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RESEARCH PAPER

Abscisic acid, cold and salt stimulate conserved metabolic regulation in the moss *Physcomitrella patens*

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ABSTRACT

- Salt and cold are major abiotic stresses that have adverse effects on plant growth and development. To cope with these stresses and their detrimental effects plants have evolved several metabolic, biochemical and physiological processes that are mainly triggered and mediated by the plant hormone abscisic acid (ABA).
- To elucidate the metabolic responses of the moss *Physcomitrella patens*, which serves as a model plant for abiotic stress adaptation, we performed GC-MS-based metabolic profiling of plants challenged for 5 and 28 h with either salt, cold or ABA.
- Our results indicate significant changes in the accumulation of several sugars including maltose, isomaltose and trehalose, amino acids including arginine, histidine, ornithine, tryptophan and tyrosine, and organic acids mainly citric acid and malonic acid. The metabolic responses provoked by ABA, cold and salt show considerable similarities. The accumulation of certain metabolites positively correlates with gene expression data whereas some metabolites do not show correlation with cognate transcript abundance.
- To place our results into an evolutionary context we compared the ABA- and stress-induced metabolic changes in moss to available metabolic profiles of the seed plant *Arabidopsis thaliana*. We detected considerable conservation between the species, indicating early evolution of stress-associated metabolic adaptations that probably occurred at the plant water-to-land transition.

INTRODUCTION

The moss *Physcomitrella patens* has become an ideal model plant for evolutionary developmental (evo-devo), molecular and stress adaptation studies. The remarkable features of *P. patens* include an ideal evolutionary position at the basis of land plants, a fully sequenced genome, a haploid-dominant life cycle, simple single cell layer morphology, well-characterised tissue types whose differentiation is controlled by plant hormones, and very efficient gene targeting by homologous recombination, which is applicable for functional gene analyses.

Environmental conditions such as drought, salinity stress, high and low temperatures, result in heavy yield losses worldwide. Taking into consideration that *P. patens* is highly tolerant to drought, salt and osmotic stresses (Frank *et al.* 2005b), understanding the underlying molecular, biochemical and physiological mechanisms may help to address the challenges that accompany global climate change. In this regard, several studies have reported analysis of stress-associated changes in gene expression (Cuming *et al.* 2007; Richardt *et al.* 2010; Beike *et al.* 2015) or changes in the proteome (Wang *et al.* 2009; Lang *et al.* 2011), while studies addressing stress-responsive metabolic changes in moss are limited (Nagao *et al.* 2005, 2006; Rother *et al.* 2006; Erxleben *et al.* 2012). The study of Erxleben *et al.* (2012) addressed drought-responsive metabolites in *P. patens* and identified five compounds (altrose, maltitol, L-proline, butyric acid and one unknown compound) that are only detected in response to drought stress, and three additional compounds (L-aspartic acid, L-norleucine, L-serine) showing increased concentrations upon dehydration. Nagao *et al.* (2005, 2006) observed the accumulation of soluble sugars associated with ABA-induced freezing tolerance in protonema tissue, while Rother *et al.* (2006) performed metabolic analysis in response to Cd^{2+} stress and identified a rapid decrease in chlorophyll and accumulation of cysteine and glutathione. Since changes in metabolites depend on changes in gene expression and subsequent changes in protein abundance or altered protein activity, analyses of metabolic changes are necessary to completely understand the entire processes enabling the adaptation of land plants to altered environmental conditions. Further, based on the *P. patens* genome, it was shown that this moss encodes an extraordinarily high percentage of genes that are related to metabolism, which could be a major reason for the high tolerance to diverse abiotic factors (Lang *et al.* 2005).

Coupled with the above-mentioned unique tolerance of *P. patens* to abiotic stresses, it is well documented that the plant hormone abscisic acid (ABA) is the central mediator of responses to various environmental stresses (Kempa *et al.* 2008). In *A. thaliana*, ABA accumulates in response to several stresses, such as salt, cold and drought (Xiong *et al.* 2001, 2002;

Ruggiero *et al.* 2004). Various studies in *A. thaliana* demonstrated that the majority of stress-induced genes, which code for proteins involved in the related metabolic processes are up-regulated by ABA. As in higher plants, *P. patens*' common stress responses are also induced upon exogenous ABA application, and the core components of ABA signalling and responses are conserved from *P. patens* to angiosperms (Sakata *et al.* 2014; Saruhashi *et al.* 2015). Further, *P. patens* acquires increased freezing, hyperosmosis and dehydration tolerance following ABA treatment (Machuka *et al.* 1999; Kamisugi & Cuming 2005; Nagao *et al.* 2005; Oldenhof *et al.* 2006; Bhyan *et al.* 2012). In addition, an increase in endogenous ABA levels is caused by osmotic stress in *P. patens* (Minami *et al.* 2003). In this respect, increasing ABA leads to elevated levels of stress-responsive metabolites, although some changes may not be regulated by ABA and might rather be stress-specific. Accordingly, the aim of this study is to explore changes in primary metabolites of the model plant *P. patens* following ABA treatment, and upon cold and salt stresses, in order to elucidate conserved and stress-specific metabolic changes in such an ancient plant species. The results should provide further insights on stress-associated metabolic adjustment for plant adaptation.

