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Microarray analysis of the moss *Physcomitrella patens* reveals evolutionarily conserved transcriptional regulation of salt stress and abscisic acid signalling

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Abstract Regulatory networks of salt stress and abscisic acid (ABA) responses have previously been analyzed in seed plants. Here, we report microarray expression profiles of 439 genes encoding transcription-associated proteins (TAPs) in response to salt stress and ABA in the salt-tolerant moss *Physcomitrella patens*. Fourteen and 56 TAP genes were differentially expressed within 60 min of NaCl and ABA treatment, respectively, indicating that these responses are regulated at the transcriptional level. Overlapping expression profiles, as well as the up-regulation of ABA biosynthesis genes, suggest that ABA mediates the salt stress responses in *P. patens*. Comparison to public gene expression data of *Arabidopsis thaliana* and phylogenetic analyses suggest that the role of DREB-like, Dof, and bHLH TAPs in salt stress responses have been conserved during embryophyte evolution, and that the function of ABI3-like, bZIP, HAP3, and CO-like TAPs in seed development and flowering emerged from pre-existing ABA and light signalling pathways.

Keywords Salt tolerance · Transcription factors · NCED · *Physcomitrella patens* · Abscisic acid · Gene expression profiling

Abbreviations

ABA	Abscisic acid
LCA	Last common ancestor
MYA	Million years ago
NCED	9-*cis*-epoxycarotenoid dioxygenase

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Introduction

Soil salinization constitutes a severe and increasing constraint that impairs crop production worldwide and has led to attempts to understand the genetic basis of salt stress responses and adaptive mechanisms in seed plants. Microarray analyses of salt-stressed *Arabidopsis thaliana* and rice plants identified hundreds of stress-responsive genes (Kawasaki et al. 2001; Kreps et al. 2002; Rabbani et al. 2003; Seki et al. 2002; Takahashi et al. 2004). Their gene products can be divided into two major groups: (1) effectors of cellular stress tolerance, and (2) regulatory molecules such as protein kinases, phosphatases, and transcriptional regulators. It has been hypothesized that a common set of genes forms the basis of salt stress responses in both salt-sensitive (glycophytes) and salt-tolerant (halophytes) plants, and that their differential regulation accounts for varying degrees of salt tolerance (Zhu 2001). Consistently, independent studies revealed constitutively elevated transcript levels of salt stress-associated genes under normal growth conditions in the halophyte *Thellungiella halophila* whereas homologs of these genes were found to be specifically up-regulated in response to salt stress in its glycophytic relative *A. thaliana* (Gong et al. 2005; Taji et al. 2004; Wong et al. 2006). The molecular mechanisms conferring salt tolerance in *T. halophila* include maintenance of cellular ion and osmotic homeostasis under high salt conditions by the control of sodium uptake and synthesis of osmoprotective substances (Inan et al. 2004; Taji et al. 2004; Volkov et al. 2004). Key enzymes and ion transporters involved in these tolerance mechanisms are differentially regulated in *T. halophila* as compared to *A. thaliana* (Kant et al. 2006).

The regulatory networks controlling abiotic stress responses have been intensively studied in *A. thaliana* (Chen and Zhu 2004; Yamaguchi-Shinozaki and Shinozaki 2006). Large-scale expression profiling of transcription factor (TF) genes showed that regulators of stress response pathways are differentially expressed in response to various abiotic stress conditions in *A. thaliana* (Chen et al. 2002). Key regulators of osmotic stress response pathways include members of the AP2/EREBP, bZIP, NAC, bHLH, MYB, HD-Zip and HD-ZF gene families (Yamaguchi-Shinozaki and Shinozaki 2006). The fundamental role of particular TFs in the abiotic stress response was substantiated by overexpression studies of single stress-responsive TFs in seed plants which caused elevated expression levels of down-stream target genes and enhanced tolerance to various abiotic stresses (Chen et al. 2008; Dubouzet et al. 2003; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Kim et al. 2004; Park et al. 2001).

The moss *Physcomitrella patens* emerged as a salt-tolerant model that is amenable to genetic analysis. *P. patens* is highly tolerant to salt, drought and osmotic stress and is capable of a tight control of ion and osmotic homeostasis (Benito and Rodriguez-Navarro 2003; Frank et al. 2005b; Lunde et al. 2007; Wang et al. 2008). A first large-scale microarray analysis of *P. patens* ESTs identified eight genes that are differentially expressed 2 h after NaCl application (Cuming et al. 2007). Furthermore, salt-induced genes have been identified in *P. patens* encoding regulatory proteins such as calmodulin binding proteins (Takezawa and Minami 2004), a Ca^{2+}-ATPase (Qudeimat et al. 2008), and the AP2/EREBP transcription factor PpDBF1 (Liu et al. 2007). These findings indicate that salt stress responses might be regulated at the transcriptional level in *P. patens*. However, substantial knowledge on salt stress response pathways in this salt-tolerant moss remains elusive.

Like desiccation tolerance, salt tolerance emerged independently in phylogenetically distant plant species. Nevertheless, the knowledge on the common mechanisms from which salt tolerance emerged is scarce compared to the knowledge on mechanisms underlying desiccation tolerance (Oliver et al. 2000). *P. patens* holds a phylogenetic key position as the bryophyte lineage diverged early in land plant evolution from the ancestor of extant seed plants (Zimmer et al. 2007). During ~450 MYA of divergent evolution extant mosses and seed plants developed different strategies for survival, the dominant gametophytic and sporophytic generation, respectively (reviewed in Lang et al. 2008). Paralleling the morphological evolution, loss and gain of functions occurred in signalling and regulatory pathways in these lineages (e.g. Veron et al. 2007). Nevertheless, previous works identified TAPs with similar functions in tissues of the *P. patens* gametophyte and *A. thaliana* sporophyte as well, such as MADS $MIKC^C$ TFs involved in the generation of reproductive organs (Singer et al. 2007) and the bHLH TFs AtRHD6 and PpRSL1 controlling root hair and rhizoid development, respectively (Menand et al. 2007). The comparative analysis of TAP gene expression profiles in response to salt stress between *P. patens* and *A. thaliana* would allow to study the evolution of salt stress response pathways in embryophytes and to infer ancestral TAP functions.

In seed plants, high salinity and drought cause an increase in intracellular levels of the phytohormone ABA (Zhu 2002). Rising ABA concentrations rely on enhanced ABA biosynthesis, diminished ABA degradation and mobilization of ABA from ABA-glucose-esters (Lee et al. 2006; Zhu 2002). Studies on ABA-deficient *A. thaliana* mutants led to the identification of abiotic stress signalling pathways, some of which are dependent on ABA, while others function independently of ABA (Yamaguchi-Shinozaki and Shinozaki 2006). Furthermore, several aspects of plant growth and development are regulated by ABA, e.g. the acquisition of desiccation tolerance during seed maturation (Finkelstein

et al. 2002). In mosses, ABA and drought induce the differentiation of protonema cells into brachycytes (brood cells) which represent vegetative propagules for survival under adverse environmental conditions (Decker et al. 2006; Goode et al. 1993; Schnepf and Reinhard 1997; Tintelnot 2006). ABA is also involved in abiotic stress adaptation of *P. patens*, since exogenously applied ABA increases freezing tolerance (Minami et al. 2003) and increased endogenous ABA levels were detected upon osmotic stress treatment (Minami et al. 2005). While the transcriptional regulation of ABA response pathways has been intensively studied in seed plants (Finkelstein et al. 2002), the knowledge on ABA-mediated gene expression in *P. patens* is restricted to the conserved ABA-mediated activation of ABA-responsive (ABRE) *cis*-elements (Kamisugi and Cuming 2005; Knight et al. 1995; Qudeimat et al. 2008).

Here, we report on the first gene expression analysis focusing on *P. patens* TAPs, comprising transcription factors (TFs) and other transcriptional regulators (TRs) (Richardt et al. 2007). To characterize the transcriptional regulation of salt stress and ABA responses and to identify genes that might be involved in this regulation we monitored early expression changes of 439 *P. patens* TAP genes upon NaCl and ABA treatment, respectively. To investigate the molecular mechanism underlying the osmotic stress-induced increase of ABA (Minami et al. 2005), we furthermore analyzed the expression of genes encoding key enzymes of ABA biosynthesis. Publicly available gene expression data of *A. thaliana* allowed us to subsequently compare the generated gene expression profiles of *P. patens* to salt stress- and ABA-responsive expression of TAP genes in a seed plant to elucidate components of regulatory pathways that evolved during land plant radiation.

