RESEARCH PAPER

Proteome analysis of *Physcomitrella patens* exposed to progressive dehydration and rehydration

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Abstract

*Physcomitrella patens* is an extremely dehydration-tolerant moss. However, the molecular basis of its responses to loss of cellular water remains unclear. A comprehensive proteomic analysis of dehydration- and rehydration-responsive proteins has been conducted using quantitative two-dimensional difference in-gel electrophoresis (2D-DIGE), and traditional 2-D gel electrophoresis (2-DE) combined with MALDI TOF/TOF MS. Of the 216 differentially-expressed protein spots, 112 and 104 were dehydration- and rehydration-responsive proteins, respectively. The functional categories of the most differentially-expressed proteins were seed maturation, defence, protein synthesis and quality control, and energy production. Strikingly, most of the late embryogenesis abundant (LEA) proteins were expressed at a basal level under control conditions and their synthesis was strongly enhanced by dehydration, a pattern that was confirmed by RT-PCR. Actinoporins, phosphatidylethanolamine-binding protein, arabinogalactan protein, and phospholipase are the likely dominant players in the defence system. In addition, 24 proteins of unknown function were identified as novel dehydration- or rehydration-responsive proteins. Our data indicate that *Physcomitrella* adopts a rapid protein response mechanism to cope with dehydration in its leafy-shoot and basal expression levels of desiccation-tolerant proteins are rapidly upgraded at high levels under stress. This mechanism appears similar to that seen in angiosperm seeds.

Key words: 2D-DIGE, dehydration tolerance, LEA proteins, moss, proteome profiling, rehydration, *Physcomitrella patens*.

Introduction

Bryophyte-like organisms were the first terrestrial plants that emerged more than 450 million years ago (Kenrick and Crane, 1997). Evolving from an aquatic, green algae-like ancestor, development of morphological and physiological features was principally aimed at adaptation to water deficiency for land colonization by these plants (Kenrick and Crane, 1997; Bateman et al., 1998; Qiu et al., 2006). As a representative bryophyte, *Physcomitrella patens* (hereafter *Physcomitrella*) has been recognized as a model system for the study of basic cytology and development in plant biology, as well as for understanding the molecular mechanisms during evolution that permitted the original bryophyte to cope with terrestrial habitats (Rensing et al., 2008). Like other mosses, the life cycle of *Physcomitrella* is characterized by an alternation of two generations. Morphologically, dominant gametophytic generation is subdivided into two distinct developmental stages, i.e. protonema and gametophore. The former is composed of filamentous cells; the latter is made up of leafy-shoot tissues. It has been demonstrated that *Physcomitrella* can survive severe dehydration of their vegetative organs (e.g. leafy-shoots), which is uncommon in higher vascular plants (Frank et al., 2005; Oldenhof et al., 2006; Cuming et al., 2007). However, little is known about the molecular features responsible for the dehydration tolerance of leafy-shoots, the green haploid vegetative tissues, of *Physcomitrella*.

In general, tolerance to dehydration or complete desiccation is very common among mosses. Vegetative tissues of some species can withstand complete desiccation (drying to...
a water content of 10% or less), and then resume normal growth upon rehydration. A well-studied experimental model is moss, *Tortula ruralis*. It was reported that *T. ruralis* can tolerate a complete loss of free protoplasmic water (Wood and Oliver, 2004). Its cellular protection system was demonstrated to be essentially constitutive and contained key components of the system characterized within vascular plants, such as dehydrin protein and sugar (Oliver, 1991; Wood and Oliver, 2004). Moreover, the synthesis of proteins required for defence and repair occurred only under rehydration conditions (Oliver, 1991). Summarizing these results, Wood and Oliver (2004) proposed that vegetative tolerance in moss plants involved constitutive cellular protection coupled with a repair mechanism that is induced upon rehydration. However, the hypothesis seems to be controversial (Cuming et al., 2007; Proctor et al., 2007).

Compared with *T. ruralis*, vegetative tissues of *Physcomitrella* cannot tolerate complete desiccation, protonemal tissues in particular (Oldenhof et al., 2006; Cuming et al., 2007; Khandelwal et al., 2010). Interestingly, its gametophore colonies survive in desiccated environments (Wang XQ et al., 2009). The tolerance discrepancies among tissues and species need to be clarified. Therefore, it is very necessary to explore the molecular details that contribute to the dehydration-tolerance response in various tissues of *Physcomitrella*. A survey based on microarray revealed that, in protonemal tissue of *Physcomitrella*, 130 genes are specifically induced by dehydration (Cuming et al., 2007). The set of induced genes includes many members encoding late embryogenesis abundant (LEA) proteins and other homologues of angiosperm genes expressed during drought stress. In gametophore colonies of *Physcomitrella*, Frank et al. (2005) identified 19 genes responsive to dehydration using cDNA macroarray. These genes include homologues that are known to be associated with abiotic stress in different species of vascular plants (Frank et al., 2005). Proteomic analysis of the gametophore tissues revealed that, under desiccation conditions, the abundance of an additional 71 proteins is altered to cope with desiccation events (Wang XQ et al., 2009). Summarizing the previous results, the strategy followed by *Physcomitrella* for coping with dehydration/desiccation seems to be to mobilize the defence and repair system upon dehydration, which is in contrast to that seen in *T. ruralis*, but is similar to that seen in angiosperms. However, only a few dehydration-responsive genes/proteins belonging to a few functional categories have been identified in *Physcomitrella* (Frank et al., 2005; Wang XQ et al., 2009). Furthermore, when microarray data of RNA expression in protonemal tissue (Cuming et al., 2007) were compared with proteomics data from a gametophore colony (Wang XQ et al., 2009), huge discrepancies were observed between responses at the RNA and protein levels. Therefore, the mechanisms of dehydration/desiccation tolerance in vegetative tissues of *Physcomitrella* remain unclear.

Leafy-shoots of *Physcomitrella* are haploid organisms with stem, leaves, and filamentous rhizoids, which are morphologically analogous to the shoot of angiosperms. It was observed that these haploid plants can recover from severe dehydration. The molecular mechanisms underlying this phenotype remain poorly understood. Here, 2-D DIGE, plus traditional 2-DE coupled with mass spectrometry, was applied to identify dehydration- and rehydration-responsive proteins in *Physcomitrella* leafy-shoots. The earlier procedure for protein extraction (Cui et al., 2005, 2009) was modified to minimize losses and to optimize it for quantitative proteomics. The proteomic results were further confirmed using RT-PCR analysis of total RNA. Analysis of all the results taken together reveals a possible mechanism of cellular protection during dehydration in *Physcomitrella* leafy-shoots.