MATERIAL AND METHODS

Plant material

Physcomitrella patens (Hedw.) Bruch & Schimp, ecotype 'Gransden 2004' was used in this study (Rensing *et al.* 2008). A protonema culture was grown in liquid minimal medium (250 $mg \cdot l^{-1}$ KH_2PO_4, 250 $mg \cdot l^{-1}$ KCl, 250 $mg \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, 1000 $mg \cdot l^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$ and 12.5 $mg \cdot l^{-1}$ $FeSO_4 \cdot 7H_2O$) under standard growth conditions described previously (Frank *et al.* 2005a). Every 4 days the liquid medium was exchanged and protonema tissue was disrupted for 10 s with an Ultra-Turrax device (IKA, Staufen, Germany). The samples were treated for 5 and 28 h with 25 μM ABA or 250 mM NaCl, or were incubated at 0 °C. To guarantee comparability between controls and treated samples, all samples of each replicate were precultured and grown in parallel under long-day conditions (16 h light/8 h darkness, with light from 07:00 to 23:00 h) with a light intensity of 80 μmol·photons $m^{-2} \cdot s^{-1}$. For the 5-h time point, treatments were started at 10:00 h and harvested along with the control samples at 15:00 h. Treatments of samples for 28 h were started at 11:00 h and harvested at 15:00 h on the next day along with the control samples, *i.e.* they were grown for 12 h in light, 8 h in darkness and 8 h in light. After harvesting all samples were immediately frozen in liquid nitrogen.

Plant extracts for metabolite profiling *via* GC-MS analysis

Plant material (20 mg FW) was extracted in 700 μl 100% methanol +30 μl internal standard solution (0.2 $mg \cdot ml^{-1}$ ribitol) at 70 °C for 15 min. Following centrifugation at 20,800 × *g* for 10 min, 375 μl chloroform and 750 μl water were added and mixed well with the supernatant. Following a second centrifugation at 15,300 × *g* for 15 min, the clear supernatants (150 μl) were dried overnight under vacuum. Residues were then derivatised for 120 min at 37 °C in 40 μl of 20 $mg \cdot ml^{-1}$ methoxyamine hydrochloride in pyridine, followed by a 30-min treatment at 37 °C with 70 μl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). The GC-MS system used was a gas chromatograph coupled to a time-of-flight mass spectrometer (Pegasus III; Leco, St. Joseph, MI, USA). An autosampler system (PAL) injected the samples. Helium was used as carrier gas at a constant flow rate of 2 $ml \cdot s^{-1}$ and gas chromatography was performed on a 30-m DB-35 column. The injection temperature was 230 °C and the transfer line and ion source were set to 250 °C. The initial temperature of the oven (85 °C) was increased at a rate of 15 $°C \cdot min^{-1}$ up to a final temperature of 360 °C. After a solvent delay of 180 s, mass spectra were recorded at 20 $scans \cdot s^{-1}$ with m/z 70–600 scanning range. Chromatograms and mass spectra were evaluated using Chroma TOF 1.0 (Leco) and TagFinder 4.0 software (Roessner *et al.* 2001; Schauer *et al.* 2005). The characteristics of GC-TOP-MS metabolites are shown in Table S1.

Extraction of RNA, cDNA synthesis and quantitative RT-PCR

Total RNA was extracted from 100 mg protonema tissue using TRIzol reagent (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. RNA samples were treated with DNaseI (NEB, M0303S) for 30 min at 37 °C to remove remaining genomic DNA, followed by DNaseI inactivation for 10 min at 65 °C. First strand cDNA was synthesised from 2 μg total RNA using oligo-dT and M-MuLV reverse transcriptase (NEB, M0253S) in the presence of 1× M-MuLV reaction buffer and 500 μM of each dNTP in a final volume of 20 μl. Reverse transcription was carried out at 42 °C for 1 h, and reverse transcriptase was inactivated at 65 °C for 20 min. All cDNAs were stored at −20 °C until use. The cDNAs were diluted to a concentration corresponding to 5 $ng \cdot \mu l^{-1}$ of the input RNA, and 5 μl of the diluted cDNA was used per qRT-PCR reaction. The transcript abundance was determined with SYBR green fluorescence in an iQ5 real time cycler (Bio-Rad, Hercules, CA, USA) with an initial 5 min incubation at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 58 °C and 15 s at 72 °C. Data were collected after each cycle and the melting curve of the product was calculated to assess its specificity at the end of the qPCR reaction. All qRT-PCR reactions were performed in three biological and three technical replicates, and normalisation was performed with the constitutively expressed housekeeping gene *PpEF1α* encoding the translation elongation factor 1α. Fold changes in gene expression between the samples were calculated applying the $2^{-\Delta\Delta CT}$ method. The nucleotide sequences of oligonucleotide used in this study are listed in Table S2.

Comparative analysis of genes associated with differentially regulated metabolites

Based on literature studies, we generated a list of *A. thaliana* genes that encode enzymes involved in the regulation of primary metabolites and searched for orthologous genes in the *P. patens* genome using the 'g:Profiler orthologue search' software (Reimand *et al.* 2016). Those genes that could be associated with the differential accumulation of metabolites detected in our study were analysed with respect to their transcriptional changes using published gene expression data (Khraiwesh *et al.*

2015) in order to correlate transcriptional regulation to the respective changes of the related metabolites (Table S3).

RESULTS

Metabolic profiling in *P. patens*

Profiling changes in primary metabolites were conducted with *P. patens* plants that were treated with ABA (25 μM) or exposed to salt (250 mM NaCl) or cold stress (0 °C) for 5 and 28 h. The samples were grown under an 16/8 h light/dark photoperiod with a light intensity of 80 μmol·photons·m^{-2}·s^{-1}. To ensure comparability between the treatments and the corresponding controls, all untreated controls and treated samples were harvested at the same time of day at 15:00 h, *i.e.* 8 h after the onset of light. In total 73 metabolites were confidently detected, and non-supervised PCA was performed to elucidate the metabolic changes in response to the different treatments. All replicates (n = 5) of each treatment clustered together, which confirmed the reliability and reproducibility of the experiments (Fig. 1). Table S4 contains the raw values of all metabolites obtained from the five replicates and *t*-test *P*-values of all comparisons. The deduced fold changes of primary metabolites in response to the different treatments are shown in Fig. 2, and histograms of the absolute values of all metabolites based on the averages of five replicates are presented in Figures S1–S4. Significant changes can be observed for several sets of compounds, including amino acids, organic acids and sugars. For some metabolites we observed differences in their accumulation between the 5 and 28-h control samples. However, this was only detected for metabolites with very low abundance, such as histidine, ornithine, tryptophan and malonic acid. Nevertheless, when compared to the changes between the control samples, we observed obvious changes in their accumulation in response to ABA, salt and cold treatments (Figures S1–S4).

