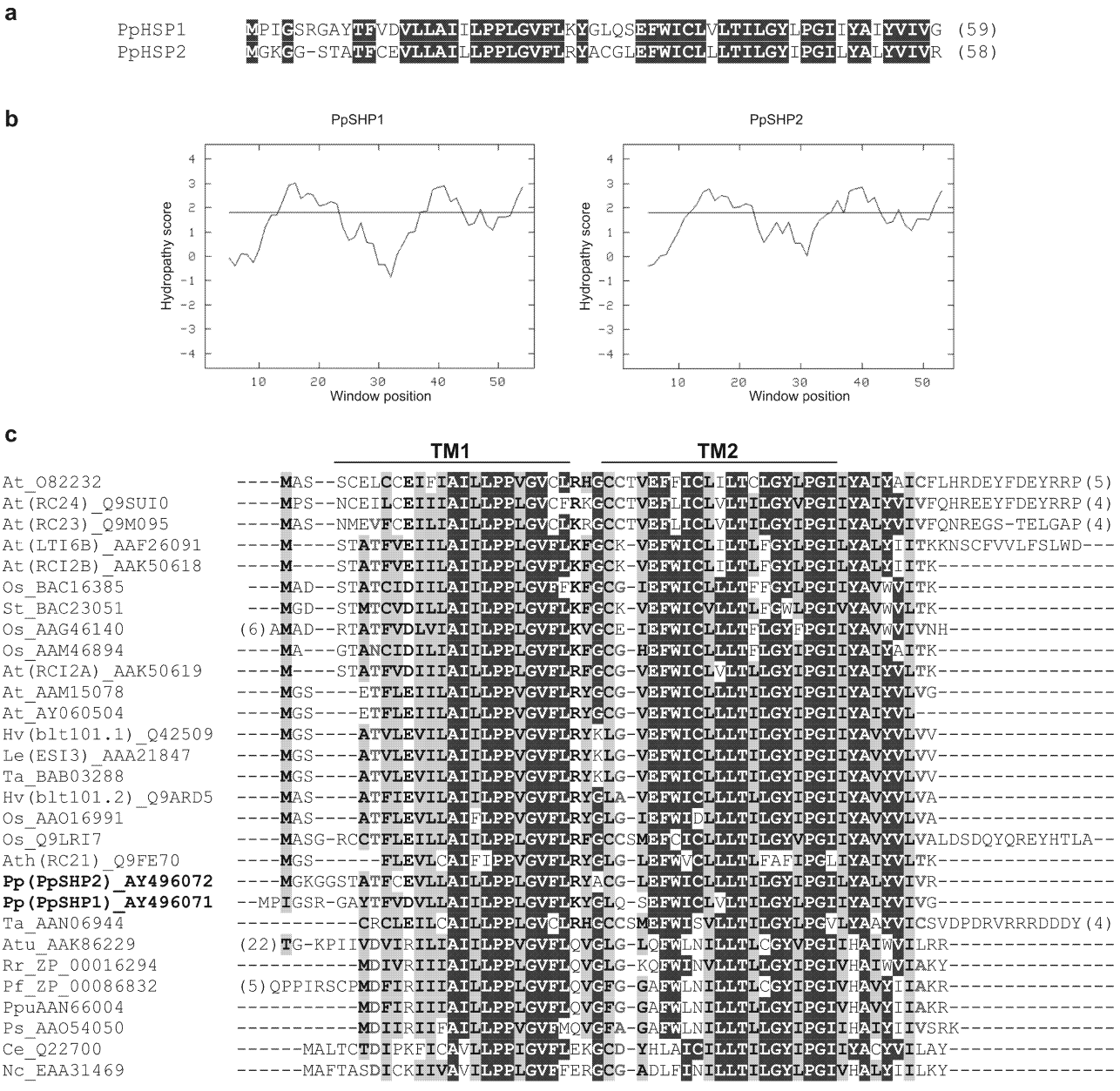

Fig. 1 a Amino acid sequence comparison of PpSHP1 and PpSHP2. *Black boxes* Identical amino acid residues. **b** Hydropathy plot of PpSHP1 and PpSHP2 showing two membrane-spanning regions for both proteins. **c** Comparative amino acid sequence analyses of 29 proteins belonging to the class of small hydrophobic proteins. Amino acid positions that are shared by at least 70% of the sequences are indicated by *black boxes*. Conserved amino acid exchanges are marked by *gray boxes*. The two membrane-spanning regions that are predicted for all proteins are indicated by *black lines* above the alignment (*TM1*, *TM2*). The two *Physcomitrella* sequences are marked in *bold*. The denotation of the proteins indicates the species and, if possible, the name of the protein (*in brackets*) and the accession number. *At Arabidopsis thaliana*, *Os Oryza sativa*, *St Solanum tuberosum*, *Hv Hordeum vulgare*, *Le Lophopyrum elongatum*, *Ta Triticum aestivum*, *Pp Physcomitrella patens*, *Atu Agrobacterium tumefaciens*, *Rr Rhodospirillum rubrum*, *PfPseudomonas fluorescens*), *Ppu Pseudomonas putida*, *Ps Pseudomonas syringae*, *Ce Caenorhabtidis elegans*, *Nc Neurospora crassa*

