Running head: Protein Profile of Desiccation-Tolerance in *P. patens*

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Exploring the Mechanism of *Physcomitrella patens* Desiccation Tolerance through a Proteomic Strategy

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Abstract

The moss, *Physcomitrella patens*, has been shown to tolerate abiotic stresses, including salinity, cold, and desiccation. To better understand this plant’s mechanism of desiccation tolerance, we have applied cellular and proteomic analyses. Gametophores were desiccated over one month to 10% of their original fresh weight. We report that during the course of dehydration, several related processes are set in motion: plasmolysis, chloroplast remodeling, and microtubule depolymerization. Despite the severe desiccation, the membrane system maintains integrity. Through two-dimensional gel electrophoresis (2-DE) and image analysis, we identified 71 proteins as desiccation-responsive. Following identification and functional categorization, we found that a majority of the desiccation-responsive proteins were involved in metabolism, cytoskeleton, defense, and signaling. Degradation of cytoskeletal proteins might result in the cytoskeletal disassembly and consequent changes in the cell structure. Late embryogenesis abundant (LEA) proteins and reactive oxygen species-scavenging enzymes are both prominently induced and they might help to diminish the damage brought by desiccation.
INTRODUCTION

Water is a key component of living organisms and one of the critical environmental factors that controls the distribution of plants on the earth. With increasing urbanization and human progress, water deficiency looms as a widespread and worsening agricultural problem. In such a scenario, the ability of plants to tolerate water deficit has important economic ramifications.

Since the first plant grew on land, plants have had to confront different kinds of extreme environmental stresses. Desiccation, defined as the complete loss of “free” water from an organism (Gaff, 1971), is an extreme stress. During life's long history, terrestrial plants have evolved a suite of mechanisms for surviving and thriving under a water-limited environment (Wood, 2005). Desiccation tolerance is the ability of plants to recover from low cellular water content (5–15%). Although such tolerance is a common among plant propagules, such as spores, pollen grains, and seeds, the vast majority of vegetative and reproductive tissues do not survive desiccation (Bewley, 1979; Oliver and Bewley, 1997; Porembski and Barthlott, 2000; Proctor and Smirnoff, 2000; Oliver et al., 2000a). Phylogenetic analyses suggest that most of the vegetative desiccation-tolerant plants belong to earlier evolutionary clades, such as algae, lichens, and bryophytes. These comprise the pioneer plants that are believed to have first colonized land. However, during the course of evolution, the ability to tolerate desiccation was not retained in most vascular plants (Tuba et al., 1998; Proctor and Smirnoff, 2000). Because bryophytes are generally one cell-layer thick and have no water-conducting organs and few stomata, water is rather easily gained or lost from the surrounding environment. When free water is depleted from the surface of the plant, the leaf cells immediately move toward equilibrium with the water potential of the surrounding air, resulting in desiccation (Oliver et al., 2000b; Hoekstra et al., 2000b).
Therefore, bryophytes needed to evolve mechanisms to survive in dry environments in order to fully exploit terrestrial habitats.

Desiccation tolerance is a complex phenomenon, which is just beginning to be understood. Many scientists believe that a thorough understanding of desiccation tolerance mechanisms will facilitate the creation of water-tolerant crop species (Bartels and Sunkar, 2005; Bohnert et al., 2006). Because of this, many studies of desiccation-tolerance mechanism have been carried out in bryophytes, which include the majority of the desiccation-tolerant plants (Smirnoff, 1992; Oliver et al., 2000a; Oliver et al., 2000b; Proctor, 2001; Oliver et al., 2005). Summarizing previous results, Bewley and Oliver (Bewley and Oliver, 1992) proposed that a plant could be desiccation tolerant if it had the ability to protect its cellular integrity in the dried state and to mobilize repair mechanisms upon subsequent hydration.

More recently, genomic-level studies have been initiated. Most of these studies have made gene expression profiles (Oliver et al., 2004; Oliver et al., 2005; Weng et al., 2005; Iturriaga et al., 2006) and proteomic studies, fewer in number, have focused on metabolic recovery following rehydration (Oliver and Bewley, 1997; Tuba et al., 1998; Proctor and Smirnoff, 2000). It has been shown that during the first two hours of hydration of dried Tortula ruralis gametophores, the synthesis of 74 proteins is up-regulated and that of 25 proteins is down-regulated (Oliver, 1991). The up-regulated proteins are known as rehydrins, and the down-regulated are termed hydrins. Few studies have been performed at the proteome level aimed at exploring the mechanisms of desiccation tolerance.

The moss, Physcomitrella patens, has been used as a model system in plant science for years because of the several advantages it offers (Frank et al., 2005; Minami et al., 2005; Quatrano et al., 2007). Adding to its usefulness, this species'
genome has recently been fully sequenced (Rensing et al., 2008), which renders it suitable for functional genomics. Here, we report that *P. patens* is desiccation tolerant, with changes in cell structure correlated with changes in the proteome. Furthermore, proteomic analysis reveals that some mechanisms of vegetative desiccation tolerance in *P. patens* are related to those that occur during seed maturation in angiosperms.

**RESULTS**

*Physcomitrella patens* is a desiccation-tolerant plant

Four-week-old *P. patens* gametophores were subjected to slow dehydration by drying in a desiccator until there was a 90% loss in their fresh weight. Usually, it takes one month to obtain desiccated *P. patens* gametophores. As a consequence of the severe water loss involved, we define this treatment as desiccation. Besides the shrinking of the *P. patens* gametophores, the color changed to dark green during desiccation treatment (Fig. 1A and B). During the treatment, chlorophyll content declined slightly, particularly as water loss became more extreme (Fig. 2). Interestingly, desiccation did not result in the death of the *P. patens* gametophores. Following the desiccation treatment, the *P. patens* gametophores were rehydrated by floating in distilled water for 2 h, and subsequently cultivated in standard growth conditions. This rehydration promoted the recovery of plants that thereafter exhibited vigorous growth and development, all but indistinguishable from that of untreated plants. In neither morphology nor fresh weight (data not shown) could we detect significant differences between control plants and those subjected to desiccation and rehydration (Fig. 1C and D). Based on these results, we conclude that *P. patens* is desiccation-tolerant.