### Materials and methods

**Plant material cultivation**

*Physcomitrella patens* ecotype ‘Gransden 2004’ was cultured on modified BCD medium (Nishiyama et al., 2000) in a growth chamber at 23 °C with a 16 h photoperiod, and a light intensity of 150 μmol m⁻² s⁻¹. To obtain uniform leafy-shoots, 2-week-old gametophore colonies (mixed tissues of leafy-shoots and protonema) were ground gently with a homogenizer. The homogenate was resuspended in liquid BCD medium (1 mM, MgSO₄, 10 mM KNO₃, 45 μM FeSO₄, 1.85 mM, KH₂PO₄, 1 mM, CaCl₂, and trace elements, pH 6.5) and was transferred to a cellophane overlay on solid BCD medium containing 0.8% (w/v) agar, supplemented with 5 mM ammonium tartrate and 0.5% (w/v) glucose for protonema cultivation. After 8 d, the cellophane overlay bearing regenerated protonemal tissues was transferred onto ammonium tartrate-free BCD medium to allow the growth of leafy-shoots. After 20 d, leafy-shoots with stem, 7–9 fully extended leaves, and rhizoids were obtained and used in the study.

**Physiological analysis of dehydration tolerance in leafy-shoots**

To determine their tolerance for water deficiency, 20-d-old leafy-shoots were subjected to a progressive dehydration stress followed by rehydration. For dehydration treatment, leafy-shoots grown on cellophane were transferred to a flat beaker with nine layers of filter papers wetted with 1 ml BCD medium to avoid exposure to an abrupt change in humidity at the onset of the treatment. The beakers were then placed in a small growth chamber with 30% relative humidity, a 16 h photoperiod, and a light intensity of 150 μmol m⁻² s⁻¹. After dehydrating naturally and progressively for 3–4 d at 23 °C, the leafy-shoots were transferred to fresh solid BCD medium and allowed to revive for 6 d. During dehydration and rehydration, both water and chlorophyll content in leafy-shoots were determined at various time points according to a previously described method (Frank et al., 2005). For every time point, six measurements were performed.

**Extraction of total protein for 2-D DIGE**

Total protein of leafy-shoots was extracted using a fractionation method (Cui et al., 2005, 2009) with modifications. Approximately 1 g of leafy-shoots was ground to a fine powder in liquid nitrogen. First, soluble proteins were extracted with buffer I (50 mM TRIS-HCl, pH 7.8, 10% glycerol, 2% β-mercaptoethanol, 1 mM PMSF, and 1 mM EDTA). After ultrasonication and centrifugation, the supernatant was collected in a test tube. In a second step, insoluble proteins associated with cellular structures and membrane were extracted with buffer II (100 mM phosphate buffer, pH 7.1, containing 0.2 M KCl, 10% glycerol, 2 mM MgSO₄, 4% (w/v)
CHAPS, 2% β-mercaptoethanol, 1 mM PMSF, and 1 mM EDTA), following ultrasonication and further extraction with 7 M urea, 2 M thiourea, and 30 mM DTT. After centrifugation, the supernatant was combined into the test tube. Subsequently, the combined supernatant was mixed with an equal volume of ice-cold phenol (Tri-buffered, pH 7.5) and centrifugation at 20 000 g for 30 min at 4 °C to separate the phenol and aqueous phases. The collected phenol phase was then mixed with 5 vols of cold, saturated ammonium acetate in methanol and left at –20 °C overnight to precipitate proteins. After centrifugation, the protein pellet was rinsed twice with cold acetone containing 13 mM DTT and then lyophilized. The resulting protein samples were used for quantitative DIGE analysis and traditional 2-DE. Protein content was estimated using a modified Bradford method as described previously by Cui et al. (2009). In addition, the total protein content of leafy-shoots was also determined using a modified Kjeldahl analysis (Gornall et al., 1949; Wang YX et al., 2009).

**Protein CyDye labelling**

Proteins from both types of samples, untreated control leafy-shoots (20-d-old) or the dehydrated (3 d dehydration) and rehydrated leafy-shoots (6 d rehydration) were labelled with DIGE-specific Cy3 or Cy5 according to the manufacturer’s instructions (GE Healthcare) with the following modifications. Briefly, after resuspension in lysis buffer (7 M urea, 2 M thiourea, and 4% CHAPS) and adjusting the pH to 8.5, 50 μg of proteins were mixed with 300 pmol of CyDye and incubated on ice in the dark for 30 min. The labelling reaction was stopped by the addition of lysine. In the experiments, biological replicates were labelled reciprocally with either Cy3 or Cy5, and an internal standard was generated by pooling equal amounts of proteins from each sample labelled with DIGE-specific Cy2.

**2-D DIGE and image analysis**

Pairs of Cy3- and Cy5-labelled protein samples and a Cy2-labelled internal standard were mixed together in a 1:1:1 ratio and then subjected to IEF and SDS-PAGE as described previously by Cui et al. (2009). Measurements were done in four replicates. The resulting gels were scanned using Typhoon 9400 Imager (GE Healthcare). The images were analysed using DeCyder v6.5 software according to the manufacturer’s user guide. Spot intensities were normalized based on the internal standard labelled with Cy2. Differentially-expressed spots were selected based on the P value of the t test (P <0.05); the presence in all replicates; and spot abundance ratio of >1.5.

**Traditional 2-DE and image analysis**

Traditional 2-DE was used to analyse the change of proteins extracted from the above plant samples again. For isolectric focusing (IEF), 750 μg of proteins were re-suspended in IEF buffer and then loaded on to an 18 cm immobilized pH gradient (IPG) strip with a linear pH gradient ranging from 4 to 7. Following SDS-PAGE, gels were stained with Coomassie Brilliant Blue (CBB), and image and data analysis were carried out using our published protocol (Cui et al., 2009). At least nine gels derived from three biological replicates of each sample were compared. A spot abundance ratio of >1.5 was set as a threshold to identify differentially-expressed proteins in this study.

**In-gel tryptic digestion, mass spectrometry, and database search**

The spots of interest were picked from corresponding preparative CBB stained gels and submitted to in-gel digestion with trypsin (Cui et al., 2009). Extracted peptides were analysed by MALDI-TOF/TOF on a mass spectrometer, Ultraflex III from Bruker Daltonics (Bremen, Germany) as previously described by Chen et al. (2010). For the acquisition of mass spectra, 0.5 μl samples were spotted onto a MALDI plate, followed by 0.5 μl matrix solution (4 mg ml⁻¹ α-cyano-4-hydroxycinnamic acid in 35% acetonitrile and 1% TFA). Mass data acquisitions were piloted by flexcontrol software v3.0 using batched-processing and automatic switching between MS and MS/MS modes. All MS survey scans were acquired over the mass range m/z 800–4500 in the reflection positive-ion mode and accumulated from 2000 laser shots with an acceleration of 23 kV. The MS spectra were externally calibrated using PeptideCalibStandard II (Bruker Daltonics) (1046.542, 1296.685, 1347.735, 1619.822, 2093.086, 2465.198, and 3147.471), and internally calibrated using trypsin autolytic products (m/z 842.509, 1045.564, 1940.935, and 2211.104) resulting in mass errors of <30 ppm. The MS peaks were detected with a minimum signal/noise (S/N) ratio ≥20 and cluster area S/N threshold ≥25 without smoothening and raw spectrum filtering. Peptide precursor ions corresponding to contaminants including keratin and the trypsin autolytic products were excluded in a mass tolerance of ±0.2 Da. The filtered precursor ions with a user-defined threshold (S/N ratio ≥50) were selected for the MS/MS scan. Fragmentation of precursor ions was performed using the LIFT positive mode. MS/MS spectra were accumulated from 4000 laser shots. The MS/MS peaks were detected on a minimum S/N ratio ≥50 without fragmentation of precursor ions. Interatomic distances deduced by searching in NCBI-nr databases of green plants. For unambiguous identification of proteins, a matching of more than five peptides and a sequence coverage >15% was considered adequate.

