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ORIGINAL ARTICLE

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Physcomitrella patens is highly tolerant against drought, salt and osmotic stress

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Abstract In order to determine the degree of tolerance of the moss *Physcomitrella patens* to different abiotic stress conditions, we examined its tolerance against salt, osmotic and dehydration stress. Compared to other plants like *Arabidopsis thaliana*, *P. patens* exhibits a high degree of abiotic stress tolerance, making it a valuable source for the identification of genes effecting the stress adaptation. Plants that had been treated with NaCl tolerated concentrations up to 350 mM. Treatments with sorbitol revealed that plants are able to survive concentrations up to 500 mM. Furthermore, plants that had lost 92% water on a fresh-weight basis were able to recover successfully. For molecular analyses, a *P. patens* expressed sequence tag (EST) database was searched for cDNA sequences showing homology to stress-associated genes of seed plants and bacteria. 45 novel *P. patens* genes were identified and subjected to cDNA macroarray analyses to define their expression pattern in response to water deficit. Among the selected cDNAs, we were able to identify a set of genes that is specifically up-regulated upon dehydration. These genes encode proteins exerting their function in maintaining the integrity of the plant cell as well as proteins that are known to be members of signaling networks. The identified genes will serve as molecular markers and potential targets for future functional analyses.

Keywords Abiotic stress · *Physcomitrella* · Stress-related genes · Stress tolerance

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Abbreviations ABA: Abscisic acid · EST: Expressed sequence tag

Introduction

Investigation of the molecular mechanisms involved in the abiotic stress response of plants has made substantial progress in recent years (for reviews, see Knight and Knight 2001; Zhu 2001a, 2001b; Seki et al. 2003). Many genes that are associated with the abiotic stress response have been isolated based on their differential expression pattern but their functional analysis remains fragmentary. Another experimental approach to characterize key regulators of the abiotic stress response made use of *A. thaliana* lines carrying stress-inducible promoters fused to the firefly luciferase reporter gene. These lines had been subjected to undirected chemical or physical mutagenesis and were subsequently screened for altered luciferase activity (Liu et al. 2000; Shi et al. 2000; Lee et al. 2001; Xiong et al. 2001a, 2001b; Chinnusamy et al. 2003). This forward genetic screen and the positional cloning of the affected genes led to the identification of candidate genes involved in various molecular mechanisms associated with plant stress responses. Besides these studies on abiotic stress using *A. thaliana*, diverse plant species have been chosen to investigate the molecular machinery involved in stress adaptation, including the resurrection plant *Craterostigma plantagineum* (Bartels and Salamini 2001) and the salt-tolerant ice plant *Mesembryanthemum crystallinum* (Bohnert and Cushman 2000). The major reason for studying these plants is their unique ability to tolerate severe dehydration and salt stress, respectively. The overriding aim is to understand the molecular mechanisms conferring the high degree of tolerance. Even though many data have been generated during these investigations the polyploidy of these species hampers genetic analyses.

A powerful alternative to these approaches to determine the biological function of stress-related

genes could be the reverse genetics strategy by the creation of knockout plants. The moss *Physcomitrella patens* represents an excellent system in which to study gene function because the generation of targeted knockout plants is facilitated by a high frequency of homologous recombination (Reski 1998; Schaefer 2001). *P. patens* was originally chosen to study plant differentiation processes, which was facilitated by the haploid status of the moss protonema. Meanwhile, *P. patens* has become accessible to state-of-the-art molecular and genetic analyses which have promoted the moss system for analysis of almost all aspects of plant biology. Recently, Minami et al. (2003) investigated the freezing tolerance of *Physcomitrella* protonemata, which was markedly enhanced upon pretreatment with abscisic acid (ABA). Furthermore, they analyzed fourteen ABA-responsive *Physcomitrella* genes, which had been isolated by means of mRNA differential display, comparing untreated and ABA-treated protonemata. The first analysis of *Physcomitrella*'s tolerance to salt further indicated that the plants were able to tolerate NaCl concentrations up to 600 mM when the plants had been slowly adapted to increasing salt concentrations (Benito and Rodriguez-Navarro 2003). This high degree of tolerance has been ascribed to the presence of an Na^+-pump ATPase, which is usually not found in flowering plants and therefore may have been lost during the evolution of land plants. Recently, we have characterized the stress-responsive expression pattern of two *Physcomitrella* genes homologous to the *Arabidopsis RCI2A* and *RCI2B* genes (Kroemer et al. 2004). The regulation of these genes upon different stress treatments indicates that stress-related signaling pathways might have been altered during the evolutionary development of land plants.

Because further physiological data and information about the molecular events underlying the abiotic stress response in *Physcomitrella* are limited we have set up a physiological screen to monitor its ability to cope with different abiotic stress treatments. Moreover, to identify additional molecular markers associated with the stress response we have searched a comprehensive *P. patens* expressed sequence tag (EST) database (Rensing et al. 2002) for homologous genes that have been related to the abiotic stress response in different plant species. The identified cDNAs have been subjected to expression profiling experiments to detect genes that are responsive to stress conditions; a detailed analysis of their roles in the stress-related biochemical and metabolic pathways, however, exceeds the intention of this work. The identification of stress-related genes in the moss *P. patens* should provide an indication of common molecular mechanisms in response to abiotic stress that might have been evolutionarily conserved in different plant species. The presence of such stress-related genes will be the prerequisite to implement their functional analysis in *P. patens*.

