The capability to synthesize phytochelatins and the presence of constitutive and functional phytochelatin synthases are ancestral (plesiomorphic) characters for basal land plants

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Abstract

Bryophytes, a paraphyletic group which includes liverworts, mosses, and hornworts, have been stated as land plants that under metal stress (particularly cadmium) do not synthesize metal-binding peptides such as phytochelatins. Moreover, very little information is available to date regarding phytochelatin synthesis in charophytes, postulated to be the direct ancestors of land plants, or in lycophytes, namely very basal tracheophytes. In this study, it was hypothesized that basal land plants and charophytes have the capability to produce phytochelatins and possess constitutive and functional phytochelatin synthases. To verify this hypothesis, twelve bryophyte species (six liverworts, four mosses, and two hornworts), three charophytes, and two lycophyte species were exposed to 0–36 μM cadmium for 72 h, and then assayed for: (i) glutathione and phytochelatin qualitative-quantitative content by HPLC and mass spectrometry; (ii) the presence of putative phytochelatin synthases by western blotting; and (iii) in vitro activity of phytochelatin synthases. Of all the species tested, ten produced phytochelatins in vivo, while the other seven did not. The presence of a constitutively expressed and functional phytochelatin synthase was demonstrated in all the bryophyte lineages and in the lycophyte Selaginella denticulata, but not in the charophytes. Hence, current knowledge according to phytochelatins have been stated as being absent in bryophytes was therefore confuted by this work. It is argued that the capability to synthesize phytochelatins, as well as the presence of active phytochelatin synthases, are ancestral (plesiomorphic) characters for basal land plants.

Key words: Bryophytes, cadmium, charophytes, glutathione, hornworts, liverworts, lycophytes, metals, mosses, phytochelatins, phytochelatin synthase.

Introduction

Phytochelatins (PCs) are thiol-peptide compounds whose general structure is (γ-glutamate–cysteine)_n–glycine, with n usually ranging from 2 to 5 (Grill et al., 1985). Due to the thiol group of cysteine residues, PCs can bind cadmium (Cd) and other thiolphilic metals, and prevent them from circulating in the cytosol (Grill et al., 1985). PCs are synthesized from reduced glutathione (GSH), by means of the constitutively expressed cytosolic enzyme phytochelatin synthase (PCS), a γ-glutamylcysteine dipeptidyl transpeptidase (EC 2.3.2.15) (Grill et al., 1989; Vatamaniuk et al., 2004), belonging to clan CA of the papain-like cysteine proteases (Vivares et al., 2005; Romanyuk et al., 2006; Rea, 2012).

It is now well known that higher plants (Sanità di Toppi and Gabbielli, 1999; Rea, 2012), a number of marine and
freshwater algae (Chlorophyta, Chrysophyta, Phaeophyta, Rhodophyta, etc.) (Gekeler et al., 1988; Pawlik-Skowrońska et al., 2007), some fungi (Gekeler et al., 1989; Kneer et al., 1992; Bolchi et al., 2011),lichens (Pawlik-Skowrońska et al., 2002), and even some animal species (Clemens et al., 2001; Vatamaniuk et al., 2001) do actually produce PCs in response to metal stress, particularly Cd.

In contrast, it has been recognized until now that the earliest diverged extant land plants, collectively referred to as bryophytes [liverworts (Hepatophyta, sin. Marchantiophyta), mosses (Bryophyta), and hornworts (Anthocerotophyta) (Vanderpoorten and Goffinet, 2009)] do not synthesize PCs under metal stress (Bruns et al., 1999, 2001; Rother et al., 2006; Leinenweber et al., 2009; BLEUEL et al., 2011). As a matter of fact, the ‘model’ moss Physcomitrella patens, at present the only bryophyte with an entirely sequenced genome (Rensing et al., 2008), lacks the PCS gene (Kopriva et al., 2007).

However, based on the limited number of species investigated (mainly mosses, very few liverworts, and no hornworts), the conclusion concerning a generalized absence of PCs in bryophytes appears to be quite premature and not aprioristically transferable tout court to all these plants. Indeed, with regards to liverworts, the very preliminary screening carried out by Gekeler et al. (1989) proved the thalloid species Marchantia polymorpha to be a weak producer of PCs under Cd stress. Such an interesting experimental result, however, was subsequently contradicted by Bruns et al. (2001), who exposed M. polymorpha and Pellia epiphylla to Cd for up to 10 d, and did not detect any PC synthesis. Likewise, although the presence of PCs in the metal-exposed freshwater moss Rhynchosostegium riparioides and in the liverwort Lunularia cruciata was considered likely (Jackson et al., 1991; Carginale et al., 2004), no unequivocal proof of PC synthesis was ever provided in these species.

