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Desiccation Tolerance Mechanism in Resurrection Fern-Ally *Selaginella tamariscina* Revealed by Physiological and Proteomic Analysis

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Drought is one of the most severe limitations to plant growth and productivity. Resurrection plants have evolved a unique capability to tolerate desiccation in vegetative tissues. Fern-ally *Selaginella tamariscina* (Beauv.) is one of the most primitive vascular resurrection plants, which can survive a desiccated state and recover when water becomes available. To better understand the mechanism of desiccation tolerance, we have applied physiological and proteomic analysis. Samples of *S. tamariscina* were water-deprived for up to seven days followed by 12 h of rewatering. Our results showed that endogenous abscisic acid (ABA) increased to regulate dehydration-responsive genes/proteins and physiological processes. In the course of dehydration, the contents of osmolytes represented by soluble sugars and proline were increased to maintain cell structure integrity. The activities of four antioxidant enzymes (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and glutathione reductase (GR)) also increased. In contrast, both the rate of photosynthesis and the chlorophyll content decreased, and plasma membrane integrity was lost. We identified 138 desiccation-responsive two-dimensional electrophoresis (2-DE) spots, representing 103 unique proteins. Hierarchical clustering analysis revealed that 83% of the proteins were down-regulated upon dehydration. They were mainly involved in photosynthesis, carbohydrate and energy metabolism, stress and defense, protein metabolism, signaling, membrane/transport, cell structure, and cell division. The dynamic expression changes of the desiccation-responsive proteins provide strong evidence that cell structure modification, photosynthesis reduction, antioxidant system activation, and protein post-transcriptional/translational modifications are essential to the poikilochlorophyllous fern-ally *S. tamariscina* in response to dehydration. In addition, our comparative analysis of dehydration-responsive proteins in vegetative tissues from 19 desiccation tolerant and nontolerant plant species suggests that resurrection *S. tamariscina* has developed a specific desiccation tolerant mechanism. To our knowledge, this study constitutes the first detailed investigation of the protein complement in fern/fern-allies.

Keywords: proteomics • resurrection fern-allies • *Selaginella tamariscina* • desiccation tolerance

Introduction

Drought, one of the most severe abiotic stress factors affecting plant growth and productivity, has caused considerable reduction in crop yield worldwide.[1] Most plants produce structures that can deal with desiccation, such as seeds, pollen grains, and spores. In addition, some plants, including resurrection plants, have evolved a series of specialized mechanisms to tolerate desiccation in vegetative tissues in response to drought stress.[2−5] Resurrection plants are widespread but uncommon in different taxonomic groups, including bryophytes (158 species),[6] fern/fern-allies (60−70 species),[7] and angiosperms (~350 species).[4] Resurrection plants are represented by the bryophyte *Tortula ruralis*, the fern-allies *Selaginella* spp., the grass *Eragrostis nindensis*, the monocotyledonous *Xerophyta* spp., the dicotyledonous crops *Craterostig-*

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ma spp., and the woody shrub *Myrothamnus flabellifolius*.[4] Resurrection plants have the ability to survive desiccation and fully recover from a loss of ~90% of protoplasmic water in the vegetative tissues. Therefore, they are excellent model systems for investigating the mechanisms underlying dehydration tolerance.[8]

Anatomical and physiological studies on the aforementioned resurrection species have revealed that these plants have evolved the ability to overcome the drought-induced mechanical, oxidative, and destabilizing stress by relying on their morphological adaptations (e.g., leaf curling, cell volume reduction, and cell wall folding/shrinkage), hormone regulation (e.g., accumulation of abscisic acid (ABA), indole-3-acetic acid (IAA), cytokinin, and zeatin), antioxidant protection (such as the synthesis of (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and glutathione reductase (GR)), and accumulation of osmolytes (such as proline and soluble sugars).[9−11] Through analysis of genes and proteins in resurrection plants, the molecular regulatory mechanisms have started to be understood. For example, ABA regulates the expression of *Craterostigma* desiccation tolerant-1 gene (*CDT-1*)[12] and induces transcription factor homeodomain-leucine zipper (HD-ZIP),[13] desiccation-responsive gene phospholipase D-2 (*CpPLD-2*),[14] and late embryogenesis abundant (LEA) proteins.[10] In addition, transcriptional analysis of some resurrection plants has led to the identification of 10 368 expressed sequence tags (ESTs) (representing 5563 genes) in *T. ruralis*,[15] 200 cDNAs in *Craterostigma plantagineum*,[16] 424 cDNAs in *Xerophyta humilis*,[17] and 1046 ESTs (873 genes) in *Selaginella lepidophylla*.[18] By comparing these data with the *Arabidopsis* ESTs, and especially comparing the ESTs from *S. lepidophylla* with those from *S. moellendorffii*, which does not have anhydrobiotic capacity,[18,19] the unique genes and/or special regulatory processes conferring desiccation tolerance were revealed. Genes involved in photosynthesis, sugar metabolism, disease defense, transport, protein synthesis, cytoskeleton, energy production, and secondary metabolism were found to be overrepresented in resurrection plants.

Recently, proteomic approaches have shown utility in elucidating desiccation tolerance mechanisms in land plants. To date, analysis of differentially expressed proteins in response to water deficits or desiccation stress has been conducted on 18 tolerant or nontolerant plant species, and more than 720 proteins have been identified.[20−42,43] Among the studies, only a few reports were on resurrection plants.[20,23−25,43] A total of 38 dehydration-responsive proteins were identified in the Angiosperm resurrection plants. Evidence was provided of an essential detoxification mechanism in desiccation tolerance that was mediated through glutathione and remodeling of photosynthetic apparatus.[23,24] Additionally, 71 desiccation-responsive proteins, mainly involved in metabolism, cytoskeleton, stress/defense, and signaling, were identified in the primitive resurrection moss *Physcomitrella patens*.[20] All the proteomic studies suggest that multiple metabolic processes are involved in desiccation response and tolerance. Photosynthesis- and redox-related proteins, antioxidant enzymes, and heat shock proteins (HSPs)/chaperones appear to play common roles in different plants in addition to some desiccation-responsive proteins unique to certain species.[20−42,44] This implies that plants in different evolutionary positions may have developed unique strategies to cope with drought/desiccation stress. Nonetheless, in resurrection plants we know little about the molecular regulatory mechanisms underlying their desiccation tolerance. In addition, the common and distinct molecular features of desiccation tolerance and intolerance between resurrection plants and nontolerant plants are not clear.

In contrast to angiosperm species (especially economic crops and trees), in which most desiccation-related genomic and proteomic studies have been conducted, species in the fern-ally genus *Selaginella* have received little attention. *Selaginella* with about 700 species belongs to an ancient lineage of vascular plants that have been present on Earth more than twice as long as angiosperms.[45] Some species in *Selaginella* have evolved unique capabilities to withstand cellular desiccation. Phylogenetic analysis suggests that the desiccation tolerance of *Selaginalla* spp. derived from at least one independent evolution.[46] Thus, the study of its desiccation tolerance mechanism could provide novel insight into the early evolution of land plants. EST analysis of *S. lepidophylla* has shown that genes involved in transport, cell structure, secondary metabolism, protein modification, and abiotic stress account for a large portion of the genome.[18] In addition, recent physiological and transcriptional analysis of *S. tamariscina*, a closely related resurrection species of *S. lepidophylla*, found that, during dehydration, endogenous ABA dramatically increased and 22 unique genes were differentially expressed.[47] The up-regulated genes were involved in cellular protection and the down-regulated genes were photosynthesis related. Despite significant progress in this research area, the molecular mechanisms underlying desiccation tolerance in resurrection species of *Selaginalla* are not clear. Proteomic analysis is likely to reveal the underlying molecular characteristics. It may also help to define an ancient core of genes and proteins common to all vascular plants. In this study, resurrection fern-ally *S. tamariscina* (Beauv.) was used for physiological analysis and comparative proteomics. Our results revealed that desiccation tolerance was largely attributable to ABA-induced morphological modification, photosynthesis reduction, accumulation of osmolytes, and increase of antioxidation activity. The 138 dehydration-responsive proteins identified by the two-dimensional electrophoresis (2-DE) mass spectrometry (MS) approach may play important roles in the above morphological and physiological changes. Together with the results of comparative analysis of dehydration-responsive proteins in 19 plant species,[20−42] this study provides evidence that *Selaginella*, as a fern-allies with an independent evolution of desiccation tolerance, has developed specific desiccation tolerant mechanisms.[46]

Experimental Procedures

Plant Material. *S. tamariscina* (Beauv.) grows naturally on exposed rocks of hills in the region of Jingpo Lake, Heilongjiang Province, China. Plants with similar-sized aerial parts in the same habitat and plant community were selected to be homogeneous in terms of age, development, and water status. *S. tamariscina* samples with root matrix were collected and maintained in pots in a greenhouse. Plants grew under the conditions of 27 ± 2 °C, 12 h illumination with a light intensity of ~300 μmol m^{-2} s^{-1}, and a relative humidity of 60 ± 5%. Plants were grown with regular irrigation for 30 days prior to desiccation treatment. Drought conditions were induced by withholding irrigation. Over the period of one week, after water deprivation for 0 days (d), 1 d, 3 d, 5 d, 7 d, and 12 h after rewatering, the aerial parts of the respective plants were harvested.