Materials and methods

Plant material and growth conditions

The cultivation of *Physcomitrella patens* plants has been described previously by Reski et al. (1994). Plants were grown axenically either in liquid or solid Knop medium containing 250 mg l^{-1} KH_2PO_4, 250 mg l^{-1} $MgSO_4 \times 7H_2O$, 250 mg l^{-1} KCl, 1,000 mg l^{-1} $Ca(NO_3)_2 \times 4H_2O$, 12.5 mg l^{-1} $FeSO_4 \times 7H_2O$, pH 5.8; solid medium was supplemented with 1% (w/v) agar. The plants were cultured under standard conditions in a growth chamber at 25 ± 1°C under a 16/8 h light/dark photoperiod with a light intensity of 55 µmol photons m^{-2} s^{-1}. Plants grown in liquid culture were subcultured at 7-day intervals; plants grown on solid medium were transferred to fresh medium monthly. For osmotic stress and salt stress treatments, plants grown on solid Knop medium were transferred onto Knop medium supplemented with the indicated concentrations of sorbitol or NaCl, respectively. After 3 days the plants were retransferred onto standard Knop medium and grown for 2 weeks under standard growth conditions before determination of the chlorophyll content. The dehydration treatment was performed by drying the plants on solid Knop medium for the times indicated followed by a 1-h rehydration step in sterilized tap water and transfer of the plants onto standard solid Knop medium. After 2 weeks of recovery on Knop medium the plants were harvested and the chlorophyll content measured. Plant material used for the isolation of RNA for macroarray experiments and RNA gel blot analyses was obtained from liquid cultures. The dehydration treatment was performed by harvesting the plant material from the culture, removing attached medium and drying the plants at 25°C for the times indicated. During all dehydration treatments, plants were weighed at designated time intervals to determine the loss of water. Percentage loss of fresh weight was calculated based on the initial weight of the plants. For salt and sorbitol treatments, plants were transferred to Knop medium supplemented with 300 mM NaCl or 600 mM sorbitol, respectively. Abscisic acid (ABA) treatment was carried out by application of 50 µM (±)-*cis-trans* ABA to the liquid cultures.

Analysis of chlorophyll content

Stress treatments of *Physcomitrella* plants that were intended for chlorophyll measurements were performed in three independent repetitions using ten plants for each treatment. *Physcomitrella* plant material was ground up in liquid nitrogen using a ball mill to obtain a homogenous fine powder. 0.4 g of the homogenized plant material was placed in a 2-ml Eppendorf tube and the chlorophyll was extracted with 1.5 ml of 80% (v/v) acetone. The samples were vigorously mixed for 5 min,

resulting in complete extraction of the chlorophyll from the plant material. To remove cell debris the samples were centrifuged for 5 min at 14,000 *g*. For chlorophyll quantification the absorbance of the supernatant was measured at wavelengths of 645 and 663 nm. After measurement, the supernatant was completely re-transferred into the initial Eppendorf tube and the samples were dried in speed-vac centrifuge at room temperature until a stable dry weight of each sample was obtained. The total chlorophyll was calculated as follows: mg chlorophyll/g dry weight = $[(A_{663})(0.00802) + (A_{645})(0.0202)] \times 1.5$/dry weight.

Isolation of RNA

Total RNA was isolated according to Pawlowski et al. (1994). Briefly, 400 mg of plant material was ground to fine powder under liquid nitrogen and transferred into an Eppendorf tube. 500 µl of a 1:1 (v/v) mixture of phenol:extraction buffer [0.1 M LiCl, 0.1 M Tris–HCl (pH 9.0), 10 mM EDTA, 1% (w/v) 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:isoamyl alcohol (25:24:1, by vol.) and once with chloroform. The aqueous phase was isolated and the RNA was precipitated by addition of 1 vol. 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 addition of 0.1 vol. of 3 M sodium acetate (pH 5.2) and 2 vol. of ethanol. The RNA was resuspended in 40 µl H_2O. Poly(A)$^+$ RNA was isolated from total RNA using the Qiagen Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany).

cDNA synthesis

cDNA was synthesized for 1 h at 42°C in a 40-µl reaction volume containing 500 ng poly(A)$^+$ RNA; 2.5 ng *uidA* mRNA; 10 mM DTT; 1 mM each of dATP, dGTP and dTTP; 5 µM dCTP; 1.48 MBq α-[^{32}P]dCTP (110 TBq mmol^{-1}); and 200 U Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) in the appropriate reaction buffer delivered with the enzyme. The reaction was stopped by addition of 1.3 µl of 1% (w/v) SDS, 1.3 µl of 0.5 M EDTA and 4 µl of 3 N NaOH, and incubation for 30 min at 65°C followed by a 15-min incubation at room temperature. The reaction was neutralized by addition of 13 µl of 1 M Tris–HCl (pH 8.0) and 4 µl of 2 N HCl. On two occasions, 2 µl of each reaction was removed to calculate cDNA synthesis yield. The determination of incorporated radioactivity and calculation of the cDNA yield were carried out according to Sambrook and Russel (2001). The remaining synthesis reaction was precipitated by addition of 0.1 vol. of 3 M sodium acetate (pH 5.2), 0.1 vol. of yeast tRNA (10 mg ml^{-1}) and 2 vol. of ethanol. After centrifugation the synthesized cDNAs were resuspended in 100 µl H_2O.

EST search

Accession numbers of stress-related proteins were retrieved from SWISSPROT and TREMBL databases using the sequence retrieval system (http://www.expasy.ch/srs5/). The protein sequences were utilized to identify homologous proteins within the *P. patens* EST database using the BLASTP algorithm (Altschul et al. 1997). The protein sequences and their accession numbers that have been used for the EST database search are given in Table 1.

Synthesis of *uidA* mRNA

The use of the *uidA* gene as a spiking control for the normalization of hybridization signals of macroarray experiments was previously described by Smith-Espinoza (2001). A part of the *uidA* gene (accession no. S69414) was amplified using a forward primer including the T7 promoter sequence and a reverse primer including a poly(T) stretch. The primer sequences were:

i. Forward primer: 5′ *TAA TAC GAC TCA CTA TAG GG*A GGT GGA CGA TAT CAC C 3′; the italic letters indicate the T7 promoter, the underlined bases correspond to nucleotides 549–564 of the *uidA* gene.
ii. Reverse primer: 5′ TTT TTT TTT TTT CGA AGC-GGG TAG ATA TCA CAC TC 3′; the underlined bases correspond to nucleotides 781–803 of the *uidA* gene.PCR was carried out using the plasmid GH3/GUS (Li et al. 1999) as DNA template. PCR conditions were: 3 min at 94°C, 30 cycles of 30 s at 94°C, 45 s at 58°C, 45 s at 72°C and final extension for 5 min at 72°C. 1 µg of the purified amplicon was used as template in an in vitro transcription reaction carried out with the MAXIscript In Vitro Transcription Kit (Ambion, Austin, TX, USA).

cDNA macroarrays

The cDNA libraries that were generated within the EST sequencing project were cloned into a pUC18-derived vector. The inserts were amplified using primers immediately flanking the cloning sites. Aliquots (about 10 ng) of the amplicons were spotted in duplicate onto nylon membranes using a 96-pin replicator. For normalization of the arrays, 0.1, 1, 5, 10, 50 and 100 ng of the amplified *uidA* gene were included during the spotting procedure. To assess unspecific hybridization to the cloning vector, pUC18 plasmid was spotted as a control. As positive control a *P. patens* cDNA insert, which shows high