Recent studies have acquired a large body of evidence to support the hypotheses that: (i) land plants, and particularly bryophytes, have a charophytic ancestry [given in particular by the Charophyta orders Charales, Coleochaetales, and Zygnematales (McCourt et al., 2004; Qiu et al., 2006; Qiu, 2008; Becker and Marín, 2009; Wodniok et al., 2011)]; and (ii) hornworts are the sister group of tracheophytes (Qiu et al., 2006; Qiu, 2008; Villarreal et al., 2010; LIGRONE et al., 2012). Concerning these points, with regard to charophytic algae, the synthesis of PCs has until now been definitely detected just in one species, namely Micrasterias denticulata (Volland et al., 2013), but no further investigation on PC synthesis occurrence in other charophytes, more phylogenetically close to (early) land plants, has been carried out. Secondly, no unequivocal data on PC production in early tracheophytes, namely lycophytes (Lycophyta), have been published, and the experiments performed by Gekeler et al. (1989) on Cd-induced PCs in Lycopodium clavatum and Selaginella viticulosa have not received solid confirmation in other lycophytes.

Interestingly, it has been well documented that the Early Devonian Rhynie Cherts of NE Scotland (~396±12 Mya) contains the earliest and best preserved bryophytic and vascular flora (Rice et al., 1995; Kenrick and Crane, 1997; Pires and Dolan, 2012). This ancient site featured high levels of arsenic (As), mercury (Hg), gold (Au), zinc (Zn), etc., all PC inducers to varying extents (Zenk, 1996, and references therein). In view of the above, the present work assumed the main hypothesis that the capability to produce PCs and the constitutive presence of functional PCS(s) enzyme(s) might represent useful conserved traits for all bryophyte lineages. A fortiori, the general reliance of bryophytes on aerial fallout for mineral nutrition, and hence their potential vulnerability to heavy metal pollution (Tyler, 1990; Harmens et al., 2012, and references therein), demand further caution in generalizing conclusions about an overall lack of PCs (and PCds) in these plants.

To verify this hypothesis, investigation was focused not only on mosses, the most studied group within bryophytes, but also on liverworts, placed in the basal almost position amongst land plants (Qiu et al., 2006; Qiu, 2008; CRANDALL-STOTLER et al., 2009), and on hornworts, sisters of tracheophytes (Qiu et al., 2006; Qiu, 2008; Becker and Marín, 2009; Wodniok et al., 2011), and in lycophytes, a very ancient lineage that diverged shortly after evolution of vascular tissues in land plants, and also present in the Early Devonian Rhynie Cherts (Rice et al., 1995; Kenrick and Crane, 1997; Pires and Dolan, 2012).

To provide a clear understanding of the taxa investigated in this work, a simplified cladogram is shown in Fig. 1.

Materials and methods

Experimental set-up and species employed

The material employed in the experiments [charophytes; bryophyte gametophytes; lycophyte sporophytes (roots)] consisted of non-axenic cultures (see the species list below), collected in Italian locations (Apuan Alps, Massucceci Lake, surroundings of Parma, and Salerno) in February–October of 2012 and 2013. In addition, specific monocultures of Coleochaete scutata were obtained from the Carolina Biological Supply Company, Burlington, NC, USA, and set up as the other cultures. All material was abundantly rinsed with double-distilled water, carefully checked for the absence of all potential soil/biological contaminants and endosymbiotic organisms (i.e. Cyanobacteria, Glomeromycota, etc.) under a stereomicroscope (WILD, Heerbrugg, Switzerland) and an Olympus BH2 microscope ×40 (Olympus Italia, Segrè, Italy), and then independently cultivated as specific monoculture in a growth chamber under the conditions detailed below.

All together, 17 species were investigated (Fig. 1): three charophytes (Charophyta), namely Spirogyra sp. Link, Chara vulgaris L., and Coleochaete scutata Bréb.; six liverworts (Hepatophyta), namely Conocephalum conicum (L.) Dumort., Marchantia polymorpha L., Pellia epiphylla (L.) Corda, Radula complanata (L.) Dumort., Aneura pinguis (L.) Dumort., and Scaupania undulata (L.) Dumort.; four mosses (Bryophyta), namely Sphagnum palustre L., Polytrichastrum formosum (Hedw.) G.L.Sm., Hypnum cupressiforme Hedw., and Fontinalis antipyretica Hedw.; two hornworts (Anthocerotophyta), namely Athroceros punctatus L. and Phaeoceros laevis (L.) Prosk.; and two lycophytes, namely Huperzia selago (L.) Bernh. ex Schrak. and Mart. and Selaginella denticulata (L.) Spring. As reference organisms for testing PC production and PCs presence/size/activity, monocultures of Nostoc sp. (Cyanobacteria), Physcomitrella patens (Bryophyta), and Arabidopsis thaliana (Magnoliophyta) in vitro...
plants were grown in the same controlled conditions as the above material.