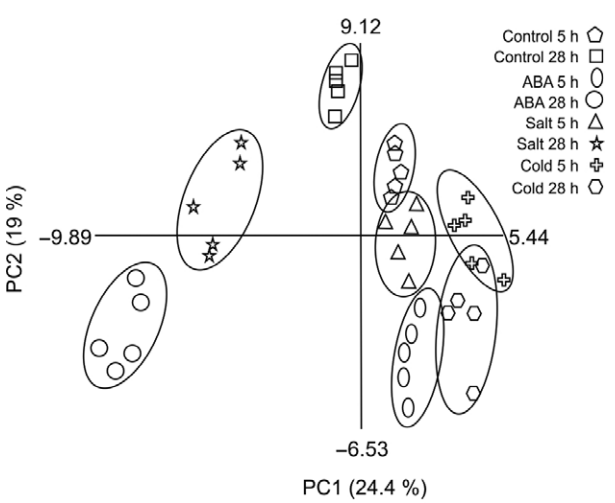

Fig. 1. Principal components analysis (PC1 *versus* PC2) of primary metabolites of *P. patens* subjected to ABA (25 μM), salt (250 mM NaCl) or cold (0 °C) for 5 and 28 h to visualise variations among replicates and conditions. PCA was performed on the normalised absolute values of all detected metabolites from five replicates. Each sample is represented by a separate geometric shape, to the right of the figure, and the corresponding five biological replicates are grouped together within oval shapes.

Metabolic changes in amino acids and polyamines

Amino acids, which are the building blocks of proteins, serve various other functions in plants, including a role both for the amino acids themselves and for their derivatives in plant osmoregulation. In our experiment, most amino acids, with few exceptions, behave in a similar manner following ABA treatment, and after exposure to salt or cold stresses.

Amino acids that increased significantly following 5 h of ABA treatment include histidine, isoleucine, proline, serine, tryptophan, tyrosine and valine. Further, at 28 h following ABA treatment a large number of amino acids showed significant increases, in particular histidine, isoleucine, ornithine, tryptophan and tyrosine. In contrast to the increase in levels of the above-mentioned amino acids, levels of other amino acids and polyamines, such as alanine, GABA, putrescine and glutamine, were reduced at 28 h (Fig. 2, Figure S1, Table S4).

Upon salt treatment, most amino acids tend to increase, including tryptophan, ornithine, isoleucine and leucine. In addition, a significant increase in proline content was observed after 5 h of treatment, but its level returned to that of control plants after 28 h. Further, the elevated levels of isoleucine, leucine and tyrosine following salt treatment persisted over the two time points. In contrast, levels of alanine, asparagine, GABA, glutamine and serine were reduced upon the salt treatment (Fig. 2, Figure S1, Table S4).

In respect of cold treatment, significant increases in the levels of histidine, ornithine, tryptophan, phenylalanine and tyrosine were observed. Other amino acids that showed highly elevated levels are beta-alanine, glutamine, glycine, isoleucine, leucine, lysine, proline and valine. In contrast, levels of cysteine and serine were significantly reduced, either at both time points or at least at one of them (Fig. 2, Figure S1, Table S4).

Metabolic changes in organic acids

Organic acids are also involved in stress responses of plants and may further contribute to maintain cation–anion equivalence. Significant changes in the levels of organic acids were observed following ABA, salt and cold treatments. The most obvious significant changes were recorded from ABA-treated plants, in particular changes in citric acid and malonic acid. In addition, citric, malic, malonic, pyroglutamic and pyruvic acids showed significant increases in salt-stressed plants. In contrast, slight decreases in the levels of adipic, aspartic, glutaric2-oxo, nicotinic and threonic acids were noted for at least one time point. In cold-stressed plants, the most pronounced changes were detected for malonic, pyroglutamic and citric acids (Fig. 2, Figure S2, Table S4).

Metabolic changes in sugars

Sugars are crucial for stress adaptation since they improve the osmoregulation capacity of plants. In addition, soluble sugars also act as nutrients and signalling molecules and, as such, play roles in modifying gene expression and protein patterns. Among the most obvious significant changes in sugars for samples of the 5-h time point were those in isomaltose, maltose and trehalose. Furthermore, changes in the levels of sucrose, glucose, galactinol and fructose were significant in response to at least one of the treatments. ABA treatment caused transitory