Table 1 Results of the *Physcomitrella patens* EST database search

Query	Species	Function	Protein length (aa)	E value	EST sequence length (bp)	% Identity/similarity of x amino acids	Accession number
SCOF-1 (P93166)[a]	Soybean	Zinc finger transcription factor	240	8 e-11	689/C[b]	38/49 120 aa	AJ566705
ABI3 (Q01593)	*A. thaliana*	Transcriptional activator	720	3 e-33	665/S	71/88 124 aa	AJ566720
				3 e-34	1,172/C	67/84 105 aa	AJ566739
ALA1 (P98204)	*A. thaliana*	Aminophospholipid ATPase	1,158	1 e-67	656/S	123/147 193 aa	AJ566713
				6 e-28	618/C	57/85 152 aa	AJ566710
				2 e-25	797/S	62/96 145 aa	AJ566732
DREB1A (O82131)	*A. thaliana*	AP2 domain transcription factor	216	1 e-15	1,568/C	42/49 85 aa	AJ566723
				2 e-15	740 / S	48/60 124 aa	AJ566734
CBF1 (P93835)	*A. thaliana*	AP2 domain transcription factor	213	1 e-15	1,018/C	42/50 85aa	AJ566704
DREB1C (O65613)	*A. thaliana*	AP2 domain transcription factor	216	4 e-20	669/S	53/64 101 aa	AJ566727
DREB2A (O82132)	*A. thaliana*	AP2 domain transcription factor	335	3 e-13	1,853/C	32/39 64 aa	AJ566716
				4 e-13	862/C	31/38 59 aa	AJ566736
GS2 (P14655)	Rice	Glutamine synthetase	428	1 e-156	1,790/C	256/296 371 aa	AJ566706
betA (P17444)	*E. coli*	Choline dehydrogenase	556	1 e-4	1,170/C	64/113 285 aa	AJ566725
				1 e-3	1,482/C	25/30 60 aa	AJ566721
betB (P17445)	*E. coli*	Betaine aldehyde dehydrogenase	489	2 e-59	1,403/C	128/177 324 aa	AJ566707
OsCDPK7 (Q9FXQ3)	Rice	Ca^{2+}-dependent protein kinase	551	1 e-159	1,759/C	267/321 398 aa	AJ566711
				4 e-96	1,221/C	186/224 270 aa	AJ566722
DBF2 (P22204)	Yeast	Kinase	572	3 e-25	530/S	67/98 175 aa	AJ566701
				1 e-24	1,272/C	69/102 205 aa	AJ566712
				1 e-16	586/S	55/86 205 aa	AJ566700
ANP1 (O22039)	*A. thaliana*	MAP kinase kinase kinase	661	1 e-26	1,704/C	67/121 213 aa	AJ566699
				3 e-23	617/S	48/56 65 aa	AJ566730
				5 e-21	858/C	52/85 146 aa	AJ566738
AtMPK3 (Q39023)	*A. thaliana*	MAP kinase	370	1 e-108	1242/C	192/226 274 aa	AJ566731
				2 e-80	767/C	136/175 213 aa	AJ566729
COR TMC-AP3 (O82688)	Barley	Chloroplastic amino acid-selective channel protein	144	4 e-35	1,040/C	73/93 135 aa	AJ566743
				3 e-34	1,012/C	68/93 133 aa	AJ566742
				1 e-33	580/S	67/92 132 aa	AJ56670
AtProDH (P92983)	*A. thaliana*	Proline dehydrogenase	499	1 e-41	542/C	84/116 173 aa	AJ566702
				4 e-40	719/S	79/119 194 aa	AJ566733
Cryoprotective osmotin like protein (Q9FT35)	*Solanum dulcamara*	Cryoprotective protein	248	1 e-34	1,187/C	86/108 228 aa	AJ566719
				4 e-27	760/C	66/88 157 aa	AJ566726
COR47 (P31168)	*A. thaliana*	Cold- and dehydration-induced protein	265	2 e-6	729/S	33/39 75 aa	AJ566740
				2 e-6	1,243/C	33/39 75 aa	AJ566728
HSP17.6 (Q96489)	Tomato	Small heat-shock protein	158	3 e-21	1,214/C	52/77 136 aa	AJ566737
				7 e-17	1,148/C	45/59 104 aa	AJ566718
				7 e-14	1,325/C	34/48 84 aa	AJ566717
WPM-1 (P93615)	Wheat	ABA induced plasma membrane protein	182	3 e-27	764/C	62/86 143 aa	AJ566724
				3 e-27	855/C	61/83 142	AJ566714
				8 e-27	960/C	58/83 140 aa	AJ566715
OsP5CS (O04226)	Rice	Delta1-pyrroline-5-carboxylate synthetase	716	1 e-123	1,020/C	222/270 336 aa	AJ566703
SODA (P11796)	*Nicotiana plumbaginifolia*	Mn Superoxide dismutase	228	9 e-73	1,028/C	140/157 216 aa	AJ566741
				9 e-27	1,126/C	81/111 203 aa	AJ566735
				3 e-24	1,173/C	69/97 178 aa	AJ566709

[a]The accession numbers of the protein sequences are given in parentheses
[b]Defines if the hit in the *P. patens* EST database represents a contig (*C*; clustered sequence of at least two EST sequences) or singleton (*S*; consisting of a single EST sequence)

homology to the ribosomal protein L21 and has been shown to be constitutively expressed (Reski et al. 1997), was spotted onto the membranes. The DNA was denatured for 5 min in 0.4 N NaOH, 3 M NaCl and neutralized for 5 min in 6× SSC twice. The DNAs were cross-linked to the membrane by UV-irradiation at 0.12 J cm^{-2}. The arrays were pre-hybridized for 4 h at 65°C in 10 ml of 0.5 M Na-phosphate buffer (pH 7.2), 1 mM EDTA, 7% (w/v) SDS and 100 μg ml^{-1} denatured salmon sperm DNA. Before hybridization the buffer was exchanged and 50 ng of radioactively labeled cDNA was added. Hybridization was performed at 65°C overnight. Unbound probe was removed by rinsing the membranes briefly with 40 mM Na-phosphate (pH 7.2) and 0.1% (w/v) SDS and washing twice with the same buffer for 30 min at 65°C. Hybridization signals were detected by exposing the membranes to an imaging plate and subsequent scanning of the plate using the Personal Molecular Imager FX System (Bio-Rad, Munich, Germany) imaging device. The quantification of the signal intensities as well as the subtraction of local background values were carried out using the Quantity One software package from Bio-Rad. The mean value of hybridization signals derived from duplicate spots was used for data analysis. The hybridization signals of independent experiments were normalized using the *uidA* controls present on each membrane. The induction or repression of the individual cDNAs was calculated as follows. Signal intensities after hybridization with cDNA derived from untreated plants were set to one. The signal intensities obtained after hybridization with the cDNAs from the dehydrated samples were divided by the corresponding value of the untreated sample (signal intensity dehydration/signal intensity untreated = x-fold induction/repression).