Materials and methods

Plant materials and stress treatments

The cultivation of *P. patens* was performed in liquid mineral medium [250 mg l^{-1} KH_2PO_4, 250 mg l^{-1} $MgSO_4 \times 7H_2O$, 250 mg l^{-1} KCl, 1000 mg l^{-1} $Ca(NO_3)_2 \times 4H_2O$, 12.5 mg l^{-1} $FeSO_4 \times 7H_2O$, pH 5.8 with KOH] (Reski and Abel 1985) as described previously (Frank et al. 2005a). Freshly disrupted protonemata were sub-cultured for 5 days in a 10 L bioreactor (0.3 vvm aeration, 500 rpm, pH 7.0) (Hohe and Reski 2002) to a dry weight of 150 g/ml, transferred to fresh medium and cultured over night in Erlenmeyer flasks on shakers at 125 rpm. For NaCl and ABA treatments the medium was supplemented with 250 mM NaCl or 10 μM (±)-*cis-trans* ABA, respectively, 5 h after the onset of the light phase. Untreated control samples were adjusted to 100 μM KOH which served as ABA solvent. After 30 and 60 min the moss material of three flasks per treatment was harvested, pooled and frozen in liquid nitrogen. All treatments were performed in three independent biological replicates. Images of *P. patens* protonemata before and after the treatments (Supplementary Table S1) were taken using a Zeiss Axioplan 2 microscope with an Axiocam MRc5.

RNA extraction and microarray hybridization

Total RNA was isolated (Trizol; Invitrogen Corp., Carlsbad, USA and RNeasy Mini Kit; QIAGEN, Hilden, Germany) and 4 μg were used for linear amplification (ampULSe; Kreatech, Amsterdam, The Netherlands). Four μg of copy RNA were Cy5-labelled using the ULS technology (ampULSe) and hybridized to 12 K custom oligonucleotide microarrays (Combimatrix Corp.; Mukilteo, WA, USA). Prehybridization, hybridization, imaging and stripping of microarrays were performed according to the manufacturer's instructions with four hybridization-stripping rounds per array. Cy5 fluorescence intensities were collected at 5 μm resolution (GenePix 4200A; Molecular Devices, Ismaning/München, Germany) and median pixel intensities of the spots were quantified (Microarray Imager v5.7.1; Combimatrix Corp.). Defective spots were excluded manually based on deviating mean and median pixel intensities.

Microarray design

Using the Combimatrix probe design suite, at least six independent 35-mer oligonucleotide probes were designed for 1,427 *P. patens* virtual transcript sequences (Lang et al. 2005; Rensing et al. 2005) encoding putative TAPs, as well as for several internal control genes (Combimatrix design no. 3959). The sequences represent the previously reported seed set of the PlanTAPDB resource (Richardt et al. 2007). Prior to probe design, redundant sequences were removed based on pairwise alignments (>90% identity across 90% length). Upon availability of the *P. patens* genome sequence, the assembled EST sequences were mapped to gene models v1.1 (Rensing et al. 2008) by spliced alignments (GenomeThreader v1.0.0, 90% identity, 85% coverage of the assembled transcript) (Gremme et al. 2005). Corresponding gene models could be assigned for 1,200 assembled transcripts. Among those, 16 mapped to tandemly arrayed genes or homologous gene models of high identity and, thus, their accessions were concatenated to represent a single probe target (e.g. "Phypa_108139–Phypa_92627", Supplementary Table S2). Another 15 assembled transcripts mapped to multiple, directly adjacent and hence probably partial gene models, from which the longest gene model was selected as representative. Furthermore, the microarray probes were filtered for a single specific match in the *P. patens* genome sequence v1.1 (BLASTN, 97% identity, max. 2 mismatches)

(Altschul et al. 1997). Twenty-one percent of the probes were excluded due to multiple or missing matches in the genome resulting in 1,165 probe sets of four to seven probes.

Analysis of gene expression data

Gene expression data were processed and analyzed using Expressionist Analyst Pro v4.5 (Genedata, Basel, Switzerland). Background subtraction was carried out using the average of the lowest 5% of spot fluorescence intensities. To remove non-linearities in log-log plots of different arrays expression data of all arrays were subjected to Lowess normalization ($f = 0.1$) (Cleveland 1979) to a common baseline array (average fluorescence intensity of all arrays) based on least-invariant sets of probes. The latter were defined by rank difference thresholds selecting approximately 10% of the probes per array. For each gene summarized expression estimates were calculated by the condensation of probe sets applying Median Polish with default settings (Irizarry et al. 2003). As suggested by Choe et al. (2005) and Pearson (2008), the summarized expression estimates were once more subjected to the Lowess normalization (see above). Differential expression of genes was assessed using the regularized Bayesian unpaired *t*-test CyberT (Baldi and Long 2001) and correction of *P*-values for multiple testing by calculating the false discovery rate (Benjamini and Hochberg 1995). Genes with *q*-values ≤ 0.05 and a minimum twofold change of expression were considered to be differentially expressed. Fold changes were calculated from the average expression values detected in treated and control samples. Significant differences of selected populations of differentially regulated TAP genes between *P. patens* and *A. thaliana* were assessed using Fisher's exact test.

For the expression analysis of AtGenExpress data, Affymetrix CEL files were downloaded from The *Arabidopsis* Information Resource (TAIR) (accession numbers: ABA, ME00333; salt, ME00328; http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp). Normalized expression data were obtained using gcRMA with default settings (Wu et al. 2004) and subjected to statistical analyses as described above. To compare results from ABA-treated seedlings to those from salt-stressed roots and shoots, the latter two were combined according to Ma et al. (2006). In this case, only genes with induced or repressed expression in both samples were subjected to further analysis (for separate expression data of roots and shoots see Supplementary Table S3). All conditions are represented by two biological replicates. Differential expression was analyzed as described above.

Annotation of TAPs

A reliable prediction of TAPs is based on the identification of particular protein domains present in their amino acid sequences (Qian et al. 2006). Previously reported TF classifications (Guo et al. 2008; Riano-Pachon et al. 2007) as well as consensus domain compositions of PlanTAPDB TF and TR families (Richardt et al. 2007) were manually curated and TAP family classification rules deduced. To obtain comparable annotations of *P. patens* and *A. thaliana* TAP microarray features, the classification rules were applied to the complete sets of proteins predicted in the *P. patens* and *A. thaliana* genome (Phypa v1.1, TAIR v7) based on TAP protein domain patterns detected via HMMER searches (E $\leq$ 10E-4) (http://hmmer.wustl.edu/).

Out of 1,506 predicted *P. patens* TAP genes, 439 are represented by probe sets on the microarray (Supplementary Table S2). In total, the *P. patens* TAP genes belong to 89 TAP families, the majority of which (74) is represented on the microarray, covering 35% of their members on average. The Affymetrix ATH1 microarray harbours 1,911 of the 2,290 predicted *A. thaliana* TAP genes (Supplementary Table S3) and includes members of all 95 classified TAP families with an average coverage of 86.5%.

RNA gel blot and semi-quantitative RT-PCR

For validation of microarray expression profiles and expression analysis of 9-*cis*-epoxycarotenoid dioxygenase (NCED) genes, protonemata were grown and treated as described above. For the dehydration treatment the liquid medium was removed and protonemata were exposed to air in a Petri dish. Samples were taken after 1 h for the validation experiments and after 1, 2, 3, and 6 hours for the NCED expression analysis. Semi-quantitative RT-PCR analysis from three independent biological replicates was performed as previously described (Martin et al. 2009) using gene-specific primers for *Phypa_163123* (forward, GCAATGTGGGAAATTCTTGG; reverse, GATCCGA AGCTTGTCATTCC), *Phypa_168363* (forward, GTTACA GCTGTTTCCTCAGAATGC; reverse, GGAAAATCTCT CGCCAAGCTAT), *Phypa_178653* (forward, TCCAAA GAGGTGTTGGATGC; reverse, AACCTTCCGGAGAT CAGACC), *Phypa_28324* (forward, ACGAAGAGCAC TCCGAACATATC; reverse, AATGGCAGCTAGAGCG TATGAT), and *Phypa_29828* (forward, GGAATGCTTC ATAGAGGAAGTACG; reverse, GCGCTCAAGATTCCT AGTAAGTTC). NCED and *Phypa_135214* EST sequences were amplified from the vector backbone using M13 forward and reverse primers for hybridization of RNA gel blots as previously described (Frank et al. 2005b).

Identification of NCEDs and phylogenetic analyses

P. patens NCED EST sequences were identified by TBLASTN searches in the EST database pp03/04 (www.cosmoss.org) using AtNCED1-6 and 9 as queries. Upon

availability of the *P. patens* genome sequence (Rensing et al. 2008), NCED gene models were identified by BLASTP, mapped to the *P. patens* EST NCED sequences and used for phylogeny inference.