Water Content and ABA Determination. After the plant samples were collected, they were cut into pieces and fresh

weight was recorded. The collected samples were used for immediate analysis or stored at −80 °C. If frozen samples were used, they were thawed. Dry weight was obtained by baking at 105 °C for 15 min, then subsequent drying at 80 °C until the weight stabilized. The water content in the samples was calculated as the difference between fresh weight and dry weight divided by the fresh weight. Extraction and quantification of endogenous ABA was performed as described by Yang et al.[48]

Determination of Relative Electrolyte Leakage and Malondialdehyde Contents. Membrane permeability was measured by relative electrolyte leakage (REL). Fresh aerial tissues (0.5 g) were rinsed three times with Milli-Q water. The tissues were cut and completely immersed in 20 mL of Milli-Q water, then degassed for 20 min. The electrical conductivity of the solution (E1) was determined using a conductivity instrument (DDS-11A). The sample tubes were subsequently incubated in boiling water (100 °C) for 10 min and allowed to cool to ambient temperature, and then the electrical conductivity of the solution (E2) was measured. In addition, the electrical conductivity of Milli-Q water (E3) was also measured. The REL was calculated using the equation: REL (%) = (E1 − E3)/(E2 − E3) × 100%.

Lipid peroxidation was estimated by measuring the contents of malodialdehyde (MDA). The aerial tissues were ground in liquid nitrogen and extracted using 3 mL of 50 mM PBS (pH 7.5). After being cooled at 4 °C for 4 h, the samples were centrifuged at 13000*g* at 4 °C for 30 min and the supernatants were collected. Next, 3 mL of trichloroacetic acid/thiobarbituric acid (TCA-TBA) solution (5% w/v TCA and 0.5% w/v TBA) was added to 1 mL of the supernatant. The reaction solution was incubated for 30 min at 95 °C followed by cooling down to ambient temperature. The absorbance was detected under 532, 450, and 600 nm using a spectrophotometer (Ultrospec 2100 pro UV/Visible, GE Healthcare). The MDA content was calculated according to Li et al.[49]

Total Soluble Sugar and Proline Analysis. Total soluble sugar and proline were extracted and determined according to a method developed by Li et al.[49]

Photosynthesis and Chlorophyll Measurement. The net photosynthetic rate (Pn) and stomatal conductance (Gs) of the samples were determined using a portable photosynthesis system LI-COR 6400 (LI-COR Inc.) at 10:00 a.m. The chlorophyll contents were determined using a method described by Lichtenthaler et al.[50]

Activity Analysis of Antioxidant Enzymes. Activities of four antioxidant enzymes (SOD, CAT, POD, and GR) were determined. The aerial tissues were grounded in 6 mL of extraction buffer 1 (50 mM PBS, pH 7.8 for SOD and CAT assays) or 6 mL extraction buffer 2 (100 mM PBS, pH 7.0 for POD and GR assays) at 4 °C. The homogenates were collected and centrifuged at 15000*g* at 4 °C for 20 min. SOD was assayed on the basis of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). A 6 mL reaction solution of SOD consisted of 50 mM PBS (pH 7.8), 130 mM methionine, 750 μM nitro blue tetrazolium chloride (NBT), 100 μM EDTA-Na_2, 20 μM riboflavin, and 0.1 mL of enzyme extract. The reaction solution was incubated for 10 min under fluorescent light with an intensity of 50 μmol m^{-2} s^{-1} for 20 min. The absorbance was determined at 560 nm using a spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required to inhibit photochemical reduction of NBT by 50%. CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 by the method described by Change and Maehly.[51] The decline in A_{240} was recorded, and the activity was expressed as the number of μmol of H_2O_2 catalyzed by a unit of CAT per min. POD activity was analyzed using a modified Rao et al. method.[52] The reaction solution contained 100 mM PBS (pH 7.0), 50 mM *o*-methoxyphenol, 40 mM H_2O_2, and 0.1 mL of enzyme extract. GR activity was determined according to the method described by Halliwell et al.[53] The reaction solution consisted of 50 mM Tris-HCl, 0.5 mM GSSG, 5 mM $MgCl_2$, and 0.2 mM NADPH. GR activity was determined to be 340 nm within 3 min and expressed as the number of μmol of NADPH oxidization.

Protein Sample Preparation. The samples of plants at various stages (0 days after dehydration (DAD), 1 DAD, 3 DAD, 5 DAD, 7 DAD, and 12 h after rehydration (HAR)) were ground to powder in liquid nitrogen in a chilled mortar. After 10 mL of Tris pH 8.8 buffered phenol (Sigma) and 10 mL of extraction buffer (0.1 M Tris-HCl pH 8.8, 0.9 M sucrose, 10 mM EDTA, 0.4% β-mercaptoethanol) were added, the samples were homogenized for 15 min. The solutions were transferred to Falcon tubes and agitated for 30 min at 4 °C, followed by centrifugation at 15 000*g* for 30 min at 4 °C. The phenol phase was removed to new tubes, and rest was extracted again by using 4 mL of extraction media and 4 mL of phenol. The two extractions were combined and precipitated by adding 5 vol of 0.1 M ammonium acetate in methanol and incubating at −20 °C overnight. The precipitate was collected by centrifugation in 20000*g* for 20 min at 4 °C and washed twice with 0.1 M ammonium acetate in methanol, ice-cold 80% acetone, and 70% ethanol. The resulting pellets were dissolved in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 4% proteinase inhibitor cocktail, 2% (v/v) pH 4−7 ampholytes) for 1 h, and used for 2-DE immediately or stored in aliquots at −80 °C. Protein concentration was determined using a Quant-kit (GE Healthcare) according to manufacturer's instructions. Bovine serum albumin was used as the standard.

2-DE and Protein Abundance Analysis. Protein samples were analyzed using 2-DE according to a method described by Dai et al.[54] The experiments were repeated three times using protein samples independently prepared from different samples. Images were acquired by scanning each stained gel using an ImageScanner III (GE Healthcare) at a resolution of 300 dpi and 16-bit grayscale pixel depth, and then analyzed with ImageMaster 2D software (version 5.0) (GE Healthcare). The normalized relative percent volume (RV) values of the protein spots in triplicate biological repeats for each sample were subjected to further statistical analysis. The significance of differentially expressed proteins was determined by *t*-test. Proteins were considered to be differentially expressed if they displayed at least a 1.5-fold change in RV values and a p value < 0.05.

Protein Identification by MALDI-TOF/TOF MS. The differentially expressed spots were excised from the 2-DE gels and digested as previously described.[54] The MS spectra were acquired using a 4800 Proteomics analyzer (MALDI TOF/TOF mass spectrometer, Applied Biosystems). A Mass standard kit (Applied Biosystems) and a standard BSA digest (Sigma-Aldrich) were used for MS and MS/MS calibrations and fine-tuning the resolution and sensitivity of the system. The mass error was below 30 ppm at both MS and MS/MS mode and the resolution was more than 25 000.

Database Searching. The MS/MS spectra generated from the 4800 Proteomics analyzer was searched against the NCBInr

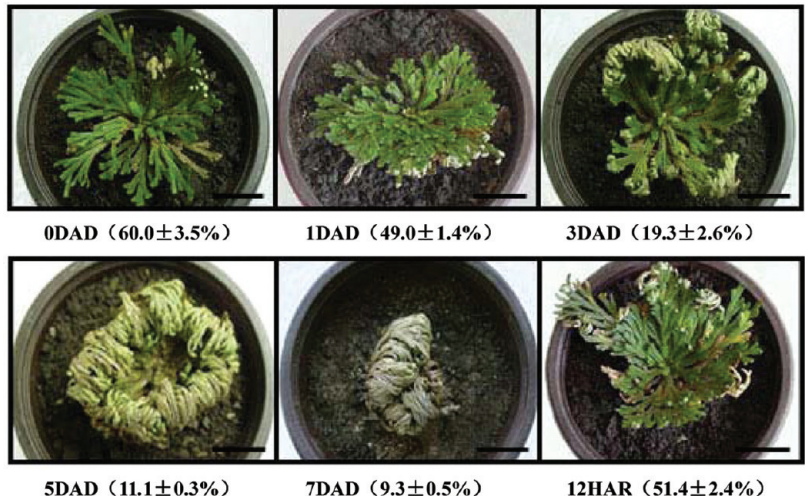

Figure 1. Morphological changes and water contents (in brackets) in aerial parts of *S. tamariscina* upon dehydration and rehydration. The plants were subjected to up to 7 days of water deprivation followed by rewatering for 12 h. The samples exhibited gradual curling associated with water reduction in the course of dehydration and then became fully open after 12 h of rehydration. Values (means ± SD) were determined from three replicates ($n = 3$). Bar = 3.5 cm.

protein databases (http://www.ncbi.nlm.nih.gov/) (701 710 sequences entries in NCBI on July 25, 2009) using a Mascot Dameon software (Matrix Science). The taxonomic category was green plants. Monoisotopic and protonated molecular ions were submitted the search. The searching criteria were mass accuracy of ±0.3 Da, one missed cleavage site allowed, carbamidomethyl (C) set as a fixed modification, and oxidation of methionine as a variable modification. To obtain high confident identification, proteins had to meet the following criteria: (1) the top hits on the database searching report, (2) a probability-based MOWSE score greater than 43 ($p < 0.01$), and (3) more than two peptides matched and nearly complete y-ion series and complementary b-ion series present. The ion series should correspond to peaks with high relative intensity.