RNA gel blot analyses

20 μg of total RNA of each sample was separated in a 1% (w/v) denaturing agarose gel and transferred onto a nylon membrane as described by Sambrook and Russel (2001). The membranes were hybridized with radioactively labeled probes of *P. patens* homologs of *betA* (accession number AJ566721), *COR47* (accession number AJ566740), *OsCDPK7* (accession number AJ566711), *WPM-1* (accession number AJ566715) and *COR TMC-AP3* (accession number AJ566742). For the *P. patens* homologs of *betA*, *COR47* and *COR TMC-AP3* the cDNA hybridization probes were obtained by PCR amplification using standard M13 forward and reverse primers. The cDNA probes of the *P. patens* homologs of *OsCDPK7* and *WPM-1* were generated using gene-specific primers to amplify sections of the 3′ untranslated regions. The primers that were used were 5′-TAGTAGCTCCTGGTTACCAA-3′ and 5′-AATGAAAACATTTGAGATCC-3′ for the *OsCDPK7* homolog and 5′-TCGGCTGCTATCGCTGGGTA-3′ and 5′-TGTCTCCGGGAGTGTTAGCG-3′ for the *WPM-1* homolog. Hybridization was carried out under high-stringency conditions as described previously (Bartels et al. 1990). After hybridization the membrane was exposed to an imaging plate. The plate was scanned using the Personal Molecular Imager FX System (Bio-Rad) imaging device.

Results

Physcomitrella patens displays a high degree of tolerance against salt and osmotic stress

To assess the degree of tolerance of *P. patens* against salt and osmotic stress, in vitro-cultured plants were exposed for 3 days to medium containing increasing concentrations of NaCl and sorbitol, respectively. We performed an initial screening to monitor the resistance of *P. patens* plants to NaCl and sorbitol stress using concentrations ranging from 50 mM to 1 M. In vitro-cultured plants were chosen to ensure highly standardized experimental conditions. After the treatment the plants were released to standard growth medium and were screened for survivors. Up to 300 mM of NaCl and 500 mM of sorbitol we did not observe any differences between stressed and untreated plants whereas higher concentrations of both stress factors affected the recovery of the plants. For a detailed analysis of the tolerance to NaCl and sorbitol we carried out stress treatments at concentrations of 250, 300, 350 and 500 mM NaCl and 400, 450, 500 and 750 mM sorbitol, respectively. Again, plants were exposed to medium containing salt or sorbitol for 3 days. After the treatments, plants were allowed to recover on standard growth medium for 2 weeks. To monitor the influence of salt and sorbitol on plant growth we determined the chlorophyll content immediately after the stress treatment and after 2 weeks of recovery under standard growth conditions. These measurements were performed with three independent repetitions (Fig. 1). Plants that had been exposed to salt concentrations up to 350 mM were able to recover whereas plants exposed to 500 mM NaCl were completely bleached 2 weeks after the treatment. Even though the chlorophyll content of the plants that were treated with upto 350 mM NaCl decreased in comparison to the untreated control plants (Fig. 1a), no bleached areas could be observed by microscopic inspection. Plants that had undergone treatments with sorbitol were able to tolerate concentrations up to 500 mM. Up to 500 mM sorbitol, plants recovered successfully, which was indicated by an increase in the chlorophyll concentration upon recovery on standard growth medium (Fig. 1b).

Physcomitrella patens tolerates severe dehydration stress

P. patens plants were subjected to dehydration stress to determine their capability to cope with severe water loss.

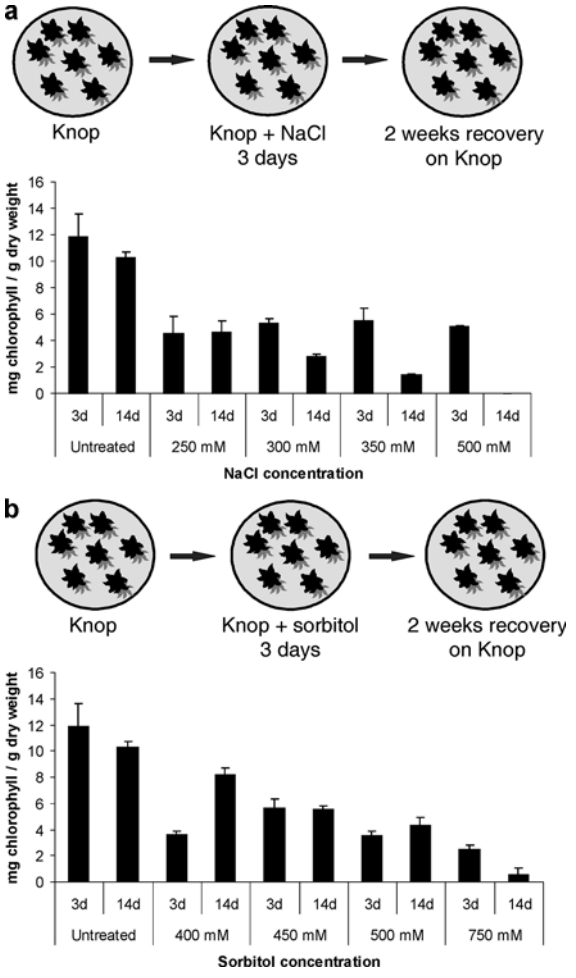

Fig. 1a,b Osmotic stress treatment of *Physcomitrella patens* plants. **a** NaCl treatment. The *upper panel* illustrates the experimental setup. Plants were transferred for 3 days onto Knop medium containing the indicated NaCl concentrations. Plants were then re-transferred onto Knop medium without NaCl and grown under standard conditions. The chlorophyll content was determined immediately after the stress treatments (*3d*) and after 2 weeks (*14d*) of recovery under standard growth conditions. The values shown are means ± SD ($n = 3$). **b** Sorbitol treatment. The *upper panel* illustrates the experimental setup (compare with **a**). The chlorophyll content was determined immediately after the stress treatments (*3d*) and after 2 weeks (*14d*) of recovery under standard growth conditions. The values shown are means ± SD ($n = 3$)