**RNA extraction and semi-quantitative RT-PCR**

Total RNA was isolated from *Physcomitrella* leafy-shoots using the E.Z.N.A. Plant RNA Kit (Omega Bio-tek, Inc.) according to the manufacturer’s protocol II. Semi-quantitative RT-PCR was carried out as previously described (Chen et al., 2010). Reverse transcription reactions were carried out using Oligo(DT) primers and the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The exponential phase of RT-PCR was determined by measuring the aliquots of PCR products taken after different number of PCR cycles. The primer sequences used for each gene are provided in Supplementary Table S1 at JXB online. *Physcomitrella patens* cDNA was used as a positive control.

**Results and discussion**

**Leafy-shoots can survive around 75% water loss**

To assess the dehydration tolerance of *Physcomitrella*, leafy-shoots were subjected to a progressive dehydration stress followed by rehydration. Figure 1 shows changes in both water and chlorophyll contents during dehydration and rehydration. After dehydration for 3 d, only 22.6% of the water content was retained in the leafy-shoots. In other words, the leafy-shoots had lost around 75% of their water content by the third day of dehydration treatment. A further prolongation by one day of the dehydration treatment led to 94% water loss (Fig. 1A). In contrast to the rapid decrease in water content, only a slight decrease in chlorophyll content was observed in leafy-shoots after 3 or 4 d of dehydration (Fig. 1B). Subsequently, water recovery experiments were performed. As shown in Fig. 1, during the rehydration phase there was complete recovery of the water
content in leafy-shoots that had been dehydrated for 3 or 4 d. By contrast, the chlorophyll content appeared to decrease during rehydration. Recovery of the chlorophyll content was only partial in plants that had been subjected to a 3-d dehydration phase, while in those subjected to 4-d dehydration, the chlorophyll content decreased rapidly and continuously during the rehydration phase (Fig. 1b).

A rapid loss of chlorophyll during rehydration has previously been reported in *Physcomitrella* (Frank et al., 2005).

The observation of tolerance to approximately 75% of water loss in leafy-shoots of *Physcomitrella* is consistent with earlier observations in protonemal tissues of *Physcomitrella* (Cuming et al., 2007), but is slightly lower than the estimated 90–95% in gametophore colonies of *Physcomitrella* (Frank et al., 2005; Oldenhof et al., 2006; Wang XQ et al., 2009). In contrast to desiccation-tolerant moss species such as *T. ruralis* (Oliver et al., 2005), *Atrichum androgynum* (Mayaba et al., 2001, 2002), and *Polytrichum formosum* (Proctor et al., 2007), the leafy-shoots used in the current investigation could not tolerate the completely air-dried state nor could they recover after remoistening (Fig. 1). Hence, *Physcomitrella* has been categorized as a moss species with an intermediate tolerance between desiccation-tolerance and desiccation-sensitive.

**Protein content and extraction efficiency**

Using a modified Kjeldahl analysis, the protein content in leafy-shoots was determined to be only 6.8±0.4 mg g⁻¹ of fresh tissue. To minimize protein loss and to obtain highly resolved 2-DE gels, in this study, soluble and insoluble proteins were extracted separately from *Physcomitrella* leafy-shoots according to a previously described method (Cui et al., 2005) with a few modifications. This fractionation procedure coupled with phenol extraction (see the Materials and Methods) consistently yielded 6.4±0.7 mg of total proteins g⁻¹ fresh leafy-shoots based on Bradford’s assay. This indicates that 94.1% of proteins in leafy-shoots could be extracted by this procedure. This protocol, optimized for moss protein extraction, also yielded a high resolution of protein spots in 2-DE gels.

**Proteome profiles of Physcomitrella leafy-shoots in response to dehydration and rehydration**

Physiological assays for the content of water and chlorophyll demonstrated that *Physcomitrella* leafy-shoots could recover from around 75% of water deficiency, which is uncommon in shoots of most vascular plants. To understand the molecular details of the dehydration resistance response better, proteins were extracted from *Physcomitrella* leafy-shoots at different times during the dehydration (3 d) and subsequent rehydration (6 d) treatments and subjected to a comprehensive proteomics analysis. 2-DE maps were generated for untreated control leafy-shoots, dehydrated and rehydrated leafy-shoots using two complementary techniques, i.e. quantitative 2-D DIGE and traditional 2-DE stained with CBB. The parallel experiments permitted both the identification of dehydration- or rehydration-responsive proteins that could not be visualized earlier due to protein dye bias (Cui et al., 2009), and an evaluation of the consistency of results generated by the different techniques.

Figure 2 represents DIGE images of total proteins from *Physcomitrella* leafy-shoot, displaying significant alteration of proteome profiles during dehydration and rehydration. To eliminate bias due to technical variations, each gel included an internal standard prepared by pooling aliquots of all the samples within the experiment. A total of 2308 spots were detected and analysed. Of these 100 were dehydration-responsive protein spots (Fig. 2A) and 82 rehydration-responsive protein spots (Fig. 2B) that were significantly up-regulated or down-regulated in leafy-shoots subjected to 3 d dehydration and rehydration for 6 d. The differentially-expressed protein spots were consistent with those from traditional 2-DE analysis. Apart from these, an additional 12 dehydration-responsive and 22 rehydration-responsive protein spots (spot ratio >1.5) were also
visualized on triplicate 2-D gels stained with CBB (see Supplementary Fig. S1 at JXB online) as previously described by Cui et al. (2009). Thus a total of 216 differentially expressed protein spots, including 112 dehydration-responsive and 104 rehydration-responsive, were identified in Physcomitrella leafy-shoots (Fig. 2A, B). As expected, there is a considerable overlap between the two sets of proteomic data, with 74 protein spots showing differential expression in response to both dehydration and rehydration.

Identification of differentially-expressed moss proteins by MALDI-TOF/TOF MS

The differentially-expressed protein spots (n=142) were manually excised from CBB-stained preparative gels and subjected to MALDI TOF/TOF MS analysis. By combining information of peptide mass fingerprinting (PMF) and peptide sequence tags, 116 protein spots were identified successfully, corresponding to 87 genes. All proteins identified in this work are well matched with known sequences of Physcomitrella species (see Supplementary Table S2 at JXB online). This achievement could be largely due to the updated information of genome sequences of Physcomitrella (Rensing et al., 2008) and the efficient algorithm based on MALDI TOF/TOF MS. However, most proteins were assigned to the group of predicted proteins in current release (v1.6). By sequence comparisons with recent entries in the NCBI-nr database, 63 of the differentially-expressed proteins could be annotated since they share significant sequence homology with known proteins. The remaining 24 proteins were assigned to the group of predicted proteins. These are proteins of unknown function, responsive to dehydration or rehydration stress in plants. These results are summarized in Tables 1 and 2. The MS/MS data set for all protein spot are provided in Supplementary data SM1 and Supplementary Table S2 at JXB online.