	Fold changes					
	ABA 5 h	ABA 28 h	Salt 5 h	Salt 28 h	Cold 5 h	Cold 28 h
Amino acids						
Alanine	0.79	0.30*	0.93	0.65*	2.47*	1.09
Alanine, beta	1.49*	0.77*	1.66*	0.74*	1.15	3.41*
Arginine	2.35*	7.81*	1.50*	2.43*	1.48*	4.34*
Asparagine	1.28	0.61*	0.99	0.54*	1.15	1.30*
Cysteine	0.84*	1.69*	0.69*	1.59*	0.61*	0.39*
GABA	0.83	0.29	1.82*	0.51	2.64*	1.73
Glutamine	0.77	0.44*	1.19*	0.67*	2.11*	3.67*
Glycine	1.60*	1.17*	1.16*	1.16*	1.69*	3.31*
Histidine	2.83*	10.34*	1.50	3.48*	1.43	8.85*
Homoserine	1.48	2.14*	1.25	1.57*	1.05	1.27
Isolucine	3.52*	4.12*	1.93*	3.13*	1.94*	3.99*
Leucine	4.34*	1.81*	2.33*	2.28*	2.86*	5.91*
Lysine	2.01	3.20*	1.40*	2.15*	2.06*	2.84*
Methionine	1.33*	2.66*	1.02	2.06*	1.00	1.19
Ornithine	2.65*	24.22*	1.57*	4.22*	1.59*	9.62*
Phenylalanine	2.58*	1.32*	1.49*	1.70*	1.58*	5.03*
Proline	2.38*	0.79	1.61*	1.00	4.00*	2.50*
Putrescine	1.93*	0.61*	1.24	1.90	1.66	1.36*
Serine	2.86*	0.71*	1.14*	0.65*	0.65*	0.40*
Threonine	1.07	1.78*	1.01	1.71*	0.87*	1.07
Tryptophan	4.40*	30.35	2.21*	4.45*	1.91*	6.37*
Tyramine	0.97	0.75*	0.93	0.99	1.04	1.01
Tyrosine	3.05*	6.68*	1.80*	2.00*	1.86*	4.38*
Valine	1.91*	1.88*	1.40*	1.71*	1.54*	3.14*
Organic acids						
Adipic acid	0.95	1.67*	0.85	1.48*	0.74*	0.68*
Aspartic acid	1.05	0.42*	1.14	0.67*	0.90	1.00
Benzoic acid	1.15*	1.24	1.01	1.02	1.25	1.07
Citric acid	1.52*	9.10*	1.54*	1.85*	0.74*	2.97*
Fumaric acid	1.41	0.92	1.00	1.06	1.08	1.01
Galactonic acid	0.85	0.66*	0.94	1.14	0.78	0.97
Glutamic acid	1.02	1.17*	0.97	1.51*	0.96	1.27*
Glutaric acid, 2 oxo	1.50*	1.43*	0.83	1.90*	0.84*	1.60*
Glyceric acid	1.01	0.53*	0.99	1.09	0.92	0.89
Malic acid	0.76	0.72*	0.95	1.00	0.97	0.53 *
Malonic acid	3.50*	4.54*	1.72*	1.79*	8.66*	10.38*
Nicotinic acid	1.00	0.78*	0.92	0.92	1.03	0.94
Pyroglutamic acid	1.31	1.40	1.19	2.58*	1.82*	2.60*
Pyruvic acid	1.17	1.29*	1.10	1.83*	1.12	1.21
Succinic acid	1.18	0.84	1.16	1.15	0.87	0.61
Threonic acid	0.81*	0.28*	0.87	0.78*	0.95	0.93
Sugars and sugar alcohol						
Erythritol	1.24*	1.55	1.14	1.51	1.13	0.89
Fructose	1.13	1.59	2.15*	1.74	0.82	0.83
Fucose	1.14	1.04	0.99	1.02	1.39	1.24
Galactinol	0.97	1.21*	1.01	1.10	1.16	1.15
Glucose	1.12	1.77	2.24*	1.91	0.81	0.83
Isomaltose, or similar	7.75*	0.73	2.71*	0.90	1.92*	27.44*
Maltose	6.93*	0.86	4.20*	1.16	2.57*	55.47*
Raffinose	1.03	1.57*	0.95	1.35*	0.97	0.97
Rhamnose	0.97	0.96	0.94	1.13	1.03	1.02
Sucrose	1.19	1.48*	0.92	1.70*	0.96	1.05
Threitol	1.15	1.34*	1.07	1.39	1.11	0.93
Trehalose, alpha, alpha	4.33*	0.70	2.85*	0.88	1.91*	24.63*
Unknon, similar to raffinose	2.93*	13.45*	1.26*	4.19*	1.10	1.66*
Unknown-sugars	1.94*	2.36*	1.11	0.84	1.76*	2.18*
Unknown-sugars	4.87*	0.72*	3.17*	1.30	2.06*	35.18
Others						
Adenine	1.02	0.77	1.08	0.83	0.91	0.97
Adenosine-5-monophosphate	1.14	1.01	0.86	1.09	1.07	1.35
Allantoin	0.96	0.96*	0.94	1.05	0.91	0.99
Dehydroascorbic acid	1.24	2.60*	0.88	1.19	1.12	0.91
Dopamine	0.99	0.85*	0.88	1.39*	1.03	1.20*
Ethanolamine	1.26	1.10	1.17	1.03	1.41	0.84
Fructose-6-phosphate	1.15	1.39*	1.47*	1.84*	1.31*	1.97*
Glucose-6-phosphate	1.21	1.65*	1.55*	2.00*	1.39*	2.34*
Glycerol	0.96	0.92	0.93	0.97	0.99	0.85
Glycerol-2-phosphat	1.11	0.60	1.00	0.36*	1.15	1.33
Glycerol-3-phosphate	0.69*	0.54*	0.82	1.22	0.88	0.73*
Inositol-1-phosphate	1.02	0.97*	1.04*	0.67*	1.22*	0.80*
Insitol, myo	0.89*	0.65*	0.86*	0.97	0.92	0.93
Malic acid, 2-methyl	1.12*	0.49*	0.93	1.24*	1.23*	0.85*
Octadeconoic acid, n	1.03	1.11	0.97	1.06	1.37	1.13
Phosphoric acid	1.06	1.06	1.11	1.07	1.22	1.14
Unknown	0.63	0.48	1.03	0.75	1.91*	3.38*
Urea	0.89	0.86	0.77	1.17	0.82	0.74*

Fig. 2. Heat map of the metabolic profiles of *P. patens* subjected to ABA, NaCl or cold for 5 and 28 h, including fold changes with respect to the untreated wild type at each time point. A negative score (depicted in red) represents decreased levels, while a positive score (depicted in purple) indicates increased levels. The intensity of the colour is correlated with the absolute values of the detected changes in levels of the individual metabolites. Asterisks indicate *t*-test $P \leq 0.05$ calculated from five replicates.

changes in levels of isomaltose, maltose and trehalose since these sugars increased after 5 h but declined again after 28 h of treatment. In contrast, changes in the levels of fructose, galactinol, glucose, raffinose, sucrose and threitol in response to ABA treatment were slow and were higher at 28 h compared to 5 h. Upon cold stress, there were large changes in the levels

of maltose and trehalose over the whole period. Sugars were the group of metabolites that showed the largest increase upon cold stress, as can be seen from the changes in levels of trehalose, isomaltose and maltose (Fig. 2, Figure S3, Table S4).