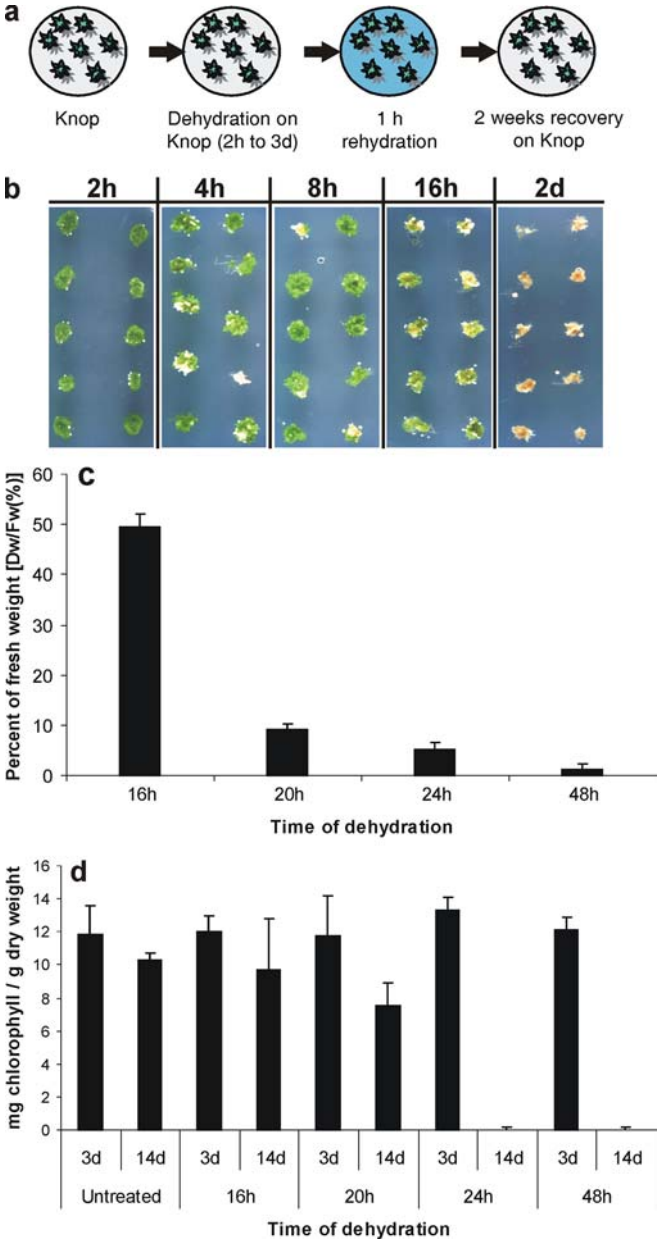

Fig. 2a–d Dehydration treatment of *P. patens* plants. **a** The experimental setup. Plants were transferred onto Knop medium and dried for the indicated time points. Plants were rehydrated for 1 h in sterile tap water and transferred onto Knop medium. **b** *P. patens* plants were dehydrated for the indicated time points. Photographs of the plants were taken after 2 weeks release on Knop medium. **c** Water loss during the dehydration treatment. Percentage loss of fresh weight was calculated based on the initial weight of the plants. **d** Chlorophyll content of control plants and dehydrated plants. The chlorophyll content was determined immediately after the stress treatments (*3d*) and after 2 weeks (*14d*) of recovery under standard growth conditions. The values shown are means ± SD ($n = 3$)

To determine the limits of tolerance of *P. patens* to severe water loss, plants were initially subjected to dehydration stress for several time points ranging from 2 h to 72 h. For this, plants were dried on solid medium followed by subsequent rehydration by floating in tap water. The rehydrated plants were then transferred to fresh medium and cultivated under standard growth conditions. *P. patens* plants that lost up to 85% of their fresh weight during the dehydration treatment were able to recover upon rehydration and release to standard growth medium. To narrow down the degree of tolerance to dehydration stress we performed the same experiment by drying the plants for 16, 20, 24 and 48 h (Fig. 2). To obtain statistical quantitative data the chlorophyll content of the stressed plants was determined immediately after the dehydration treatment and after 2 weeks of recovery under standard growth conditions. After 20 h of dehydration the plants had lost around 92% of their fresh weight. These plants were

able to return to normal growth upon standard growth conditions. Even though the chlorophyll content of the stressed plants did not reach the values of the untreated control plants they were able to survive these conditions, as can be seen by comparison with plants that had been dried for 24 h and 48 h in which we were unable to measure any remaining chlorophyll after the recovery period. As gametophores as well as protonemal tissue were able to recover after the dehydration treatment it is supposed that both tissues display the same degree of tolerance against desiccation.

Stress-related genes in *P. patens*

Different genes with diverse biological functions have been associated with the response to abiotic stress in plants. Using a cDNA macroarray approach we intended to identify members of stress-related genes in *P. patens*. For this we selected genes that are associated with abiotic stress in different species to search for homologs in the *P. patens* EST database. 22 protein sequences were used to perform homology searches within a clustered *P. patens* EST database. We were able to identify EST sequences showing homologies to all search sequences. The sequences that have been identified either comprise contig sequences, which are built up from at least two EST sequences, or they represent singletons, built up from a single EST. The extent of homology between the EST sequences and the protein sequences used for the database search, as well as the EST accession numbers, are given in Table 1.