genes from other plant species, we observed that the expression of *PpSHP1* and *PpSHP2* was responsive to all of the stress treatments applied. We detected a marked increase in the transcript levels of both genes after 2 h of exposure to dehydration, salt, sorbitol and cold treatment. We also observed that *PpSHP2* appears to be more sensitive to dehydration and salt stress than to the other stresses. The responsiveness of both genes to ABA was found 4 h post-application of the hormone. The induction of the two *Physcomitrella* genes by different abiotic stresses suggests either a common signaling pathway, which is activated after the perception of all stress types, or overlapping pathways that are linked at the stage of transcriptional activation of both genes. Moreover, in

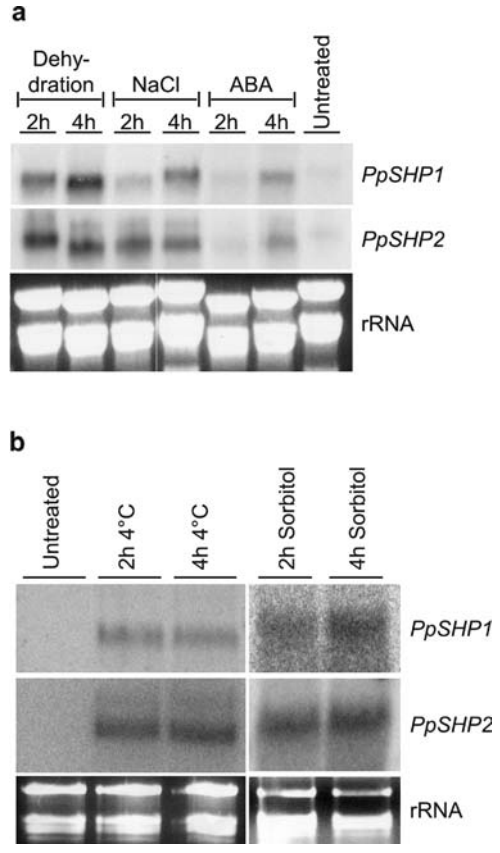

Fig. 2 **a** Expression analyses of *PpSHP1* and *PpSHP2* in response to dehydration, NaCl (250 m*M*) and ABA (50 μM). Treatments were carried out for 2 h and 4 h. From each sample 20 μg of total RNA was loaded onto the gel. The *lowermost panel* shows the ethidium bromide-stained rRNA bands of one representative gel, indicating equal loading of the RNA samples. **b** Expression analyses of *PpSHP1* and *PpSHP2* in response to cold and sorbitol (600 m*M*). Treatments were carried out for 2 h and 4 h. From each sample 20 μg of total RNA was loaded onto the gel. The *lowermost panel* shows the ethidium bromide-stained rRNA bands of one representative gel, indicating equal loading of the RNA samples

contrast to related genes analyzed in seed plants, the *Physcomitrella* genes were inducible by all of the stress treatments investigated, which suggests that stress-related regulatory pathways might have been diversified during the evolution of land plants.

Discussion

A class of genes coding for small hydrophobic proteins has been found in many organisms, including animals, plants, fungi and bacteria. The amino acid sequence of these proteins share a high degree of homology. Based on the existence of two hydrophobic regions within this primary structure, these proteins are thought to be membrane-integral proteins exhibiting two membrane-spanning domains. We isolated two genes from the moss *P. patens* that belong to this gene family. Sequence analyses revealed that the moss genes *PpSHP1* and *PpSHP2* perfectly match the predicted protein topologies of these proteins, including the presence of two putative membrane-spanning regions. Comparative analyses with 27 protein sequences from other organisms showed that the highest degree of identity of the proteins is located within the two transmembrane regions. The high conservation of these proteins implies that they may fulfill similar functions in different organisms that separated early during the evolutionary process. A number of plant genes from this gene family have been studied with respect to their involvement in the response to abiotic stresses. All of the homologous plant genes analyzed to date have been shown to be responsive to abiotic stress treatments (Goddard et al. 1993; Gulick et al. 1994; Capel et al. 1997; Brown 1998). To our knowledge until now no expression studies have been performed with genes from non-plant organisms. The first evidence that this class of proteins exhibits analog functions was provided by Nylander et al. (2001). These authors were able to complement the sodium- sensitive phenotype of the yeast *SNA1* deletion mutant by expression of the homologous *RCI2A* gene from Arabidopsis in the mutant background. Although this experiment proved the hypothesis that the high conservation of this class of genes reflects common functional mechanisms, the molecular mode of action of these proteins remains still unknown. A functional characterization in homologous plant species by over-expression or knockout approaches may present a further step to the unraveling of this question. As the small hydrophobic proteins are encoded by small gene families in the different plant species it will be interesting to learn if reverse genetic strategies will be successful with respect to probable gene redundancy.