Changes in other metabolites

In addition to the metabolite classes mentioned above, we detected considerable changes in the concentration of metabolites belonging to other classes. One such is dehydroascorbic acid, showing significantly increased levels upon ABA treatment that even doubled over 28 h. However, neither abiotic stress had a major effect on the level of this metabolite. In contrast, both stresses led to significantly higher levels of glucose-6-phosphate and fructose-6-phosphate at both 5 and 28-h time points. Compared to the 5-h time point, the metabolite levels were higher after 28 h, while ABA caused a significant increase only after 28 h. As for changes in the levels of other metabolites, such as adenine, ethanolamine, glycerol-3-phosphate, inositol-1-phosphate, myo-inositol, 2-methyl malic acid and urea, no consistent trends could be observed (Fig. 2, Figure S4, Table S4).

Based on these results, it is evident that *P. patens*, unlike higher plants, has evolved diverse metabolic regulations to cope with stresses. For example, cold stress (at 28-h time point) led to the accumulation of maltose (55.47-fold), ornithine (9.62-fold), malonic acid (10.38-fold), histidine (8.85-fold), tryptophan (6.37-fold) and leucine (5.91-fold), whereas ABA treatment led to the accumulation of tryptophan (32.35-fold), ornithine (24.22-fold) histidine (10.34-fold) and citric acid (9.10-fold). Such diverse metabolic regulation might be an indicator for a 'generalist' approach (discussed below).

Specific regulation of ABA, salt and cold

Since many abiotic stresses provoke an increase in ABA, which acts as the major stress hormone mediating molecular adaptations in land plants including mosses (Cuming *et al.* 2007), we analysed whether differentially accumulating metabolites respond in a stress-specific manner or if they are also modulated by exogenous ABA application. For this, we compared the patterns of metabolites that showed at least 1.5-fold differential accumulation in response to the stress treatments. We generated Venn diagrams to depict their overlapping and/or specific response caused by the different treatments (Fig. 3). A list of the individual metabolites depicted in the Venn diagrams is presented in Table S5. These results indicate that ABA, salt and cold share a considerable number of up-regulated metabolites that respond in a similar manner, suggesting that the respective metabolic changes are mediated in an ABA-dependent manner. In addition, we found several metabolites that respond specifically to cold or salt, suggesting stress-specific and ABA-independent regulation, respectively. We also identified metabolites that respond in a similar manner to two of the treatments, pointing to partially overlapping regulatory pathways (Fig. 3).

Expression analysis of genes related to regulated metabolites

The observed changes in metabolites can be caused by transcriptional regulation of the genes encoding the respective

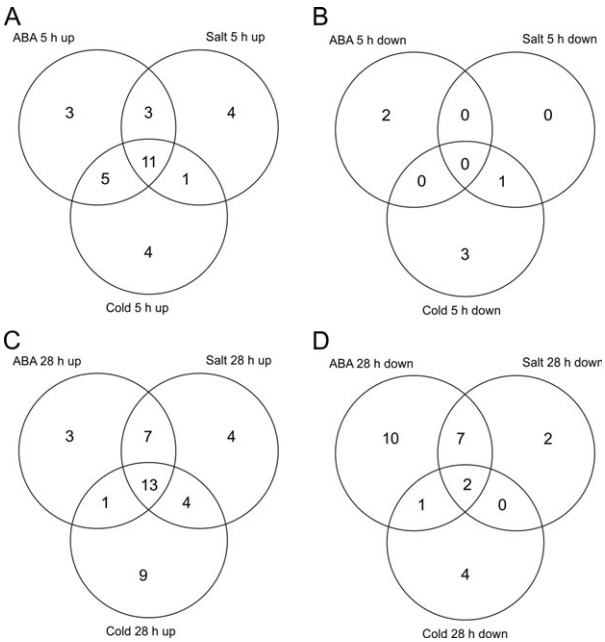

Fig. 3. Venn diagrams depicting the overlap or specificity of metabolite changes in response to the different treatments (ABA, NaCl or cold). The Venn diagrams were generated from values presented in Fig. 2. (A) up-regulated metabolites after 5 h, (B) down-regulated metabolites after 5 h, (C) up-regulated metabolites after 28 h, (D) down-regulated metabolites after 28 h.

metabolic enzymes and/or by post-translational activation or deactivation of these enzymes. To analyse whether transcriptional regulation of the corresponding genes is a predominant mechanism, we performed gene expression analysis by qRT-PCR with a set of genes that encode proteins potentially underlying the detected metabolic changes. These genes were selected on the base of their involvement in the biosynthesis of the primary metabolites that are differentially regulated by stresses or ABA treatments. Most of the genes belong to gene families that have multiple members in *P. patens* (Table S3). Accordingly, the following genes were analysed: ATP phosphoribosyl transferase (*Pp1s3_614V6.1*, histidine biosynthesis), pyrroline-5-carboxylate synthetase (*Pp1s22_40V6.1*, proline accumulation), phosphoglycerate dehydrogenase (*Pp1s11_357V6.1*, serine synthesis), phospholipid cytidylyltransferase (*Pp1s36_263V6.1*, phosphatidylethanolamine biosynthesis), cytoplasmic aconitate hydratase (*Pp1s253_16V6.1*, catalyses citrate to isocitrate), ornithine sigma aminotransferase (*Pp1s126_82V6.1*, ornithine metabolism), acetyl glutamate kinase (*Pp1s146_98V6.1*, arginine/ornithine/proline biosynthesis) and arginase 1 (*Pp1s72_148V6.1*, catalyses arginine to ornithine) (Fig. 4).

