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ORIGINAL PAPER

Metabolite profiling of the moss *Physcomitrella patens* reveals evolutionary conservation of osmoprotective substances

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Abstract The moss *Physcomitrella patens* is suitable for systems biology studies, as it can be grown axenically under standardised conditions in plain mineral medium and comprises only few cell types. We report on metabolite profiling of two major *P. patens* tissues, filamentous protonema and leafy gametophores, from different culture conditions. A total of 96 compounds were detected, 21 of them as yet unknown in public databases. Protonema and gametophores had distinct metabolic profiles, especially with regard to saccharides, sugar derivates, amino acids, lignin precursors and nitrogen-rich storage compounds. A hydroponic culture was established for *P. patens*, and was used to apply drought stress under physiological conditions. This treatment led to accumulation of osmoprotectants, such as altrose, maltitol, ascorbic acid and proline. Thus, these osmoprotectants are not unique to seed plants but have evolved at an early phase of the colonization of land by plants.

Keywords Bryophyte · Drought stress · Lignins · Metabolomics · Osmoprotectant · Proline

Abbreviation
GC–MS Gas chromatography–mass spectrometry

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Introduction

The moss *Physcomitrella patens* is a plant model organism since it offers gene-targeting options for reverse genetics (Kamisugi et al. 2006; Strepp et al. 1998), has a simple single-cell layer morphology in well-characterised tissue types (Reski 1998) and the differentiation is controlled by plant hormones (Decker et al. 2006). Further, the *P. patens* genome was fully sequenced (Rensing et al. 2008), and is regarded as a stepping stone in plant evolution between algae and seed plants (Lang et al. 2008).

As a start into moss systems biology, studies on transcript profiling (Cuming et al. 2007; Richardt et al. 2010) and on proteomics (Heintz et al. 2004, 2006; Lang et al. 2011; Wang et al. 2009) have been performed, whereas knowledge about the metabolite composition of this species is still scarce (Schulte et al. 2006). Nevertheless, metabolite profiling is

essential, especially in plants, for an understanding of biological systems (Fernie et al. 2004; Weckwerth 2009).

It is known that bryophytes (liverworts and mosses) are rich in secondary metabolites (Krzaczkowski et al. 2008; McCleary et al. 1960; Sakai et al. 1988), some of them with commercial value (reviewed in Beike et al. 2010). Likewise, the analysis of the *P. patens* transcriptome revealed an extraordinary high percentage of gene products involved in secondary metabolism (Lang et al. 2005). None of these studies, however, described changing metabolite concentrations in response to developmental and/or environmental changes, although such studies would further our insights into metabolic adaptations during transition from water to land or during adaptation to abiotic stresses as previously shown in other plants (Hosp et al. 2007; Kaplan et al. 2004; Rizhsky et al. 2004). As a start into such analyses we report on metabolite profiling of two major *P. patens* tissues from different culture conditions revealing a remarkable conservation of osmoprotectants during land plant evolution.

Materials and methods

Plant materials

For subsequent analyses, the Gransden 2004 strain of *P. patens* (Hedw.) Bruch & Schimp. whose genome was fully sequenced (Rensing et al. 2008) was used. Protonemata were cultivated in liquid plain mineral medium (Knop medium) under standard conditions as described previously (Frank et al. 2005; Reski and Abel 1985). To establish gametophore cultures in liquid medium, gametophores grown on agar plates (see Frank et al. 2005 for details) were transferred to liquid medium and disrupted for 5 s with an Ultra-Turrax device (IKA, Staufen, Germany). The medium was renewed every 14 days. To set up an axenically grown hydroponic gametophore culture, protonema was transferred to a polypropylene gauze (mesh size 250 µm and thread size 215 µm; Zitt Thoma GmbH, Freiburg, Germany) that had previously been stringed on a glass ring of 4.5 cm in diameter. The ring was placed in an autoclavable Magenta® vessel with polycarbonate body and polypropylene closure (dimensions: 77 × 77 × 77 mm; Sigma-Aldrich, Seelze, Germany) containing liquid Knop medium taking care that the gauze was kept wet by the medium. Protonema was plated on the gauze 7 days after subculturing and was grown for 8 weeks by exchanging the medium weekly.