For phylogenetic analysis of plants NCEDs as well as four TAP families, protein sequences were aligned using MAFFT linsi (Katoh et al. 2005). The alignments were manually curated using JalView (Clamp et al. 2004) to cover conserved regions including the protein domain(s) underlying the initial characterization of the TAP gene families (Supplementary Fig. S4). Phylogenetic trees were inferred using different methods. Bayesian inference (BI) was carried out using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) with eight gamma-distributed rate categories (four chains, two runs). Trees were visualized using FigTree v1.1.2 (http://tree.bio.ed.ac.uk/software/figtree/). Support values shown at the nodes represent BI posterior probabilities.

Results and discussion

Response of *P. patens* to NaCl and ABA

In this study we describe the analysis of TAP gene expression in *P. patens* protonemata that were exposed to 250 mM NaCl or 10 μM ABA for 30 and 60 min, respectively. Molecular and physiological responses under these conditions have been previously characterized and it was shown that *P. patens* protonemata and gametophores are able to survive exposure to 500 mM NaCl (Frank et al. 2005b; Saavedra et al. 2006). Altered expression of *P. patens* genes has previously been reported after treatment with 250 mM NaCl for 60 min (Frank et al. 2007; Qudeimat et al. 2008), yet earlier responding genes remained unknown. We found that a treatment with 250 mM NaCl for 30 min is sufficient to induce cellular plasmolysis (Supplementary Fig. S1) and thus might trigger cellular adaptation responses. Cultivation of protonema in liquid medium supplemented with 100 μM ABA results in the formation of brachycytes and tmema cells, showing that ABA under these conditions invokes morphological changes (Decker et al. 2006; Tintelnot 2006). It is also known that exogenous application of ABA at a concentration of 10 μM confers ABA-inducible gene expression in *P. patens* protonema within 15–60 min (Cuming et al. 2007; Minami et al. 2003). We therefore applied ABA at a concentration of 10 μM to study ABA-dependent expression of TAP genes in *P. patens*. As expected, ABA had no effect on the cell morphology after 30 min and 60 min of the treatment (Supplementary Fig. S1).

Salt stress- and ABA-responsive expression of TAP genes in *P. patens*

In order to identify *P. patens* TAP genes that may play a role as early regulators in salt stress responses and ABA signalling, we performed microarray expression profiling of protonemata (consisting of chloronema and caulonema cells) exposed to elevated salt concentrations (250 mM NaCl) or the phytohormone ABA (10 μM), respectively. This approach has been proven valid in many previous studies (see "Introduction"). However, differentially expressed genes might be involved in both specific regulatory pathways and more global responses of the plant cell.

Among 439 *P. patens* TAP genes represented on the TAP microarray, statistical analysis of normalized expression levels compared to those of untreated control samples led to the identification of 14 and 56 TAP genes displaying a differential expression within 60 min of exposure to high salinity and ABA, respectively (Fig. 1a). Although only a fraction of the *P. patens* TAP genes is represented on the microarray, we detected rapid induction of TAP genes by salt stress and/or ABA belonging to TAP families of *A. thaliana* key regulators (e.g. AP2/EREBP, bHLH, bZIP, HD-Zip class I, and NAC) (Table 1) (Yamaguchi-Shinozaki and Shinozaki 2006). The highest rates of induction by salt stress and/or ABA (> 5-fold) were observed for genes belonging to the AP2/EREBP, bZIP, ABI3/VP1, bHLH, and C2C2-Dof TAP families (Table 1). Members of these gene families have previously been associated with salt stress responses or ABA-mediated gene regulation in seed plants (Parcy et al. 1994; Yamaguchi-Shinozaki and Shinozaki 2006; Yanagisawa 2004).

These results already suggest that components of the transcriptional networks controlling salt stress and ABA responses might have been evolutionarily conserved since the divergence of the bryophyte and vascular plant lineages. Even though significant changes in the physiological response of *P. patens* such as photosynthesis rates and the formation of reactive oxygen species have not been observed after 12 h of treatment with elevated salt concentrations (Frank et al. 2007; Qudeimat et al. 2008), we cannot exclude that changes in gene expression may result from secondary effects. High salt exposure probably constitutes a stringent condition resulting in cellular injury, which might induce additional cellular responses known from salt-treated seed plants such as repair, detoxification and growth control (Zhu 2001). Nevertheless, we expect transcriptional changes of TAP genes in response to high salt concentrations which are directly associated with salt-responsive signalling pathways after perception of the stress signal. Furthermore, exogenous application of ABA may lead to artificially increased endogenous ABA levels triggering, besides endogenous signalling cascades, unspecific responses.

Our results demonstrate that in *P. patens*, as known from seed plants (Yamaguchi-Shinozaki and Shinozaki 2006), TAP genes are transcriptionally regulated within 1 h in

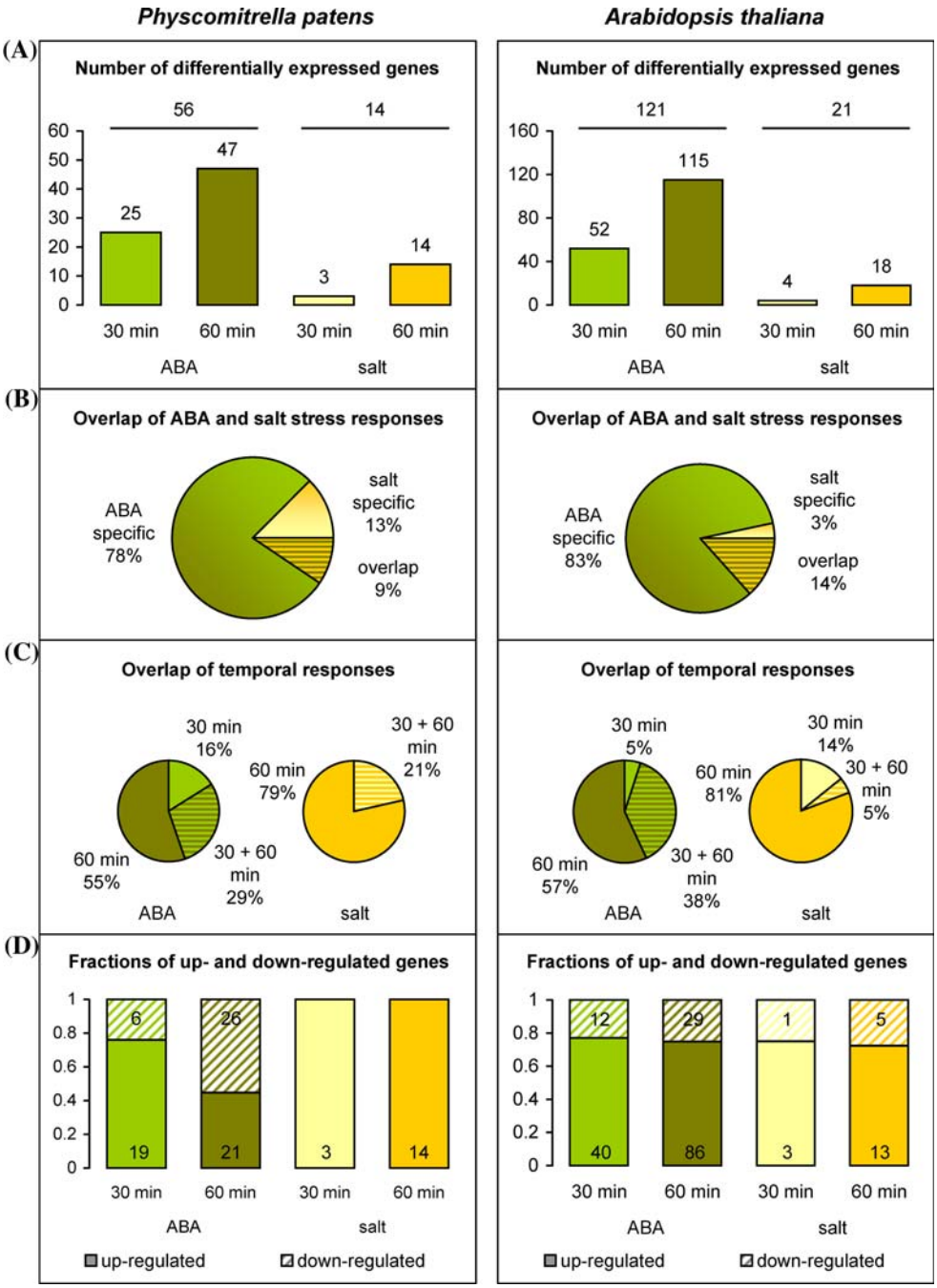

Fig. 1 Global patterns of early ABA- and salt stress-responsive TAP gene expression in *P. patens* and *A. thaliana*. TAP genes differentially expressed in *P. patens* protonemata and *A. thaliana* seedlings, roots, and shoots in response to ABA and high salinity were identified using CyberT and FDR correction of *P*-values ($q < 0.05$); only genes with a minimum twofold change were considered. Results from *A. thaliana* root and shoot salt stress datasets were combined according to Ma et al. (2006). **a** Absolute numbers of differentially expressed TAP genes. **b** Fractions of overlapping and stimulus-specific responses. **c** Temporal patterns of transient (after 30 min only), constitutive (after both 30 and 60 min) and delayed (after 60 min only) responses. **d** Relative proportions of up- and down-regulated genes