Functional classification of the dehydration- and rehydration-responsive proteins

Following the functional definitions of Bevan et al. (1998) and the established features of metabolism (Buchanan et al., 2000), the differentially-expressed proteins identified in this work could be classified into seven functional groups (Fig. 3). Except for 24 novel proteins with unknown functions, the majority of proteins were assigned to four categories, i.e. seed maturation, disease/defence, protein folding and stability, as well as energy. Interestingly, the proteins with the first three functional categories were mostly up-regulated. More strikingly, proteins in the functional category of seed maturation represented the largest group of up-regulated proteins, accounting for 29% and 18% of the identified dehydration- and rehydration-responsive proteins, respectively. This strongly suggests that leafy-shoots of Physcomitrella probably adopt a dehydration tolerance mechanism similar to that established for angiosperm seeds.

Among the differentially-expressed proteins, a majority were up-regulated as shown in Table 1 and Fig. 3. During dehydration, the abundance of 78 protein spots increased and that of only 15 decreased; while during rehydration, the abundance of 69 protein spots increased and that of 15
Table 1. Functional categories of dehydration- and rehydration-responsive proteins in leafy-shoots of *Physcomitrella*. Average values for the fold change (up-regulation or down-regulation) are shown for each protein spot on 2DE.

| No | Accession no | Gene product | Spot number | Up-regulation folds<sup>a</sup> | Down-regulation folds<sup>a</sup> |
|----|--------------|--------------|-------------|-------------------------------|----------------------------------|
|    |              |              |             | Drought | Rehydration | Drought | Rehydration |
| Seed maturation | | | | | |
| 1 | XP_001769448 | Group 3 LEA protein | 207 | 3.97 | 1.99 |
|   |              |              | 206 | 3.43 | |
|   |              |              | 205 | 2.65 | |
|   |              |              | 204 | 3.00 | |
|   |              |              | 203 | 2.09 | |
|   |              |              | 217 | 7.72 | 1.92 |
|   |              |              | 216 | 13.70 | |
|   |              |              | 215 | 4.32 | |
|   |              |              | 801 | 2.51 | |
| 2 | EDQ69797<sup>c</sup> | Group 3 LEA protein | 243 | 5.87 | 2.91 |
|   |              |              | 240 | 5.24 | 2.26 |
|   |              |              | 630 | 2.70 | 1.61 |
|   |              |              | 805 | 2.72 | |
|   |              |              | 807 | 1.64 | |
| 3 | EDQ50092<sup>c</sup> | Dehydrin | 643 | 1.59 | 1.57 |
|   |              |              | 256 | 1.94 | 2.13 |
|   |              |              | 251 | 2.90 | |
|   |              |              | 252 | 3.01 | |
|   |              |              | 250 | 3.75 | 1.53 |
| Disease/defence | | | | | |
| 12 | EDQ62837<sup>d</sup> | Phycocyanin | 439 | 2.08 | 7.60 |
| 13 | AAV65396<sup>d</sup> | Phycocyanin | 408 | 2.37 | 7.40 |
| 14 | XP_001782104 | Phycocyanin | 685 | 1.53 | 2.78 |
| 15 | XP_001769177<sup>c</sup> | Phosphatidylethanolamine binding protein | 284 | 9.13 | 3.13 |
| 16 | XP_001760409 | Arabinoxylan protein | 449 | 4.42 | 2.26 |
| 17 | EDQ72147 | Phospholipase D | 209 | 2.38 | 2.19 |
|   |              |              | 210 | 2.49 | 2.42 |
| 18 | EDQ55079 | Dehydroascorbate reductase | 277 | 12.53 | 12.51 |
| 19 | EDQ56799 | GDP-D-mannose-3’,5’-epimerase | 904 | –1.75 | |
|   |              |              | 905 | –1.52 | |
| 20 | EDQ63468 | Aldo-keto reductase | 694 | 10.86<sup>b</sup> | 4.30<sup>b</sup> |
| 21 | EDQ58371<sup>c</sup> | Aldo-keto reductase | 691 | 2.39<sup>b</sup> | |
| 22 | EDQ80964 | Thioredoxin peroxidase | 279 | 6.28 | 4.87 |
| 23 | XP_001757965 | 2-Cys peroxiredoxin | 735 | –1.59 | |
| 24 | ABF66648 | Lipoygenase-2 | 615 | 1.58<sup>c</sup> | |
| 25 | XP_001769187<sup>c</sup> | Aldehyde dehydrogenase | 627 | 1.50<sup>c</sup> | |
| 26 | XP_001785650 | Aldehyde dehydrogenase | 636 | 1.51<sup>c</sup> | |
| 27 | XP_001758337 | Benzoquinone reductase | 679 | 1.61 | |
| 28 | XP_001762556 | Benzoquinone reductase | 680 | 1.54 | |
| 29 | XP_001784490 | Quinone oxidoreductase-like protein | 720 | –1.90<sup>b</sup> | –2.26<sup>b</sup> |
Table 1. Continued