Protonema that mainly comprised of chloronema and caulonema cells, but only rarely buds, was harvested from nine biological replicates. Gametophores that had been growing for 8 weeks either in liquid medium or in hydroponic culture were harvested for five biological replicates each. Four biological replicates with hydroponic gametophore cultures were grown for 8 weeks and subjected to drought stress by lowering the water levels ~1 cm below the gaze for 2 weeks prior to harvest.

All samples were immediately frozen in liquid nitrogen. Before extraction of polar compounds, 50 mg fresh weight tissues were lyophilized for 3 days.

Plant extracts for metabolite identification

The extraction of polar compounds was carried out according to Fiehn (2006), with the following modifications: the lyophilized tissue was ground using a ball mill (TissueLyser, Qiagen, Hilden, Germany) for 90 s at 30 Hz. Polar compounds were extracted in 1.5 ml 87% methanol. Ribitol (Sigma, Deisenhofen, Germany) was added to each sample as internal standard. The samples were extracted for 15 min at 70°C and at 1,400 rpm on a thermoshaker (Thermomixer compact, Eppendorf, Hamburg, Germany). Insoluble tissue was removed by centrifugation (Centrifuge 5804R, Eppendorf, Hamburg, Germany) at 12,000×*g* for 5 min. 800 µl of the supernatant were dried under vacuum overnight. For derivatization 20 mg methoxyamine (Merck, Darmstadt, Germany) was dissolved in 1 ml pyridine (Merck, Darmstadt, Germany). To each sample 25 µl methoxyamine/pyridine was added and incubated for 90 min at 30°C and at 1200 rpm on a thermoshaker. Silylation was performed with 40 µl *N*-methyl-*N*-trimethylsilylfluoroacetamide (MSTFA, Sigma Deisenhofen, Germany) and incubation for 30 min at 37°C and at 1200 rpm (thermoshaker). For GC–MS analysis, 55 µl of the samples plus 25 µl alkane standard (dissolved in pyrimidin at final concentration at 0.25 mg/ml, Neochema, Bodenheim, Germany) solutions, which were added to provide retention time anchor points, were transferred into a 100 µl GC vial.

Mass spectrometric analysis

The derivatised samples were stored at least for 2 h before injection with an autosampler into a GC–quadrupole MS system (GC: 7890A; MS: 5975C; Agilent Technologies, Waldbronn, Germany) operating in electron impact ionisation mode. 1 µl of the extract was injected in splitless mode with an injector temperature of 230°C. Separation of metabolites was performed on a fused silica capillary column (column length 30 m, HP-5 ms; Agilent Technologies Waldbronn, Germany) coated with a 0.25 µm (5%-phenyl)-methylpolysiloxane stationary phase with a temperature gradient starting from 80°C and increasing by 5°C/min to 320°C. A mass to charge ratio range of 70–500 was scanned with the quadrupole mass detector at a rate of 12 scans/s. Detection of metabolites was performed by setting the ion source filament energy to 70 eV.

Data analysis

For raw data processing the GC–MS manufacturer's software was used. Data were deconvoluted and peak areas quantified using the AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). Peak identifications were carried out by matching retention indices and mass spectral similarity against the NIST 05 mass spectral library (http://www.nist.gov/srd/nist1a.cfm) and user-defined metabolite library based on the Golm metabolome database (Kopka et al. 2005; http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html). A minimum mass spectrum match factor of 75 was applied and the retention index window was set to 20 with a maximum penalty of 15. Only net match values >65 were considered. The following pure single compounds (Sigma-Aldrich, Seelze, Germany) were determined in external standard runs for the mass spectra comparison: altrose, fructose, galactose, isomaltose, maltose, sucrose, talose, galactinol, maltitol, myo-inositol, alanine, asparagine, glutamic acid, glutamine, glycine, norleucine, proline, serine, valine, acetic acid, ascorbate, butyric acid, citric acid, ocalic acid, malic acid, succinic acid, allantoin, glucose-6-phosphate, camposterol and stigmasterol.