**Cellular structure in desiccated *P. patens***

As a desiccation-tolerant plant, *P. patens* must be able to limit damage to a repairable
level and to maintain the integrity of cellular structures during desiccation. In the desiccated state, the central large vacuoles were broken into many small vesicles and the cytoplasm appeared far denser than it did prior to treatment (Fig. 3A and B). In spite of the dramatic shrinkage of the cell, the plasma membrane seemed to maintain its integrity, as judged from complete profiles encircling the protoplast and by clear differences in density between the cytoplasm and periplasm. During desiccation, chloroplasts appeared more spherical compared to their usual ellipsoidal shape (Fig. 3A and B), consistent with morphologies reported previously to occur in vascular plants subjected to drought and salt stresses (Abdelkader et al., 2007; Barhoumi et al., 2007). Furthermore, during desiccation, starch grains, which filled this organelle prior to desiccation, were lost. Grana gradually became less prominent, and plastoglobuli-like structures appeared (Fig. 3C). Besides the vacuole and chloroplast, other organelles were not obviously disrupted.

Proteome profile of *P. patens* during desiccation

To gain further understanding of the molecular mechanisms that contribute to the survival of gametophores of *P. patens*, we extracted total proteins from control and desiccated gametophores and separated them using 2-DE. Representative 2-DE images were established for both the control and treated samples in a pH range of 4 to 7 and a molecular-mass range of 10 to 100 kDa (Fig. 4). There were obvious differences between the gels of control and desiccated plants, which indicated that desiccation dramatically changed the profile of expressed proteins of the gametophores.

To analyze the changes in the proteome profile, we compared gels of each sample, digitized after staining with coomassie blue as described in Materials and Methods. Data from three biological replicates were collected and only those spots
used that were detected in all three replicate gels. Based on this criterion, more than 1,300 protein spots could be detected for each sample. Statistical analysis of the data from all replicates enabled us to identity 71 protein spots that were significantly altered in response to the desiccation. Among them, 25 spots represented down-regulated proteins (spots D1-D25; Fig. 4), 33 spots represented up-regulated proteins (spots U26-U58; Fig. 4), and 13 spots represented de-novo-induced proteins (spots I59-I71; Fig. 4). Quantitative data confirm that the abundance of these proteins altered significantly (Fig. 5).

The desiccation response is a complicated physiological process, in which many biochemical processes might be initiated or inhibited. So, it is reasonable to expect changes in the abundance of many proteins; however, only 71 out 1300 spots changed intensity by more than two-fold. This surprising stability of the desiccated proteome suggests that those 71 proteins that did change are worthy of further attention for elucidating the mechanism of desiccation tolerance.

**LC-MS/MS protein identification and functional categorization**

To categorically identify the nature of proteins involved in desiccation tolerance, we used an LC-MS/MS approach. All 71 differentially displayed protein spots were excised from preparative 2-D gels, digested with trypsin, and analyzed through LC-MS/MS. We searched the amino acid sequences against the databases using the SEQUEST software as described before (Wang et al., 2008). All of the differentially expressed proteins could be identified, with just 19 of them categorized as unknown or putative proteins (Table 1). Following the functional definitions of Bevan et al. (1998), the down-regulated proteins fell into six categories whereas up-regulated (including induced) proteins required only five (Fig. 6). Except for the unknown category, only two categories were shared by down-regulated and up-regulated
proteins: metabolism and cytoskeleton/structure. Proteins of the functional categories of detoxification, protein synthesis, and transcription were present only in the down-regulated group, whereas those in defense and signaling categories were specific to the up-regulated group (Fig. 6 and Table 1).

**Changes of metabolic proteins**

Twenty-five proteins categorized within the metabolism group were altered dramatically in response to desiccation treatment (Table 1, Fig. 6). Among these proteins, eight were down-regulated and 17 were either induced or up-regulated. Proteins of this category are involved in basic metabolic process such as photosynthesis, sugar catabolism, or amino acid metabolism. Generally, the proteins involved in anabolism are down-regulated, and those involved in catabolism are up-regulated (or induced) by desiccation (Table 1).

Photosynthesis is one a major and characteristic metabolic process of green plants. Under desiccation conditions, both the large (spot D14) and small subunits (spots D23, D25) of ribulose bisphosphate carboxylase (Rubisco), which is a major photosynthetic enzyme, decreased steeply in abundance (Fig. 5). Desiccation also down regulated chloroplast ATP synthases (spots D3, D19). Nonetheless, the light-harvesting chlorophyll a/b-binding protein 2 (spots I59, I60) and a major chlorophyll-binding protein (spot I61) were induced. Interestingly, the observed molecular weights of these three chlorophyll-binding proteins were higher than their theoretical weights by about 44 kDa. The reason for this increase is not clear but might result from an unusual post-translational modification.

Several proteins that are involved in sugar catabolism were found to be up-regulated during desiccation. These proteins include alpha-glucan water dikinase (spot I62), transketolase (spot U33), rhamnose biosynthetic enzyme 1 (spot U43),
fructokinase (spots U44, U45), and glyceraldehyde-3-phosphate dehydrogenase (spots U47, I64).

In addition to the above-mentioned proteins, some proteins involved in the metabolism of fatty acids, amino acids, and nucleotides also changed in abundance in response to the desiccation treatment. These proteins include enoyl-CoA hydratase (spot I69), 3-isopropylmalate dehydrogenase (spot D5), S-adenosylmethionine synthetase (spot D6), ketol-acid reductoisomerase (spot U27), aminoacylase (spot U40), subtilisin-like proteases (spot I63), and phosphoribosyl diphosphate synthase (spots I65, I71) (Table 1, Figs. 4 and 5).

**Changes in cytoskeletal proteins**

Several cytoskeletal proteins were identified as desiccation-responsive. Except for ftsZ1 (spot U46), the changed cytoskeletal proteins were all down-regulated by desiccation. These were β-tubulin (spot D1), α-tubulin (spot D2), actin (spot D4), and myosin heavy chain (spot D11). Both the tubulins are constituents of microtubules, actin is the basic unit of microfilaments, and myosin is a motor protein that associates with actin. The decrease of these cytoskeletal proteins indicates that the cytoskeleton might be disassembled in response to desiccation. This hypothesis was supported by observations with fluorescence microscopy, which show a lattice work of microtubules surrounding the chloroplasts in control gametophores but no filamentous structures in desiccated gametophores (Fig. 7).