After selection and cleaning, the youngest parts of the samples (n ranging from 6 to 24; Table 1) were transferred into sterilized plant culture vials (Phytacon™, filled with 100 ml of sterile modified Chiaudani–Vighi (Chiaudani and Vighi, 1977) culture medium (pH 6.0), with a final composition as follows: NaNO₃ 2.55 mg l⁻¹, K₂HPO₄ 0.10 mg l⁻¹, MgCl₂ 0.57 mg l⁻¹, MgSO₄·7H₂O 2.94 mg l⁻¹, CaCl₂·2H₂O 0.44 mg l⁻¹, NaHCO₃ 1.50 mg l⁻¹, FeSO₄ 5.58 µg l⁻¹, H₂BO₃ 18.55 µg l⁻¹, MnCl₂ 26.43 µg l⁻¹, ZnCl₂ 3.28 µg l⁻¹, CoCl₂ 80 ng l⁻¹, CuCl₂ 0.9 ng l⁻¹, Na₂MoO₄·2H₂O 730 ng l⁻¹, omitting the ethylenediaminetetraacetic acid (EDTA) in order to avoid undesirable Cd chelation. Then, all vials were placed in a growth chamber at 20 ± 1 °C, under a photoperiod of 16 h light [photosynthetic photon flux density (PPFD) 60 µmol m⁻² s⁻¹/8 h dark. With regard to the species to be analysed for their thiol-peptide content and PCS inactivation, half of the samples were treated at 72 h with 36 µM Cd, in the form of 3CdSO₄·8H₂O (C. conicum also with 72 µM Cd and for 144 h), whereas the other half (controls) were given deionized water in an identical volume as the Cd solution. Moreover, the bryophyte cultures were daily sprayed with Cd at the same concentration as that in the medium (as well as deionized water in controls), because of their well-known capability to absorb elements also from the atmosphere (Tyler, 1990). At the end of the treatments, all plants were carefully rinsed with deionized water, gently blotted dry with filter paper, wrapped in aluminium foil, frozen in liquid nitrogen, and briefly stored at −80 °C for further analyses.

**Thiol-peptide characterization by mass spectrometry**

The identity of putative GSH and PCs was verified by electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS). Liquid chromatographic elution was carried out using post-column derivatization with 300 µM Ellman’s reagent (5,5’-dithiobis(2-nitrobenzoic acid); DTNB) (Sigma-Aldrich), detected at 412 nm (PerkinElmer detector mod. 200), and quantified by calibration curves for standard SH groups. Identification of GSH and individual PCs was based on the comparison of their retention times with standard GSH (Merck, Darmstadt, Germany) and PC samples from *Silene vulgaris*.
Table 1. Content of glutathione (GSH) and phytochelatins (PCs) in charophytes, bryophytes (gametophytes), and lycophytes (roots), non-exposed (control) or exposed to 36 μM Cd for 72 h (in Conocephalum conicum also 36 μM Cd for 144 h and 72 μM Cd for 72 h)

GSH and PC concentrations (means ± SE) are expressed in nmol g⁻¹ FW.