A relative quantification of metabolite peaks was done by calculation of the area of the total ion signal. The area value for each metabolite was then normalized for the area measured for the internal ribitol standard and corrected for blank values where only solvents and derivatisation reagents were measured. If a metabolite was detected in three out of five (for the unstressed gametophore samples) or in six out of nine (for the protonema sample) biological replicates the metabolite was included in the calculation and comparison. Otherwise it was discarded as to be specific for the sample. For the stressed gametophores from hydroponic culture, the threshold was set to three out of four replicates. A mean area was calculated out of the replicates in one sample for a comparison of the metabolite contents between samples. To detect significant differences in the metabolite content between two samples, an ANOVA ($p < 0.05$) was performed.

Results and discussion

We compare metabolite profiles from *P. patens* protonema grown in liquid culture (Fig. 1a), gametophores grown in liquid culture (Fig. 1b), gametophores grown in hydroponic culture (Fig. 1c), and from gametophores after 2 weeks of physiological drought stress. In total, 96 different polar compounds could be detected by relative matching with mass spectra libraries and the retention index (RI) (Table 1). This number is in the range found with similar protocols in seed plants (Hosp et al. 2007; Kaplan et al. 2004; Roessner et al. 2001). From the total of 96 different compounds, 91 were found in unstressed moss tissues, whereas 5 were specific for drought-stressed gametophores.

Protonema

From 96 detected compounds, 49 appeared in protonema from which 23 metabolites were found exclusively in this tissue and not in gametophores. Differences in the metabolite spectra of the distinct tissue types were detectable in the sugar and amino acid composition as well as for compounds belonging to the energy metabolism (Figs. 2, 3; Table 1).

Protonemata contain the monosaccharides, such as glucose, galactose and galactopyranoside but lack the entire set of disaccharide sugars except isomaltose. In addition, five polyhydroxy acids like galactonic and gluconic acid were detected in protonema but not in gametophores. Since the generation of such oxidised sugars is dependent on strong oxidising agents (Ioannidi et al. 2009), these findings reveal physiological differences between protonemata and gametophores.

Statistical analyses of the amino acid amounts in the various tissue samples revealed that there are also significant differences between protonema and gametophores from liquid cultures for the amount of asparagine, serine and norleucine, the content in the first two being higher in the protonema than in the gametophores.

Among the metabolites of the energy metabolism the cyclic amine pyrrolidine, also known as tetrahydropyrrole,

Fig. 1 Culture conditions used for metabolite profiling: protonema tissue in liquid culture (**a**), gametophores in liquid culture (**b**) and gametophores in hydroponic culture (**c**). *Scale bars* 1 cm

Table 1 List of metabolites detected in *Physcomitrella patens* under different culture conditions and under drought stress