Hierarchical Cluster Analysis. Self-organizing maps (SOMs) hierarchical clustering analysis of the protein expression profiles was performed based on $\log_2$ transformed fold induction RV across spots using Cluster software (version 3.0).[55]

Results

Resurrection Phenomenon of *S. tamariscina*. *S. tamariscina* has the ability to remain alive in a dehydration state and then resume normal growth after rehydration. In order to study the morphological changes of *S. tamariscina* in response to desiccation, plants were subjected to seven days of water deprivation. The resurrection phenomenon of the plants was observed at 0 DAD, 1 DAD, 3 DAD, 5 DAD, 7 DAD and 12 HAR, respectively. In well-irrigated condition (0 DAD), the aerial parts of plants fully opened and the water content reached 60.0 ± 3.5%. In the course of dehydration, the aerial parts of plants exhibited gradual morphological rolling and wilting (Figure 1). At 1 DAD, there was little visible damage to the aerial parts of plants. Gradually, the plant curled up and the crown decreased by approximately 40% at 3 DAD and 50% at 5 DAD. The corresponding water content in the samples was reduced to 19.3 ± 2.6% at 3 DAD and 11 ± 0.3% at 5 DAD. At 7 DAD, when the water content dropped to the lowest level (9%), the aerial parts of plant curled into a ball. When water was provided again, the aerial parts fully opened at 12 HAR and the water content recovered to 51% (Figure 1). This obvious resurrection phenomenon is an active strategy for avoiding dehydration damage.

Photosynthesis Inhibited during Dehydration. Photosynthesis is generally believed to be sensitive to dehydration and rehydration. In this study, the amounts of total chlorophyll, chlorophyll a, and chlorophyll b in the samples declined gradually from 0 DAD to 7 DAD, and increased in 12 HAR (Figure 2A). However, the ratios of chlorophyll a/b were maintained at relatively stable levels during dehydration and rehydration. In addition, the net photosynthetic rate (Pn) and stomatal conductance (Gs) values were reduced significantly at 1 DAD, and Pn reached almost zero, accompanied by stomatal closure, at 3 DAD, 5 DAD and 7 DAD (Figure 2B,C). All the results indicated that water deficit in the rolling and wilting samples led to stomatal closure and reduction of photosynthetic activity, which may help plants to cope with dehydration by reducing damage from reactive oxygen species (ROS).

Plasma Membrane Integrity and Osmolyte Accumulation during Dehydration. To determine the levels of membrane integrity and permeability of the cell membrane in the *S. tamariscina* samples, the status of REL and MDA were monitored in all stages of dehydration and rehydration (Figure 3A,B). RELs were found to be maintained at a relatively constant level in the samples from 0 DAD, 1 DAD, or 3 DAD, but sharply increased from 5 DAD and 7 DAD. After rehydration (12 HAR), RELs showed recovery (Figure 3A). Similarly, MDA contents did not show obvious differences at 0 DAD, 1 DAD, and 3 DAD, but increased at 5 DAD and 7 DAD, and declined to normal at 12 HAR (Figure 3B). These results indicated that membrane integrity was maintained at the earlier stages of dehydration (1 DAD and 3 DAD), even when the water content in samples was already reduced to 19.3 ± 2.6% at 3 DAD (Figure 1). Although membrane integrity was lost at 5 DAD and 7 DAD when the water content was less than 11 ± 0.3%, it was rapidly repaired during rehydration at 12 HAR.

The accumulation of compatible solutes and osmolytes (such as proline and sugar) under osmotic stress can help to maintain

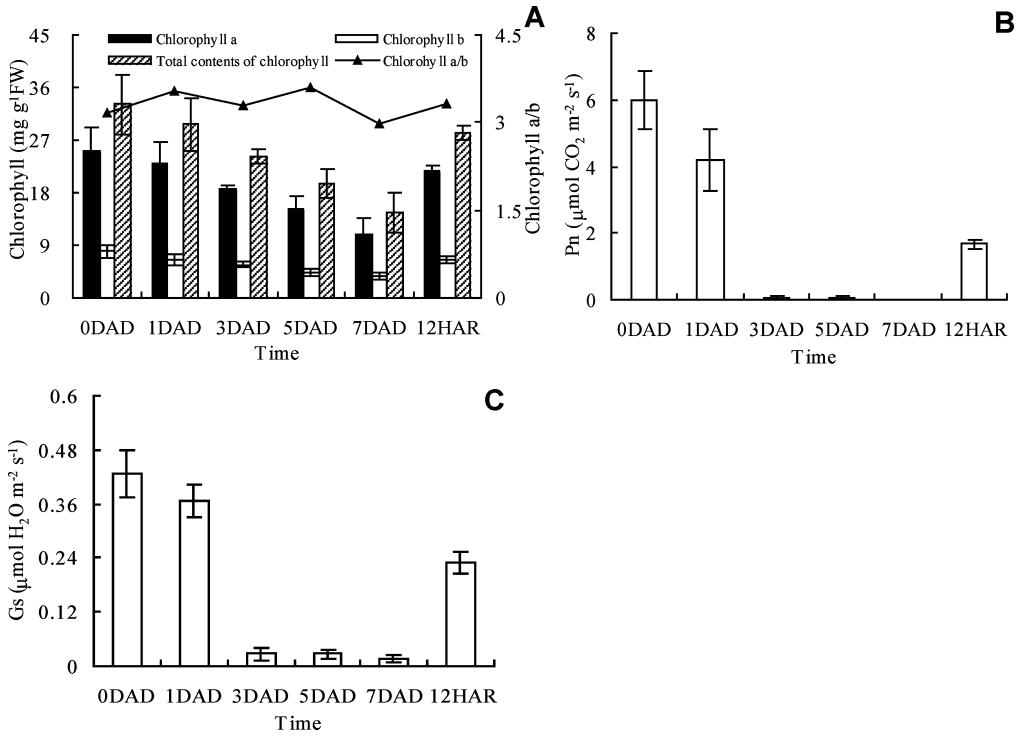

Figure 2. Effect of dehydration and rehydration on chlorophyll contents (A), net photosynthetic rate (Pn) (B), and stomatal conductance (Gs) (C) in *S. tamariscina*. Values (means ± SD) were determined at 0 days after dehydration (0 DAD), 1 DAD, 3 DAD, 5 DAD, 7 DAD, and 12 h after rehydration (HAR) of 7 DAD from three replicates ($n = 3$) at each stage.

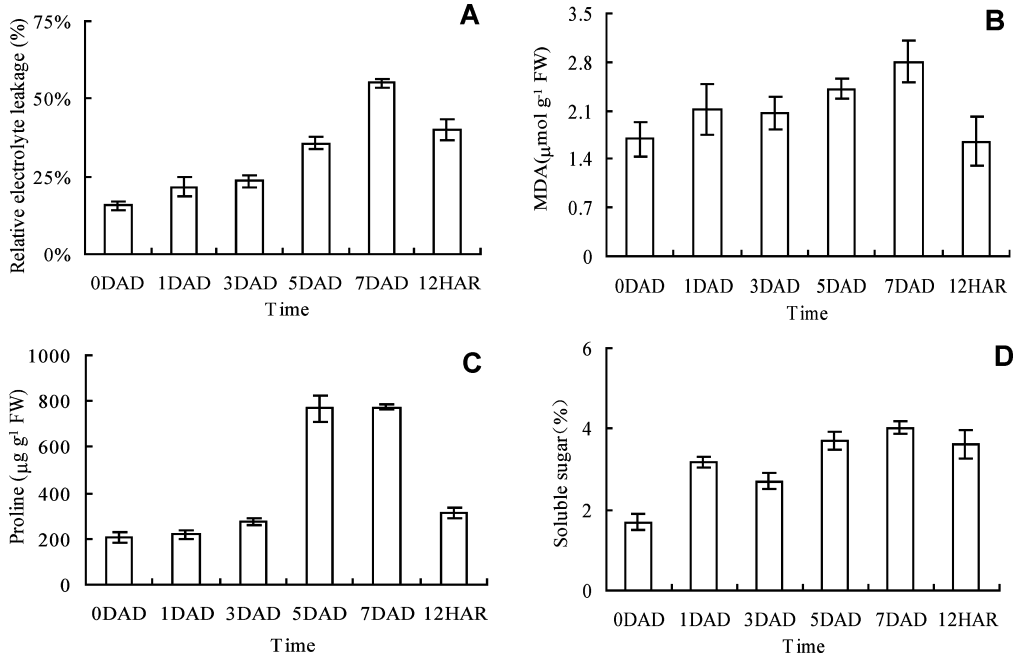

Figure 3. Effect of dehydration and rehydration on cell membrane stability and osmolytes in *S. tamariscina*. (A) relative electrolyte leakage (REL); (B) malondialdehyde (MDA); (C) proline; (D) soluble sugars. Values (means ± SD) were determined at 0 days after dehydration (0 DAD), 1 DAD, 3 DAD, 5 DAD, 7 DAD, and 12 h after rehydration (HAR) of 7 DAD; all with three replicates ($n = 3$) at each stage.

osmotic balance and protein stabilization.[2,46] We analyzed proline levels and sugar content in the samples. Proline levels were relatively stable at 0 DAD, 1 DAD, 3 DAD, and 12 HAR, but increased significantly at 5 DAD and 7 DAD (Figure 3C). This change was coincident with the results of REL and MDA (Figure 3A,B), suggesting the important role of proline under conditions of serious osmotic stress. In contrast, the amount of soluble sugars in the samples upon dehydration and rehydration was markedly higher than normal (Figure 3D). This implied that soluble sugars in *S. tameriscina* play important roles in osmotic adjustment and other processes of protection.