response to salt stress and ABA. The differentially regulated TAP genes almost exclusively belong to TF, rather than TR, gene families (Supplementary Table S5), suggesting that NaCl- and ABA-responsive expression of effector genes is controlled via transcriptional regulation of DNA-binding TFs. The identified 64 differentially expressed TAPs extend the list of water-stress associated *P. patens* genes from a previous microarray analysis (Cuming et al. 2007) by covering earlier time points and thus additional putative signalling responses. This substantially enlarges our knowledge of the transcriptional regulation of salt stress and ABA responses in the salt-tolerant moss and serves as a starting point for further functional analyses.

Validation of *P. patens* expression profiles

Besides *P. patens* TAP genes, we included eleven control genes in the expression profiling analysis (Supplementary Table S2), which were previously shown to be induced by abiotic stress and ABA (W. Frank, unpublished results) (Kroemer et al. 2004). The array data confirmed the

Table 1 *P. patens* differentially expressed TAP genes

Gene model v1.1	TAP family classification	Gene name	Public accession number(s)	Description	Estimated fold change			
					ABA 30 min	ABA 60 min	NaCl 30 min	NaCl 60 min
Specifically salt-induced TAPs								
Phypa_27022	AP2/EREBP				–	–	2.1	4.9
Phypa_29828	AP2/EREBP	PpACP1[a]	CF811725	(prt279 SSH cDNA)	–	–	–	2.5
Phypa_47830	AP2/EREBP				–	–	–	2.2
Phypa_160018	AP2/EREBP				–	–	–	2.1
Phypa_173329	tify				–	–	–	2.7
Phypa_176040	AP2/EREBP				–	–	–	2.2
Phypa_195459	AP2/EREBP				–	–	–	5.4
Phypa_229640	bHLH				–	–	–	5.2
Salt- and ABA-induced TAPs								
Phypa_28324	AP2/EREBP				52.6	33.0	2.2	10.8
Phypa_49506	AP2/EREBP				4.2	2.3	–	2.1
Phypa_126548	AP2/EREBP				6.1	3.5	6.9	15.7
Phypa_153324	C2C2-Dof	PpDof1[b]			2.3	–	–	2.4
Phypa_168363	ABI3/VP1	PpABI3B[c]	BAE80315	Transcription factor ABI3-like protein	10.2	8.6	–	2.8
Phypa_172697	AP2/EREBP				4.7	–	–	2.9
Specifically ABA-induced TAPs								
Phypa_27666	CCAAT-HAP3	PpHAP3F[d]		CCAAT-box binding factor HAP3-like protein	–	3.3	–	–
Phypa_29817	bZIP				–	2.4	–	–
Phypa_38844	C2C2-Dof	PpDof11[b]			3.5	6.0	–	–
Phypa_39201	ABI3/VP1				–	2.1	–	–
Phypa_49171	bHLH				4.4	5.0	–	–
Phypa_59557	GRAS	PpGAL2/ DELLAL1[e]/ DELLAa[f]	XP_001774314, ABU63413	GAI1-like protein 2, DELLA-like protein	–	2.6	–	–
Phypa_87648	MYB-related				2.1	–	–	–
Phypa_90560	MYB-related				–	2.2	–	–
Phypa_112295	SNF2				–	2.1	–	–
Phypa_113756	AP2/EREBP				2.6	2.2	–	–
Phypa_117713	AP2/EREBP				2.4	–	–	–
Phypa_121441	C2C2-CO-like				–	2.0	–	–
Phypa_135214	C2C2-Dof	PpDof10[b]			2.1	2.3	–	–
Phypa_140222	NAC				2.1	2.3	–	–
Phypa_141045	Dicer and Argonaute	PpAGO1c	XP_001774237	Argonaute family member	–	3.3	–	–
Phypa_142112	AP2/EREBP				2.0	–	–	–
Phypa_158812	ABI3/VP1	PpABI3A[c]	BAE80314	Transcription factor ABI3-like protein	2.5	2.6	–	–
Phypa_160508	bZIP				5.0	5.2	–	–
Phypa_226476	C2C2-CO-like	PpCOL3[g]	CAI64585	CONSTANS-like 3 protein	2.4	2.2	–	–
Phypa_233854	C2C2-CO-like				2.0	2.1	–	–
Phypa_234989	HD-Zip	PpHB6[h]	BAA93465	Class I HD-Zip protein PpHB6	2.6	–	–	–
Specifically ABA-repressed TAPs								
Phypa_29819	AP2/EREBP				–	−2.5	–	–
Phypa_48206	bHLH				–	−2.2	–	–

Table 1 continued

Gene model v1.1	TAP family classification	Gene name	Public accession number(s)	Description	Estimated fold change			
					ABA 30 min	ABA 60 min	NaCl 30 min	NaCl 60 min
Phypa_49174	bHLH				–	−2.9	–	–
Phypa_71211	HD-Zip	PpHB4[h]	BAA93463	Class I HD-Zip protein PpHB4	−2.3	–	–	–
Phypa_82320	PLATZ				–	−2.2	–	–
Phypa_104526	MADS	PPM6	XP_001753230, CAI39204	MIKC* MADS-domain protein PPM6	–	−2.2	–	–
Phypa_114045	MYB				–	−2.1	–	–
Phypa_116740	GRAS				−2.3	−4.5	–	–
Phypa_120059	GRAS				–	−2.1	–	–
Phypa_127355	HD-Zip				–	−2.1	–	–
Phypa_130556	bHLH				–	−2.4	–	–
Phypa_135927	RB				–	−2.1	–	–
Phypa_144279	GRAS				–	−2.4	–	–
Phypa_148211	MADS	PPMA12	XP_001780172	MIKC* MADS-domain protein PPMA12	–	−2.5	–	–
Phypa_163821	AP2/EREBP				–	−3.5	–	–
Phypa_166576	AP2/EREBP				−2.0	–	–	–
Phypa_167487	bHLH	PpRSL1[i]	ABO84930	Rhd6-like 1 protein	–	−2.0	–	–
Phypa_169585	bHLH				–	−3.7	–	–
Phypa_172669	bHLH				–	−2.8	–	–
Phypa_173530	AP2/EREBP				−2.4	–	–	–
Phypa_174493	HD-Zip	PpHB7[h]	BAC54164	Class I HD-Zip protein PpHB7	–	−2.3	–	–
Phypa_176868	GRAS				−2.7	−3.5	–	–
Phypa_186168	MADS	PPMA10	XP_001767102	MIKC MADS-domain protein PPMA10	–	−2.6	–	–
Phypa_189022	AP2/EREBP				−5.2	−2.5	–	–
Phypa_189179	GRAS				–	−2.5	–	–
Phypa_196844	AP2/EREBP				–	−3.3	–	–
Phypa_209063	bHLH				–	−2.4	–	–
Phypa_217835	TRAF				–	−2.2	–	–
Phypa_233986	G2-like				–	−3.6	–	–

Differential expression of *P. patens* TAP genes after 10 μM ABA and 250 mM salt treatment was determined via statistical testing using the CyberT (Baldi and Long 2001). *P*-values were corrected for multiple testing by calculating the false discovery rate (Benjamini and Hochberg 1995). Significant changes in transcript abundance are shown (FDR q-value < 0.05, estimated fold change > 2). TAP family classification is based on the presence of protein domain patterns (see "Materials and methods"). Literature describing the phylogenetic and/or functional characterization of individual genes is cited next to the gene name

[a] Cho et al. (2007)

[b] Shigyo et al. (2007)

[c] Marella et al. (2006)

[d] Xie et al. (2008)

[e] Hirano et al. (2007)

[f] Yasumura et al. (2007)

[g] Zobell et al. (2005)

[h] Sakakibara et al. (2001)

[i] Menand et al. (2007)

induction of all control genes in response to ABA and NaCl, since we detected increased transcript levels within 60 min after the onset of the treatment. Furthermore, we validated the induction of four identified salt- and/or ABA-responsive TAP genes in *P. patens* by semi-quantitative RT-PCR or RNA gel blot (Fig. 2) confirming induction rates as low as 2.1-fold. One of these genes (*Phypa_28324*) encoding an AP2/EREBP TF has also been found to be induced upon drought stress in a previous microarray study (Cuming et al. 2007). In addition, we also confirmed the constitutive expression of two non-induced TAP genes by semi-quantitative RT-PCR (Fig. 2). Taken together, the internal controls as well as the independent validation of TAP gene expression substantiate the reliability of the *P. patens* microarray expression data and indicate a high sensitivity for the detection of changes in mRNA levels.