	RI	KEGG ID	Protonema	Gametophores from			*q*-val
				Liquid culture	Hydroponic culture	Drought stress	
Sugars							
Allose[a]	1871	C01487		1.18E−03	1.88E−03		
Altrose[a]	1896	C06464				2.14E−03	
Fructose	1874/2318	C01496		1.24E−02	2.00E−02	1.23E−02	
Galactose	1984	C01582	2.23E−02		7.27E−03		
Glucopyranoside, alpha-D-	1880		3.12E−01			1.64E−01	
Glucose	1890/2323	C00293	2.71E−02	2.35E−02	1.78E−02		
Gulose[a]	1878	C06465		2.09E−03	4.42E−03	5.58E−03	
Idose	1907	C06466			2.25E−03		
Isomaltose	2883	C00252	1.15E−03		1.55E−03	1.14E−03	
Lactose	2690	C00243		1.63E−03	1.32E−03		
Maltose	2766	C00208		8.96E−04	6.73E−04	7.26E−03	
Sucrose	3035/3075	C00089		1.15E−03			
Talose	1902	C06467		5.80E−03	7.91E−03		
Trehalose	2744	C01083		4.61E−02	8.39E−02		
Polyhydroxy acids							
Allonic acid	1962		5.19E−04				
Galactonic acid	1992	C00880	3.96E−03				
Gluconic acid	1997	C00257	3.83E−03				
Mannonic acid	2012		1.16E−03				
Xylonic acid	1705		1.10E−03				
Sugar conjugates							
Galactinol	2986	C01235		4.48E−03	1.08E−03		
Galactosylglycerol[a]	2310			1.73E−03			
Maltitol	2836					8.47E−04	
Xylitol	1717	C00379	1.10E−003				
Polyols							
Hexitol	1920			5.14E−04	6.04E−04		
Myo-inositol	2091	C00137	5.54E−02	1.90E−02	1.68E−02	1.15E−02	
Pentitol	1700		1.81E−03	2.12E−03	1.10E−02		
Threitol	1494	C16884	5.69E−04		9.61E−04		
Amino acids							
Alanine, L-	1382	C00041	3.68E−03	1.34E−03	1.90E−03		
Asparagine, L-	1681/1871	C00152	3.56E−03	1.03E−03	8.85E−04		a, b
Aspartic acid, L-	1519/1739	C00049	1.17E−02	7.78E−03	3.84E−03	4.38E−03	c
Butyric acid, 4-amino-[a]	1327	C00334		1.12E−03			
Glutamic acid, L-	1626	C00025		6.75E−03	4.56E−03		
Glutamine, L-	1782	C00064			4.43E−03	5.48E−03	
Glycine	1328	C00037	8.99E−03	2.77E−03	8.34E−04		
Norleucine, L-[a]	1352	C01933	3.05E−02	4.98E−03		6.01E−03	a, c
Norvaline, L-	1260	C01826	6.63E−04				
Proline, L-	1324	C00148				7.09E−03	
Serine, L-	1380	C00065	3.04E−02	1.32E−02	6.57E−03	9.86E−03	a, b, c
Threonine, L-	1321/1403	C00188	6.38E−02	1.89E−01	8.26E−02	1.60E−02	a, e
Tryptophan, L-	2226	C00078	1.10E−03				
Valine, L-	1222	C00183	2.23E−03				