<table>
<thead>
<tr>
<th>Charophytes</th>
<th>GSH (control)</th>
<th>GSH (Cd-exposed)</th>
<th>PC₃ (Cd-exposed)</th>
<th>PC₄ (Cd-exposed)</th>
<th>PC₅ (Cd-exposed)</th>
<th>Total PCs (Cd-exposed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spirogyra sp. (n=10+10)</td>
<td>105.5±9.7</td>
<td>144.7±16.2</td>
<td>2.7±0.9</td>
<td>1.9±0.7</td>
<td>ND</td>
<td>4.6±0.8</td>
</tr>
<tr>
<td>Chara vulgaris (n=10+10)</td>
<td>92.5±10.5</td>
<td>99.7±7.5</td>
<td>4.1±0.3</td>
<td>3.8±0.6</td>
<td>2.7±0.7</td>
<td>10.6±0.8</td>
</tr>
<tr>
<td>Coleochaete scutata (n=5+5)</td>
<td>222.3±12.1</td>
<td>212.2±7.7</td>
<td>9.5±0.4</td>
<td>16.8±1.2</td>
<td>14.1±0.7</td>
<td>40.4±0.8</td>
</tr>
<tr>
<td>Liverworts</td>
<td></td>
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<tr>
<td>Conocephalum conicum (n=6+6)</td>
<td>1.4±0.2</td>
<td>6.8±2.4**</td>
<td>6.1±3.5</td>
<td>1.2±0.5</td>
<td>0.8±0.3</td>
<td>8.1±1.4</td>
</tr>
<tr>
<td>Marchantia polymorpha (n=4+4)</td>
<td>2.9±0.2</td>
<td>3.6±0.8</td>
<td>1.8±0.4</td>
<td>1.3±0.9</td>
<td>ND</td>
<td>3.1±0.6</td>
</tr>
<tr>
<td>Pellia epiphylla (n=3+3)</td>
<td>132.2±36.8</td>
<td>89.4±26.5</td>
<td>1.0±0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Radula complanata (n=3+3)</td>
<td>2.7±1.1</td>
<td>6.3±2.1</td>
<td>1.2±0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Scapania undulata (n=3+3)</td>
<td>20.5±3.5</td>
<td>17.8±2.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Mosses</td>
<td></td>
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</tr>
<tr>
<td>Sphagnum palustre (n=6+6)</td>
<td>23.4±3.1</td>
<td>34.2±2.1*</td>
<td>4.5±0.3</td>
<td>5.8±0.2</td>
<td>3.0±0.7</td>
<td>13.3±0.8</td>
</tr>
<tr>
<td>Polytrichastrum formosum (n=4+4)</td>
<td>121.7±12.7</td>
<td>367.7±25.6**</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hypnum cupressiforme (n=4+4)</td>
<td>302.9±46.1</td>
<td>123.6±28.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fontinalis antipyretica (n=3+3)</td>
<td>25.7±7.0</td>
<td>148.0±19.1*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hornworts</td>
<td></td>
<td></td>
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<tr>
<td>Anthoceros punctatus (n=5+5)</td>
<td>2.8±0.7</td>
<td>1.9±0.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Phaeoceros laevis (n=12+12)</td>
<td>4.5±0.9</td>
<td>1.8±0.2*</td>
<td>1.6±0.3</td>
<td>3.3±0.9</td>
<td>ND</td>
<td>4.9±0.6</td>
</tr>
<tr>
<td>Lycophytes</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Huperzia selago (n=5+5)</td>
<td>337.4±23.6</td>
<td>275.9±31.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Selaginella denticulata (n=5+5)</td>
<td>67.7±10.6</td>
<td>126.4±15.9*</td>
<td>3.8±1.6</td>
<td>3.1±1.0</td>
<td>6.9±1.3</td>
<td></td>
</tr>
</tbody>
</table>

Asterisks indicate significant differences in GSH concentrations between controls and Cd-exposed samples at *P < 0.05 or **P < 0.01, according to the Kruskal–Wallis non-parametric test; n for each experimental set (controls+Cd-exposed) is specified in parenthesis. ND, no PCs detected by HPLC and ESI-MS analyses.

temperature, 300 °C; and tube lens, 110 V. MS experiments were carried out in the 200–1500 mass-to-charge ratio (m/z) range. MS/MS experiments were performed in the ion-trap collision cell with a normalized collision energy of 35 arbitrary units and an isolation width of 1 m/z; the product ions were analysed with an Orbitrap analyzer, with the m/z set as a function of GSH and PC molecular mass.

Phytochelatin synthase activity assays

Bryophyte and lycophyte PCS activities were assayed in 250 mg of fresh Cd-untreated material, from gametophytes of C. conicum, P. epiphylla, R. complanata, A. pinguis, S. undulata, S. palustris, P. formosum, F. antipyretica, and P. laevis; and sporophytes (roots) of H. selago and S. denticulata (and A. thallana plants as a positive control), grown as described above. The PCS activity was not tested in the charophytes, in the liverwort M. polymorpha, in the moss H. cupressiforme, and in the hornwort A. punctatus, essentially due to the lack of fresh material at the time of the revision of the present work. All the material was assayed in vitro, mainly following the protocol of Wojas et al. (2008), with some slight modifications. Each sample was put in a 1.5 ml Eppendorf tube (filled with 350 mg of glass microbeads with a diameter of 0.2 mm), placed in liquid nitrogen, and ground to a powder with a TAC 200/S amalgamator (Linea TAC s.r.l., Asti, Italy; oscillation frequency: 4200 strokes min⁻¹ for 10 s); the use of the amalgamator gave a better yield compared with the mortar extraction. The powder was then added to 700 μl of extraction buffer (Wojas et al., 2008) and homogenized for 10 s with another cycle of amalgamator shaking. The homogenized samples were then centrifuged twice at 4 °C/13 000 g for 10 min, and 400 μl of the supernatants added to 100 μl of the reaction buffer, as in Wojas et al. (2008), containing 100 μM Cd and the protease inhibitor cocktail ‘complete mini EDTA-free’ (Roche Italia, Milan, Italy). After an incubation time of 90 min at 35 °C, and termination of the reaction with 125 μl of 20% trichloroacetic acid, the measurement of the PCS activity was immediately performed by HPLC–ESI-MS, set up as described in the previous paragraph. The PCS activity was determined from the integrated total PC peak areas, and expressed in pmol PCs min⁻¹ g⁻¹ FW, according to calibration curves set up with known concentrations of standard GSH (Merck, Darmstadt, Germany), and to Ogawa et al. (2010).