| No | Accession no | Gene product | Spot number | Up-regulation folds\(^a\) | Down-regulation folds\(^a\) |
|----|--------------|--------------|-------------|---------------------------|----------------------------|
|    |              |              |             | Drought                   | Rehydration                |
|    |              |              |             | Drought                   | Rehydration                |
| 30 | XP_001773059 | Translation initiation factor 5A | 686 | 2.76 |  |
| 31 | XP_001771324 | Translation initiation factor 4A | 648 | 3.20\(^b\) | 3.24\(^b\) |
| 32 | XP_001780334 | Putative chaperonin 60 beta precursor | 806 | 3.75 | 1.81 |
| 33 | XP_001783048 | Heat shock protein 70 | 621 | 1.56 |  |
| 34 | XP_001772650 | Heat shock protein 70-2 | 618 | 1.90\(^b\) |  |
| 35 | XP_001781229 | Heat shock protein 70-3 | 624 | 2.54\(^b\) |  |
| 36 | XP_001779894 | Heat shock protein | 702 |  | −1.65 |
| 37 | XP_001770511 | Heat shock protein 90/ GRP94 | 607 | 1.53 | 1.56 |
| 38 | XP_001775725 | Heat shock protein Hsp100 | 610 | 1.53\(^b\) | 1.62\(^b\) |
| 39 | EDQ74403 | Putative chloroplast FtsH protease | 234 | 1.99 |  |
| 40 | XP_001769853 | Putative FtsH-like protein Pftf precursor | 631 | 2.00 |  |
| 41 | EDQ59103 | Putative serine carboxypeptidase II | 402 | 2.10 | 1.93 |
| 42 | XP_001769359 | Gamma interferon inducible lysosomal thiol reductase | 812 | 3.18 | 2.64 |
| 43 | XP_001784758 | Protein disulphide isomerase-like | 910 | 5.51\(^b\) | 4.77\(^b\) |
| 44 | XP_001756944 | Calnexin 1 | 640 | 1.59 |  |
| 45 | EDQ55432\(^c\) | Carbonic anhydrase | 312 | −2.67 | −1.91 |
| 46 | EDQ82463\(^c\) | Rubisco activase | 304 | −2.17 |  |
| 47 | XP_001779467 | Rubisco activase | 305 | −1.66 |  |
| 48 | XP_001776035 | Rubisco activase | 908 | −1.68 |  |
| 49 | NP_904194 | Rubisco large subunit | 709 | −2.22 |  |
| 50 | XP_001760166 | Sedoheptulose bisphosphatase | 722 | −1.53 | −1.74 |
| 51 | XP_001765395 | 23 kDa subunit of oxygen-evolving system of photosystem II | 745 | −1.70 |  |
| 52 | XP_001752812 | Chlorophyll a/b-binding protein | 668 | 1.75\(^c\) |  |
| 53 | XP_001775294 | Photosystem II stability/assembly factor HCF136 | 690 | 1.90 |  |
| 54 | EDQ57614\(^d\) | Phosphoglycerate kinase | 431 | 1.57 | 2.25 |
| 55 | XP_001776961 | Transketolase | 614 | 2.78 | 2.08 |
| 56 | XP_001764998\(^d\) | Transketolase | 901 | −1.69 |  |
This implies that there is active protein synthesis in leafy-shoots of *Physcomitrella* during dehydration and rehydration. This contrasts with the previous view from the desiccation-tolerant moss, *T. ruralis*, wherein the synthesis of stress-responsive proteins occurred mainly during post-desiccation rehydration (Wood and Oliver, 2004), in agreement with observations in vascular plants (Moore *et al.*, 2009).

### Table 1. Continued

| No | Accession no | Gene product | Spot number | Up-regulation folds<sup>a</sup> | Down-regulation folds<sup>a</sup> |
|----|--------------|--------------|-------------|---------------------------------|-----------------------------------|
|    |              |              |             | Drought | Rehydration | Drought | Rehydration |
| 57 | XP_001783115<sup>c</sup> | Glyceraldehyde 3-phosphate dehydrogenase | 661         | 1.88<sup>b</sup>             |                     |
| 58 | XP_001773841 | Phosphoglucomutase | 903         |                     | -1.65                   |
| 59 | XP_001756785 | Putative ATP synthase | 672         | 1.84<sup>c</sup>         |                     |

### Other and unknown proteins

| No | Accession no | Gene product | Spot number | Up-regulation folds<sup>a</sup> | Down-regulation folds<sup>a</sup> |
|----|--------------|--------------|-------------|---------------------------------|-----------------------------------|
|    |              |              |             | Drought | Rehydration | Drought | Rehydration |
| 60 | EDQ82483     | Nucleotide diphosphate kinase 2 protein | 315         |                     | -1.68                   |
| 61 | XP_001778544 | Glutamine synthetase | 716         |                     | -1.58                   |
| 62 | XP_001755833 | Cytosolic glutamine synthetase | 713         |                     | -1.64<sup>b</sup>         |
| 63 | XP_001764953 | Arginase | 909         |                     | -2.29                   |
| 64 | XP_001760386 | Dissocation-related protein | 655         | 2.31<sup>b</sup> | 1.79<sup>b</sup>         |
| 65 | EDQ50069     | Predicted protein | 223         | 14.95             | 8.46                   |
|    |              |              | 218         | 20.77             | 7.99                   |
| 66 | XP_001782231 | Predicted protein | 450         | 12.27             | 4.92                   |
| 67 | XP_001772850 | Predicted protein | 689         | 10.42             | 3.72                   |
|    |              |              | 266         | 5.44              | 3.95                   |
| 68 | XP_001784742 | Predicted protein | 442         | 10.29             | 2.74                   |
|    |              |              | 443         | 6.25              | 1.89                   |
| 69 | XP_001756904 | Predicted protein | 412         | 9.95              | 3.98                   |
| 70 | EDQ78135     | Predicted protein | 416         | 6.21              | 4.47                   |
| 71 | XP_001772198 | Predicted protein | 667         | 9.13              | 1.59                   |
| 72 | XP_001774835 | Predicted protein | 424         | 7.36              | 6.25                   |
| 73 | XP_001765013 | Predicted protein | 682         | 6.94              | 6.45                   |
| 74 | EDQ62252     | Predicted protein | 272         | 5.05              | 2.90                   |
| 75 | XP_001754730 | Predicted protein | 208         | 4.29              | 5.50                   |
| 76 | XP_001778432 | Predicted protein | 747         | 3.38<sup>d</sup>  | 2.44<sup>d</sup>         |
| 77 | EDQ6935<sup>c</sup> | Predicted protein | 405         | 3.14              | 2.72                   |
| 78 | XP_001762972<sup>c</sup> | Predicted protein | 808         | 2.27              |                     |
| 79 | XP_001779748 | Predicted protein | 435         | 2.24              | 2.13                   |
| 80 | EDQ50096     | Predicted protein | 428         | 1.68              | 2.43                   |
| 81 | XP_001775296<sup>c</sup> | Predicted protein | 608         | 1.70              | 1.62                   |
| 82 | XP_001770325 | Predicted protein | 638         | 1.96              |                     |
| 83 | XP_001756363 | Predicted protein, ABA inducible | 448         | 1.90              |                     |
| 84 | XP_001762972<sup>c</sup> | Predicted protein | 269         | 1.75              |                     |
| 85 | EDQ73558     | Predicted protein | 817         | 2.59              |                     |
| 86 | XP_001766107 | Predicted protein | 696         | 1.51<sup>d</sup>  |                     |
|    |              |              | 625         | 2.36<sup>d</sup>  |                     |
| 87 | XP_001757198 | Predicted protein | 733         |                     | -1.58                   |

<sup>a</sup> Mean value from analysis of DIGE replicates unless otherwise stated.
<sup>b</sup> Mean value from analysis of triplicate CBB stained gels.
<sup>c</sup> Dehydration-responsive genes (n=19) identified previously by microarray analysis in protonemal tissues of *Physcomitrella* (Cuming *et al.*, 2007).
<sup>d</sup> Dehydration-responsive gene (n=2) identified previously in gametophore colonies of *Physcomitrella* (Hoang *et al.*, 2009; Saavedra *et al.*, 2006).
LEA proteins are the largest group of proteins up-regulated under dehydration stress

During dehydration and rehydration, a large number of LEA proteins (27 proteins spots) were strongly induced, presumably to cope with the huge water variation in leafy-shoots (Table 1). Quantitative DIGE analysis showed that, during dehydration, 27 protein spots presented a strong increase in abundance, with an average of 4.2-fold increase (range, 1.6–13.7-fold); while during the phase of rehydration 15 protein spots showed 1.5–6.2-fold increase in abundance (Fig. 4). Altogether 27 LEA protein spots were identified as products of 11 LEA genes in Physcomitrella. Of these, nine LEA proteins (21 spots) were annotated as unknown function from leafy-shoots of Physcomitrella as identified by MALDI TOF MS/MS. Average values for the fold change (up-regulation or down-regulation) in the abundance of the proteins are shown.