Table 1 continued

	RI	KEGG ID	Protonema	Gametophores from			*q*-val
				Liquid culture	Hydroponic culture	Drought stress	
Acids							
Acetic acid	1830	C00033	3.02E−04		4.36E−04		
Aminohexanoic acid, 6-hydroxy-2-	1636		3.05E−02			7.38E−02	
Ascorbic acid	1944	C00072	1.22E−03			5.50E−04	
Butyric acid	1626	C00246				2.15E−03	
Caffeoylquinic acid	3116			1.19E−02	1.61E−02	8.15E−03	
Caffeic acid	2141	C01197		5.35E−04	4.89E−04		
Citric acid	1820	C00158	5.11E−03	2.39E−02	7.19E−03		
Dehydroascorbic acid[a]	1857	C05422			9.71E−04		
Erythronic acid	1541		5.19E−02	7.95E−02	4.23E−02	4.84E−02	
Erythro-pentonic acid	1543		6.01E−04				
Oxalic acid	1122	C00209		4.39E−03	1.71E−03	6.79E−03	
Isocitric acid	1824	C00311		3.81E−03	1.01E−03		
Maleic acid	1333	C01384		4.84E−04	1.41E−02		
Malic acid	1486	C00711	2.13E−01	2.60E−01	4.71E−02	4.53E−02	
Pentonic acid[a]	1768			4.75E−04			
Piperidine carboxylic acid, 2-[a]	1363	C00408	2.10E−03				
Malonic acid	1221	C00383		4.51E−04			
Propanoic acid	1197	C00163		3.55E−03			
Pyroglutamic acid	1530	C01879	1.57E−02	1.37E−02	9.15E−03		
Pyrrolidone carboxylic acid, 2-	1534		1.86E−02				
Succinic acid	1346	C00042	6.46E−03	7.38E−02	1.22E−03		a, d
Tetracosanoic acid	2835	C08320	1.27E−02				
Threonic acid[a]	1569	C01620	4.41E−02	5.05E−02	7.48E−04		
N-compounds							
Allantoin[a]	2093	C01551	2.56E−02	3.44E−02	1.77E−02	6.47E−03	
Nicotianamine	1472	C05324		1.84E−03	8.20E−04		
Urea	1272	C00086		6.36E−03	9.56E−03		
Cyclic amines							
Porphine	1002			3.15E−03	2.39E−03		
Pyrrolidine	1141		2.88E−03				
Phosphates							
Glucose-6-phosphate	2332	C00092	2.03E−03	1.54E−03	1.25E−03		
Myo-inositol phosphate	2475	C01177	3.34E−03				
Phosphoric acid	1292	C00009	3.72E−02			2.18E−02	
Lactone							
Furanone, 2(3H)-	1778	C08571	1.31E−03				
Others							
Butanal	1375	C01412	2.85E−03				
Isophosphinoline	1834			1.53E−03	1.51E−03		

	RI	Protonema	Gametophores from			*q*-val
			Liquid culture	Hydroponic culture	Drought stress	
Unknown compounds						
EITTMS_N12C_ATHL_3499.7_1135EC24_	3502		4.55E−03			
EITTMS_N12C_ATHR_1358.9_1135EC06_	1356		6.72E−03			
EITTMS_N12C_ATHR_1440.5_1135EC06_D3	1443	6.59E−03	5.15E−04			

Table 1 continued

	RI	Protonema	Gametophores from			*q*-val
			Liquid culture	Hydroponic culture	Drought stress	
EITTMS_N12C_ATHR_1967.6_1135EC44_G	1967	1.12E−03	1.44E−03	1.27E−03		
EITTMS_N12C_ATHR_2055.3_1135EC44_[a]	2048		2.11E−03			
EITTMS_N12C_ATHR_2057.9_1135EC44_	2059		2.55E−03			
EITTMS_N12C_ATHR_2062.8_1135EC44_	2065		9.60E−04			
EITTMS_N12C_ATHR_2491.7_1135EC44_	2495		2.33E−04			
EITTMS_N12C_ATHR_2777.5_1135EC44_	2777		4.28E−01	2.41E−01		
EITTMS_N12C_ATHR_2914.2_1135EC44_	2916		4.44E−04			
EITTMS_N12C_NTAR_1969.2_1135EC27_G	1973		8.42E−02	3.50E−02		
EITTMS_N12C_STUL_2725.5_1135EC28_G	2727	9.32E−04	2.72E−04	8.89E−06		
EITTMS_N12C_STUR_2277.7_1135EC29_[a]	2287		1.99E−03	1.18E−03	1.21E−03	
EITTMS_N12C_ATHR_2072.0_1135EC25_	2072			1.73E−03		
EITTMS_N12C_NTAL_2505.0_1135EC26_	2504			1.34E−03		
EITTMS_N12C_STUO_2759.3_1135EC31_	2759			6.74E−04		
EITTMS_N12C_STUR_1832.7_1135EC29_	1834			8.09E−04		
EITTMS_N12C_ATHR_2027.2_1135EC44_	2029	1.00E−03				
EITTMS_N12C_ATHR_2066.3_1135EC44_[a]	2066	7.93E−04		5.14E−04		
EITTMS_N12C_ATHR_2103.6_1135EC44_	2102	7.22E−04				
EITTMS_N12C_ATHR_2988.6_1135EC44_	2992				3.64E−02	