Little correspondence was observed between changes in transcript abundance and changes of the related metabolite, probably because metabolic homeostasis involves complex transcriptional and post-transcriptional mechanisms. However, we detected an increase in beta-amylase expression in response to cold stress that correlates well with the observed increased level of maltose sugars, the products of amylase-mediated starch hydrolysis. At the 5-h time point, we detected significant fold increase of 4.9-, 2.8-, 8.3- and 9.9-fold for all four beta-amylase genes *Pp1s106_57V6.1*, *Pp1s23_21V6.1*, *Pp1s233_4V6.1*

and *Pp1s317_42V6.1*. And after 28 h, the observed fold increases were 8.3-, 1.08-, 7.9- and 5.3-fold for *Pp1s106_57V6.1*, *Pp1s23_21V6.1*, *Pp1s233_4V6.1* and *Pp1s317_42V6.1*, respectively. Furthermore, the consistent up-regulation of all four beta-amylase genes correlates well with the increasing accumulation of maltose in the 5-h treatments of ABA and salt (Fig. 5).

For a more comprehensive analysis of metabolite to gene expression correlations we further made use of a recently published large-scale gene expression study that was based on next generation RNAseq profiling of *P. patens* transcriptomes in response to ABA, salt and cold (Khraiwesh *et al.* 2015). For this, we searched for genes encoding enzymes that might be involved in the differential accumulation of metabolites and deduced their transcriptional changes from the study performed by Khraiwesh *et al.* (2015) to correlate transcriptional regulation to the respective changes of the related metabolites (Table S3). Also, this approach revealed diverse correlations between gene expression and metabolic changes. For example, after 5 h of cold stress valine accumulates 1.5-fold and two out of four valine synthase genes show a positive correlation (1.54-, 2.13-fold increase), whereas the other two are negatively correlated (each 1.4-fold down-regulation). Another example affects the glutamate dehydrogenase family that is involved in the biosynthesis of GABA. GABA accumulates 2.4-fold upon 5 h of cold treatment, but only one out of four glutamate dehydrogenase genes is 6.87-fold up-regulated, while two genes are down-regulated (1.25- and 1.9-fold), and the expression of another gene remained unchanged.

However, we also detected genes and gene families that are positively correlated with the metabolic changes. For example, the alanine aminotransferase gene family, with six members in total, is positively correlated to the accumulation of alanine after cold treatments. The complete set of differentially regulated metabolite-related genes that correlate positively to the accumulation of metabolites is highlighted in Table S3. For some gene families we even detected negative correlations, such as for the tryptophan synthase genes that are down-regulated