Comparison of salt stress- and ABA-induced TAP transcript changes between *P. patens* and *A. thaliana*

Regulators of salt stress and ABA-responses are well studied in seed plants (Finkelstein et al. 2002; Yamaguchi-Shinozaki and Shinozaki 2006) and comprehensive gene expression data are available. To compare salt stress- and ABA-responsive TAP gene expression in the moss *P. patens* and a seed plant, we analyzed gene expression data of *A. thaliana* in comparison to the generated *P. patens* expression profiles. We used datasets of the AtGenExpress gene expression atlas (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp) that derive from *A. thaliana* seedlings treated with 150 mM NaCl and 10 μM ABA for 30 and 60 min, respectively. These datasets were previously used for the analysis of salt stress- and ABA-responsive gene expression (Denby and Gehring 2005; Ma et al. 2006; Nemhauser et al. 2006).

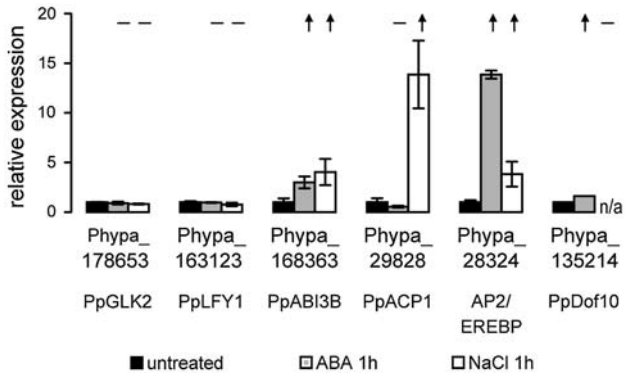

Fig. 2 Validation of microarray results. Expression profiles of two TAP genes with constitutive expression and four differentially expressed TAP genes were confirmed by semi-quantitative RT-PCR (*PpGLK2* (Yasumura et al. 2005), *PpLFY1* (Tanahashi et al. 2005), *PpABI3B* (Marella et al. 2006), *PpACP1* (Cho et al. 2007), *Phypa_28324*) or RNA gel blot (*PpDof10* (Shigyo et al. 2007)). Expression changes in protonemata treated for 60 min with 250 mM salt and 10 μM ABA, respectively, are shown. Expression of *Phypa_135214* upon high salinity was not tested, as the microarray results indicated no differential expression. *Bar* charts show relative expression levels normalized against the constitutive expression of the control gene encoding ribosomal protein L21 (*PpRPL21*). Relative expression under untreated conditions was set to one. *Error bars* show the 2x standard error of three independent replications of RT-PCRs. *Gene names* or TAP family classification are indicated beneath the gene model names. The *arrows* and *hyphen* above the plot indicate whether differential expression was detected within the microarray expression data and which of the conditions resulted in larger expression changes of the respective transcript (Supplementary Table S2)

The statistical analysis of 1,911 annotated *A. thaliana* TAPs present on the Affymetrix ATH1 microarray identified 21 and 121 genes responsive to high salinity and ABA, respectively (Fig. 1a). Among these, our analysis confirmed the induction of well known NaCl- and ABA-responsive marker genes encoding AtDREB1A-C, AtDREB2A, AtABF3, AtMYC2, AZF2, ATHB6, ATAF1, ANAC019, and ANAC072/RD26 (Lu et al. 2007; Sakamoto et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2006) in *A. thaliana* seedlings (Supplementary Table S6). Furthermore, 86% of the ABA-regulated TAP genes were also identified in a previous analysis of the AtGenExpress 30, 60 and 180 min ABA datasets (Nemhauser et al. 2006), demonstrating a good correlation with the data presented here.

In concordance with previous results (Richardt et al. 2007), the domain-based TAP gene classification revealed a nearly complete overlap of TAP families in the genomes of *P. patens* and *A. thaliana*, facilitating a comparison of their TAP gene expression patterns. Only for seven small TAP families (GeBP, NOZZLE, PBF_2-like, SAP, ULT, BBR/BPC, DBP) encoded by the *A. thaliana* genome we did not identify the corresponding homologs in *P. patens*. Both, the *P. patens* and *A. thaliana* TAP families are partially represented on the two microarray platforms (Supplementary Table S5). Nevertheless, based on the identified differentially expressed TAP genes, a relative comparison of the expression patterns in response to salt stress and ABA is feasible.

In *A. thaliana*, the number of TAP genes responding to exogenously applied ABA considerably exceeds the number of genes which are responsive to high salinity (Fig. 1a). These findings corroborate the current knowledge from *A. thaliana*, since ABA mediates multiple abiotic stress responses and regulates additional processes (Finkelstein et al. 2002). As a similar result is observed for *P. patens* (Fig. 1a), we suggest that ABA is involved in additional responses in the moss as well. Compared to the immediate-to-early changes in gene expression at 30 min, an enhancement of transcriptional changes occurs after

60 min in *P. patens* and *A. thaliana* apparently involving a second set of TAP transcripts (Table 1 and Supplementary Table S6), which might be regulated by the immediate-to-early responding TAPs. Furthermore, in both species, the extent of overlap between ABA- and salt stress-responsive genes is comparable (9 and 14%, Fig. 1b). The slightly higher fraction of salt-specific changes observed in *P. patens* (13 vs. 3% for *A. thaliana*, $P = 0.019$) could be due to the specialization of *A. thaliana* organs in sensing of and responding to salt stress (e.g. Jia et al. 2002; Ma and Bohnert 2007). In all cases, changes in transcript abundance in response to both ABA and salt point into the same direction (repression or induction) suggesting that ABA is able to mimic the salt stress response, most likely by acting as a second messenger in salt stress associated signalling pathways.

However, the temporal and directional distributions of ABA- and salt-induced transcript changes also depict differences in TAP gene response patterns between *P. patens* and *A. thaliana*. In contrast to *A. thaliana*, in *P. patens* all early (30 min) salt-induced genes are still and even further up-regulated after 60 min (Fig. 1c), indicating persistent effects of salt stress on *P. patens* TAP gene expression. Additionally, in *P. patens*, the set of transiently (exclusively after 30 min) ABA-regulated genes is enlarged (16 vs. 5% in *A. thaliana*, $P < 0.05$) (Fig. 1c). Moreover, the TAP genes responding to ABA after 60 min show significantly more repression in *P. patens* (55 vs. 25 in *A. thaliana*, $P < 0.05$) (Fig. 1d). These results indicate an immediate integration of ABA-transduced signals followed by the downregulation of many TAP genes, and suggest that the systematic response might occur faster in *P. patens* protonema than in *A. thaliana*. On one hand this might be explained by a rapid uptake of ABA due to the direct exposure of protonemata to ABA-containing medium. However, these single cell wide filaments that lack any protecting tissues imply a specific need for fast adaptive responses for survival in adverse environments. This possibly reflects the situation of early land plants colonizing terrestrial habitats.

Impact of differential expression of TAP genes on the degree of salt tolerance in land plants

The differential expression of genes has been proposed to account for the varying degrees of salt tolerance in glycophytes and halophytes (Zhu 2001). To challenge this hypothesis, we compared the fractions of the identified salt stress-responsive TAP genes in the salt-tolerant moss *P. patens* and *A. thaliana*. A larger fraction of the analyzed TAP genes changed their expression upon salt stress in *P. patens* as compared to *A. thaliana* (3.2 vs. 1.1%). These results suggest that the regulation of TAP and subsequent effector gene expression is important for the high salt tolerance of *P. patens*. Consistently, many genes have been found to be differentially expressed in response to high salinity in a highly salt-tolerant cultivar of *Oryza sativa*, while their expression remained unchanged in a salt-sensitive cultivar (Chao et al. 2005). By contrast, stress-associated genes are constitutively overexpressed in *T. halophila*, while transcriptionally induced in the glycophyte *A. thaliana*, pointing towards a stress-anticipatory readiness of this halophytic plant (Gong et al. 2005; Taji et al. 2004; Wong et al. 2006).