Table 2. Dehydration- and rehydration-responsive proteins of unknown function from leafy-shoots of Physcomitrella as identified by MALDI TOF MS/MS. Average values for the fold change (up-regulation or down-regulation) in the abundance of the proteins are shown.

| No | Spot number | Experimental Mr/pl | Up-regulation folds<sup>a</sup> | Down-regulation fold<sup>a</sup> |
|----|-------------|---------------------|-------------------------------|---------------------------------|
|    |             |                     | Drought | Rehydration | Drought | Rehydration |
| 1  | 220         | 71.62/5.13          | 14.47   | 7.78         |          |             |
| 2  | 826         | 14.92/5.39          | 7.22    | 5.33         |          |             |
| 3  | 809         | 26.09/5.42          | 5.63    | 2.37         |          |             |
| 4  | 400         | 20.44/4.85          | 3.62    | 2.98         |          |             |
| 5  | 649         | 43.53/5.96          | 3.20    |              |          |             |
| 6  | 810         | 23.31/5.58          | 2.82    | 2.3          |          |             |
| 7  | 821         | 21.85/5.38          | 2.76    | 3.01         |          |             |
| 8  | 296         | 21.50/4.87          | 2.60    | 2.04         |          |             |
| 9  | 613         | 72.57/5.99          | 2.51    | 2.05         |          |             |
| 10 | 803         | 62.63/5.67          | 2.32    |              |          |             |
| 11 | 683         | 20.86/5.06          | 2.24    | 1.97         |          |             |
| 12 | 662         | 33.34/6.46          | 2.21<sup>b</sup> | 3.06<sup>b</sup> |          |             |
| 13 | 688         | 15.04/5.27          | 2.13    | 1.67         |          |             |
| 14 | 811         | 17.42/5.26          | 2.11    | 3.13         |          |             |
| 15 | 804         | 82.66/4.88          | 1.79    |              |          |             |
| 16 | 616         | 79.60/6.33          | 3.44<sup>b</sup> |            |          |             |
| 17 | 820         | 23.72/5.59          | 3.63    |              |          |             |
| 18 | 818         | 64.52/6.14          | 2.87    |              |          |             |
| 19 | 816         | 64.72/5.94          | 2.24    |              |          |             |
| 20 | 906         | 17.06/5.58          |        | –4.69        |          |             |
| 21 | 902         | 62.95/5.46          | –1.71   |              |          |             |
| 22 | 708         | 50.00/5.34          | –1.98<sup>0</sup> | –1.88<sup>0</sup> |          |             |
| 23 | 750         | 40.05/6.32          | –1.50<sup>0</sup> | –1.81<sup>0</sup> | –1.81<sup>0</sup> |             |
| 24 | 739         | 15.31/4.74          | –1.88   |              |          |             |
| 25 | 714         | 40.94/5.62          | –1.63   |              |          |             |
| 26 | 701         | 84.83/5.06          | –1.88   |              |          |             |

<sup>a</sup> Mean value from replicated DIGE analysis.
<sup>b</sup> Mean value from analysis of triplicate CBB stained gels.

Fig. 3. Distribution of functional categories of the differentially-expressed proteins in Physcomitrella leafy-shoots in response to dehydration (A) and rehydration (B).
group 3 LEA characterized by multiple repeated 11-mer sequences (Dure, 1993, 2001); the remaining two (6 spots) were identified as group 2 LEA (dehydrins) characterized by sequences corresponding to K-domain (EKKGIMDKI-KEKLPG) and/or Y-domain (V/TDEYGNP) (Close et al., 1989, 1993; Close, 1996; Campbell and Close, 1997). However, there were no group 1 LEA proteins revealed in this study. Further details on the LEA proteins are provided in Supplementary data SM1 at JXB online.

Interestingly, it was found that three LEA proteins in Physcomitrella (XP_001769448, EDQ69797, and EDQ50092) display multiple isoforms that appeared to respond dynamically to the process of dehydration and rehydration. These isoforms of LEA proteins occupied an acidic region with high molecular weight on gels (Fig. 2). One of these appears as five spots of 77 kDa and four spots of lower mass in a pI range of 5.35 and 5.48 (Fig. 2, main spots 207 and 217). Although protein isoforms have been frequently observed on 2-D gels, relatively little is known about isoforms of LEA protein (Tolleter et al., 2007). The correlation between their multiple isoforms and the dehydration/rehydration conditions could provide an important clue for in-depth study of biological function of these LEA proteins.

About 90% of LEA proteins identified here have not been reported in previous proteome studies of Physcomitrella (Wang XQ et al., 2009; Sarnighausen et al., 2004; Cho et al., 2006). This could be due to several reasons including the plant material used, the efficiency of protein extraction, and the MS technique. However, the present proteomics data on leafy-shoots is in excellent agreement with the microarray results of Physcomitrella protonemal tissue (Cuming et al., 2007). Sequence comparison confirmed that seven LEA genes that were up-regulated in dehydrated protonemal tissues encoded 15 LEA protein spots which were seen on the 2-D DIGE gels of dehydrated/rehydrated leafy-shoots (Table 1; Fig 2).

Most of LEA proteins had a detectable basal level of expression in untreated control leafy-shoots. There were 13 protein spots corresponding to the products of seven LEA genes which were expressed in untreated control plants as seen on 2-D gels (see Supplementary Fig. S1 at JXB online; Fig. 4). The expression profiles of these were studied using a semi-quantitative RT-PCR analysis (Fig. 6). Nine of the 11 LEA genes were significantly transcriptionally induced in response to dehydration and rehydration. Only two genes encoding protein spots 403 and 684 showed a smaller change at the transcript level. The majority of LEA genes had a detectable basal level of expression in control leafy-shoots, and their synthesis was induced when dehydration stress was applied (Fig. 6). A detectable basal level of expression of LEA proteins has not been reported in previous investigations on Physcomitrella (Frank et al., 2005; Oldenhof et al., 2006; Cuming et al., 2007; Wang XQ et al., 2009). These unexpected findings partially support the controversial hypothesis based on a series of studies on desiccation-tolerance in moss T. ruralis, that the mechanism of desiccation tolerance in bryophytes probably involves a constitutive level of cellular protection (Wood and Oliver, 2004; Oliver et al., 2005).