Unknown metabolites refer to mass spectra, which matched with non-identified compounds from the Golm metabolome database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html). They are listed with the names from the Golm metabolome database for data completeness. *E*-values correspond to mean peak areas from GC–MS analyses of metabolites. Significant differences in metabolite content between two samples (quantification values, *q*-val) were quantified using ANOVA ($p < 0.05$). (a) Protonema versus liquid gametophores, (b) protonema versus hydroponic gametophores, (c) protonema versus drought-stressed gametophores, (d) liquid versus hydroponic gametophores, (e) liquid versus drought-stressed gametophores, (f) hydroponic gametophores versus drought-stressed gametophores

RI retention index of the analyte(s)

[a] Net match values amounted to between 65 and 75; for all other compounds net match values were >75

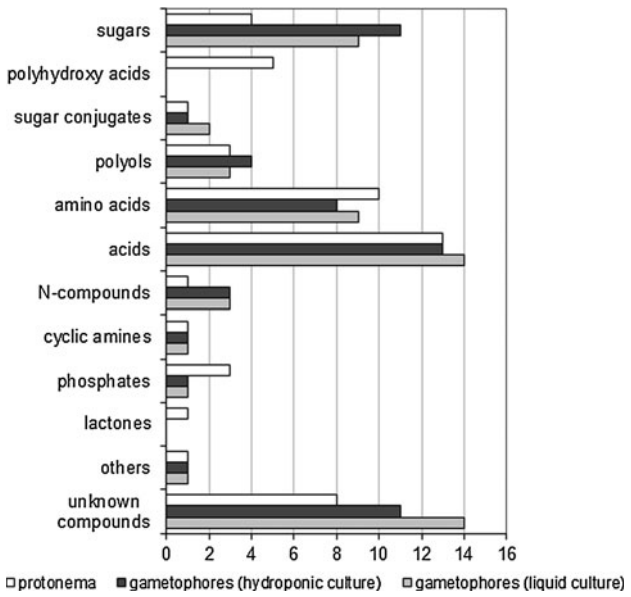

Fig. 2 *Bar chart* showing the number of metabolites identified in protonema and gametophores tissues grouped in functional classes

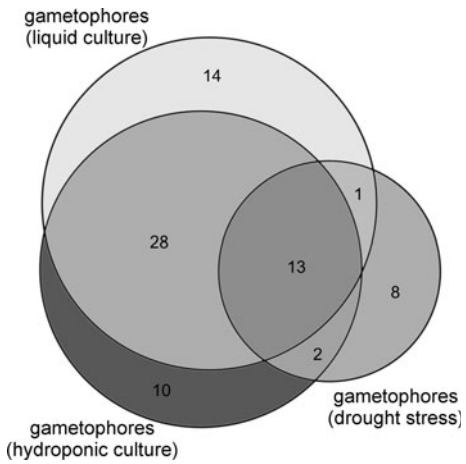

Fig. 3 *Venn diagram* showing the number of metabolites in the three tested culture conditions of gametophore tissues

was only detected in protonema. This compound had been found in leaves of tobacco and carrot. Its ring structure is present in numerous natural alkaloids such as nicotine and

is also a central structure in the amino acids proline and hydroxyproline (Kajikawa et al. 2009).

Gametophores

While protonemata grow in close contact with the soil, gametophores erect in the air and are therefore more exposed to abiotic stresses. In line with this, the metabolite spectra from protonemata and gametophores differed markedly. Intriguingly, the profiles of gametophores were rather similar to one another, independent from culture conditions (Fig. 3; Table 1). From gametophores grown in liquid culture 56 metabolites were detected while in hydroponically cultured gametophores 53 metabolites were found. Several compounds specific to gametophores have strong osmoprotectants activity.