Expression profiling of stress-related genes

Many of the genes that were selected for the EST database search have been shown to be specifically induced upon abiotic stress conditions. To analyze the identified cDNAs with respect to their expression pattern under abiotic stress conditions we chose a macroarray approach. As some of the proteins that had been used to search the EST database led to the detection of more than one homolog in *Physcomitrella* (Table 1) we used 45 different cDNAs for further analyses. The cDNA inserts of 45 EST clones representing putative stress-associated cDNAs from *P. patens* were amplified by PCR and spotted onto nylon membranes. In cases where the homology search of the *P. patens* EST database led to the identification of a contig sequence, we used the clone that contributed the longest sequence stretch to the contig sequence for PCR amplification. The membranes carrying these cDNA inserts were hybridized with radioactively labeled cDNAs that had been synthesized from untreated *P. patens* tissues and tissues exposed to various stages of desiccation. To assure the comparability of the different hybridization experiments the bacterial *uidA* gene was included as a spiking control for normalization of the membranes. Among the cDNAs present on the membranes we were able to identify 25 cDNAs showing hybridization signal levels above background. The results of the expression profiling experiment and the changes in the expression of the individual genes are summarized in Table 2. Among the analyzed cDNAs, 19 showed enhanced expression levels after the dehydration treatment. Six cDNAs exhibited no changes in expression as a result of the various treatments or were slightly down-regulated by dehydration. Among the cDNAs that showed an enhanced expression level during the dehydration treatments we found genes with putative regulatory functions within the dehydration-induced signal transduction pathways and genes performing putative protective functions within the plant cell. The *P. patens* genes showing the highest expression levels upon dehydration treatment encode proteins that have been implicated in the cold adaptation of seed plants. These genes were homologs of *COR47*, encoding an LEA-like protein from *A. thaliana* (Gilmour et al. 1992), *WPM-1*, encoding a transmembrane polypeptide from wheat (Koike et al. 1997), and *COR TMC-AP3* from barley, coding for a chloroplastic amino acid-selective channel protein (Baldi et al. 1999). One *P. patens* homolog of the rice *OsCDPK7* gene (Saijo et al. 2000), encoding a calcium-dependent protein kinase, also showed high induction of its expression by the dehydration treatment. The remaining *P. patens* genes that are induced by water deficit encompass homologs of *ABI3* (Giraudat et al. 1992), *DREB2A* (Shinwari et al. 1998), *AtMPK3* (Mizoguchi et al. 1993) from *A. thaliana*, *HSP17.6* from sunflower (Almoguera and Jordano 1992), *SODA* from *Nicotiana plumbaginifolia* (Bowler et al. 1989), *OsP5CS* from rice (Igarashi et al. 1997), and *bet A* and *bet B* from *Escherichia coli* (Holmström et al. 2000).

Northern blot analysis of stress-associated genes

In order to verify the macroarray results by RNA gel blot analysis we chose five genes that have shown a dehydration-inducible expression pattern. These genes were *P. patens* homologs of *betA* (*PpbetA*; AJ566721), *COR TMC-AP3* (*PpCOR TMC-AP3*; AJ566742), *OsCDPK7* (*PpCDPK7*; AJ566711), *COR47* (*PpCOR47*; AJ566740) and *WPM-1* (*PpWPM-1*; AJ566715) (the accession numbers of the genes are given in parentheses; see Table 2). Total RNA from untreated, 2-h-dried, 4-h-dried and 8-h-dried plants was blotted onto a membrane and hybridized with the respective cDNA probes (Fig. 3). The transcripts of all genes showed a marked induction after 2 h of dehydration treatment. The transcript of the *P. patens* homolog of *WPM-1* was not detectable in untreated tissues, whereas the homologs of *COR47*, *betA*, *OsCDPK7* and *COR TMC-AP3* showed a basal expression level in the untreated plants, which was markedly up-regulated in response to drought. Thus, the Northern blot experiments reproduce the general trend observed with the cDNA arrays. Many

Table 2 Quantification results of the expression profiling experiments

EST with homology to:	Function	Fold induction/repression			Accession number
		2 h of dehydration	4 h of dehydration	8 h of dehydration	
SCOF-1	Zinc finger transcription factor	#[a]	#	#	AJ566705
ABI3	Transcriptional activator	1.25	4.82	2.27	AJ566720
		1.40	9.65	1.99	AJ566739
ALA1	Aminophospholipid ATPase	#	#	#	AJ566710
		#	#	#	AJ566713
		#	#	#	AJ566732
DREB1A	AP2 domain transcription factor	#	#	#	AJ566734
		#	#	#	AJ566723
CBF1	AP2 domain transcription factor	#	#	#	AJ566704
DREB1C	AP2 domain transcription factor	#	#	#	AJ566727
DREB2A	AP2 domain transcription factor	#	#	#	AJ566716
		0.78	2.44	1.88	AJ566736
GS2	Glutamine synthetase	0.76	0.31	0.7	AJ566706
betA	Choline dehydrogenase	1.09	1.41	1.93	AJ566725
		0.63	1.99	2.17	**AJ566721**[b]
betB	Betaine aldehyde dehydrogenase	0.85	0.38	0.96	AJ566707
OsCDPK7	Ca^{2+}-dependent protein kinase	4.51	12.93	2.37	**AJ566711**
		2.24	2.49	1.40	AJ566722
DBF2	Kinase	#	#	#	AJ566700
		1.39	0.96	1.19	AJ566712
		1.16	0.7	1.03	AJ566701
ANP1	MAP kinase kinase kinase	#	#	#	AJ566730
		#	#	#	AJ566699
		1.34	0.99	1.12	AJ566738
AtMPK3	MAP kinase	1.91	0.61	0.83	AJ566729
		2.72	8.64	1.97	AJ566731
COR TMC-AP3	Chloroplastic amino acid-selective channel protein	15.67	11.67	15.73	**AJ566742**
		7.19	6.15	8.75	AJ566708
		18.40	10.19	11.24	AJ566743
AtProDH	Proline dehydrogenase	#	#	#	AJ566702
		#	#	#	AJ566733
Cryoprotective osmotin-like protein	Cryoprotective protein	#	#	#	AJ566726
		#	#	#	AJ566719
COR47	Cold- and dehydration-induced protein	191.73	157.56	154.34	AJ566728
		67.97	68.74	84.37	**AJ566740**
		27.40	30.46	53.26	AJ566740
HSP17.6	Small heat-shock protein	#	#	#	AJ566717
		#	#	#	AJ566718
		2.87	0.68	5.82	AJ566737
WPM-1	ABA-induced plasma membrane protein	6.45	8.94	9.42	AJ566724
		1	0.94	1.27	AJ566714
		53.95	93.99	100.27	**AJ566715**
OsP5CS	Delta1-pyrroline-5-carboxylate synthetase	2.53	2.88	1.19	AJ566703
SODA	Mn Superoxide dismutase	2.71	2.60	3.26	AJ566741
		#	#	#	AJ566709
		#	#	#	AJ566735