Western blots of phytochelatin synthases

Proteins were extracted from at least 150 mg of fresh tissues of C. vulgaris and C. scutata; gametophytes of C. conicum, P. epiphylla, R. complanata, A. pinguis, S. undulata, S. palustris, P. formosum, F. antipyretica, P. laevis and (P. patens as a negative control); and sporophytes of P. laevis, H. selago (roots), and S. denticulata (roots), by using 300 μl of lysis buffer, containing 50 mM thiourea, 7 M urea, 0.5% Triton X-100, 1% dithiothreitol (DTT), 2% (w/v) polyvinylpyrrolidone (PVPP), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.2% β-mercaptoethanol. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as a standard. Aliquots of protein extracts (~20 μg of total protein per well) were separated using a 12% SDS–polyacrylamide gel (Laemmli, 1970), and electrotransferred at 100 V for 60 min to a nitrocellulose membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), using the Mini Trans-Blot cell.
apparatus (Bio-Rad Laboratories, CA, USA). Protein loading and transfer efficiency of extracts were verified by Ponceau-S staining. Western blot analyses were performed with a polyclonal antibody (diluted 1:5000 in blocking buffer and probed for 2 h) raised against *A. thallana* PCS1. As references for the immunoblotting specificity/detection, recombinant *A. thallana* PCS1 (AtPCS1, 56 kDa) (Ruotolo et al., 2004), root samples of *in vitro* grown *A. thallana* seedlings, and monocultures of the cyanobacterium *Nostoc* sp., were employed. No western blots of apo-*Glomeromycota* as accidental contaminants were performed, since these fungi do not posses any PCS (as shown by a search on the GenBank database for annotated PCS orthologues on 15 November 2013) and do not produce PCs (Bolchi et al., 2011). Immunoactivity was visualized using an anti-rabbit IgG antibody conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences AB) and a chemiluminescence western blotting detection system (Pierce ECL Plus Western blot Substrate; Pierce, Rockford, IL, USA), according to the manufacturer’s instructions.

**Statistics**

Statistical analyses were performed by SPSS Statistics, version 19. Comparisons amongst independent samples were done by the Kruskal–Wallis non-parametric test (*P* < 0.05 and *P* < 0.01).

**Results**

**Bryophytes, charophytes, and lycophytes synthesize cadmium-induced phytochelatins in vivo**

All charophytes investigated here were shown to possess relatively high levels of GSH, whose concentrations were not significantly affected by Cd treatment (Table 1). Two species, namely *C. vulgaris* and *C. scutata*, produced PCs under Cd stress up to PC$_4$, the latter species being the best PC producer amongst all the species tested in this work (Table 1). In contrast, *Spirogyra* sp. synthesized only traces of PCs, up to PC$_3$ (Table 1). In order to confirm the identity of the HPLC-detected thiol-peptides in charophytes, samples were subjected to ESI-MS and MS/MS analyses. Representative *C. vulgaris* and *C. scutata* ESI-MS chromatograms for GSH in controls, and for GSH and PCs in Cd-exposed material are shown in Supplementary Fig. S1 available at *JXB* online.

As regards bryophytes, amongst the liverworts belonging to the class Marchantiopsida (Fig. 1), in *C. conicum* the GSH level under Cd exposure was much higher than in controls (*P* = 0.004, Table 1), and PCs (PC$_2$, PC$_3$, and PC$_4$) were detected, even with longer exposure time (144 h) or higher Cd concentration (72 μM) (Table 1). In *M. polymorpha*, no differences in GSH levels between controls and Cd-exposed samples were found, and PC$_2$ and PC$_3$, but not PC$_4$, were produced (Table 1). With regard to the Jungermanniopsida (comprising Jungermanniidae and Metzgeriidae) (Fig. 1), the highest levels of GSH amongst all liverworts investigated here were found in *P. epiphylla*, with no differences between controls and Cd-exposed samples (although there was a non-significant, but manifest downward trend under Cd stress) (Table 1); as far as PCs are concerned, only PC$_2$ was produced *in vivo* by this liverwort (Table 1). In *R. complanata*, the levels of GSH were much lower than those of *P. epiphylla*, and no significant differences were observed in GSH levels between controls and Cd-exposed samples; with regard to PCs, in *R. complanata* only PC$_3$ was induced *in vivo* by Cd (Table 1). In *A. pinguis* and *S. undulata*, no differences in GSH levels were found between controls and Cd-treated samples (Table 1), and no PC *in vivo* production was detected.