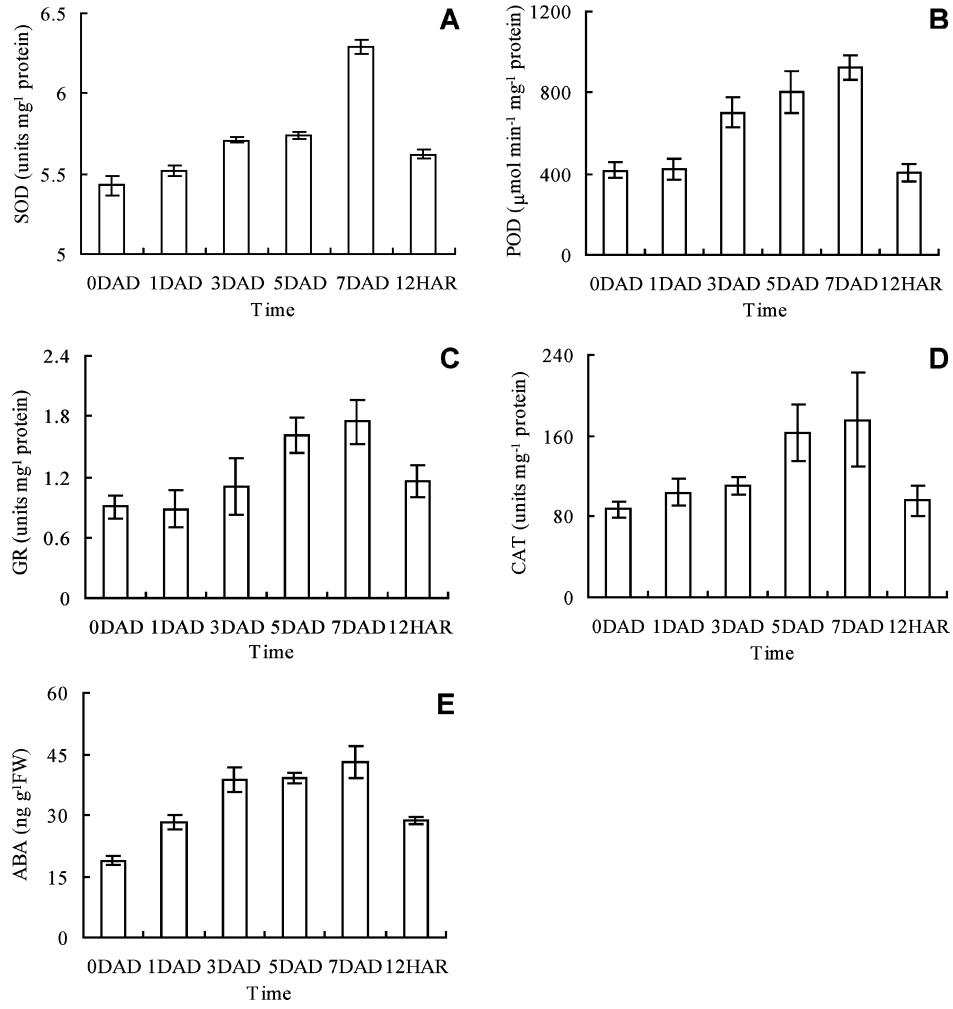

Figure 4. Effect of dehydration and rehydration on the activities of antioxidant enzymes and accumulation of abscisic acid (ABA) in *S. tamariscina*. (A) Superoxide dismutase (SOD); (B) peroxidase (POD); (C) glutathione reductase (GR); (D) catalase (CAT); (E) ABA contents. Values (means ± SD) were determined at 0 days after dehydration (0 DAD), 1 DAD, 3 DAD, 5 DAD, 7 DAD, and 12 h after rehydration (HAR) of 7 DAD with three replicates ($n = 3$) at each stage.

Activity of Antioxidant Enzymes. We analyzed the activities of four antioxidant enzymes (SOD, POD, CAT, and GR) to evaluate the effect of dehydration on plant antioxidation system. As shown in Figure 4A−D, the activities of all four enzymes increased gradually and consistently during dehydration and returned to normal levels at 12 HAR. This result indicated that antioxidation mechanisms had been triggered and supported the notion that the dehydration damage to the plants was mainly caused by oxidative stress, that is, the accumulation of ROS.[9]

ABA Increased during Dehydration Stress. ABA is an important phytohormone that regulates plant acclimation to dehydration. ABA has been shown to be associated with the expression of dehydration-related genes/proteins and is involved in dehydration signal transduction in resurrection plants.[13,56] In this study, endogenous ABA contents in the samples increased about 2.3-fold during desiccation from 18.8 ± 1.1 ng g^{-1} at 0 DAD to 42.9 ± 3.7 ng g^{-1} at 7 DAD. After rewatering, ABA contents in 12 HAR samples showed a decrease toward normal levels (Figure 4E). ABA accumulation under dehydration conditions may induce the expression changes of regulatory and metabolic proteins important for desiccation tolerance.[1]

Identification, Functional Categorization and Hierarchical Cluster Analysis of Differentially Expressed Proteins. To investigate the differentially expressed proteins in *S. tamariscina* under dehydration conditions, the protein profiles of samples at all six stages of dehydration and rehydration were obtained (Figure 5). On the gels of each sample, more than 1100 protein spots were detected. Image analysis revealed 653 reproducibly matched protein spots across samples of the different stages, including 269 protein spots presented in less than six stages. Among them, 209 reproducibly matched spots showed at least a 1.5-fold change in abundance ($p < 0.05$) under dehydration and rehydration conditions. The 209 differentially expressed protein spots were in-gel digested and submitted for identification. A total of 159 gel spots were identified using MALDI TOF/TOF MS and Mascot database searching with stringent criteria (Figure 5; Supporting Information, Tables S1, S2, and S3). The rest of the spots were not identified due to poor MS spectra or the database limitations. Among the 159

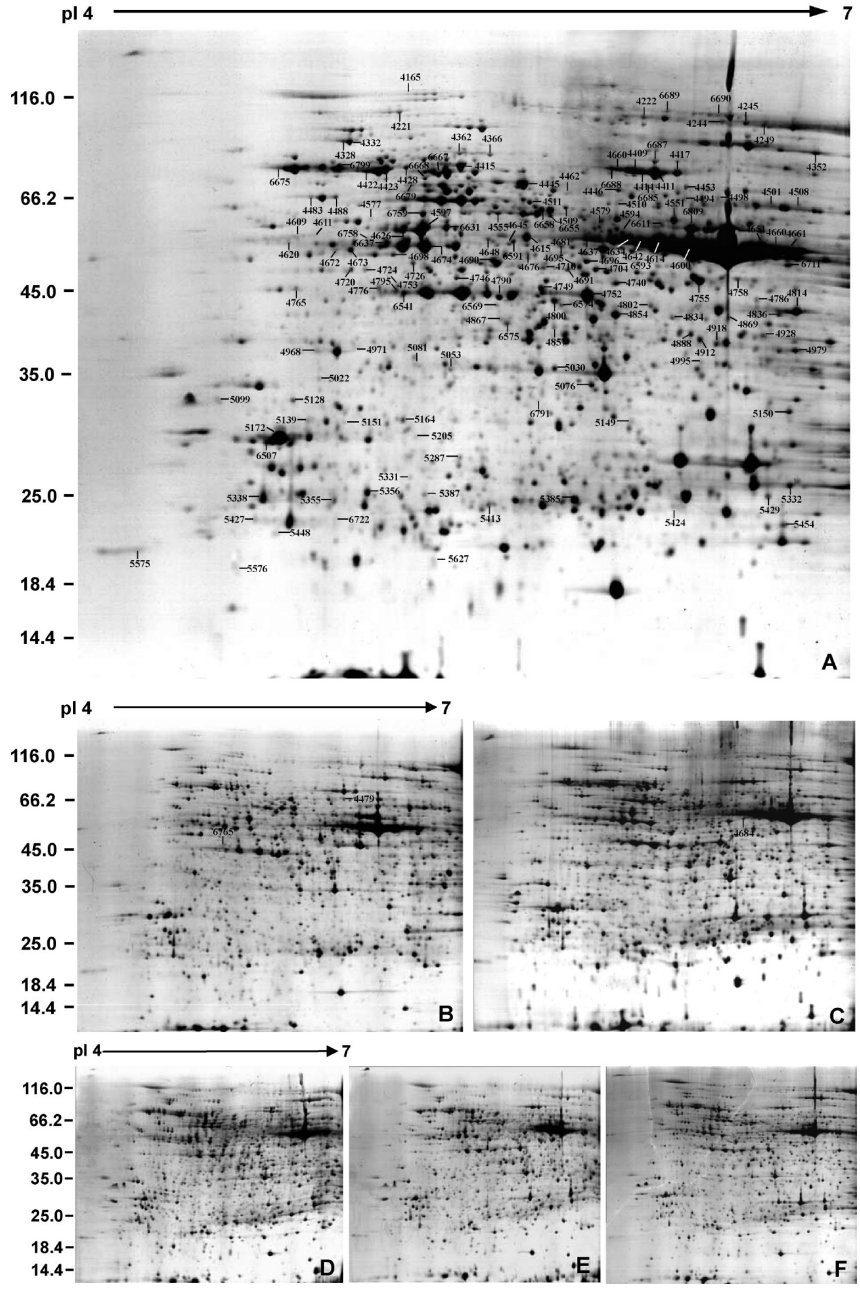

Figure 5. Coomassie Brilliant Blue (CBB)-stained 2-DE protein maps. Protein was extracted from *S. tamariscina* upon dehydration and separated on 24 cm IPG strips (pH 4−7 linear gradient) through isoelectric focusing (IEF) in the first dimension, followed by 12.5% SDS-PAGE gels in the second dimension. A total of 138 differentially expressed proteins identified by mass spectrometry were marked with numbers on the representative gels. Detailed information can be found in Supporting Information, Table S1. (A) 0 days after dehydration (0 DAD); (B) 1 DAD; (C) 3 DAD; (D) 5 DAD; (E) 7 DAD; (F) 12 h after rehydration (HAR) of 7 DAD.