**Proteins involved in defense and signaling**

Upon desiccation, many proteins related to defense and signaling underwent large changes in abundance. Interestingly, all these changed defense or signaling proteins were up-regulated (Table 1 and Fig. 5). These might be instrumental in protecting the cells from incurring damage during the desiccation treatment. We identified three
signaling proteins: calcium-dependent protein kinase-like protein (spot U28), ser/thr-specific protein kinase-like protein (spot U32), and IAA/AUX protein (spot U48). The defense proteins included heat shock proteins (HSPs, spots U29, U35, U36, U37, and U38; mainly HSP70), luminal binding protein (spot U55), the endoplasmic reticulum molecular chaperone, which also belongs to the HSP70 family (Rothman, 1989), and some anti-oxidation proteins (spots U53, U54, U57, I66, and I68). The anti-oxidation proteins, including ascorbate peroxidase (APX; spots U53, I66), thylakoid-bound ascorbate peroxidase (spot U54), peroxiredoxin (Prx; spot U57), and 2-Cys peroxiredoxin (2-Cys Prx; spot I68) were up-regulated and induced. These proteins are important scavengers of reactive oxygen and are implicated in defense against oxidative damage. Besides these canonical defense proteins, other proteins included in this group are a dehydrin (spot U34) and physcomitrin (spot I70).

DISUSSION

The majority of the vegetative desiccation-tolerant plants belong to the algae, lichens, and bryophytes, all of which belong evolutionarily to early arising clades within the plant kingdom (Oliver et al., 2000a). A recent survey has shown that about 158 species of mosses are vegetative desiccation-tolerant (Wood, 2007). In this study, we show that the model moss, *P. patens*, is also desiccation-tolerant. It recovers following a severe desiccation treatment. The ultrastructural and proteomic studies can help to reveal the strategy applied by this moss species to survive desiccation.

Alterations in cellular structure help to limit desiccation damage

Desiccation is a phenomenon of severe water loss. To be desiccation-tolerant, a plant must meet three criteria: 1) constrain the damage to a repairable level; 2) maintain its cellular integrity under the desiccation conditions; 3) immediately mobilize the repair
mechanisms upon the following hydration (Bewley, 1979). Upon dehydration, the vacuoles of three desiccation-tolerant angiosperm species were reported to be filled with non-aqueous materials (Farrant, 2000). Additionally, in Xerophyta humilis, most of the cytoplasm was filled with vacuoles, which were argued to help the cell prevent plasmolysis; whereas, Craterostigma wilmsii maintained small vacuoles and prevented plasmolysis through invagination and folding of the cell wall (Farrant, 2000). A third species, Myrothamnus flabellifolius, exhibited a pattern that was intermediate between the above-mentioned two species, and was in fact similar to that of P. patens observed here where the central vacuole became vesiculated and, although plasmolysis was not prevented, the plasma membrane was not obviously damaged (Fig. 3).

Additionally, we found that desiccation resulted in the up-regulation of rhamnose biosynthetic enzyme 1 (spot U43). This enzyme catalyzes the synthesis of rhamnose, which is a prominent component of cell wall pectin. In angiosperms, pectins have been linked to communication between cell wall and cytoplasm particularly during periods of stress (Humphrey et al., 2007), although it remains to be demonstrated to what extent this holds for bryophytes. Evidently, P. patens survives desiccation with several strategies; how the moss combines these strategies still needs to be determined.

Based on the changes in their photosynthesis apparatus, the vegetative desiccation-tolerant plants can be divided into homoiochlorophyllous and poikilochlorophyllous types (Tuba et al., 1998). In the dried state, plants of the former type retain their chloroplast structure whereas plants of the latter break them down (Proctor and Tuba, 2002). P. patens belongs to the homoiochlorophyllous group: Chlorophyll levels remained high and chloroplast structure was largely preserved,
although changed to a spherical form with less well stacked thylakoids. Furthermore, greening was rapid after hydration, which is characteristic of homoiochlorophytes (Tuba et al., 1993). In this respect, the breakdown of the photosynthetic apparatus induced by desiccation was different from that occurring in senescent leaves, in which the chloroplast is dismantled completely (Tuba et al., 1993). The changes in the \textit{P. patens} chloroplast structure may be viewed perhaps as part of a meticulously executed protection strategy of the plant facilitating its survival under non-optimum conditions.

An important player in the reorganization of the photosynthetic apparatus might be FtsZ (spot U46) (Kiessling et al., 2000; McAndrew et al., 2001; Kiessling et al., 2004). It has been suggested that a cytoskeleton-like FtsZ network in chloroplasts aids in maintaining the chloroplast structure. Overexpression of FtsZ has been demonstrated to lead to alterations of the chloroplasts in arabidopsis leaves (El-Kafafi el et al., 2008) that are similar to those observed here for desiccated \textit{P. patens} gametophores.

The cytoskeleton forms a dynamic framework that maintains cell shape and organelle motility (Smith, 2003). Recently, the cytoskeleton has been implicated in sensing osmotic stress (Komis et al. 2008). Our observation of the depolymerization of microtubules (Fig. 7) and the disappearance of tubulin (spots D1, D2, D4), along with evidence presented earlier (Pressel et al., 2006; Proctor et al., 2007), indicate that the microtubules might be incompatible with the desiccated state. Interestingly, angiosperm embryos also lose microtubules at maturation (de Castro et al. 1995).

**Metabolism adjustment in response to desiccation**

Alpert and Oliver (2002) suggested that during the drying process the desiccation-tolerant plant needed to shut down its metabolism sequentially. In this study, we found that anabolism-related proteins, mainly photosynthetic proteins,
decreased (Table 1). Moreover, the thylakoid was less well stacked (Fig. 3), the chlorophyll content lowered, and some chlorophyll-binding proteins unusually modified (Fig. 1, Table 1). It is reasonable to conclude that photosynthesis was suppressed during the onset of desiccation. Tuba et al. (1996, 1997) detected inhibition of photosynthesis in *X. scabrida* during drying. It seems that the reduction in the rate of photosynthesis is a common early response even in homiochlorophytes, presumably because of the lack of demand for reducing power.