As far as mosses are concerned, in *S. palustre*, belonging to the very ancient Sphagnopsida moss clade (Fig. 1) (Newton et al., 2009), the GSH level under Cd stress was higher than in controls (*P* = 0.048), and the production of PC$_2$, PC$_3$, and PC$_4$ was detected (Table 1). In contrast, in *P. formosum*, *H. cupressiforme*, and *F. antipyretica*, no PC production under Cd stress was evidenced, but marked differences as regards the GSH content between control and Cd-exposed samples were observed (Table 1). In particular, *P. formosum* and *F. antipyretica* showed large increases in GSH levels under Cd stress compared with controls (*P* = 0.015 and *P* = 0.042, respectively), whereas *H. cupressiforme* displayed an opposite trend (Table 1).

In the two hornworts studied (*A. punctatus* and *P. laevis*), the thiol-peptide production in response to Cd stress varied qualitatively between the two species. In *A. punctatus*, no significant differences in GSH levels between controls and Cd-exposed plants were measured, and no PC synthesis was detected (Table 1). In contrast, in *P. laevis* the GSH concentration under Cd stress significantly diminished (*P* = 0.023), whereas synthesis of PC$_2$ and PC$_3$ was induced (Table 1).

Finally, in the two lycophytes, namely *H. selago* and *S. denticulata*, the thiol-peptide levels varied to a large extent between them. In *H. selago*, high constitutive levels of GSH, without variation under Cd stress, were detected, and no PC synthesis was measured *in vivo* (Table 1). In contrast, in *S. denticulata* the GSH concentrations were lower than in *H. selago*, but they significantly increased (*P* = 0.031) under Cd stress, which also induced the synthesis of PC$_2$ and PC$_3$ (Table 1).

Representative *C. conicum*, *S. palustre*, and *P. laevis* HPLC and ESI-MS chromatograms for GSH in controls, and for GSH and PCs in Cd-exposed samples, are shown in Fig. 2. Moreover, representative *C. conicum* MS/MS product-ion spectra and the relative fragmentation patterns for GSH, PC$_2$, PC$_3$, and PC$_4$ are shown in Fig. 3. Finally, ESI-MS chromatograms of GSH and PCs produced by *P. epiphylla* and *S. denticulata* are shown in Supplementary Fig. S2 available at *JXB* online.

**Basal land plants possess constitutive and functional phytochelatin synthases**

The gametophytes of the liverworts *C. conicum* and *S. undulata*, and of the moss *S. palustre*, showed the constitutive presence of a putative PCS band of almost the same molecular mass, that is ~36 kDa (Fig. 4, lanes 5–7); in contrast, the gametophytes of the hornwort *P. laevis* and the sporophytes (roots) of the lycophyte *S. denticulata* were shown to possess, respectively, a lighter and a heavier (than those above) putative PCS band, with molecular masses of ~28 kDa and ~40 kDa (Fig. 4, lanes 3 and 9). A band of ~28 kDa was also immunodetected in the sporophytes of *P. laevis* (Fig. 4, lane 2). All the basal land plant PCSs were lighter than the PCSs from *A. thaliana* (roots and recombinant), whose bands were shown to possess a molecular mass of ~56–58 kDa (Fig. 4, lanes 4 and 10).
As expected, no PCS band was detected in *P. patens* (Fig. 4, lane 8). Western analysis of the other species, including the charophytes, did not reveal the presence of any immunoreactive band. Also, in all the above blots, no presence of aspecific bands was detected, thus allowing it to be excluded that accidental contamination with other species had occurred. In particular, no putative PCS-like bands were immunodetected in extracts from monocultures of *Nostoc* sp. (Fig. 4, lane 1).

Furthermore, the assayed bryophyte and lycophyte species had *in vitro* PCS activity varying to different extents (Table 2), but in general much lower than that measured in *A. thaliana* plants *in vitro*. In fact, if the latter activity was fixed equal to 100%, the relative PCS activities of bryophytes and lycophytes (extracted and incubated in identical conditions to those of *A. thaliana*) ranged from a minimum of 1.0% in *S. palustre*, to a maximum of 13.1% in *H. selago* (Table 2). In general, the highest PCS activities were assayed in the two lycophytes, followed in decreasing order by the liverworts, the hornwort *P. laevis*, and the moss *S. palustre*. No PCS activity was detected in the mosses *P. formosum* and *F. antipyretica*. Omission of Cd supply in the extraction and reaction buffers resulted in no detectable or very low synthesis of PCs.