identified protein spots, 138 contained a single protein each (Tables 1 and 2; Supporting Information, Tables S1 and S2), and the remaining 21 spots had two or three proteins each (20 spots with two proteins and 1 spot with three proteins each) (Supporting Information, Table S3). Among the 138 spots, 115 spots present in all the six stages, and the rest 23 were detected less than six stages. On the basis of the Gene Ontology term for each protein, results of BLAST alignments, and the literature, the 138 identities (IDs) representing 103 unique proteins (UPRs) were classified into 14 functional categories: photosynthesis, carbohydrate and energy metabolism, metabolism, stress and defense, transcription related, protein synthesis, protein folding, protein degradation, signaling, membrane and transport, cell structure, cell division/differentiation and fate, miscellaneous, and function unknown (Figure 6; Supporting Information, Tables S1 and S2).

To better understand the coordinately regulated proteins upon dehydration and rehydration, hierarchical clustering was applied to the 138 proteins identities (Figure 7). Two main clusters were formed: cluster I included 24 identities whose abundances increased upon dehydration; cluster II contained 114 down-regulated identities. The cluster II contained three main subclusters. The first subcluster comprised 23 identities, which were up-regulated at 1DAD and then down-regulated after 3DAD. The second subcluster contained 10 identities which were up-regulated at 5DAD and down-regulated at other

Table 1. Drought/Dehydration-Responsive Proteins in Different Evolutionary Groups Identified by Proteomic Studies

		resurrection species				angiosperm		
no.[a]	unique proteins/protein families (UPs/UPFs) name[b]	bryophyte[c]	fern-ally[d]	angiosperm[e]	gymnosperm[f]	trees in angiosperm[g]	Gramineae & Fabaceae[h]	other species[i]
	01 Photosynthesis (11)							
1	RuBisCO LSU	1 (1)	18 (2)	1 (1)	8 (2)	17 (7)	18 (14)	7 (7)
2	RuBisCO binding protein		2 (1)			5 (5)		
3	RuBisCO activase		3 (3)		1 (1)	8 (6)	6 (3)	
4	chlorophyll a/b-binding protein	3 (2)	2 (2)				2 (2)	
5	OEC			3 (3)		2 (2)	2 (2)	
6	OEE		4 (2)		4 (2)	6 (4)	2 (2)	
7	ferredoxin-NADP reductase					4 (3)	1 (1)	
8	fructose-1,6-bisphosphatase		1 (1)				1 (1)	
9	sedoheptulose-1,7-bisphosphatase		2 (2)			1 (1)		
10	phosphoribulokinase	2 (1)	2 (1)					
11	high chlorophyll fluorescence 136		1 (1)	1 (1)				
	02 Carbohydrate and Energy Metabolism (13)							
12	ADP-glucose pyrophosphorylase		1 (1)			1 (1)		
13	UDP-glucose-4-epimerase		1 (1)				1 (1)	
14	GDP-mannose 3,5-epimerase		1 (1)	1 (1)				
15	ribose-5-phosphate isomerase		1 (1)			1 (1)		
16	transketolase	1 (1)	5 (1)	1 (1)		7 (2)	1 (1)	
17	aldolase	1 (1)	1 (1)				1 (1)	
18	triosephosphate isomerase	2 (2)				2 (1)	4 (3)	
19	GAPDH	2 (1)				3 (3)	5 (4)	
20	phosphoglycerate kinase	1 (1)	2 (2)			6 (3)		
21	enolase		4 (2)	1 (1)			1 (1)	
22	isocitrate dehydrogenase		2 (2)				2 (2)	
23	ATP synthase		7 (5)	2 (2)		11 (9)	3 (3)	
24	alcohol dehydrogenase			1 (1)		1 (1)		
	03 Metabolism (9)							
25	glutamine synthetase		2 (2)			1 (1)		
26	GSAT		1 (1)	1 (1)			1 (1)	
27	methionine synthase		1 (1)				1 (1)	
28	*S*-adenosylmethionine synthetase					1 (1)	1 (1)	
29	glycine dehydrogenase					3 (1)	1 (1)	
30	nucleoside diphosphate kinase	1 (1)					1 (1)	1 (1)
31	cytochrome P450	1 (1)	1 (1)				1 (1)	
32	caffeoyl CoA-O-methyltransferase				2 (1)		1 (1)	
33	isoflavone reductase					1 (1)	2 (2)	
	04 Stress and Defense (12)							
34	superoxide dismutase				1 (1)		6 (5)	1 (1)
35	ascorbate peroxidase	3 (2)		1 (1)		3 (3)	1 (1)	
36	peroxidase					2 (2)	1 (1)	
37	glutathione peroxidase			1 (1)	2 (1)		2 (2)	
38	glutathione transferase			1 (1)			1 (1)	
39	peroxiredoxin	2 (2)		1 (1)		2 (2)	4 (3)	1 (1)
40	catalase		1 (1)				2 (2)	
41	ferritin	1 (1)					1 (1)	
42	dehydrin	1 (1)					2 (2)	
43	disease resistance protein					4 (3)	1 (1)	
44	auxin-responsive protein	1 (1)					1 (1)	
45	desiccation-related protein			1 (1)		1 (1)		
	05 Transcription Related (8)							
46	transcription factor		1 (1)				3 (3)	
47	NAC					1 (1)		1 (1)
48	glycine-rich RNA binding protein	1 (1)		1 (1)		1 (1)	8 (1)	
49	histone					1 (1)	2 (2)	
50	maturase			1 (1)		1 (1)	1 (1)	
51	retroelement		1 (1)	1 (1)		1 (1)		
52	reverse transcriptase		2 (2)			1 (1)		
53	helicase			1 (1)		1 (1)		
	06 Protein Synthesis (2)							
54	elongation factor			3 (1)		1 (1)	4 (3)	
55	ribosomal protein			1 (1)		4 (4)		

Table 1. Continued

no.[a]	unique proteins/protein families (UPs/UPFs) name[b]	resurrection species: bryophyte[c]	resurrection species: fern-ally[d]	resurrection species: angiosperm[e]	gymnosperm[f]	angiosperm: trees in angiosperm[g]	angiosperm: Gramineae & Fabaceae[h]	angiosperm: other species[i]
	07 Protein Folding and Transporting (2)							
56	HSP/chaperone	5 (5)	9 (6)	3 (3)	1 (1)	25 (21)	11 (11)	4 (4)
57	AAA ATPase		3 (2)				3 (3)	
	08 Protein Degradation (2)							
58	protease	1 (1)	3 (3)	1 (1)			2 (2)	
59	proteasome		1 (1)			2 (2)	3 (3)	
	09 Signaling (6)							
60	Ser/Thr protein kinase	1 (1)					1 (1)	
61	14-3-3 protein		1 (1)				1 (1)	
62	GTP-binding protein					1 (1)	1 (1)	
63	Ran		1 (1)				1 (1)	
64	NB-ARC domain containing protein					1 (1)	1 (1)	
65	protein phosphatase			1 (1)			1 (1)	
	10 Membrane and Transport (4)							
66	ABC transporter			1 (1)				1 (1)
67	H^+-ATPase	1 (1)	2 (2)	1 (1)		2 (2)	3 (3)	
68	Exo70 exocyst complex					1 (1)	1 (1)	
69	membrane protein	1 (1)		1 (1)				
	11 Cell Structure (2)							
70	actin	1 (1)	1 (1)		1 (1)		1 (1)	
71	tubulin	2 (2)	4 (4)			1 (1)		
	12 Cell Division, Differentiation and Fate (1)							
72	chromosome segregation protein		2 (2)			1 (1)		1 (1)
	13 Miscellaneous (1)							
73	Kelch repeat-containing protein	1 (1)					1 (1)	
	14 Unknown (0)							
	total	24, 37 (33)[j]	37, 97 (65)[j]	26, 33 (31)	8, 20 (10)[j]	41, 139 (106)[j]	53, 130 (111)[j]	8, 17 (17)[j]