The coherence between the responses at the protein and transcript levels indicates that LEA proteins are the largest group of proteins that accumulated significantly during dehydration in Physcomitrella. So far, hundreds of LEA proteins have been isolated from vascular and non-vascular plants (see reviews in Dure et al., 1989; Close, 1997; Cuming, 1999; Shih et al., 2008), as well as from microbes and animals (Volker et al., 1994; Browne et al., 2002; Gal et al., 2004; Tunnacliffe et al., 2005; Wang et al., 2007). In most cases, LEA transcripts only appear under stress conditions or in embryos of plant seeds, and are therefore suggested to be associated with desiccation.
tolerance (see reviews in Bray, 1994; Ingram and Bartels, 1996; Cuming, 1999; Hundertmark and Hincha, 2008; Shih et al., 2008). Numerous studies support the notion that the dehydration-induced LEA proteins function as cellular protectants to stabilize cellular components by preventing protein aggregation/denaturation and plasma membrane fusion caused by water loss (Chakrabortee et al., 2007; Shih et al., 2008). However, several other studies have revealed that LEA proteins may be constitutively expressed in plants such as pea (Robertson and Chandler, 1994), birch (Rinne et al., 1999), Arabidopsis (Nylander et al., 2001), citrus (Sanchez-Ballesta et al., 2004), the moss T. ruralis (Wood and Oliver, 2004), similar to Physcomitrella shown in the present study. It is possible that a certain basal level of expression of LEA proteins is required for growth and development of leafy-shoots under unstressed conditions, or may represent a constitutively expressed defence system that is required at the onset of dehydration. The precise function of LEA proteins remains to be determined.

Leafy-shoots display an impressive set of defence mechanisms

Apart from LEA proteins, our proteomics data revealed 13 other proteins that are likely to be involved in the defence
mechanisms of *Physcomitrella*. Of these, four proteins (seven spots) known to be associated with membrane components and membrane stabilization were highly expressed in dehydrated/rehydrated leafy-shoots. These are actinoporins (spots 439, 408, 685), phosphatidylethanolamine binding protein (spot 284), phospholipase D (spots 209, 210), and arabinogalactan protein (spot 449). Actinoporin is a homologue of pore-forming cytotoxin protein studied extensively in sea anemone (Hoang et al., 2009). In leafy-shoots, three protein spots corresponding to actinoporins were found to increase in abundance during dehydration, with an average increase of 2-fold and to an average increase of about 6-fold during rehydration (see Fig. 2; Table 1). RNA blot analysis revealed that actinoporins of *Physcomitrella* were induced by dehydration stress (Hoang et al., 2009). These have been proposed to be adapted to bind phospholipid molecules via an atypical Trp cluster (Hoang et al., 2009). On the basis of the observed highest abundance of actinoporins in rehydrated leafy-shoots, it is suggested that these proteins with a known hemolytic activity (Hoang et al., 2009) play a key role in the recovery of the normal function of membranes.

Phosphatidylethanolamine-binding protein (PEBP, spot 284) was another phospholipid-associated protein identified in this study, which showed a remarkably high level of induced expression in dehydrated and rehydrated leafy-shoots (9-fold or 3-fold, respectively; see Fig. 2; Table 1). Recently, four genes encoding PEBP have been annotated in the *Physcomitrella* genome (Hedman et al., 2009). PEBP (spot 284) shows a low sequence similarity to all of them and, therefore, may be a new member of the PEBP family. PEBP family members are involved in many important biological processes, including membrane fluidity and membrane biogenesis (Moore et al., 1996; Frayne et al., 1998), anti-apoptotic responses (Zhang et al., 2007), and the regulation of ABA signalling during seed germination (Xi et al., 2010). It is possible that the PEBP proteins that appear to be highly expressed in *Physcomitrella* play a significant role in dehydration tolerance.

Arabinogalactan protein (AGP, spot 449) and phospholipase D (PLD, spots 209, 210) appeared to accumulate steadily throughout the dehydration/rehydration cycle (Table 1). It is well known that AGP is a typical glycosylphosphatidylinositol (GPI) anchored protein tightly bound to the outer side of plasma membranes in flowering plants (Schultz et al., 2000; Eisenhauer et al., 2003). Under osmotic stress due to high salt, a massive up-regulation of AGP is thought to act as a buffer zone to prevent direct interaction of the naked membrane with the wall matrix (Lamport et al., 2006; Seitz et al., 1999). The observed higher expression of AGP (>2-fold) in *Physcomitrella* leafy-shoots subjected to dehydration and rehydration suggests that this mechanism of membrane stabilization seen in flowering plants has been retained in the less evolved species. Interestingly, two isoforms of a key enzyme, phospholipase D (PLD, spots 209, 210) that controls AGP release under stress conditions (Munnik et al., 2000; Rueland et al., 2002) were also identified. To summarize, the above proteins associated with membrane components and membrane stabilization may be an indication that *Physcomitrella* has an ancient and efficient defence system to protect membranes against dehydration stress.

Among proteins in the defence category, the highest induction was found to be dehydroascorbate reductase (spot 277), which exhibits >12-fold higher expression during both dehydration and rehydration (Table 1). Dehydroascorbate reductase linked to ascorbate metabolism is one of the key enzymes responsible for the removal of active oxygen species (AOS). Other enzymes involved in AOS metabolism, for example, thioredoxin peroxidase (spot 279) and 2-Cys peroxiredoxin (spot 735), were also identified in the study (Table 1). In addition, it was found that expression of three proteins involved in quinone redox cycling was altered in the course of dehydration/rehydration, including two benzoquinone reductases (BR, spots 679, 680) and quinone oxidoreductase (QR)-like protein (spot 720). Several reports have supported the importance of these enzymes in protection against oxidative stress (Ernst et al., 1987; Brock and Gold, 1996; Matvienko et al., 2001). Therefore, in *Physcomitrella*, ascorbate metabolism and redox cycling of quinones could be dominant modules in its anti-oxidation defence system.

**Importance of protein biosynthesis and quality control systems**

Expression of 13 proteins that function in protein biosynthesis and quality control was enhanced in dehydrated/rehydrated leafy-shoots. These comprise eukaryotic initiation 4A (spot 648), eukaryotic initiation factor 5A-2 (spot 686), various HSPs (>60 kDa, seven spots), proteases (three spots), gamma interferon inducible lysosomal thiol reductase (GILT, spot 812), protein disulphide isomerase-like (PDI, spot 639), and calnexin 1 (spot 647) (Table 1). The first two proteins function in translation machinery and were up-regulated 2–3-fold in dehydrated leafy-shoots, suggesting that the protein biosynthesis in *Physcomitrella* was active during dehydration. This observation is in contrast to that in *T. ruralis*, wherein the synthesis of proteins during dehydration is highly unlikely due to the loss of polyribosomes (Oliver, 1991; Oliver and Bewley, 1997). It is suspected that the phase discrepancy in protein synthesis among bryophytes is related to their capability of dehydration tolerance.