Respiration was probably affected to a lesser extent than was photosynthesis. Tuba et al. (1996) detected respiration in *X. scabrida* even near the end of the drying period. We find increases in some respiration-related enzymes, such as fructokinase (spot U44) and glyceraldehyde-3-phosphate dehydrogenase (spots U47, I64). The ability to respire despite severe water loss may provide the plant enough energy to adjust its cellular structure and physiological status to counteract the effects of desiccation. Furthermore, increase of these respiration-related enzymes might be preparation for future growth recovery. Additionally, catabolism of polysaccharides, such as starch, was enhanced, at least at the early stage of desiccation. The α-glucan water dikinase (spot I62) catalyzes starch phosphorylation, which is required for starch degradation (Mikkelsen et al., 2004). Therefore, the accumulation of this protein is consistent with degradation of starch grains under desiccation (Fig. 3; Table 1). On one hand starch degradation provides energy for the cell and on the other hand provides carbon skeletons for osmotic adjustment, stabilizing the membrane system and preventing the crystallization of cellular solutes (Hoekstra et al., 2001).

**Other proteins that help to resist desiccation**

As we described above, all the defense and signaling proteins identified in our study were up-regulated by desiccation. This suggests that these proteins play important
roles in ensuring the survival of desiccated *P. patens*. The late-embryogenesis abundant (LEA) proteins were first found in plant seeds (Dure et al., 1981), and subsequently also found in vegetative tissues under different stresses, such as salinity, cold, and drought (Hundertmark and Hincha, 2008). Previous studies have shown that LEA-encoding genes are strongly enhanced in the desiccation-tolerant plants during drying (Piatkowski et al., 1990; Hundertmark and Hincha 2008); therefore they might be important for the acquisition of desiccation tolerance (Bartels and Sunkar, 2005).

Dehydrins (also classified as Group 2 LEA) have been shown to act as key components of dehydration tolerance (Close, 1996, 1997; Zhu et al., 2000; Allagulova Ch et al., 2003). Dehydrins are hypothesized to function by preventing the aggregation of dehydration-sensitive proteins and by stabilizing large-scale hydrophobic interactions, such as those found in membranes and hydrophobic patches of proteins (Tunnacliffe and Wise, 2007).

Heat shock proteins (HSPs) maintain partner proteins in a folding-competent, folded, or unfolded state, to minimize aggregation of non-native proteins, or to target non-native or aggregated proteins for degradation and removal from the cell (Bukau and Horwich, 1998). We detected HSP70s as the major HSPs that play a role in *P. patens’* response to desiccation (Table 1) and cold (data not shown). Recently, these proteins have also been shown to be important for high salinity (Wang et al., 2008). Genome analysis has shown that the HSP70 family has expanded to nine cytosolic members in *P. patens*, whereas all algal genomes sequenced to date encode one single cytosolic HSP70 (Wang et al., 2004). Evolutionally, it seems that HSP70s are a crucial part of plants spreading on land, presumably ensuring resistance to various abiotic stresses.
In addition to the above-mentioned proteins, we also found certain anti-oxidative proteins were up-regulated (or induced) (Table 1). Mechanisms for scavenging reactive oxygen play major roles and act in several organelles, particularly in extreme environments, including dehydration (Smirnoff, 1998, Apel and Hirt, 2004). The efficiency of the scavenging system is closely related to the plant’s ability to resist various abiotic stresses (Smirnoff, 1998). Ascorbate peroxidase (spots U53, U54, I66) and peroxiredoxin (spots U57, I68) are two major enzymes that are up-regulated and are plausibly involved in scavenging of reactive oxygen. In addition, ferritin (spot U56), a protein that is highly conserved and plays a critical role in iron storage and homeostasis, was up-regulated (Theil, 1987; Balla et al., 1992; Harrison and Arosio, 1996). The storage function of ferritins has been associated with a cytoprotective antioxidant effect (Balla et al., 1992; Juckett et al., 1995). Presumably, the increase of all these proteins helps *P. patens* cells to minimize the damage reactive oxygen, and hence to maintain cellular integrity.

**CONCLUSION**

As early land-growing plants, bryophytes like *P. patens* pioneered responses to numerous extreme stresses during their life cycle. Furthermore, most bryophyte organs being single cell sheets or filaments exacerbates the risks of incurring stress-related damage. Nevertheless *P. patens* survives robustly under various environmental stresses, including high salt, low temperature, and desiccation. Here, we show that the proteome changes during desiccation are few and in selected functional categories as expected from a well choreographed response. The challenge now is to discover how each of these groups actually moves the plant toward its goal of survival. We expect that careful proteomic analysis can continue to play an
important role in these discoveries.

MATERIALS AND METHODS

Plant materials and growth conditions

*Physcomitrella patens* (Hedwig) ecotype ‘Gransden 2004’ was grown in modified BCD medium containing 0.5% (w/v) glucose and 0.75% (w/v) agar (Ashton et al., 1979). Gametophores were cultured under standard conditions (23 °C) with a light cycle of 16 h light/8 h darkness and a light intensity of 55 μmol s⁻¹ m⁻². Four-week-old gametophores were used for experiments. Gametophores were desiccated by placing them in a sealed, transparent container containing activated silica gel and held in the growth chamber.

Determination of water content and chlorophyll content

To determine water content, gametophores were weighted at harvest and then dried in an oven at 105°C for 3 days and weighed again. Water content was calculated as the difference between fresh and dry weights divided by fresh weight, and expressed as a percentage. To determine chlorophyll content, freshly harvested samples were extracted in a one to one mixture of acetone and ethanol, as described by Chen (1984), and the absorbance of the extract was measured at 652 nm with a spectrophotometer (GE Healthcare BIO-Science). Chlorophyll content was calculated based on Arnon (1949).

Sample preparation for microscopy

For transmission electron microscopy, fresh tissues were taken and immediately immersed in 2.5% glutaraldehyde fixative in phosphate buffer (0.1 M, pH 7.0) for 10 min, and then in fresh fixative at 4°C for 3 h in darkness. Specimens were
subsequently buffer washed, post-fixed in 1% OsO₄ solution at 4°C for 5 h, dehydrated in a graded ethanol series followed by propylene oxide, and embedded in LX 122 Epon. Sections were post-stained for 5 min in saturated aqueous uranyl nitrate and 5 min in lead citrate. Specimens were examined with a Philips EM-420 electron microscope.