[a] # Signal intensity values that were not significantly above background
[b] cDNAs that were chosen for RNA gel blot analyses are in bold and underlined

genes that are specifically activated upon dehydration also show an up-regulation upon other abiotic stresses. To obtain information about cross-induction by salt and osmotic stress we analyzed the expression pattern of these genes also in response to 300 mM NaCl and 600 mM sorbitol. Furthermore, we applied 50 μM ABA to the cultures to determine if the genes are regulated in an ABA-dependent manner (Fig. 3). In the case of *PpCDPK7* we were not able to detect a transcript under the chosen conditions, which suggests an ABA-independent dehydration-specific regulation of this gene. In contrast, *PpCOR47* and *PpCOR TMC-AP3* were up-regulated by both salt and osmotic stress. These genes were also inducible by exogenous application of ABA, suggesting ABA-dependent stress-responsive overlapping signaling pathways. *PpbetA* and *PpWPM-1* showed a more defined expression pattern. The induction of *PpbetA* was limited to the 2-h ABA treatment and 8 h of NaCl treatment. In contrast to the induction of this gene upon dehydration the expression follows a defined temporal pattern. This is also the case for *PpWPM-1*, which was found to be up-regulated only upon 2 h of sorbitol treatment. These results indicate that all investigated genes are induced by dehydration,

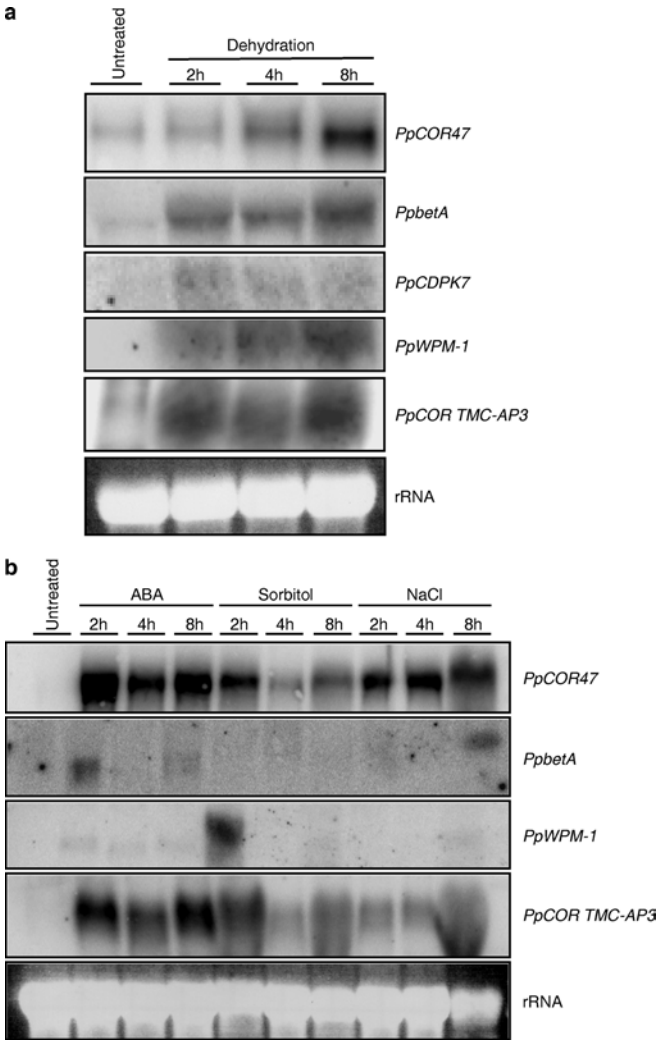

Fig. 3a, b RNA gel blot analysis of *P. patens* cDNAs. **a** 20-µg samples of total RNA derived from untreated and dehydrated plants were loaded on a 1% denaturing agarose gels and transferred to nylon membranes. The membranes were hybridized with the *P. patens* cDNA clones homologous to *COR47* (*PpCOR47*; AJ566740), *betA* (*PpbetA*; AJ566721), *OsCDPK7* (*PpCDPK7*; AJ566711), *WPM-1* (*PpWPM*-1; AJ566715) and *COR TMC-AP3* (*PpCOR TMC-AP3*; AJ566742). The *lowermost panel* shows the ethidium bromide-stained rRNA bands of one representative gel, indicating equal loading of the RNA samples. **b** 20-µg samples of total RNA derived from untreated plants and plants that had been treated with 300 mM NaCl, 600 mM sorbitol and 50 µM ABA were loaded on 1% denaturing agarose gels and transferred to nylon membranes. The membranes were hybridized with the *P. patens* cDNA clones homologous to *COR47* (*PpCOR47*; AJ566740), *betA* (*PpbetA*; AJ566721), *WPM-1* (*PpWPM*-1; AJ566715) and *COR TMC-AP3* (*PpCOR TMC-AP3*; AJ566742). The *lowermost panel* shows the ethidium bromide-stained rRNA bands of one representative gel, indicating equal loading of the RNA samples

as expected from the array data. However, the regulation of these genes in response to other abiotic factors and to the stress-mediating hormone ABA differs among the characterized genes. Whereas *PpCOR47* and *PpCOR TMC-AP3* are controlled by overlapping signaling pathways activated by various stress factors, *PpCDPK7*, *PpbetA* and *PpWPM-1* are subject to a more constricted regulation by pathways activated by a limited number of stresses.