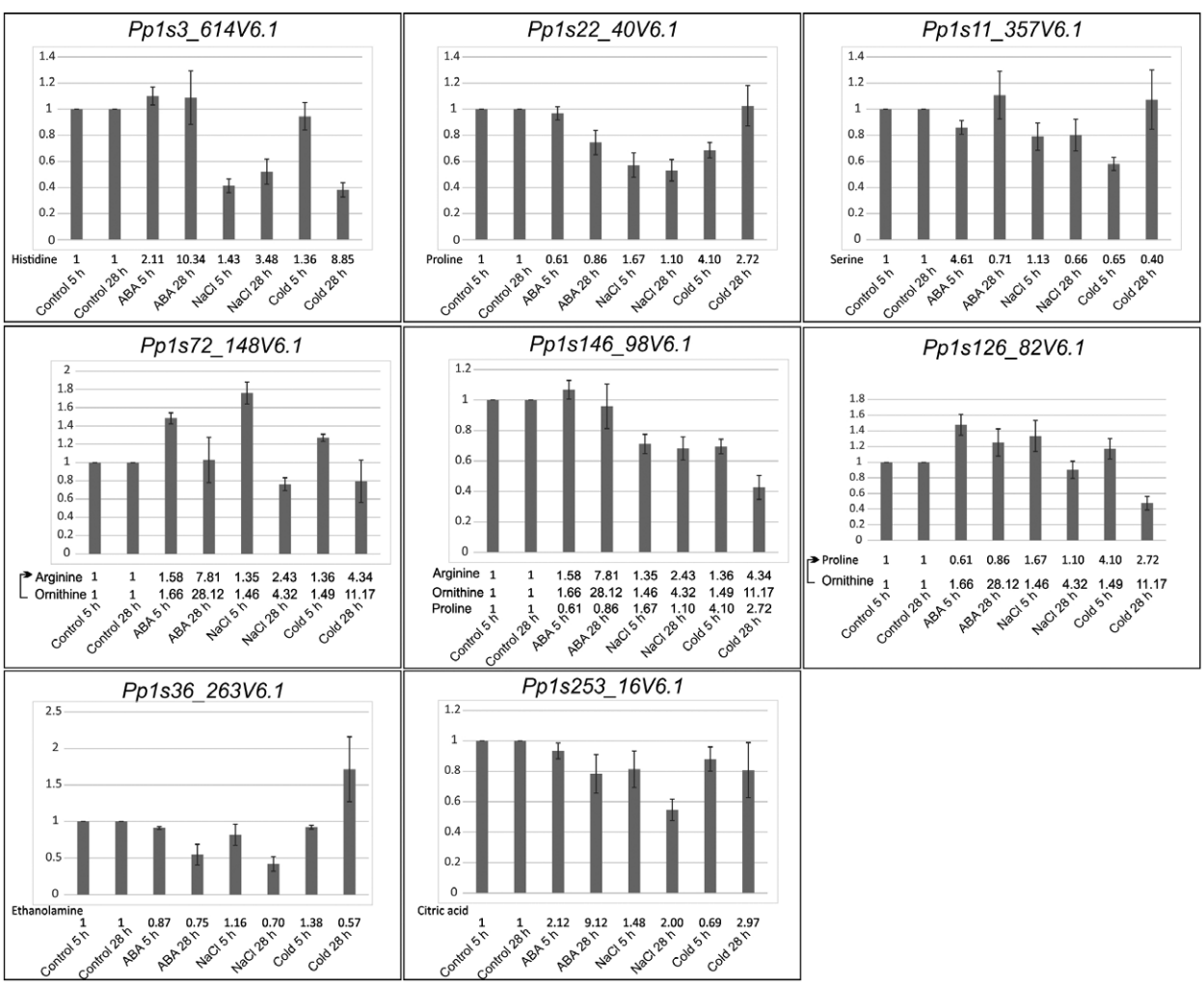

Fig. 4. Expression analysis by quantitative RT-PCR of selected candidate genes that may be involved in the synthesis of salt-, cold- and ABA-regulated metabolites. *EF1*α value normalised relative fold changes are shown for each gene. Fold changes of the respective metabolites are indicated beneath the qRT-PCR data for each gene. Based on sequence homology, the genes encode the following enzymes: *Pp1s3_614V6.1*: ATP phosphoribosyl transferase, *Pp1s22_40V6.1*: pyrroline-5-carboxylate synthetase, *Pp1s11_357V6.1*: phosphoglycerate dehydrogenase, *Pp1s36_263V6.1*: phospholipid cytidylyltransferase, *Pp1s253_16V6.1*: cytoplasmic aconitate hydratase, *Pp1s126_82V6.1*: ornithine sigma aminotransferase, *Pp1s146_98V6.1*: acetyl glutamate kinase, *Pp1s72_148V6.1*: arginase 1. Error bars indicate SE calculated from three biological replicates.

in response to ABA, salt and cold, even though tryptophan levels increase with these treatments. Based on the results revealing positive as well as negative correlations, together with diverse expression patterns even within the same gene family, we conclude that the identification of distinct correlated gene–metabolite pairs seems to be the exception, suggesting complex regulation of metabolic changes.

DISCUSSION

Changes in metabolic profiles of *P. patens* upon ABA, salt and cold treatments are both significant and multiphasic. The major trends are: (1) the rapid accumulation of a large set of metabolites from two major groups, namely sugars (of utmost importance fructose, maltose, isomaltose, trehalose and raffinose), and amino acids (of utmost importance arginine, histidine, isoleucine, leucine, ornithine, phenylalanine, proline, tryptophan and tyrosine); and (2) the slow response, which included different sets of metabolites such as organic acids, mainly citric acid, pyroglutamic acid and malonic acid. The accumulation of osmolytes is well known upon exposure of plants to abiotic stresses (Urano *et al.* 2009; Erxleben *et al.* 2012; Obata *et al.* 2015). The early responses of certain metabolites might occur *via* affecting enzymatic activity rather than gene expression (Cramer *et al.* 2011). In this sense, Krasensky & Jonak (2012) stated that changes in central carbohydrate metabolism upon stress are complex and rapid, and it is highly conceivable that the highly elevated metabolites might not only act as osmolytes, but also in the stabilisation of proteins and membranes (Saradhi *et al.* 1995; Hong *et al.* 2000).

Concerning changes in sugars, it is well known that metabolism of starch is very sensitive to environmental conditions, and starch is rapidly metabolised to provide soluble sugars. Accordingly, it is highly probable that a massive degradation of starch occurred, followed by the accumulation of maltose, upon ABA and stress treatments, as reported by Krasensky & Jonak (2012) for the depletion of starch following salt stress and ABA treatment in *A. thaliana*. Also in *P. patens*, drought stress leads to increasing levels of maltose (Erxleben *et al.* 2012). In addition, Thalmann *et al.* (2016) found that ABA controls the activity of β-amylase1 (BAM1) and α-amylase3 (AMY3) under osmotic stress conditions in *A. thaliana*. Following the accumulation of maltose, it is likely that this serves as a precursor for both sucrose acting as a major osmolyte and fructose, which were found to accumulate at a later phase (24% and 61% increases, respectively).

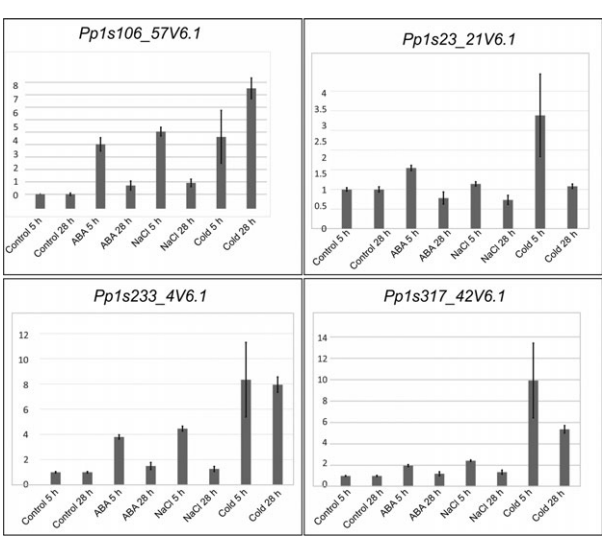

Fig. 5. Expression analysis of four beta-amylase genes (*Pp1s106_57V6.1, Pp1s23_21V6.1, Pp1s233_4V6.1, Pp1s317_42V6.1*) using quantitative RT-PCR from control plants and plants treated with ABA, or exposed to salt or cold stress for 5 and 28 h. The relative mRNA abundance was normalised to the *EF1*α transcripts. Error bars indicate SE calculated from three biological replicates.

Nagao *et al.* (2006) analysed soluble sugars using HPLC in *P. patens* treated with ABA and salt. They observed an approximately twofold increase of sucrose with ABA from 12 to 48 h, while salt stress caused an approximately 1.5-fold increase after 24 h. Upon ABA application we have observed 1.19-fold increase of sucrose after 5 h and 1.54-fold increase after 28 h, while salt treatment caused a 1.83-fold increase after 28 h. Thus, the overall changes in sucrose levels deduced in both cases showed similar increasing accumulations that do not differ drastically.