An enhancement in the synthesis of proteins involved in processes for quality control (protein folding and stability, proteolysis) is a fundamental molecular response to various adverse environmental conditions (Hartl, 1996; Wickner et al., 1999; Cui et al., 2005, 2009). These processes are aimed at maintaining proteins in their functional conformations and prevent protein aggregation. Several molecular chaperones (>60 kDa), such as Hsp 60, Hsp 70, Hsp 90, and Hsp100 (spots 806, 621, 618 607, 610), have been identified but no small molecular mass Hsps. In addition, several proteases such as chloroplast FtsH protease (spot 234), FtsH-like protein Pftf precursor (spot 631), and putative
serine carboxypeptidase II (spot 402) were also dehydration-responsive (Table 1). Obviously, proteins belonging to the functional category, protein quality control, play an important role in the responses of Physcomitrella to dehydration stress. However, the fold up-regulation of these proteins was only an average of 1.99-fold, lower than that of proteins in categories such as seed maturation (average, 3.83-fold) and defence (average, 4.41-fold) (Table 1). By contrast, the functional category of protein quality control is relatively highly induced in vascular plants in the tolerance to diverse stresses (Cui et al., 2005, 2009). Thus, it is difficult to establish a cause-and-effect relationship between the lower expression of the protein quality control system with the higher capability of dehydration tolerance exhibited in Physcomitrella.

In this study, it was found that the abundance of gamma interferon inducible lysosomal thiol reductase (GILT, spot 812) and protein disulphide isomerase-like (PDI, spot 639) was increased more than 2-fold in dehydrated/rehydrated leafy-shoots (Table 1). GILT and PDI belong to the thiol reductase family, which is involved in the reduction, oxidation, and isomerization of protein disulphide bonds in cells (Lundstrom-Ljung and Holmgren, 1998; Bogunovic et al., 2008). It has long been known that protein conformational fluctuations by thiol/disulphide transfer are involved in multiple biological processes including stress tolerance (Moriarty-Craige and Jones, 2004), as seen here for Physcomitrella exposed to dehydration/rehydration stress.

Changes in photosynthesis and energy metabolism

During dehydration and rehydration, the largest group of down-regulated proteins consisted of those involved in photosynthesis. The abundance of carbonic anhydrase (CA, four spots), an enzyme catalysing CO₂ into bicarbonate (Khalifah, 1971; Moroney et al., 2001), decreased throughout dehydration and rehydration treatments (Table 1). Similarly, expression of Rubisco activase (three spots) and Rubisco large subunit (two spots) also decreased. Since these enzymes function in the initial reactions of CO₂ assimilation, the observed decreased levels of these enzymes strongly suggested that the uptake of CO₂ might be affected under dehydration/rehydration conditions. However, key enzymes/proteins in the Calvin cycle and light reaction of photosynthesis did not exhibit significant changes in abundance on 2-D gels during dehydration. In this functional category, abundance of only four proteins (spots 668, 690, 722, 745, Table 1) was altered slightly during rehydration. The fact that the abundance of the major enzymes/proteins in the photosynthetic machinery was largely unperturbed during the dehydration/rehydration stress could be an important cellular and molecular basis for coping with drought stress in Physcomitrella.

On the other hand, there was a change in the abundance of four proteins involved in glycolysis and the pentose phosphate pathway (Table 1), namely, phosphoglycerate kinase (spots 431, 430), transketolase (spots 614, 901), glyceraldehyde 3-phosphate dehydrogenase (spot 661), and phosphoglucomutase (spot 903). Also, there was an increase in the abundance of a putative ATP synthase (spot 672). These observations may be an indication of an alteration in energetic status within cells of Physcomitrella during dehydration and rehydration.

Other metabolism and proteins of unknown function

During dehydration, expression of nucleoside diphosphate kinase (NDPK, spot 315) decreased. NDPKs are ubiquitous enzymes found in all organisms and cell types, and have distinct biological function in catalysing the synthesis of nucleoside triphosphates (Choi et al., 1999). Abundance of three enzymes involved in both glutamine and arginine metabolism declined during rehydration (Table 1): two glutamine synthetases (spots 716 and 713) and an arginase (spot 909). The reduced levels of these enzymes reflect the repression of a fundamental nucleoside metabolism in leafy-shoots during dehydration/rehydration. Apart from the differentially-expressed proteins that are involved in distinct metabolic pathways, 27 spots corresponding to 24 different proteins (encoded by 24 unique genes) with no functional annotation also showed significant changes in abundance during dehydration and rehydration (Table 1). They are predicted or hypothetical proteins in the Physcomitrella genome database, 19 of which were reported for the first time as dehydration/rehydration-responsive proteins. A predicted protein with two isoforms (represented by spots 223 and 218) showed the highest induction of 14.95- to 20.77-fold during dehydration; another two proteins represented by spots 689 and 442, were also elevated by 10.42-fold and 10.29-fold under the same conditions. In addition, nine predicted proteins were up-regulated more than 5-fold. These proteins of unknown functions that are highly expressed under dehydration/rehydration conditions may be of interest for an in-depth study of dehydration tolerance in leafy-shoots of Physcomitrella.

Concluding remarks

Leafy-shoots of Physcomitrella appear to possess a complex molecular mechanism to cope with huge variations in water availability. By a comprehensive proteomic analysis supported by the confirmation of transcription of selected protein spots, major dynamic changes in protein synthesis were revealed in the haploid vegetable tissues of Physcomitrella during dehydration and rehydration. In contrast to other moss plants, Physcomitrella leafy-shoots appear to be well prepared to utilize their preformed defence system under dehydration and rehydration conditions, as indicated for example, by the basal level of expression of a substantial number of LEA proteins even in the presence of an adequate water supply. This unexpected finding, together with 87 dehydration/rehydration-responsive proteins identified in the study, reveal some of the essential molecular details of dehydration responses in Physcomitrella. To our knowledge, most proteins identified here have not previously been described in Physcomitrella.
The experimental results presented here support the view that dehydration tolerance in moss plants has certain points of similarity with desiccation tolerance in angiosperm seed maturation. The proteomic analysis leads to the conclusion that leafy-shoots of Physcomitrella adopt a dual protective strategy against dehydration, which consists of maintaining a basal level of synthesis of stress-tolerance proteins and enhancing their synthesis further under stress. Such mechanisms may be poorly represented or absent in higher vascular plants that cannot survive in severe cellular dehydration. On the other hand, it was found that certain key molecular players that are known to contribute significantly to dehydration tolerance were induced in the leafy-shoots, indicating that these have been well conserved through evolution.

The data presented here provide a strong and reliable basis for future studies to elucidate the functional significance of specific proteins associated with plant adaptation to dehydration tolerance, a feature that is essential for land colonization.

**Supplementary data**

Supplementary data can be found at *JXB* online.

Supplementary Table S1. A list of all primers used in the study.

Supplementary Table S2. The MS/MS data set for all protein spot with peptide sequence tags.

Supplementary Fig. S1 Dynamic proteome profiles generated from traditional 2-DE.

Supplementary data SM1. Supplementary data SM1. Material on the identification of LEA proteins.

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