Discussion

The moss *P. patens* has recently been chosen to study gene function by reverse genetics, which is facilitated by the high efficiency of homologous recombination in this species (Reski 1998; Schaefer 2001). The genes of interest are disrupted by transformation of *P. patens* protoplasts using a DNA construct containing a cDNA or genomic fragment that is interrupted by the insertion of a selectable marker gene. During homologous recombination the genomic locus is replaced by the knockout construct, resulting in a loss-of-function mutation of the respective gene. This powerful technique paves the way to study fundamental questions in plant biology. We intend to use *P. patens* to analyze the molecular mechanisms underlying its adaptation to environmental changes. For the first time we have defined the tolerance of *P. patens* plants to various abiotic stresses. Compared with other plants, *P. patens* is highly tolerant to salt, osmotic and dehydration stresses. For instance, NaCl concentrations above 100 mM result in severe impairment of *A. thaliana* plants (Sunkar et al. 2003). In contrast we have observed that *P. patens* plants are able to tolerate NaCl concentrations up to 350 mM. Benito and Rodriguez-Navarro (2003) have performed similar experiments in which *P. patens* plants have been slowly adapted to increasing salt concentrations. As a result of this slow adaptation they were able to grow *P. patens* at salt concentrations up to 600 mM. Compared to our studies the increased tolerance to salt stress that is observed upon slow adaptation to increasing salt concentrations suggests a process similar to the adaptation to low temperatures known as cold acclimation. The physiological analysis of *P. patens* after treatments with sorbitol also indicated a high degree of tolerance to osmotic stress. Plants were able to survive on medium containing 500 mM sorbitol. The analyses of the effect of dehydration stress also revealed remarkable tolerance of *P. patens* plants. Plants that had lost 92% of their fresh weight during the dehydration treatment were still able to recover upon rehydration. Based on these results, *P. patens* can be considered as a drought-tolerant species as defined by Hoekstra et al. (2001). These authors define drought tolerance as the tolerance to moderate dehydration. This corresponds to about 23% water on a fresh-weight basis where no bulk cytoplasmic water is present. Among bryophytes, only the moss *Tortula ruralis*, representing a so-called resurrection plant (Oliver et al. 1993), has been described as being even more tolerant to dehydration. *T. ruralis* can be considered as desiccation tolerant, because it is able to survive further dehydration.

As a first approach to identify genes involved in the stress response we chose expression profiling experi-

ments using cDNA macroarrays. The expression profiling identified a set of 19 genes that were specifically induced by dehydration. The set of induced genes includes two homologs of the *COR47* gene (also termed *Rd17*) from *A. thaliana* encoding a dehydrin protein that is up-regulated in response to cold and dehydration (Gilmour et al. 1992). We also observed a dehydration-induced expression of three homologs of the *cor tmc-ap3* gene from barley, which has been reported to be induced by cold (Baldi et al. 1999). This gene encodes a putative chloroplastic amino acid-selective channel protein. Two cDNAs with homology to the *WPM-1* gene, which was shown to be cold-induced in wheat (Koike et al. 1997), were highly induced by water deficit in *P. patens*. The protein contains four putative transmembrane-spanning regions. Even though the regulation of these classes of genes is well characterized, their biological function remains unclear. Targeted knockouts of these genes in *P. patens* could elucidate their participation in the adaptation to abiotic stress.

Apart from those *Physcomitrella* genes showing homology to genes from other plants whose function remains to be clarified, we have found genes that were shown to have fundamental roles in protecting the cell against the harmful conditions arising during exposure to various stresses. Two homologs of the *betA* genes from *E. coli* were found to be induced by the dehydration treatment. The encoded protein, choline dehydrogenase, oxidizes choline to betaine aldehyde, which is further oxidized to glycine betaine by the same enzyme. Glycine betaine is known to be a compatible solute displaying osmoprotective functions. Over-expression of this gene in tobacco resulted in enhanced tolerance against salt stress (Holmström et al. 2000). The transcript encoding another enzyme involved in the synthesis of an osmoprotective compound was also found to be up-regulated by drought stress in *P. patens*. The encoded protein shows homology to the delta1-pyrroline-5-carboxylate synthetase from rice, which is inducible by salt, dehydration and cold treatment, and is involved in the biosynthetic pathway of proline (Igarashi et al. 1997). A cDNA encoding a protein with homology to the superoxide dismutase SODA from *Nicotiana plumbaginifolia* (Bowler et al. 1989) also showed induction upon water stress in *P. patens*. Superoxide dismutase is involved in scavenging activated oxygen species, which have been implicated in a number of physiological disorders in plants, including abiotic stress. Over-expression of the *SODA* gene from *N. plumbaginifolia* in alfalfa led to an enhanced tolerance against freezing stress (McKersie et al. 1993).

Another group of cDNAs that have been identified to be up-regulated in *P. patens* in response to dehydration stress encode proteins involved in the signal transduction network. We have found cDNAs homologous to different classes of plant protein kinases. Particularly, transcripts coding for proteins homologous to the calcium-dependent protein kinase OsCDPK7 from rice (Saijo et al. 2000) and the MAP kinase AtMPK3 (Mizoguchi et al. 1993) from *A. thaliana* were found to be inducible in *P. patens* upon water deficit. Both genes can be transcriptionally induced by a variety of different stimuli. *AtMPK3* is induced by touch, cold and salinity (Mizoguchi et al. 1996), whereas *OsCDPK7* is responsive to salt and cold stress. Upon over-expression in transgenic rice plants, *OsCDPK7* was shown to be a positive regulator commonly involved in the tolerance to both stresses.

The characterization of the expression pattern of a set of five *Physcomitrella* genes upon different treatments and exogenous application of ABA suggests the existence of specific signaling pathways that are either specific to a particular stress or represent overlapping signaling networks activated by different abiotic stress factors. We have recently investigated two stress-inducible genes from *Physcomitrella* homologous to the *Arabidopsis RCI2A* and *RCI2B* genes with respect to their response to abiotic stress, suggesting that at least some stress-related signaling pathways might have been changed during land-plant evolution (Kroemer et al. 2004).

Our studies on the abiotic stress tolerance of *P. patens* have shown that this land plant is able to cope with severe salt, osmotic and dehydration stress. The high degree of tolerance found in *P. patens* makes it a valuable source for characterization of genes involved in the stress adaptation response. The *P. patens* EST database search and the expression data obtained by the macroarray experiments clearly show that common mechanisms to counteract the impairment of cellular functions caused by exposure to abiotic stress have been evolutionarily conserved. Even though the branches of bryophytes and seed plants separated 450 million years ago, they share basic mechanisms of the abiotic stress response. Thus, the molecular mechanisms for stress tolerance of the moss could be promising targets to improve the stress tolerance of crop plants. The analysis of the *P. patens* transcriptome has shown that roughly 24% of the genes cannot be annotated to date (Rensing et al. 2002). The analysis of their expression pattern in response to abiotic stress may lead to the identification of novel stress-related genes in plants. Although these genes are not found in higher plants, they may be valuable targets for crop plant improvement.

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