[a] Numbers of unique proteins/protein families. [b] This column is a list of overlapping desiccation/drought-responsive unique proteins/protein families (UPs/UPFs) and functional categories. The data were generated from reclassification and annotation of 720 protein identities in 19 plant species in response to drought/desiccation stresses by domain searching and similarity comparison according to the GO criteria. [c] The numbers and numbers in brackets in this column show the numbers of protein identities (IDs) and unique proteins (determined by proteins with different gi number) represented by these protein IDs, respectively. And it is the same for the other five columns. The proteins in this column were detected in a resurrection bryophyte *Physcomitrella patens*.[20] [d] In this column, the data were generated in this study and the proteins were identified from a resurrection fern-allies, *Selaginella tamariscina*. [e] In this column, the proteins were identified from Angiosperm resurrection plants, including *Boea hygrometrica*[23] and *Xerophyta viscosa*.[24,25] [f] In this column, the proteins were identified from species of Gymnosperm, including pine (*Pinus pinaster*)[21] and Norway spruce (*Picea abies*).[22] [g] In this column, the proteins were identified from tree species of Angiosperm, including three species of poplar (*Populus euramericana*,[26] *P. cathayana*,[27] and *P. euphratica*,[28] holm oak (*Quercus ilex*),[29,30] and grape (*Vitis vinifera*).[31] [h] In this column, the proteins were identified from species in Gramineae and Fabaceae, including rice (*Oryza sativa*),[32,34] wheat (*Triticum aestivum*),[35] maize (*Zea mays*),[36] tall wheatgrass (*Elymus elongatum*),[37] peanut (*Arachis hypogaea*),[38,39] and chickpea (*Cicer arietinum*).[40] [i] In this column, the proteins were identified from species of Angiosperm, including sugar beat (*Beta vulgaris*)[41] and seabuckthorn (*Hippophae rhamnoides*).[42] [j] In this column, the first number shows the number of overlapping unique proteins/protein families (UPs/UPFs), the second number shows the number of protein identities (IDs) in each plant groups, and the number in bracket shows the number of unique proteins (determined by proteins with different gi number) represented by these protein IDs identified in each plant groups; RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase; LSU: large subunit; OEC: oxygen-evolving complex; OEE: oxygen-evolving enhancer protein; NAC: nascent polypeptide-associated complex; HSP: heat shock protein; Ran: Ras-related nuclear proteins; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GSAT: glutamate 1-semialdehyde amino-transferase.

stages. The remaining 81 identities were down-regulated and clustered into the third subcluster. This analysis revealed that most desiccation-responsive proteins identities (83%) were down-regulated upon dehydration, suggesting metabolic quiescence of the plants to cope with dehydration.

Proteome Characteristics in Response to Dehydration. The 138 dehydration-responsive proteins covered various signaling and metabolic pathways, which were deemed to be important to cell structure adaptation, photosynthesis reduction, osmolytes accumulation, and the increase of antioxidation activity (Supporting Information, Tables S1 and S2). Among the 14 functional categories, photosynthesis (27%), carbohydrate and energy metabolism (22%), and protein synthesis, folding, and degradation (16%) were over-represented. In particular, 37 photosynthesis-related proteins were clearly affected, including 9 protein IDs (4 UPRs) of photosystem II proteins and 28 protein IDs (6 UPRs) of dark reaction-related proteins. Most of their RVs were reduced during desiccation and had not returned to normal levels at 12 HAR. Representative proteins include RuBisCO LSU, chlorophyll a/b binding protein, and OEE. These data showed that photosynthesis was greatly reduced under drought stress.

Carbohydrate and energy metabolism-related proteins accounted for 22% of all the protein IDs. Most of the proteins were up- or down-regulated at 1 DAD but stayed at a stable level or increased from 3 DAD to 12 HAR. Seven members of ATP synthase family in particular exhibited relatively high expression under dehydration, which was likely to be the fundamental requirement for desiccation tolerance (Supporting Information, Tables S1 and S2). In addition, 7 IDs (3 UPRs) of glycolysis-related enzymes, 7 IDs (3 UPRs) of pentose phosphate pathway enzymes, and 5 ID/UPRs of other carbohydrate

Table 2. Non-Overlapping Drought/Dehydration-Responsive Proteins in the Aerial Parts of *Selaginella tamariscina*

no.[a]	unique proteins/protein families (UPs/UPFs) name[b]	nonoverlapping drought/dehydration-responsive proteins name[c]	species	gi number	thr. MW (Da)/pI[d]	exp. MW (Da)/pI[e]
		01 Photosynthesis				
1	manganese-stabilizing protein (MSP)	unnamed protein product, containing pfam01716 manganese-stabilizing protein (MSP)/photosystem II	*Spinacia oleracea*	21283	35,021/5.6	31,111/4.8
		unnamed protein product, containing pfam01716 manganese-stabilizing protein (MSP)/photosystem II	*S. oleracea*	21283	35,021/5.6	44,108/6.1
		02 Carbohydrate and Energy Metabolism				
2	sucrose synthase	OSJNBb0026I12.4, containing cd03800 GT1 sucrose synthase domain	*Oryza sativa* (japonica cultivar-group)	38346957	97,677/8.0	10,347/6.5
3	reversibly glycosylated protein	reversibly glycosylated protein	*Phaseolus vulgaris*	38194918	40,167/5.8	42,145/6.3
4	phosphogluconate dehydrogenase	6-phosphogluconate dehydrogenase2	*Zea mays*	162463403	52,919/6.2	50,132/6.6
5	thiamine pyrophosphate (TPP) domain containing protein	hypothetical protein, containing cd02012 thiamine pyrophosphate (TPP) domain	*Vitis vinifera*	147788852	74,122/6.4	23,987/5.1
6	quinone oxidoreductase	quinone oxidoreductase	*A. thaliana*	9758467	34,658/6.5	32,747/6.6
7	electron carrier/iron−sulfur cluster binding	electron carrier/iron−sulfur cluster binding	*A. thaliana*	18398564	36,311/9.3	32,890/5.1
		03 Metabolism				
8	adenosylhomocysteinase	adenosylhomocysteinase	*Medicago truncatula*	29691168	53,163/5.7	53,131/5.8
9	acetyl-CoA carboxylase	acetyl-CoA carboxylase	*Glycine max*	8886469	58,736/7.2	50,883/6.1
10	aldo/keto reductase family protein	aldo/keto reductase family protein	*A. thaliana*	42571931	27,479/6.3	42,910/6.3
		aldo/keto reductase family protein	*A. thaliana*	42571931	27,479/6.3	39,218/6.7
		04 Stress and Defense				
11	thioredoxin	thioredoxin-like 5	*A. thaliana*	5006625	13,567/6.2	44,201/5.8
12	Erv1/Alr family protein	Erv1/Alr family protein	*A. thaliana*	18402827	21,586/8.5	25,801/5.4
13	purothionin	alpha-1-purothionin	*Triticum aestivum*	401182	13,516/4.8	48,270/6.5
14	NBS-LRR resistance-like protein	NBS-LRR resistance-like protein J71	*P. vulgaris*	14348619	121,981/6.4	47,175/5.9
15	late embryogenesis abundant (LEA) domain	late embryogenesis abundant (LEA) domain	*A. thaliana*	15227697	20,004/9.3	64,134/6.6
		05 Transcription Related				
16	far-red impaired response protein	putative far-red impaired response protein	*Oryza sativa* Japonica Group	25553698	133,091/6.6	32,962/5.8
		06 Protein Synthesis				
17	initiation factor	eukaryotic initiation factor 4A	*O. sativa* Japonica Group	303844	46,902/5.3	50,318/5.6
		08 Protein Degradation				
18	peptidase	OSJNBb0003B01.14, containing pfam02902 peptidase C48 C-terminal catalytic domain	*O. sativa* (japonica cultivar-group)	58531981	190,830/6.0	68,979/5.4
		leucine aminopeptidase	*Gossypium barbadense*	32401908	9,053/8.0	59,696/5.7
19	serine carboxypeptidase	Os01g0629600, containing pfam00450 serine carboxypeptidase domain	*O. sativa* (japonica cultivar-group)	115438723	48,161/6.6	20,660/4.4
		Os01g0629600, containing pfam00450 serine carboxypeptidase domain	*O. sativa* (japonica cultivar-group)	115438723	48,161/6.6	46,015/5.7
		09 Signaling				
20	GDP dissociation inhibitor	GDP dissociation inhibitor	*Nicotiana tabacum*	2501850	49,671/5.4	50,287/6.0
21	M protein repeat-containing protein	M protein repeat-containing protein, containing pfam07765 KIP1-like protein domain	*A. thaliana*	15233570	192,840/5.2	34,847/4.7
22	ACT-UUR-ACR-like domain containing protein	Os12g0152700, containing cd04873, ACT-UUR-ACR-like domain	*O. sativa* (japonica cultivar-group)	115487380	30,480/5.1	30,774/5.3
23	prosystemin	prosystemin	*Solanum tuberosum*	2911286	22,041/4.7	49,914/6.0