We detected in gametophores many neutral soluble monosaccharides (allose, fructose, glucose, gulose and talose) and disaccharides (lactose, maltose and trehalose). Interestingly, glucose was specific for gametophores grown in liquid, whereas galactose, idose and isomaltose were found in gametophores from hydroponic culture.

Two other sugar alcohols, galactinol and galactosylglycerol, were detected in the liquid gametophore culture of *P. patens* while galactinol was also detected in the hydroponic gametophore samples. Although found in the unstressed tissue samples, galactinol which is an immediate precursor of the sugar raffinose, has a protective role against oxidative damage in plants (Nishizawa et al. 2008) and is involved in heat and cold stress responses (Kaplan et al. 2004).

In addition, the sugar alcohols or polyols, such as hexitol, pentitol, threitol and myo-inositol were found in many gametophore samples. Their specific role in osmoprotection has been reported for many seed plants (Hoekstra et al. 2001) and led to metabolic engineering with the goal to enhance stress tolerance in seed plants by targeted accumulation of these compounds (Rontein et al. 2002). Recently, the involvement of components of the carbohydrate metabolism like glucose-phosphate, myo-inositol-phosphate, myo-inositol and galactinol during starch degradation and raffinose production were shown to be responsible for thermotolerance in seed plants, independent of heat and cold acclimation processes (Guy et al. 2008). The accumulation of some of these metabolites can be observed in the unstressed gametophore samples, but neither in the protonema nor in the stressed gametophore sample. The presence and accumulation of a large amount of glycolytic intermediates and sugar phosphates in the unstressed samples is more likely to be related to their important function in metabolism and for signalling processes than a result of their mere (osmo-) protective function.

A large portion of identified metabolites belong to pathways of energy metabolisms. Known from the citrate cycle, citric and isocitric acid as well as succinic acid were found in the unstressed tissue samples of gametophores and protonema, but not in drought-stressed gametophores. The highest amounts of these metabolites were found in gametophores grown in the liquid culture suggesting that the rates of carbohydrate and lipid oxidation under these conditions are elevated. The same is true for malic acid, which is found in all the analysed tissue samples. Several other acids measured in the samples are known to evolve during the breakdown of carbohydrates (e.g. oxalic acid, threonic acid), amino acids or fatty acids (e.g. propanoic acid). Overall, the amount of such metabolites was highest in the liquid gametophore cultures and lowest in the gametophore subjected to drought stress. Among the detected compounds are also ascorbic acid and its oxidised form dehydroascorbic acid. Both these metabolites are sugar acids and have potential antioxidant capacities (Smirnoff and Wheeler 2000; Hoekstra et al. 2001; Ioannidi et al. 2009). Wolucka and van Montagu (2003) postulated that the de novo synthesis of ascorbic acid (vitamin C) could be achieved via the gulose pathway. This monosaccharide is present in all gametophore samples but not in the protonema.

Interestingly, several nitrogen-rich compounds were measured in unstressed gametophores such as urea, nicotianamine and the ureide allantoin, the latter one also being found in the drought-stressed gametophores as well as in protonema. Urea and allantoin are involved in the purine metabolism and, as already shown for legumes, could play an important role in nitrogen metabolism (Filippi et al. 2007). Nicotianamine on the contrary might function as an Fe(II) scavenger to protect cells from oxidative stress (von Wiren et al. 1999) and has been demonstrated to play an essential role in growth, flower development and fertility of seed plants (Takahashi et al. 2003). Thus, it may be involved in fertility of mosses also.

One compound, caffeoylquinic acid, is a cinnamate conjugate derived from the phenylpropanoid pathway. One of them, chlorogenic acid, has been shown to be an intermediate in the biosynthesis of lignin (Damiani et al. 2005; Mondolot et al. 2006; Xu et al. 2009; Weng and Chapple 2010). The presence of chlorogenic acid in moss (e.g. sphagnum) has been reported previously (Montenegro et al. 2009). From an evolutionary point of view *P. patens* is the first land plant which possess the complete lignin biosynthesis pathway with nine out of ten gene families present in its genome except from the ferulate-5-hydroxylase (Xu et al. 2009).