**Immunofluorescence staining, confocal microscopy, and image analysis**

The following antibodies were used: a monoclonal anti-tubulin antibody (Sigma, DM 1A T9026) and goat anti-mouse IgG-FITC (Sigma, IF0030). Gametophores were fixed for 1 h in PME buffer (50 mM PIPES, 5 mM EGTA, 2 mM MgSO₄, pH 6.5) containing 4% paraformaldehyde. The gametophores were cut into fragments and then washed with PME 3×10 min, then treated with 1% Triton X-100 for 30 min and washed with PME 3 by 10 min. Blocking was performed in PBS with 1% BSA overnight and then the samples were stained with monoclonal anti-tubulin antibody at 4°C overnight. Washing 3 times with PBS was followed by incubations with the secondary antibody for 2 h at room temperature in darkness, followed by 3×10 min washes with PBS. The fragments were then mounted in Elvanol under a cover slip. Images were acquired by a Leica SP2 confocal microscope using a 60X water objective. All images presented were assembled using the Photoshop 7.0 software package (Adobe Systems, San Jose, CA).

**Protein extraction and 2-DE**

Proteins were extracted using a phenol extraction procedure. Plant tissue (2 g) was ground to fine powder in liquid nitrogen and homogenized on ice for 30 min with 6 ml ice-cold extraction buffer (250 mM sucrose, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM PMSF, 1 mM DTT). Then an equal volume of ice-cold Tris-HCl (pH
7.5)-saturated phenol was added, and the mixture was re-homogenized for 30 min on ice. After centrifugation (20 min, 15,000 g, 4°C), the phenol phase was collected and re-extracted 2 times with extraction buffer as described above. Proteins were precipitated from the final phenol phase with three volumes of 100 mM ammonium acetate in methanol overnight at -20°C. The pellets were rinsed 3 times with ice-cold acetone containing 13 mM DTT and then lyophilized. The resulting pellets were dissolved in a rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.5% v/v IPG buffer, 1% w/v DTT) at room temperature. 2-D electrophoresis was carried out as follows: dry IPG strips (13 cm long, pH 4-7 linear) were rehydrated for 12 h in 250 μl rehydration buffer containing 800 μg protein samples. Isoelectric focusing was conducted at 20°C with an Ettan IPGphor system (GE Healthcare BIO-Science). Focusing was performed in four steps: 300 V for 1 h, 600 V for 1 h, 1000 V for 1 h, and 8000 V for 6 h. Focused strips were then equilibrated by first incubating them in an equilibration solution (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl, pH 8.8, and 1% w/v DTT) for 15 min, followed by incubation in 4% w/v iodoacetamide in the same equilibration solution for 15 min. For the second dimension, the proteins were separated on 15% SDS polyacrylamide gels. Protein spots were stained with coomassie brilliant blue R-250.

**Image and data analysis**

The 2-DE gels were scanned at a resolution of 600 dots per inch (dpi) with a UMAX Power Look 2100XL scanner (Maxium Tech, inc., Taiwan, China). The transparency mode was used to obtain a grayscale image. The image analysis was performed with an ImageMaster 2D Platinum version 5.0 (GE Healthcare BIO-Science). The optimized parameters were as follows: saliency 2.0, partial threshold 4, and minimum area 50. We quantified spots by determining the ratio of the volume of a single spot to
the whole set of spots. Only those with that chanced by more than two fold in each of the three replicates were used for further analysis.

**In-gel digestion and protein identification**

Protein spots were manually excised from the gel, and in-gel digestion by trypsin was performed according to Shen et al. (2003) with some modifications. Gel slices were washed with 25% v/v ethanol and 7% v/v acetic acid for 12 h (or overnight) at room temperature, and de-stained with 50 mM NH₄HCO₃ in 50% v/v methanol for 1 h at 40°C. Proteins were reduced with 10 mM DTT in 100 mM NH₄HCO₃ for 1 h at 60°C, and alkylated with 40 mM iodoacetamide in 100 mM NH₄HCO₃ for 30 min at room temperature in the dark. The gel pieces were minced and lyophilized, then rehydrated in 25 mM NH₄HCO₃ with 10 ng sequencing-grade modified trypsin at 37°C overnight. After digestion, the peptides were collected and the pellets were washed with 0.1% TFA in 50% v/v acetonitrile three times to collect the remaining peptides. The solution containing eluted peptides was desalted by ZipTipC 18P.

Liquid chromatography (LC) was performed on a surveyor LC system (Thermo Finnigan, San Jose, CA). The C18 column was obtained form Column Technology Inc. (Fremont, CA,). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The tryptic peptide mixtures were eluted using a gradient of 2-98% B over 180 min. The MS/MS was performed on a LTQ linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray interface and operated in positive ion mode. The capillary temperature was set to 170°C and the spray voltage was at 3.4 kV. The acquired MS/MS spectra were compared against the NCBI *Arabidopsis thaliana* and *P. patens* protein database using the TurboSEQUEST program in the BioWorks 3.1 software suite (Thermo). An accepted SEQUEST result had to have a cCn score of at least 0.1
(regardless of charge state). Peptides with a +1 charge state were accepted if they were fully tryptic digested and had a cross correlation (Xcorr) of at least 1.9. Peptides with a +2 charge state were accepted if they had an Xcorr ≥2.2. Peptides with a +3 charge state were accepted if they had an Xcorr ≥3.75.

**Supplemental Data**

**Supplemental Table** Peptides sequence matched of desiccation responsive proteins

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FIGURE LEGENDS

Figure 1. *P. patens* gametophores undergoing desiccation and rehydration. A, Control. B, Desiccated gametophores. C, Gametophores after 2-h-rehydration. D, Gametophores after one-month-rehydration.

Figure 2. Changes of the chlorophyll content during desiccation. Figure plots means ± SD from three replicate experiments.

Figure 3. TEM micrographs of control and desiccated gametophores. A, Control gametophore. B, Desiccated gametophore. C, Enlargement of the image in B. Ch, Chloroplast; CW, Cell wall; LD, Lipid drop; PM, Plasma membrane; N, Nucleus; V, Vacuole. Bar = 2 µm.