Table 2. Continued

no.[a]	unique proteins/protein families (UPs/UPFs) name[b]	nonoverlapping drought/ dehydration-responsive proteins name[c]	species	gi number	thr. MW (Da)/pI[d]	exp. MW (Da)/pI[e]
		10 Membrane and Transport				
24	embryogenesis transmembrane protein	embryogenesis transmembrane protein-like	*O. sativa* Japonica Group	55297355	32,531/9.0	66,399/5.3
		12 Cell Division, Differentiation and Fate				
25	centromere protein	putative centromere protein, containing RecF/RecN/SMC N terminal domain	*O. sativa* (japonica cultivar-group)	50428640	171,656/5.4	80,830/6.2
		13 Miscellaneous				
26	ankyrin repeats (ANK) domain containing protein	OSJNBa0084K20.9, containing cd00204 ankyrin repeats (ANK) domain	*O. sativa* (japonica cultivar-group)	21740668	70,508/8.8	49,822/5.9
27	executer	executer 1 (EX1)	*A. thaliana*	18418256	76,488/5.2	25,841/5.9
28	plant protein family domain containing protein	Os06g0545900, containing pfam03138, DUF246, Plant protein family domain	*O. sativa* (japonica cultivar-group)	115468436	66,097/9.1	25,154/6.2
29	RING-finger domain containing protein	conserved hypothetical protein, containing cd00162 really interesting new gene (RING)-finger domain	*Ricinus communis*	223533124	32,612/8.6	47,439/6.5
		14 Unknown				
30	unknown (gi115459326)	Os04g0507500, no putative function domain	*O. sativa* (japonica cultivar-group)	115459326	159,819/6.4	28,296/5.5
31	unknown (gi23495765)	hypothetical protein, no putative function domain	*O. sativa* Japonica Group	23495765	13,245/10.5	41,437/6.6
32	unknown (gi28411806)	hypothetical protein, no putative function domain	*O. sativa* Japonica Group	28411806	28,173/5.6	103,877/6.4
33	unknown (gi37991896)	hypothetical protein, no putative function domain	*O. sativa* (japonica cultivar-group)	37991896	11,283/6.7	82,303/5.5
34	unknown (gi4512672)	hypothetical protein, no putative function domain	*A. thaliana*	4512672	7,397/6.1	75,196/5.5
35	unknown (gi47496995)	hypothetical protein, no putative function domain	*O. sativa* Japonica Group	47496995	30,147/11.8	20,708/4.7
36	unknown (gi50355731)	hypothetical protein, no putative function domain	*O. sativa* (japonica cultivar-group)	50355731	25,130/5.3	108,785/6.4
37	unknown (gi56783976)	hypothetical protein, no putative function domain	*O. sativa* Japonica Group	56783976	17,624/10.6	48,240/6.3

[a] Numbers of nonoverlapping unique proteins/protein families(UPs/UPFs). [b] This column contains a list of nonoverlapping desiccation/drought-responsive UPs/UPFs and functional categories in the aerial parts of *Selaginella tamariscina*. [c] In this column is a list of nonoverlapping desiccation/drought-responsive proteins. [d] Theoretical molecular weight (Da) and pI as calculated from the gels. [e] Experimental molecular weight and pI as calculated from the gels.

metabolic enzymes were differentially expressed. Remarkably, enzymes closely related to sugar metabolism, such as GT1 sucrose synthase, ADP-glucose pyrophosphorylase, reversibly glycosylated protein, and GDP-mannose 3,5-epimerase, were up-regulated from 3 DAD to 7 DAD (Supporting Information, Tables S1 and S2). This suggested that sugar metabolism was enhanced under desiccation in order to maintain cell structure by providing carbon skeletons for osmotic adjustment, stabilizing membrane system, and preventing cellular solute crystallization.[57]

Protein metabolism and modification were also influenced by desiccation. Most of the proteins involved in protein metabolism were down-regulated at the early stages of desiccation and significantly up-regulated later. These proteins were involved in protein synthesis (i.e., eukaryotic initiation factor 4A), protein folding (i.e., five isoforms of HSP70, two isoforms of HSP83, HSP101, chaperonin, and two translational endoplasmic reticulum ATPase), and protein degradation (i.e., two kinds of protease,three peptidase, and 26S protease regulatory subunit 6A). Additionally, 14 out of 103 differentially expressed UPRs exhibited at least two isoforms on the 2D gels (Figure 5 and Supporting Information, Table S4). There were five photosynthesis-related proteins (RuBisCO LSU, RuBisCO LSU binding protein, two forms of OEE, and manganese-stabilizing protein), four carbohydrate and energy metabolism-related enzymes (enolase, ATP synthase CF1 alpha subunit, transketolase 1, and phosphoribulokinase), aldo/keto reductase, serine carboxypeptidase, two HSPs, and transitional endoplasmic reticulum ATPase (Supporting Information, Table S4). Therefore, the post-translational modification of proteins involved in photosynthesis, energy metabolism, protein folding, and degradation probably play essential roles in plant dehydration tolerance.[20,40,58−61]

In addition to the aforementioned nine HSPs/chaperonines and three AAA ATPases, which can assist in protein folding or unfolding in response to drought stress, *S. tamariscina* also utilized other stress-related proteins to cope with dehydration. Four redox-related proteins, thioredoxin-like 5, alpha-1-purothionin, Erv/Alr family protein, and CAT, were induced in some or all of the stages of dehydration. These enzymes

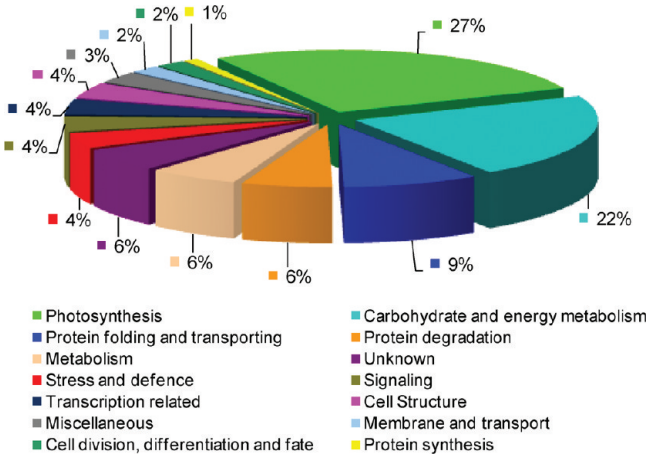

Figure 6. Functional categorization of the identified proteins in *S. tamariscina*. Please refer to Supporting Information, Table S1 for details of the 14 functional groups.

regulate cellular redox reactions so that plants could maintain redox balance under different conditions. Furthermore, two desiccation-responsive proteins, LEA protein and NBS-LRR resistance-like protein J71,[62] were induced gradually upon dehydration (Supporting Information, Tables S1 and S2). LEA proteins have a high number of polar residues and perhaps can protect other proteins and macromolecules by conferring a preferential hydration during the earlier stages of dehydration and use their own hydroxylated residues to substitute for the loss of water.[57] NBS-LRR family proteins are well-known resistance-related proteins using their LRR domain for ligand recognition and protein−protein interaction.[63,64] These stress-related proteins induced by desiccation can help the plant maintain and repair molecular and cellular structures during dehydration and recovery.

Our protein identification results also indicated that dehydration triggered other signaling and metabolic processes in the cells (Supporting Information, Tables S1 and S2). Six signaling-related proteins were affected by dehydration, Ran1 homologue, GDP dissociation inhibitor, ACT-UUR-ACR-like domain, M protein repeat-containing protein, prosystemin, and 14-3-3 protein. Furthermore, two vacuolar H^+-ATPase subunit B and embryogenesis transmembrane protein-like also exhibited differential expression. These proteins were involved in the ion transport and osmolyte regulation because the V-ATPase complexes function to translocate protons into the lumen and establish an electrochemical gradient for active transport of solutes into the vacuoles.[9] Moreover, cytoskeleton proteins showed abundant changes, including one actin and four tubulin subunits. The cytoskeleton forms a dynamic framework maintaining cell shape and has been implicated in sensing osmotic stress.[65] Similar dynamic cytoskeleton proteins were also discovered in resurrection moss *P. patens* when having to cope with dehydration.[66]

Drought/Desiccation-Responsive Protein Database. To compare the drought/desiccation-responsive proteins in *S. tamariscina* with those in bryophytes, gymnosperms, and angiosperms reported to date, we set up a database of drought/desiccation-responsive proteins in vegetative organs of 18 plant species[20−42] plus *S. tamariscina* (Supporting Information, Tables S5−S7). In this database, the 19 plant species were classified into seven groups: (1) bryophyte,[20] (2) fern-allies, (3) gymnosperms,[21,22] (4) resurrection angiosperm,[23−25] (5) tree species of angiosperm,[26−31] (6) angiosperm species in Gramineae & Fabaceae,[32−40] and (7) other species of angiosperms.[41,42] The database contains 720 protein IDs (representing 272 unique proteins/protein families, UPs/UPFs) and provides the information of protein functional categories and dynamic pattern in response to drought/desiccation (Tables 1 and 2; Supporting Information, Tables S6 and S7). It is helpful for better understanding the common or specific desiccation-tolerance mechanism for different plant species.