Another compound, caffeic acid, is a subunit in the synthesis of a special group of phenolic constituents, the lignans, described so far in liverworts and hornworts (Mues 2000; Scher et al. 2003). At least three caffeic acid *O*-methyltransferases are encoded by the *P. patens* genome

which might suggest the existence of lignans or other derivates of lignins. Until further evidence, the presence of lignin itself in mosses remains a matter of debate (Xu et al. 2009). In line with the erect nature of gametophores, caffeoylquinic acid and caffeic acid were detected in gametophores but not in protonema.

Drought stress

In gametophores, which were grown 2 weeks under physiological drought stress, 26 metabolites were detected; five (altrose, maltitol, L-proline, butyric acid, one unknown compound) of them specific for these culture conditions (Figs. 2, 3; Table 1).

A larger number of sugars than in the protonema were found in the stressed gametophores, e.g. the monosaccharides fructose, gulose and altrose, and the disaccharides maltose and isomaltose. Both disaccharides probably derived from the degradation of starch under drought stress. Rizhsky et al. (2004) showed that almost all these sugars accumulate in stressed *Arabidopsis* leaves. Sugars can act as a water substitute by satisfying the hydrogen-bonding requirement of polar groups on the surface of proteins, thus maintaining their native folding and activity (Crowe et al. 1987; Hoekstra et al. 2001). The important antioxidant and osmoprotectant role of carbohydrates might also result in the induction of maltitol, a sugar conjugate only detected in the stressed gametophores.

The detection of proline in the drought-stressed gametophores is an additional indication of severe stress. Already Handa et al. (1986) showed in tomato cells that the proline content was not only dependent on cell water potential but also on cell osmotic potential. The accumulation of proline is, therefore, a physiological alternative used by many organisms such as plants and yeast to overcome drought and other stresses (Takagi 2008; Treichel et al. 1984; Yoshiba et al. 1997) and an applicable metabolic engineering strategy to confer osmotolerance in plants (Kishor et al. 1995; Nuccio et al. 1999). The fact that the stressed plants accumulate proline but not sucrose is a strong indication of a drought specific response, since Rizhsky et al. (2004) could show that sucrose does only accumulate when drought and heat stress are combined. Other amino acids such as glutamine and glutamic acid have also been reported to be involved in stress resistance (Harrigan et al. 2007; Levi et al. 2011). We found both metabolites exclusively in gametophores, independent of culture conditions.

Unknown metabolites

Out of 96 polar metabolites detected in this study, 21 could not be identified (Fig. 2; Table 1); i.e. their mass spectra matched with database entries from the Golm metabolome database, but the database spectra belonged to non-identified compounds. At present it is not possible to assign these compounds to any specific class or function with the GC–MS analysis applied here. It is clear, however, that among these unknown compounds there are metabolites that appear in specific tissues or under specific culture conditions. The unknown compounds have been previously detected in leaves or roots of *A. thaliana*, *Solanum tuberosum* or *Nicotiana tabacum* and hence, their identification and assignment to metabolic pathways might provide additional insights into the evolution of metabolic pathways in plants.

Conclusion

Our metabolite profiling study is the first comprehensive analysis of metabolites from the moss *P. patens*. Despite a methodologically-driven bias towards polar compounds we found significant differences between different moss tissues and culture conditions. A whole set of metabolites involved in specific metabolic and signalling processes can now be combined with data from transcript profiling and quantitative proteomics.

The analysis of gametophores after drought stress unravelled a significant number of osmoprotective metabolites that only appear under such conditions. These are mainly in line with findings from seed plants revealing that the molecular mechanisms required to conferring stress tolerance evolved already during the conquest of land by plants.

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