This suggests that the regulation of salt tolerance in embryophytes might be divided into three levels: (1) the halophytic approach as observed in *T. halophila*, where genes are constitutively expressed, likely as an adaptation to high salinity; (2) the salt-tolerant approach as observed in *P. patens* and salt-tolerant cultivars of *O. sativa*, where genes are promptly activated upon abiotic stress; and (3) the glycophyte approach as observed in *A. thaliana*, where a decreased number of genes is responsive to salt stress.

In contrast, Cuming et al. (2007) suggested that the intermediate degree of dehydration tolerance in *P. patens* might result from constitutive expression of genes with stress-protective function, based on the fewer ABA- and salt-induced genes found in their study as compared to *A. thaliana*. The larger fraction of differentially expressed *P. patens* genes in our study might be explained by the earlier time points or to the higher number of replicate arrays (3 vs. 2), a parameter that is known to be correlated with the power for detection of differential gene expression (Pavlidis et al. 2003).

Although *P. patens* and *T. halophila* seem to possess different strategies of gene regulation (inductive versus constitutive), similar effector genes might confer their salt tolerance. Both plants utilize characteristic physiological mechanisms to prevent ion and osmotic stress and thereby increase salt tolerance, such as maintenance of ionic and osmotic homeostasis via potassium selectivity and accumulation of compatible osmolytes (Benito and Rodriguez-Navarro 2003; Inan et al. 2004; Lunde et al. 2007; Taji et al. 2004; Volkov et al. 2004; Wang et al. 2008).

The role of ABA in abiotic stress responses

In seed plants osmotic stress leads to increased intracellular ABA levels, which result from enhanced ABA biosynthesis, retarded ABA degradation, and release of ABA from ABA-glucose-esters (Lee et al. 2006; Zhu 2002). The rate-limiting step in ABA biosynthesis is catalyzed by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), a subgroup of carotenoid cleavage dioxygenases (CCDs). The specific induction of *NCED* genes upon drought has been shown in many plant species (Xiong and Zhu 2003). Intracellular

ABA levels increased twofold in *P. patens* protonemata upon osmotic stress treatment (Minami et al. 2005), suggesting a mediator role for ABA in salt stress signalling. This complies with the results of our expression analysis as nearly half of the salt stress-induced *P. patens* TAP genes also respond to exogenously applied ABA (Fig. 1b; Table 1). To gain insight into the molecular mechanisms underlying ABA accumulation upon osmotic stress in *P. patens*, we analyzed the expression of *NCED* genes under abiotic stress conditions.

Initially, we identified four putative *NCED* homologs in the *P. patens* EST database. Upon availability of the genome sequence (Rensing et al. 2008), we identified eight putative *NCED* genes and mapped the EST sequences to four of them. In the phylogenetic tree, two *P. patens* NCEDs (Phypa_57876 and Phypa_173118) are basal to the subtree containing five *A. thaliana* and further plant *NCED* genes (Fig. 3a). Four *P. patens* proteins are orthologous to AtCCD1, indicating a differential expansion of the gene family in *P. patens* and *A. thaliana*. Furthermore, *P. patens* harbours an ortholog of each AtCCD7 and AtCDD8, which are involved in the formation of a novel carotenoid-derived long-range signalling molecule affecting lateral shoot branching (Booker et al. 2004; Sorefan et al. 2003). No clear ortholog of AtCCD4 was identified in *P. patens*, suggesting that the functionally uncharacterized genes of this cluster might have been acquired in the angiosperm lineage. RNA gel blot hybridizations with the four *P. patens NCED* cDNAs revealed that the expression of two *PpNCED* genes (*Phypa_57876* and *Phypa_173118*) and two *AtCCD1* orthologs (*Phypa_58869*, *Phypa_159406*) is induced by dehydration, NaCl, and ABA (Fig. 3a, b). In contrast, in *A. thaliana* only *AtNCED3* is significantly induced upon drought stress (Iuchi et al. 2001; Tan et al. 2003) and, according to the AtGenExpress salt stress and ABA datasets, also within 1 h of NaCl or ABA treatment (Fig. 3a). AtCCD1 actually lacks the enzymatic specificity for 9-cis-epoxycarotenoids and is not involved in ABA biosynthesis (Schwartz et al. 2001). The function of the stress-inducible *AtCCD1* homologs in *P. patens* remains elusive.

Our results suggest that the regulation of carotenoid-cleaving enzymes and their involvement in abiotic stress responses diverged during the evolution of land plants. The induced expression of *P. patens NCED* genes indicates that the mechanism of stress-responsive ABA biosynthesis was already established in the LCA of bryophytes and angiosperms and might have been lost for most of the *NCED* paralogs in *A. thaliana*. The induction of ABA biosynthesis genes upon salt stress and the ABA-induced expression of salt stress-responsive TAP genes in *P. patens* suggest that ABA mediates salt-stress responses in a similar way as observed in seed plants. Consistently, a pre-treatment of

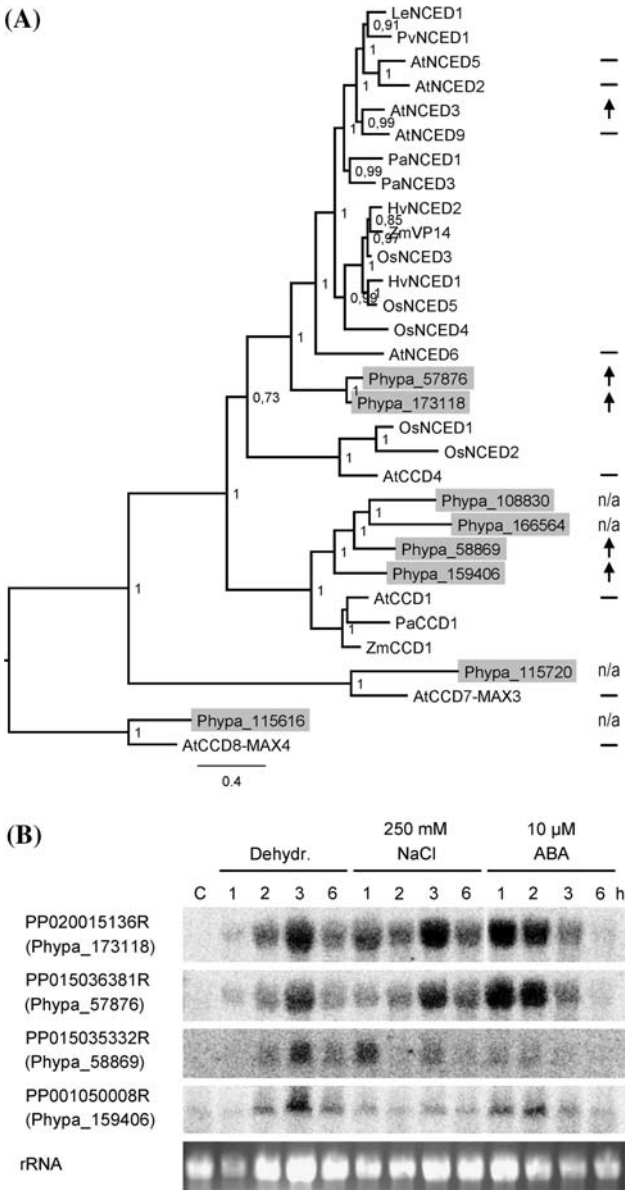

Fig. 3 Phylogeny and expression of carotenoid cleavage enzymes in plants. **a** Bayesian tree of conserved regions of plant NCED and CCD proteins. The sequences comprise nine *P. patens* gene models (*grey boxes*); *A. thaliana* and *O. sativa* genes retrieved from NCBI Genbank; ZmVP14 and ZmCCD1 from *Zea mays* (Sun et al. 2008; Tan et al. 1997); LeNCED1 from *Lycopersicon esculentum* (Burbidge et al. 1999); PvNCED1 from *Phaseolus vulgaris* (Qin and Zeevaart 1999); HvNCED1 and HvNCED2 from *Hordeum vulgare* (Millar et al. 2006); PaNCED1, PaNCED3, and PaCCD1 from *Persea americana* (Chernys and Zeevaart 2000). The *scale bar* corresponds to 20 amino acid substitutions per residue. The symbols at the right indicate transcript changes of NCED genes upon 60 min of 250 mM/150 mM NaCl or 10 µM ABA treatments, respectively, in *P. patens* (RNA gel blots, Fig. 3b) and *A. thaliana* (AtGenExpress datasets, FDR q-value < 0.05 and fold change > 2); *arrow*, induction; *hyphen*, no expression change; *n/a*, no data available. **b** RNA gel blots of four *P. patens* NCED transcripts under untreated control conditions (C), and 1–6 h of dehydration, 250 mM salt stress, or 10 µM ABA treatment, respectively. Gene models corresponding to the EST sequences used for hybridization are given in brackets

P. patens plants with ABA leads to desiccation and low temperature acclimation (Minami et al. 2003; Oldenhof et al. 2006). Furthermore, our results suggest that the increase of intracellular ABA concentration upon osmotic stress (Minami et al. 2005) and the salt acclimation of *P. patens* by step-wise increasing salt concentration in the culture medium (Benito and Rodriguez-Navarro 2003) might be accomplished through induction of *NCED* genes.