Figure 4. 2-DE of total protein extracts from control and desiccated gametophores. Upper image: control; lower image: desiccated. Desiccation-responsive proteins are indicated as follows: D, down-regulated protein; U, up-regulated protein; and I, induced protein. Gels are coomassie stained.

Figure 5. Quantification of the desiccation-responsive protein abundance. A, Down-regulated proteins. B, Up-regulated proteins. Data are means of three independent replicates ± SD.

Figure 6. Functional categorization of the desiccation responsive proteins. Of the eight categories of Bevan et al., six were represented by down regulated proteins and only five by up-regulated proteins.

Figure 7. Images of microtubules in control and desiccated gametophores. A and B, Control gametophores; C and D, desiccated gametophores. A and C are bright-field images of the whole-mount preparations imaged with epi fluorescence shown in B and D. Microtubules (indicated by red arrow) form a lattice work surrounding chloroplasts in control plants (B) but de-polymerize into dot structure (indicated by red arrow) in the desiccated plants (D). White arrows (B and D) show the chloroplast. Bright punctate staining in the control is probably starch. Bar = 10 µm.
### Table 1. Identification of dehydration stress-responsive proteins in *P. patens*

| Metabolic group          | Spot No. | Theor./exp. Mass (kDa) | Theor./exp. pI | Accession No. | NMP (%) | SC (%) | Description                                       | Species  |
|--------------------------|----------|------------------------|----------------|---------------|---------|--------|--------------------------------------------------|----------|
| **Energy and material**  | D3       | 55.3/60.7              | 5.31/5.39      | BAC85066.1     | 20      | 39.25  | ATP synthase alpha subunit                       | *P. patens* |
|                          | 55.3/60.7 | 5.31/5.39              |                | NP_904216.1    | 20      | 39.25  | ATP synthase CF1 alpha chain                    | *P. patens* |
| **metabolism** (35.21 %) | D5       | 43.4/56.7              | 5.80/4.86      | CAA71268.1     | 1       | 2.96   | hypothetical 3-isopropylmalate dehydrogenase    | *A. thaliana* |
|                          | D6       | 43.2/54.8              | 5.51/5.71      | P23686         | 1       | 3.82   | S-adenosylmethionine synthetase 1                | *A. thaliana* |
|                          |          | 43.3/54.8              | 5.67/5.71      | P17562         | 1       | 3.82   | S-adenosylmethionine synthetase 2                | *A. thaliana* |
|                          | D12      | 14.7/44.7              | 7.61/6.09      | BAC10972.1     | 5       | 50.36  | aldolase                                         | *P. patens* |
|                          | D14      | 52.7/37.7              | 6.29/6.23      | BAC85044.1     | 11      | 25.47  | rubisco large subunit                           | *P. patens* |
|                          | D19      | 59.7/36.4              | 6.18/4.86      | CAC35874.1     | 3       | 9.71   | H⁺-transporting ATP synthase beta chain-like protein | *A. thaliana* |
|                          | D23      | 23.3/17.8              | 8.74/5.50      | BAA83481.1     | 10      | 45.33  | rubisco small subunit                           | *P. patens* |
|                          | D25      | 20.1/16.6              | 8.77/6.41      | BAC87878.1     | 7       | 45.11  | ribulose bisphosphate carboxylase small chain    | *P. patens* |
|                          | U27      | 63.8/88.9              | 6.36/5.13      | CAA49506.1     | 1       | 2.88   | ketol-acid reductoisomerase                      | *A. thaliana* |
|                          | U33      | 81.5/80.1              | 5.80/5.39      | CAB82679.1     | 3       | 3.45   | transketolase-like protein                      | *A. thaliana* |
|                          | U40      | 48.6/60.8              | 6.20/5.28      | A96506         | 1       | 2.53   | probable aminoacylase                           | *P. patens* |
|                          | U42      | 44.7/70.0              | 8.08/6.68      | Q93Z66         | 1       | 4.87   | ribose-phosphate pyrophosphokinase 3            | *A. thaliana* |
|                          | U43      | 75.4/60.8              | 6.84/5.13      | Q9SYM5         | 1       | 2.09   | probable rhamnose biosynthetic enzyme 1          | *A. thaliana* |
|                          | U44      | 35.0/41.6              | 5.21/4.86      | CAB75445.1     | 1       | 3.99   | fructokinase-like protein                       | *A. thaliana* |
|                          | U45      | 37.6/43.1              | 5.51/4.91      | AAY78603.1     | 1       | 3.77   | PtkB-type carbohydrate kinase family protein    | *A. thaliana* |
|                          | U47      | 42.5/51.2              | 7.62/6.36      | CAA66816.1     | 3       | 10.61  | glyceraldehyde-3-phosphate dehydrogenase (NADP⁺) | *A. thaliana* |
|                          | I59      | 28.4/72.4              | 5.14/4.59      | BAD08519.1     | 1       | 3.75   | light-harvesting chlorophyll a/b-binding protein 2 | *P. patens* |
|                          | I60      | 28.4/72.4              | 5.14/4.64      | BAD08519.1     | 4       | 18.73  | light-harvesting chlorophyll a/b-binding protein 2 | *P. patens* |
|                          | I61      | 28.6/72.4              | 5.28/4.75      | AAA33636.1     | 4       | 12.27  | major chlorophyll binding protein               | *P. patens* |
|   |     |      |      |      |                          |                |
|---|-----|------|------|------|-------------------------|----------------|
|I62| 156.6/69.8 | 5.66/5.13| Q9SA6| 1.43| alpha-glucan water dikinase, chloroplast precursor | A. thaliana |
|I63| 82.1/69.8  | 6.21/5.29| CAB87667.1| 1.85| subtilisin-like protease-like protein | A. thaliana |
|I64| 42.5/46.1  | 7.62/6.20| CAA66816.1| 7.32| glyceraldehyde-3-phosphate dehydrogenase (NADP⁺) | A. thaliana |
|I65| 36.0/36.2  | 7.08/5.23| CAB43552.1| 6.13| phosphoribosyl diphosphate synthase | A. thaliana |
|I69| 26.4/21.6  | 7.77/4.96| CAB10400.1| 5.74| enoyl-CoA hydratase like protein | A. thaliana |
|I71| 36.0/29.5  | 7.08/5.39| CAB43552.1| 6.13| phosphoribosyl diphosphate synthase | A. thaliana |
|   |     |      |      |      |                          |                |
|Defense| U29| 216.9/85.9 | 4.81/5.39| D96796| 1.04| probable heat shock protein ,53413-59029 | A. thaliana |
|      | U34| 59.2/80.1  | 5.61/5.39| AAR13080.1| 10.11| dehydridin | P. patens |
|      | U35| 76.5/77.4  | 5.07/4.91| CAB79338.1| 5.57| HSP 70-like protein | A. thaliana |
|      | U36| 76.5/74.8  | 5.07/4.91| CAB45063.1| 1.95| HSP 70-like protein | A. thaliana |
|      | U37| 71.4/72.3  | 5.03/5.07| CAB85987.1| 18.89| dnaK-type molecular chaperone hsc70.1 | A. thaliana |
|      | U38| 71.1/72.3  | 5.14/5.23| CAA05547.1| 12.92| heat shock protein 70 | A. thaliana |
|      | U53| 27.7/36.2  | 5.66/5.71| CAD38154.1| 13.20| putative ascorbate peroxidase | P. patens |
|      | U54| 24.5/26.6  | 5.51/4.64| CAA67427.1| 3.60| thylakoid-bound ascorbate peroxidase | A. thaliana |
|      | U55| 73.6/30.5  | 5.08/5.29| Q9LK3| 2.54| luminal binding protein precursor (BiP1) (AtBP1) | A. thaliana |
|      | U56| 28.2/30.5  | 5.73/5.29| CAA63922.1| 5.88| ferritin | A. thaliana |
|      | U57| 24.1/30.5  | 6.13/5.88| O04005| 6.02| peroxiredoxin (Thioredoxin peroxidase) | A. thaliana |
|      | I66| 27.7/36.2  | 5.66/5.45| CAD38154.1| 8.00| putative ascorbate peroxidase | P. patens |
|      | I68| 29.1/25.7  | 7.74/4.59| CAA66484.2| 2.62| 2-Cys peroxiredoxin | A. thaliana |
|      | I70| 19.9/23.1  | 5.30/5.39| AAV65396.1| 23.60| phycocyanin | P. patens |
|Cytoskeleton| D1| 50.0/65.0  | 4.81/4.96| AAQ88118.1| 21.90| beta-tubulin | P. patens |
|      | D2| 49.5/60.7  | 4.99/5.13| BAC24799.1| 53.23| alpha-tubulin | P. patens |
|      | D4| 41.6/54.8  | 5.31/5.39| AAQ88109.1| 9.02| Actin | P. patens |
| Signaling (4.23%)                  | Detoxification (1.41%)       | Protein synthesis (1.41%)   | Transcription (1.41%)       | Unclear or unknown function (29.58%) |
|-----------------------------------|-------------------------------|-----------------------------|-------------------------------|-------------------------------------|
| **D11** 65.9/49.5                | **D15** 44.1/40.3             | **D16** 96.6/40.3           | **D21** 16.2/21.1             | **D58** 19.7/47.8                   |
| 5.18/5.93                        | 7.59/5.13                    | 5.51/5.07                   | 7.85/5.82                    | 6.25/5.77                           |
| C8478                            | BAD94063.1                   | CAB39730.1                  | BAC00785.1                   | E84448                              |
| 1                                | 1                            | 1                           | 1                            | 1                                   |