**Discussion**

The results of this study differ from the currently held view that basically affirms: ‘all bryophytes do not produce PCs and do not possess a PCS enzyme’ (Bruns et al., 1999, 2001; Rother et al., 2006; Kopriva et al., 2007; Leinenweber et al., 2009; Bleuel et al., 2011). In fact, PC *in vivo* production and western-immunoreactive PCS bands were found in: (i) a number of liverworts; (ii) the moss *S. palustre*; and (iii) gametophytes and sporophytes of the hornwort *P. laevis*. Cd-induced PCs were also detected *in vivo* in three charophytes, and in one lycophyte, namely *S. denticulata*, in which a PCS band was also immunodetected.

Other than PCs and PCSs, a number of the basal land plants investigated here (particularly lycophytes and liverworts) showed a detectable, and sometimes not negligible,
Phytochelatins and phytochelatin synthases in bryophytes, lycophytes, and charophytes

In vitro activity. Hence, a functional PCS enzyme is present in all the lineages of bryophytes, as well as in early tracheophytes.

Overall, the total amount of PCs produced in vivo under Cd stress by the above basal land plants appears to be much lower (sometimes by one or two orders of magnitudes or

Fig. 3. Representative Conocephalum conicum MS/MS product-ion spectra of thiol-peptides (A, m/z 308=GSH; B, m/z 540=PC2; C, m/z 772=PC3; D, m/z 1000=PC4), deduced formulae, and relative fragmentation patterns at a normalized collision energy of 35 arbitrary units (A', GSH; B', PC2; C', PC3; D', PC4). The MS/MS thiol product-ion spectra from all the other species were almost identical to that shown here.
even more) than the amount quantified in several angiosperms (Magnoliophyta), grown and tested in very similar experimental conditions by our group and colleagues (Paradiso et al., 2008; Brunetti et al., 2011; Vurro et al., 2011; Sanità di Toppi et al., 2012). Accordingly, it seems reasonable to postulate that the lower amount of PCs may be related, at least in general, to the lower basal PCS activity of land plants, compared for instance with that of A. thaliana (Table 2). A little more speculatively, it is also proposed that, from the lighter and less active (than that of A. thaliana) ‘early’ PCS of basal land plants, an evolutionarily driven trend has led to dimensional increases of the enzyme length and an improvement of its functionality, found in angiosperms. To this end, it is hypothesized that such dimensional variations might be mainly ascribed to increases in the length of the C-terminal domain (as detailed by Rea, 2012). It has in fact been proven that the A. thaliana truncated PCS1 (shorter than the full-length enzyme, since it was obtained after partial proteolysis of the C-terminal domain) possesses both a substantially decreased thermal stability and a significant decline in supporting a broad response to metal ions, particularly to Zn and Hg (Ruotolo et al., 2004); it has also recently been postulated that the C-terminal domain plays a role in protecting against the metal-induced oxidative damage possibly occurring in the N-terminal domain (Matsumoto et al., 2009). Land plants might have encountered, amongst other factors: (i) the need to counteract rapid changes in temperature, particularly high temperature excesses (Rensing et al., 2008; Banks et al., 2011) compared with the much more ‘buffered’ environment provided by water; (ii) the exigency of regulating the fluxes of metals in palaeo-soils; and (iii) the need to better protect themselves against the light-induced oxidative stress. Consequently, a longer PCS (with a better thermal stability and an improved response to metal ions and to oxidative agents) might potentially have been promoted in the course of evolution, up to the achievement of the full-length dimension of the angiosperm PCSs.

As schematically depicted in Fig. 5, the present data demonstrate that the capability for PC production under metal stress and the presence of a constitutive and functional PCS enzyme actually represent ancestral (plesiomorphic) characters. They occur not only in the dominant gametophyte generation of bryophytes (and in hornwort sporophytes), but also in the dominant sporophyte generation of basal tracheophytes. The present experiments also suggest that the capability for PC production under metal stress was, in some cases,
probably independently lost during evolution, as supported by the absence of a putative functional PCS and a detectable PC synthesis in the investigated mosses, with the exception of *S. palustre*. In this context, it would therefore appear coherent that the ‘model’ moss *P. patens* (Bryopsis) has been proven not to possess the gene for the PCS (Koprina et al., 2007), and this might also be the same for some other species investigated here. At the same time, the apparent discrepancy between the lack of *in vivo* detectable PC production in some species (i.e., *S. undulata*, *A. pinguis*, and *H. selago*) and the demonstrated activity/presence in them of the PCS enzyme might possibly be due to morphological/ultrastructural characteristics (i.e., cell wall thickness, mucilage canals, etc.) that could reduce or prevent Cd from reaching the cytosolic PCS in amounts sufficient for inducing an *in vivo* detectable synthesis of PCs.