ABA-responsive expression of TAP genes

We identified a large number of TAP genes, which are specifically regulated by ABA and might be involved in further ABA-controlled processes. Among these, the fraction of ABA-repressed genes is significantly larger in *P. patens* compared to *A. thaliana* (Fig. 1d). These *P. patens* genes belong to the TAP families AP2/EREBP, bHLH, GRAS, HD-Zip, MYB, G2-like, MADS, RB, TRAF, and PLATZ (Table 1). In *A. thaliana*, we found AP2/EREBP, bHLH, GRAS, and HD-Zip TAP genes to be repressed by ABA as well. In contrast, the differentially expressed *MYB* and *G2*-like genes were positively regulated by ABA in *A. thaliana*, and members of the MADS, RB, TRAF and PLATZ families did not show differences in expression upon ABA treatment. These findings suggest that the involvement of these TAP families in ABA responses has evolved in a distinct manner in both organisms. The function of the majority of the identified ABA-repressed TAP genes in *P. patens* remains elusive to date, while members of many of the corresponding TAP families are known to regulate growth, differentiation and development in seed plants (Montiel et al. 2004; Riechmann and Meyerowitz 1997; Riechmann and Meyerowitz 1998; Rossini et al. 2001; Schmitz and Theres 2005; Wildwater et al. 2005). The identified *P. patens* genes might be involved in similar processes, such as the ABA-induced formation of brachycytes (Decker et al. 2006; Tintelnot 2006). Two of the identified ABA-repressed TAP genes (PpHB7, PpRSL1) have been proposed to regulate the development of rhizoids in *P. patens* (Menand et al. 2007; Sakakibara et al. 2003). Considering the induction of ABA biosynthesis genes upon abiotic stresses (Fig. 3b), ABA might play a role in the integration of growth and development into abiotic stress responses in *P. patens*, as known from seed plants (Finkelstein et al. 2002).

Conservation of regulators of osmotic stress responses in vegetative tissues

DREB

In both, protonemata of the moss *P. patens* and whole seedlings of *A. thaliana*, transcription of AP2/EREBP genes is regulated by ABA and/or salt stress (Table 1 and Supplementary Table S6). Several of the *A. thaliana* NaCl- and ABA-induced genes belong to the DREB/CRT subfamily of EREBP (e.g. *AtDREB1A-C*, *AtDREB2A*), and encode osmotic- and salt stress-induced key regulators of abiotic stress responses (Yamaguchi-Shinozaki and Shinozaki 2006). Also, their transcriptional induction by ABA was previously reported (Knight et al. 2004; Li et al. 2006; Wang et al. 2006). ABA-regulation of the DREB homolog *PpDBF1* was not detected in protonemata (Supplementary Table S2), but has been shown to occur in gametophores of *P. patens* (Liu et al. 2007). All but one of the salt- and/or ABA-induced *AP2/EREBP* genes of *P. patens* (Table 1) group within or basal to an orthologous cluster containing *DREB* genes of angiosperms (Supplementary Fig. S7), indicating an ancient origin of this regulatory module and its functional conservation. This hypothesis is consistent with the proposed divergence of the AP2 and EREBP subfamilies prior to the evolutionary split of Chlorophyta and Streptophyta lineages (Shigyo et al. 2006).

C2C2 Dof

One *P. patens Dof* gene (*Phypa_153324*) is transcriptionally induced by salt stress and ABA (Table 1) and corresponds to the group A gene *PpDof1* in the phylogenetic analysis of Shigyo et al. (2007). Two *A. thaliana* group A Dof genes (*AtCDF1* and *AtCDF3*) are induced by salt stress and/or ABA as well (Supplementary Table S6), suggesting the presence of the associated regulatory module in the LCA of embryophytes and its putative function in salt stress responses and ABA signalling therein. The origin of the Dof gene family predates the split of green algae and embryophytes and genes of the ancestral group A have been found in all major plant lineages (Moreno-Risueno et al. 2007; Shigyo et al. 2007). These proteins have been associated with processes common to all green organisms such as light regulation mechanisms (e.g. AtCOG1, Park et al. 2003). Though, the function of the putative ancestral *Dof* gene remains elusive, since functional data on the single *Dof* gene of the green alga *Chlamydomonas reinhardtii* is missing. However, it is known that ABA may act as a signal to induce oxidative stress responses in *C. reinhardtii* (Yoshida 2005; Yoshida et al. 2003). Therefore, the recruitment of Dofs to regulate stress responses via ABA signalling might be a conserved mechanism in green plants.

bHLH

Furthermore, we identified one bHLH gene that is induced by high salinity in *P. patens* (*Phypa_229640*; Table 1).

bHLH is one of the biggest TF families in plants and is present across all eukaryotic kingdoms (Riano-Pachon et al. 2008). Phylogenetic analysis revealed that it constitutes one of the five putative orthologs of *AtMYC2* (Supplementary Fig. S8), which encodes a positive regulator of ABA-signalling and abiotic stress responses (Abe et al. 1997, 2003) and is transcriptionally induced by ABA in seedlings and by salt stress in roots (Supplementary Table S3 and S6). These results suggest functional conservation of MYC-like bHLH TFs as positive regulators of ABA-signalling in embryophytes. Consistent with previously reported extensive lineage-specific expansions of this large TAP family (Buck and Atchley 2003), additional paralogs have been retained in the genome of *P. patens*, which supposedly underwent neo- or sub-functionalization. Taken together, our results suggest that components of the regulatory networks of osmotic stress responses have been conserved in vegetative tissues of bryophytes and angiosperms, and hence were likely active in their LCA.

Ancestral roles of TAPs regulating seed plant development and physiology

The evolution of several signalling pathways and developmental features specific to seed plants is based on basic regulatory toolboxes already existing in the LCA of embryophytes. One well-established example is the invention of flowers, connected to the evolution of the floral regulator LEAFY (Maizel et al. 2005; Tanahashi et al. 2005) and MADS-box TFs (De Bodt et al. 2003; Riese et al. 2005; Singer et al. 2007).

Regulators of seed maturation and light signalling

ABA-regulated seed maturation in *A. thaliana* is controlled by the B3-domain TF AtABI3, which interacts with the bZIP TF AtABI5 to trans-activate ABRE *cis*-elements in promoters of seed maturation genes (Finkelstein et al. 2002). *P. patens* harbours at least nine *ABI3*-like genes (Supplementary Fig. S9). Three of the five genes that are present on the microarray are induced by ABA treatment in protonemata (*PpABI3A*, *PpABI3B*, and *Phypa_39201*; Table 1). *PpABI3B* was also found to be induced by salt stress (Table 1) and, in a previous study, to be up-regulated after 4 h of dehydration (Frank et al. 2005b). PpABI3A activates ABRE-dependent expression in an ABA-dependent manner in vegetative moss tissue and partly complements the phenotype of *A. thaliana abi3* null mutants (Marella et al. 2006). The conserved ABA-mediated activation of ABRE *cis*-elements was also shown for *LEA type-1* genes in *P. patens* (Kamisugi and Cuming 2005; Knight et al. 1995). *AtABI3* transcript levels do not increase within 60 min of salt stress or ABA treatment of seedlings (Supplementary Table S6). Its expression in seedlings is restricted to specific tissues and conditions (Brady et al. 2003; Rohde et al. 1999, 2002). However, ectopic expression of *AtABI3* confers increased freezing tolerance to vegetative tissues in response to ABA and low temperature (Tamminen et al. 2001). In specific vascular cells of maize, the *AtABI3* ortholog *ZmVP1* is induced upon drought stress (Cao et al. 2007). Hence, the salt stress- and ABA-responsive expression of *ABI3* homologs in *P. patens* protonemata suggests the involvement of *ABI3*-like genes in the vegetative osmotic stress tolerance in the LCA of bryophytes and angiosperms.