| 3.43                             | 5.61                         | 2.4                         | 6.17                         | 8.56                                |
| probable myosin heavy chain      | myrosinase-associated protein | adaptor protein complex AP-1 large subunit | glycine-rich RNA-binding protein | hypothetical protein At2g03440 |
| A. thaliana                      | A. thaliana                  | A. thaliana                 | P. patens                    | A. thaliana                         |
| **U46** 47.5/44.6                | **U28** 54.3/88.9             | **U32** 44.1/85.9           | **U48** 54.2/41.6             | **U12** 19.7/47.8                   |
| 6.04/5.07                        | 5.59/5.29                    | 5.78/5.29                   | 6.95/6.79                    | 7.25/5.77                           |
| CAB54558.1                       | CAB80488.1                   | CAC03450                    | BAB71766.1                   | E84448                              |
| 4                                | 1                            | 1                           | 1                            | 1                                   |
| 18.78                            | 2.07                         | 2.74                        | 4.97                         | 8.56                                |
| plastid division protein ftsZ1   | calcium-dependent protein kinase-like protein | ser/thr specific protein kinase-like protein | IAA/AUX protein | hypothetical protein At2g03440 |
| P. patens                        | A. thaliana                  | A. thaliana                 | P. patens                    | A. thaliana                         |
| **U13** 65.9/49.5                | **U48** 54.2/41.6             | **U16** 96.6/40.3           | **U21** 16.2/21.1             | **U35** 19.7/47.8                   |
| 5.18/5.93                        | 6.25/5.77                    | 5.51/5.07                   | 7.85/5.82                    | 6.25/5.77                           |
| Q9M630                           | E84448                       | CAB39730.1                  | BAC00785.1                   | E84448                              |
| 7                                | 1                            | 1                           | 1                            | 1                                   |
| 37.22                            | 8.56                         | 4.93                        | 34.97                        | 36.51                               |
| non-symbiotic hemoglobin         | hypothetical protein At2g03440 | chain A, crystal structure of Thil protein | unnamed protein product      | hypothetical protein At2g03440 |
| P. patens                        | A. thaliana                  | A. thaliana                 | P. patens                    | A. thaliana                         |
| **U58** 20.0/23.9                | **D13** 30.0/40.3             | **D16** 30.0/40.3           | **D20** 112.8/19.1           | **D22** 25.8/21.8                   |
| 6.32/5.77                        | 5.88/6.25                    | 5.88/6.25                   | 5.27/5.50                    | 9.44/6.25                           |
| Q9M630                           | 1RP0A                        | 1RP0B                       | CAB77756.1                   | CAB81308.1                          |
| 7                                | 1                            | 1                           | 1                            | 7                                  |
| 37.22                            | 4.93                         | 4.93                        | 0.90                         | 5.06                                |
| chain A, crystal structure of Thil protein | chain B, crystal structure of Thil protein | chain B, crystal structure of Thil protein | chain A, crystal structure of Thil protein | hypothetical protein |
| A. thaliana                      | A. thaliana                  | A. thaliana                 | A. thaliana                  | A. thaliana                         |
| **D7** 14.3/69.5                 | **D8** 15.5/47.8             | **D9** 15.5/47.8            | **D10** 19.7/47.8            | **D24** 58.8/17.2                   |
| 5.43/6.25                        | 6.47/5.55                    | 6.47/5.77                   | 7.25/5.77                    | 9.13/5.77                           |
| CAC42637.1                       | CAC43678.1                   | CAC43678.1                  | E84448                       | CAB79080.1                          |
| 2                                | 3                            | 4                           | 1                            | 1                                   |
| 36.51                            | 34.97                        | 57.34                       | 8.56                         | 3.22                                |
| unnamed protein product          | unnamed protein product      | unnamed protein product     | hypothetical protein At2g03440 | putative protein         |
| P. patens                        | P. patens                    | P. patens                   | A. thaliana                  | A. thaliana                         |
| **D17** 67.8/39.0                | **D18** 49.7/39.0             | **D19** 49.7/39.0           | **D20** 112.8/19.1           | **D24** 58.8/17.2                   |
| 5.59/5.01                        | 6.09/4.91                    | 5.27/5.50                   | 5.27/5.50                    | 9.13/5.77                           |
| G86449                           | CAB43717.1                   | CAB77756.1                  | CAB81308.1                   | CAB79080.1                          |
| 1                                | 3                            | 1                           | 7                            | 1                                   |
| 3.8                              | 11.16                        | 0.90                        | 5.06                         | 3.22                                |
| F5D14.23 protein                 | unnamed protein product      | predicted protein of unknown function | hypothetical protein         | putative protein         |
| A. thaliana                      | P. patens                    | A. thaliana                 | A. thaliana                  | A. thaliana                         |
| **D8** 15.5/47.8                 | **D9** 15.5/47.8             | **D10** 19.7/47.8           | **D12** 25.8/21.8            | **D24** 58.8/17.2                   |
| 6.47/5.55                        | 6.47/5.77                    | 7.25/5.77                   | 9.44/6.25                    | 9.13/5.77                           |
| CAC43678.1                       | E84448                       | E84448                      | CAB81308.1                   | CAB79080.1                          |
| 3                                | 1                            | 1                           | 7                            | 1                                   |
| 34.97                            | 8.56                         | 5.06                        | 3.22                         | 3.22                                |
| unnamed protein product          | hypothetical protein         | putative protein            | putative protein             | putative protein         |
| P. patens                        | A. thaliana                  | A. thaliana                 | A. thaliana                  | A. thaliana                         |
| ID  | Log2 Fold Change | NMP     | SC     | Description                                              | Species          |
|-----|-----------------|---------|--------|----------------------------------------------------------|------------------|
| U26 | 79.1/85.9       | 5.34/75 | H84791 | 1.01 hypothetical protein At2g37370                      | A. thaliana      |
| U30 | 149.1/85.9      | 4.89/5.39| F96673 | 1.14 hypothetical protein F13O11.30                      | A. thaliana      |
| U31 | 13.2/85.9       | 6.75/5.39| D84501 | 10.43 hypothetical protein At2g12170                    | A. thaliana      |
| U39 | 65.8/72.3       | 5.19/5.28| AAY25444.1| 1.87 At2g40550                                         | A. thaliana      |
| U41 | 59.9/60.8       | 6.73/5.13| CAD86489.1| 2.60 unnamed protein product                             | P. patens        |
| U49 | 56.7/38.8       | 5.65/4.86| CAB85548.1| 3.5 putative protein                                     | A. thaliana      |
| U50 | 31.9/33.8       | 5.36/6.57| CAC43713.1| 4.17 unnamed protein product                             | P. patens        |
| U51 | 34.0/33.8       | 5.90/4.86| CAC43712.1| 2.10 unnamed protein product                             | P. patens        |
| U52 | 60.7/35.0       | 8.22/5.71| C96608  | 3.83 hypothetical protein F25p12.91                      | A. thaliana      |
| I67 | 135.1/28.5      | 5.54/8.0 | F96795  | 1.49 hypothetical protein F28O16.9                      | A. thaliana      |