In addition to sugars mentioned above, the changes in galactinol and trehalose are interesting. Our results show that *P. patens* plants synthesize these in relatively low abundance, suggesting that they do not play an important role in osmoregulation, but rather seem to protect the plants from oxidative damage (Nishizawa *et al.* 2008). Trehalose levels increased after 5 h of ABA and salt treatments, which might signify its importance in slowing down protein aggregation and denaturation, as reported previously by Jain & Roy (2009).

The accumulation of amino acids might be crucial in lowering water potential in response to abiotic stresses, but also for the stabilisation of proteins, membranes and subcellular structures (Saradhi *et al.* 1995; Hong *et al.* 2000). The increase in the level of tryptophan deserves special attention. Earlier studies reported that ABA treatment induces tryptophan accumulation in *Hordeum vulgare* (Ogura *et al.* 2001), although the exact impact of such significant increases in tryptophan level is not yet clear. Tryptophan may have a major role in defence responses, as reported by Sanjaya *et al.* (2008) in *A. thaliana* upon exposure to cadmium stress. Further, it was reported that the enzymes involved in tryptophan biosynthesis are induced by stresses (Zhao *et al.* 1998) and biotic elicitors (Brader *et al.* 2001).

In addition to tryptophan, the significant increase in the level of ornithine and arginine, in particular after 28 h with all treatments, deserves further attention. It was reported that ornithine, as an intermediate compound in arginine biosynthesis, contributes to the production of compounds such as proline that serve as osmoprotectants (Kalamaki *et al.* 2009). However, our results clearly show that ornithine accumulates to high levels after 28 h of the treatments, whereas proline levels decline to levels even below those of control plants. This disconnection between the accumulation of ornithine and low proline levels might indicate that both ornithine and arginine serve roles other than proline biosynthesis. Such roles might render plants more tolerant to prolonged abiotic stresses and might act as storage or transport forms for nitrogen. Further, our results coincide with those of Yang & Kao (1999) who reported that the ornithine pathway seems to contribute little, if anything, to proline accumulation in water-stressed rice leaves. The results on proline accumulation deduced from our

study are not in line with a previous study performed in *P. patens* where proline was detected in higher amounts after drought treatment of gametophores (Erxleben *et al.* 2012). This difference might be due to the investigated tissue types since we analysed protonema tissue and Erxleben *et al.* only detected proline in stressed gametophores but could not detect it in protonema tissues. Moreover, important basic amino acids and tryptophan were not detected in the drought stress related study of Erxleben *et al.* (2012).

The third major trend is related to the accumulation of organic acids, in particular citric and pyroglutamic acids. In a study that assessed various cotton strains, it was reported that stressed plants showed several fold increases in organic acid content, with the most significant effect being the accumulation of citric acid (Timpa *et al.* 1986; Levi *et al.* 2011). As possible roles for organic acids, particularly citric acid, both osmotic adjustment and negative charge contributors for ionic balance and pH adjustment have been suggested (Guo *et al.* 2010). The only study in *P. patens* on metabolic changes upon drought stress (Erxleben *et al.* 2012) detected only a limited number of organic acids, but citric acid as well as pyroglutamic acid were not detectable, thus preventing a direct comparison with our study.

Based on the above results it is evident that *P. patens* responds to ABA treatment and abiotic stresses through increased biosynthesis of a large number of osmolytes (*e.g.* maltose, sucrose, citric acid, proline, tryptophan and ornithine). This wide and massive up-regulation of primary metabolites including major osmolytes might be related to the evolutionary position of mosses as the first plants colonising terrestrial habitats and representing the ancestors of higher plants. In contrast, the usual responses of higher plants to ABA treatment and abiotic stresses involve the up-regulation of a smaller set of osmolytes, mainly proline, glycine-betaine and disaccharides (McCue & Hanson 1992; Ishitani *et al.* 1995; Yoshiba *et al.* 1995). Rensing *et al.* (2007) stated that in this respect, mosses might have a different strategy to higher plants by being generalists, which allows them to grow in and withstand harsh habitats that are not readily accessible for seed plants. Furthermore, Knight *et al.* (1995) stated that responses of *P. patens* to stresses show remarkably high conservation with higher plants. In addition, it was reported that salt stress responses were conserved during the evolution of embryophytes (Richardt *et al.* 2010).

Despite the presumed 'generalist approach' mentioned above, a major set of compatible solutes, including GABA, glycine-betaine and polyamines (*e.g.* spermidine), were undetectable or not found to accumulate in this study of *P. patens*. The underlying reason might be related to the enzymes involved in biosynthesis of these compounds, and based on the current results, it remains to be shown if the biosynthetic pathways for these compounds in *P. patens* are simply inactive, missing or ABA-independent.

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This paper is dedicated to the memory of a former colleague Dr Basel Khraiwesh, who passed away recently. We gratefully acknowledge financial support by the German Research Foundation (DFG) to M.A.A., A.F and W.F. (SFB TR 175).

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1–S4. Histograms of mean normalised values of all metabolites (S1: amino acids, S2: organic acids, S3: sugars and sugar alcohols, S4: other compounds) detected in untreated controls and samples treated with ABA, salt or cold for 5 and 28 h. Error bars indicate SE calculated from five replicates. Dotted lines indicate adjusted scaling in order to fit individual values within the same graph.

Table S1. The characteristics of GC-TOF-MS metabolites.

Table S2. Oligonucleotides used in this study for quantification of the transcripts *via* qRT-PCR.

Table S3. Expression analysis of genes involved in the biosynthesis or degradation of differentially expressed metabolites in response to ABA, salt and cold treatment for 4 h.

Table S4. Normalised values of metabolites detected in all five replicates of each sample, and *t*-test *P*-values calculated from each comparison of untreated *P. patens* and *P. patens* treated with ABA, salt or cold for 5 and 28 h.

Table S5. List of metabolites representing the degree of overlap among samples treated with ABA, salt or cold for 5 and 28 h.

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