Even though both protein sequence and protein topology have been highly conserved in the plant homologs of this class of proteins, their expression varies with different stress treatments. For example, barley genes *blt101.1* and *blt101.2* are specifically induced by cold stress, but not by salt stress, dehydration or the exogenous application of ABA. The Arabidopsis homologs *RCI2A* and *RCI2B* are predominantly induced by cold stress, dehydration and ABA, but not by salt stress. It would seem that although the genes have been conserved throughout evolution, their regulation in response to abiotic stresses has been altered, which may be due to specific adaptations to environmental conditions. As the common ancestor of mosses and seed plants lived 450 million years ago, mosses may possess regulatory mechanisms that deviate from those controlling the expression of homologous genes found in higher plants. Contrary to the expression patterns that have been described in seed plants, the *Physcomitrella* genes *PpSHP1* and *PpSHP2* are responsive to all of the abiotic stresses that have been investigated. This implies the existence of signaling pathways that might have been lost during species diversification. The induction of *PpSHP1* and *PpSHP2* following various stress treatments may be achieved by means of a common pathway that is initiated upon the perception of the various stresses. Alternatively, the

regulation of these genes may occur by distinct stress-specific pathways that merge at the stage of transcriptional activation. The existence of such overlapping pathways have been reported for signaling cascades from several plant species (Jonak et al. 1996; Mizoguchi et al. 1996; Liu et al. 1998; Xiong et al. 1999; Kovtun et al. 2000; Shinozaki and Yamaguchi-Shinozaki 2000; Xiong and Zhu 2001). To our knowledge, the results of our investigation indicate for the first time the existence of overlapping stress-associated pathways in *Physcomitrella* that are activated upon dehydration, salt, osmotic stress, cold and ABA. Based on the diverging regulation of gene expression in *Physcomitrella* and seed plants, we hypothesize that the regulatory mechanisms present in plants were subject to diversification during the evolutionary process. As the induction of homologous genes from seed plants is limited to specific stress variants, we assume that the capacity of the underlying regulatory mechanisms may have been lost throughout evolution. An alternative hypothesis can be proposed if we consider that this class of genes is predominantly represented by small gene families. Angiosperms may have diversified these families to include members that respond to signals differently in different cell types and tissues. We have recently repeated the search for homologs of this class of genes in *Physcomitrella*, searching a newly clustered database comprising all proprietary EST data as well as all publicly available sequence entries. Our results suggest that three additional genes homologous to *PpSHP1* and *PpSHP2* may be present in *Physcomitrella*. A detailed analysis of their expression pattern upon abiotic stress treatments may provide further evidence to support one of the proposed hypotheses.

The comparatively simple morphological organization of mosses may be another reason for the cross-responsiveness of genes to different abiotic stress factors. Most of the tissues in *Physcomitrella* consist of a single cell layer. When these tissues become affected by abiotic stress conditions all of the cells have to adapt directly to the adverse conditions in order to avoid any detrimental effects caused by the stress situations. In mosses, the existence of overlapping pathways activated by multiple types of stress may contribute to the rapid establishment of biochemical mechanisms that are required to enhance tolerance to a broad range of abiotic stress conditions. However, seed plants have developed specialized tissues and organs which, in many cases, are composed of multiple cell layers. The higher order of morphological organization might also have led to the diversification of the adaptive response to adverse environmental conditions, including the regulatory pathways leading to stress-induced gene expression. Taking this hypothesis into consideration, mosses may represent an excellent model species for the isolation and characterization of genes involved in the abiotic stress response of plants. *Physcomitrella* has become a valuable model system for the functional analysis of plant genes because it is suited to generate targeted knockout mutants based on the high frequency of homologous recombination in its nuclear DNA (Strepp et al. 1998). In addition, recent experiments have shown that *Physcomitrella* is also amenable to gene silencing studies achieved by RNA interference (Bezanilla et al. 2003). Further approaches using these molecular tools may contribute to the functional characterization of genes involved in the abiotic stress response.

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