Regarding specifically the hornworts, in *P. laevis* (gametophytes and sporophytes) a PCS enzyme with an apparent molecular mass of ~28 kDa was immunodetected. Considering its molecular mass, the *P. laevis* PCS would theoretically be compatible in size with a cyanobacterial PCS-like enzyme (Harada et al., 2004; Tsuji et al., 2004; Vivares et al., 2005). As a consequence, from an evolutionary standpoint, an ancestral horizontal transfer of the PCS from an archaic (*Nostoc*-like?) cyanobacterium to an early hornwort might not be excluded in principle, with the subsequent further full acquisition, only in the hornwort, of a PC synthesis capability, since cyanobacteria do not produce PCs (Harada et al., 2004; Vivares et al., 2005), or at the most they synthesize traces of PC$_2$ alone (Tsuji et al., 2004). In contrast, a direct immunodetection in *P. laevis* of a ‘contaminant’ PCS-like *Nostoc* sp. band should be ruled out completely, as: (i) the hornwort was thoroughly cleaned of all biological (endo)contaminants under two types of microscopes; (ii) *P. laevis* produced PCs up to PC$_5$; and (iii) a PCS band of the same size was also detected in the hornwort sporophytes, not colonized by *Nostoc* sp. (Ligrone et al., 2012). In any case, in the *Nostoc* sp. monocultures set up specifically for this purpose, no PCS-like band was ever detected by the *A. thaliana* PCS1 polyclonal antibody used here (Fig. 4)

The present results also demonstrated that PCs were synthesized in charophytes (sisters of land plants), but, unexpectedly, no putative PCS bands were detected by western blots in these algae. This might be attributed to the nature of the antibody used which probably fails to recognize cyanobacterial/algal PCS epitope(s), instead recognizing only those of (early land) plant. In any case, the present study extended the investigation by Volland et al. (2013) carried out on the desmid *M. denticulata*, demonstrating that an unequivocal PC production capability is also a defining character of three further charophyte taxa, namely Spirogyra sp., *C. vulgaris*, and *C. scutata*, evolutionarily closer to land plants than *M. denticulata* (McCourt et al., 2004; Qiu et al., 2006; Qiu, 2008; Becker and Marin, 2009; Wodniok et al., 2011).

In conclusion, assuming that early bryophytes and lycophytes spread in palaeo-environments rich in PC-inducing metal(loid)s (Meharg and Hartley-Whitaker, 2002; Rice et al., 2005; Ernst et al., 2008), then the synthesis of PCs and the presence of constitutive and functional PCS enzymes, particularly in the lineages originated during the Devonian period (Fig. 5), might represent a remnant of that time. Due to their ‘benefits’, these traits have been retained up till now, perhaps with the main aim of controlling Zn [and perhaps copper (Cu)] homeostasis (Thumm et al., 1991; Tennstedt et al., 2009; Vurro et al., 2011), other than performing Cd and As detoxification.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Figure S1.** ESI-MS chromatograms of: *Chara vulgaris* (A) GSH in controls and (B) GSH, PC$_2$, PC$_3$, and PC$_4$ in Cd-exposed plants; *Coleochaete scutata* (C) GSH in controls

**Fig. 5.** Key timing events regarding charophyte and basal land plant origin and diffusion, related in particular to the lineages investigated here (chronological data are from McCourt et al., 2004; Qiu et al., 2006; Qiu, 2008; Becker and Marin, 2009; Crandall-Stotler et al., 2009; Wikström et al., 2009; Villareal et al., 2010; Banks et al., 2011; Ligrone et al., 2012). The postulated ancestral presence of constitutive and functional phytochelatin synthases and the occurrence of a phytochelatin synthesis capability are also indicated by the arrows. (This figure is available in colour at *JXB* online.)
and (D) GSH, PC₂, PC₃, and PC₄ in Cd-exposed plants. Unlabelled peaks are non-specific and do not represent signals for thiol-peptide characterization.

Figure S2. ESI-MS chromatograms of: Pellia epiphylla (A) GSH in controls and (B) GSH and PC₂ in Cd-exposed plants; Selaginella denticulata (C) GSH in controls and (D) GSH, PC₂, and PC₃ in Cd-exposed plants. Unlabelled peaks are non-specific and do not represent signals for thiol-peptide characterization.

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References


Phytochelatins and phytochelatin synthases in bryophytes, lycophytes, and charophytes