In *A. thaliana*, the induction of bZIPs from Group A (e.g. AtABI5 and ABFs/AREBs) during seed maturation and stress responses is well-known (Finkelstein et al. 2002). Nevertheless, a recent phylogenetic analysis across green plants has shown that bryophytes lack bZIPs from Group A (Corrêa et al. 2008). Interestingly, we have identified two ABA-induced *P. patens bZIP* genes (*Phypa_29817* and *Phypa_160508*; Table 1), suggesting that the interaction of ABI3 and a bZIP within the ABA response pathways precedes the LCA of embryophytes. The two ABA-responsive *P. patens* genes belong to the Group E and G of bZIPs, respectively (Supplementary Table S5 of Corrêa et al. 2008). Group G bZIP genes (*AtGBF2* and *AtGBF3*) are induced by ABA in *A. thaliana* as well (Supplementary Table S6). Only a few members of these subgroups have so far been analyzed, suggesting a role for Group E bZIPs in plant development (AtbZIP34 and AtbZIP61), and implicating Group G bZIPs into light and ABA signalling (e.g. AtGBF1 and 3, GmbZIP78) (Corrêa et al. 2008; Liao et al. 2008; Lu et al. 1996; Mallappa et al. 2006; Shen et al. 2007). The lack of Group A *bZIP* genes in *P. patens* (Corrêa et al. 2008) suggests that members of other groups, possibly E or G, could play a role similar to that of AtABI5 and ABFs/AREBs in *A. thaliana.* This assumption is further supported by the fact that PpABI3A less efficiently activates ABRE *cis*-elements in barley aleurone cells, and only weakly interacts with AtABI5 (Marella et al. 2006). Possibly the function of Group A bZIPs emerged as a specialization of Group G bZIPs during seed plant evolution and the founder gene of the ancestral group of bZIPs (Group G + J) (Corrêa et al. 2008) might have played a key role integrating light signalling and ABA responses.

Similar to the expression of ABI3-like genes, we detected ABA-induction of one *CCAAT-HAP3* gene in *P. patens* protonemata (*PpHAP3F*; Table 1). The CCAAT-HAP3 protein LEAFY COTYLEDON1 (AtLEC1) promotes AtABI3 accumulation and potentiates ABA responses in *A. thaliana* seeds (Parcy et al. 1997). *P. patens* lacks LEC1-type *HAP3* genes, but fern and lycophyte homologs exist and their expression is induced under

drought stress (Xie et al. 2008). Therefore, LEC1-type *HAP3* genes likely originated in the LCA of embryophytes and their function in seed development evolved with the origin of spermatophytes. According to the phylogeny presented in Xie et al. (2008), the ABA-induced *P. patens* non LEC1-type *HAP3* gene, *PpHAP3F*, is closely related to the single *HAP3* gene of the green alga *C. reinhardtii*. These genes are likely to represent the ancestral structure of *HAP3* genes (Xie et al. 2008). We identified one ABA- and salt stress-responsive non LEC1-type *HAP3* gene in *A. thaliana* seedlings, *AtHAP3B* (Supplementary Table S6), which has previously been reported to be induced by osmotic stress (Chen et al. 2007). These results suggest that the salt stress and ABA response networks involving non-LEC1-type *HAP3* genes were already established in the LCA of embryophytes.

AtHAP3B forms a heterotrimeric complex with AtHAP5 proteins and the flowering time regulator AtCO (CONSTANS) (Wenkel et al. 2006). In *A. thaliana*, this CCAAT-binding complex controls photoperiodic flowering under osmotic stress conditions (Chen et al. 2007). We identified three *CO*-like genes that are induced by ABA in *P. patens* protonemata (*PpCOL3*, *Phypa_141045*, and *Phypa_233854*; Table 1). Previously it has been shown that the expression of *PpCOL3* is regulated by light, similar to *CO* and *CO*-like genes in *A. thaliana* (Zobell et al. 2005), suggesting that *CO*-like genes have already been involved in light signal transduction in the LCA of bryophytes and angiosperms. The co-regulation of *CO*-like and *ABI3*-like genes by ABA in *P. patens* protonema points to an integration of ABA and light responses and is furthermore consistent with the previously reported physical interaction of AtCO and AtABI3 (Kurup et al. 2000).

Our results indicate that the core mechanism that evolved into the control of seed maturation observed in extant angiosperms was already established in the LCA of embryophytes. This mechanism might have evolved from environmental stress and light responsive regulatory networks, which were retained in both lineages and remained active in vegetative as well as in reproductive tissues.

Regulators of jasmonic acid signalling

Another *P. patens* salt-induced TAP gene encodes a member of the tify (ZIM) family (*Phypa_173329*; Table 1) (Vanholme et al. 2007). A number of *tify* genes are also called JAZ, since they are rapidly induced in response to jasmonic acid (JA) in *A. thaliana* (Thines et al. 2007). JAZ function as repressors via physical interaction with their target TFs (e.g. AtJAZ3 and AtMYC2). This interaction is reverted in a JA-dependent manner resulting in the degradation of JAZ proteins (Chini et al. 2007; Thines et al. 2007). JA signalling is connected to salt stress responses, as high salinity induces JA biosynthesis genes in *A. thaliana*, and JA seems to mediate adaptation to salt stress in barley (Jiang and Deyholos 2006; Walia et al. 2007). The identified salt-induced *P. patens* gene belongs to a paralog cluster that is sister to the cluster of AtJAZ3 and AtJAZ4 (Supplementary Fig. S10). *AtJAZ3* was previously shown to be induced in *A. thaliana* roots after 6 h of salt stress (Jiang and Deyholos 2006). The conserved salt-inducible expression of JAZ regulators in *P. patens* and *A. thaliana* substantiates the hypothesis that components of JA signalling have been established prior to the split of bryophytes and seed plants and later on acquired new or additional functions in seed plants (Chico et al. 2008; Rensing et al. 2008).

An evolutionary view on signalling crosstalk via TAPs

One of the main points of conducting comparative studies between bryophytes and angiosperms is to uncover the core networks of processes that led to the diversity of responses observed among extant plants. Our results provide indications about the cross-talk of TAPs in different processes, such as abiotic stress and phytohormone responses and light perception, in embryophytes. Members of the AP2/EREBP family were originally described as mediators of ethylene responses (Riechmann and Meyerowitz 1998), but later they were also shown to act in ABA and abiotic stress responses (Song et al. 2005). In both, *A. thaliana* and *P. patens*, we found AP2/EREBP genes that respond to ABA and/or salt stress. Although no classical ethylene responses were observed in mosses (Rensing et al. 2008), the salt-induced *AP2/EREBP* gene *PpACP1* (Table 1) has been shown to respond to ethylene (Cho et al. 2007). Hence, the crosstalk between salt stress and ethylene responses could possibly be mediated by AP2/EREBP TAPs in mosses as well and might have played an important role for the water-to-land-transition of plants. Yet, functional characterization of these TFs and further investigation of the role of ethylene in bryophytes are still needed.

Another interesting point can be raised for the bZIP and bHLH families. These two families are present in all eukaryotic lineages and have been recruited to fulfil roles in early development, comprising most diverse processes in plants, demonstrating that a low number of genes can have big plasticity due to their ability to form homo- or heterodimers. In angiosperms, much of light signal transduction occurs in a big cascade involving many bHLH and bZIP genes (Jiao et al. 2007). The core of this network seems to be present in green plants since the LCA of all chlorophytes (Riano-Pachon et al. 2008). Curiously, bZIPs from Group G are not only responsible for light signal transduction, but they are also ABA responsive. Increased ABA concentrations or salt stress lead to the accumulation

of proline, a protective osmolyte in land plants (Abraham et al. 2003). In *A. thaliana*, levels of proline are controlled via its degradation by proline dehydrogenase (ProDH), which is regulated by a bZIP TF (Satoh et al. 2004). An increase in proline content upon salt stress has also been reported for *P. patens* (Wang et al. 2008). Our results and previous studies indicate that the cross-talk between light, ABA and metabolites may have already been present in the LCA of embryophytes. The incorporation of ABA and light signalling in response to osmotic changes in the environment might have been a key feature for the conquest of the terrestrial environment.

Furthermore, salt stress responses interfere with JA signalling in seed plants (Jiang and Deyholos 2006; Walia et al. 2007). The conserved induction of one *JAZ*-like gene during salt stress in *P. patens* suggests an ancient function of JAZ regulators in salt stress responses possibly serving as the basis for their role in the crosstalk between JA signalling and salt stress responses in seed plants.

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