D1-D25, down-regulated, U26-U58, up-regulated, I59-I71, induceable expression; NMP, number of matched peptides; SC, sequence coverage.
Figure 1. *P. patens* gametophores undergoing desiccation and rehydration. A, Control. B, Desiccated gametophores. C, Gametophores after 2-h-rehydration. D, gametophores after one-month-rehydration.
Figure 2. Changes of the chlorophyll content during desiccation. Figure plots the means ± SD from three replicate experiments.
Figure 3. TME micrographs of control and desiccated gametophores. 

A, Control gametophore. B, Desiccated gametophore. C, Enlargement of the image in B. Ch, Chloroplast; CW, cell wall; LD, Lipid drop; PM, Plasma membrane; N, Nucleus; V, Vacuole. Bar = 2 μm.
Figure 4. 2-DE of total protein extracts from control and desiccated gametophores. Upper image: control; lower image: desiccated. Desiccation-responsive proteins are indicated as follows: D, down-regulated protein; U, up-regulated protein; and I, induced protein. Gels are coomassie stained.
Figure 5. Quantification of the desiccation-responsive protein abundance. A, Down-regulated proteins. B, Up-regulated proteins. Data are means of three independent replicates ± SD.
Figure 6. Functional categorization of the desiccation responsive proteins. Of the eight categories of Bevan et al., six were represented by down-regulated proteins and only five by up-regulated proteins.
Figure 7. Images of microtubules in control and desiccated gametophores. A and B, Control gametophores; C and D, desiccated gametophores. A and C are bright-field images of the whole-mount preparations imaged with epi fluorescence shown in B and D. Microtubules (indicated by red arrow) form a lattice work surrounding chloroplasts in control plants (B) but de-polymerize into dot structure (indicated by red arrow) in the desiccated plants (D). White arrows (B and D) show the chloroplast. Bright punctate staining in the control is probably starch. Bar = 10 μm.