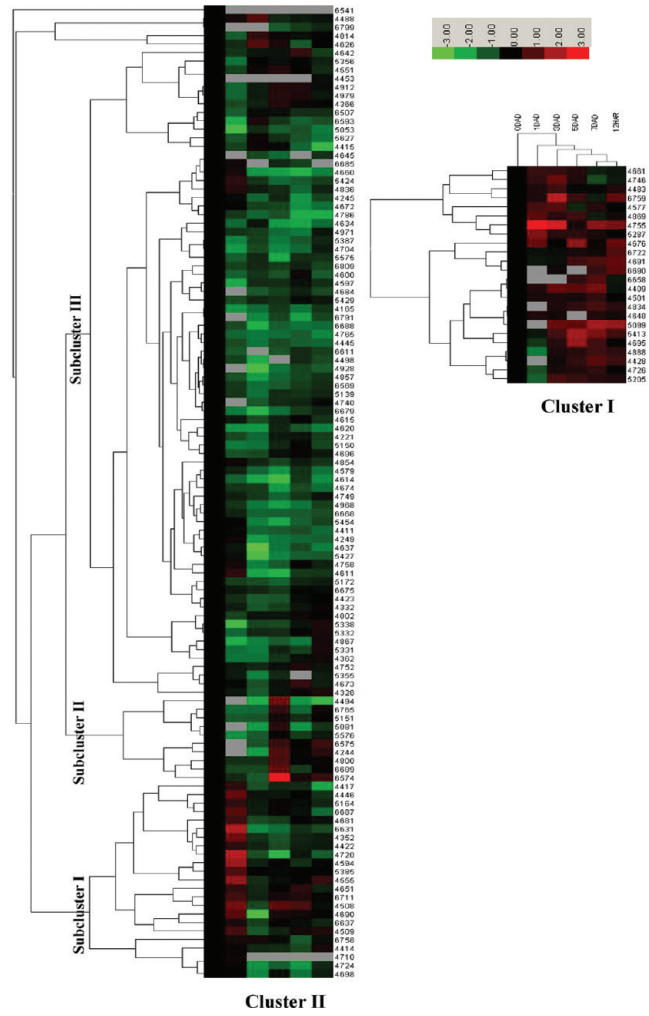

Figure 7. Hierarchical clustering analysis of the expression profiles of 138 proteins in response to dehydration and rehydration. Six columns represent treatment of 0DAD, 1DAD, 3DAD, 5DAD, 7DAD and 12HAR, respectively. The abundance ratios of protein spots were presented in Table S2, Supporting Information. The rows represent the individual proteins. The color ranges from green (3.00) for the highest down-regulation to red (3.00) for the highest up-regulation. Undetected proteins on the gels of various treatments were represented by gray color. The intensity of the colors increases with increasing expression differences, as shown in the scale bar.

Discussion

***S. tamariscina* is a Poikilochlorophyllous Desiccation Species.** Resurrection plants have evolved the ability to withstand cellular desiccation in their vegetative tissues. Plants that dismantle their photosynthetic apparatus and lose their chlorophyll content upon desiccation are termed poikilochlorophyllous (PDT) species, and plants retain their photosynthetic

apparatus in the dried state are termed homoiochlorophyllous (HDT) species. In addition, according to their tolerance to water deprivation, desiccation tolerant plants can be divided into two main categories. Category one plants can tolerate rapid desiccation, and category two can tolerate relatively slow water loss.[5] Physiological studies of a few species of angiosperms, such as *Xerophyta scabrida*,[67] *Xerophyta villosa*,[68] *X. viscosa*,[69] and *Borya nitida*,[70] confirm they were all PDT species. The desiccation tolerance mechanism in fern-ally *S. tamariscina*, however, was unclear. In this study, we conducted physiological and molecular experiments to determine its type of desiccation tolerance. *S. tamariscina* plants were collected from virtually soil-less mesa/rock, which is the common habitat for PDT species. The plants can survive seven days without watering and recover to a normal condition 12 h after rewatering. The water content in the samples reduced gradually after the start of dehydration, which led to rolling and wilting of their "fronds". The extensive reduction in the ratio of samples area to biomass during slow drying resulted in an obvious decline of photosynthesis and chlorophyll content. These are the essential features of PDT species.[46] The above findings can be further supported by the proteomic results, which revealed that 37 photosynthesis-related protein IDs were affected by dehydration (Tables 1 and 2, Supporting Information, Table S1). Additionally, the gradual accumulation of ABA, proline, and soluble sugars in the samples, as well as the minimal membrane damage, reflected the features of PDT species in coping with slow dehydration. All the data support *S. tamariscina* as a primitive poikilochlorophyllous desiccation plant that can tolerant slow water deprivation.

Activation of Antioxidant System upon Desiccation. Dehydration often leads to oxidative stress due to the accumulation of ROS mainly in mitochondria and chloroplasts. ROS can cause damage to essential cellular components (such as nucleic acids, polysaccharides, proteins, and membrane lipids)[9] and cell structure.[71] Resurrection plants have evolved various protective mechanisms.[10,11,69,72−74] The PDT plants reduced ROS formation by dismantling their chlorophyll and thylakoid membranes to avoid energy transfer from excited chlorophyll to oxygen during dehydration, and then reconstruct their photosynthetic systems after rehydration.[10] This strategy has been well studied in *Xerophyta* species.[72] In addition to structural changes, resurrection plants up-regulated various antioxidant protectants (i.e., ascorbate and glutathione) to quench ROS.[73] Such enzymes as SOD, APX, and GR usually accumulate in resurrection plants upon dehydration to help regenerate antioxidants.[69] These antioxidant enzymes have been shown to remain undenatured during desiccation so that during rehydration they can function to prevent ROS damage.[11,74] In the PDT fern-ally *S. tarmaricina*, dehydration induced rolling and wilting of the samples and the decreased chlorophyll content and shutdown of photosynthesis helped cells to avoid photo-oxidation damage. Meanwhile, the gradual increase in the activities of antioxidant enzymes (SOD, POD, CAT, and GR) in *S. tarmaricina* during dehydration helped to quench ROS. Among the four antioxidant enzymes, only CAT was up-regulated. This suggests that regulation of the activities of antioxidant enzymes plays an important role in coping with ROS. In addition, three other redox proteins, thioredoxin-like 5, alpha-1-purothionin, and Erv/Alr family protein also exhibited quantitative changes in response to dehydration.[75,76] We postulated that these proteins, together with the sugars and proline that accumulated in the samples, play important roles in regulating redox status during dehydration.[77,78]

Signaling Mechanism Underlying Dehydration Tolerance in *S. tamariscina*. ABA accumulation is one of the earliest signal responses to desiccation stress in plants.[79] ABA is known to induce the expression of several dehydration-responsive genes/proteins (such as CDT1, LEA protein, and aquaporins) in resurrection plants.[2,3,12] It was suggested that ABA-dependent and ABA-independent signaling pathways interact to induce gene expression in resurrection plants.[80] As an example, some dehydration-regulated genes/proteins like transcription factor HD-ZIP in *Craterostigma* have been found not to be induced by ABA.[13] In *S. tamariscina* under dehydration or ABA treatment, two LEA protein family genes, early light-inducible protein gene (*ELIPB*) and open stomata 1 gene (*OST1*), were strongly induced.[47] However, it was not clear that three dehydration-responsive genes, Zinc finger transcription factor Sp4 gene, embryo-specific protein 1 (*ATS1*), and FAD oxidoreductase gene, were induced by ABA. In another resurrection plant species, *S. lepidephylla*, two transcription factors (ABA-insensitive 5 and AP2 domain-containing) were detected by EST analysis; they mediate ABA-dependent gene expression leading to dehydration tolerance.[18] Using proteomic approaches, we found the changes of some expression products of these gene families or related genes accompanied by the accumulation of ABA in *S. tamariscina*. Among 138 dehydration-responsive protein IDs, LEA protein, two transcription factor-related proteins, and four redox-related enzymes were detected to be differentially expressed in response to dehydration and rehydration (Supporting Information, Tables S1 and S2) and this supports the role of ABA as a key regulator in *S. tamariscina*.

ABA has been proposed to induce accumulation of sucrose and proline,[77,81] and regulate stomatal closure[82] and redox-status in cells upon dehydration.[77] Sugar accumulation has been reported to be crucial to maintain osmolyte balance of dehydrated cells in many resurrection plants, such as *Cardiomanes plantagineum*,[83] *Sporobolus staphianus*,[84] *Myrothamnus flabellifolia*,[85] *Boea hygroscopica*,[86] and *S. lepidiphylla*.[87] Also, sugars have been shown to function as signals in the regulation of the expression of photosynthesis- and ROS-related genes.[88,89] In this study, we found an accumulation of soluble sugars and enzymes in sugar metabolism, for example, sucrose synthase, ADP-glucose pyrophosphorylase, UDP-glucose-4-epimerase, reversibly glycosylated protein (RGP), and six glycolysis/pentose phosphate pathway-related enzymes (Supporting Information, Tables S1 and S2). This supported the hypothesis that sugar accumulation may protect cell integrity by stabilizing membrane and protein structure/activity during dehydration.[90] Furthermore, some products of carbohydrate metabolism serve as precursors for cell wall synthesis.[91] Resurrection plants can utilize inherent cell wall modification to achieve stable and reversible morphological changes[92] that help to enhance dehydration tolerance.[11] In this study, glucose metabolism-related enzymes, for example, UDP-glucose-4-epimerase and RGP, exhibited abundance changes and are likely to play important roles in dehydration tolerance. Moreover, proline accumulation occurred in the samples upon dehydration. Proline is an important osmolyte for regulating redox status and scavenging ROS, as well as protecting protein and membrane structure.[77,78] In addition, stomatal closure of aerial parts under dehydration stress can also be attributed to ABA. On the basis of these data, ABA may be the primary

signaling molecule to trigger sugar and osmolyte synthesis, which then leads to changes in ROS levels and photosynthetic activity. Further investigation is needed to elucidate the detailed mechanism and signaling networks underlying dehydration tolerance in *S. tamariscina*.

Metabolic Down-Regulation in *S. tamariscina* in Response to Desiccation Appeared to Be a Specific Mechanism of Desiccation Tolerance. In land plants, desiccation tolerance is common in reproductive organs/tissues (pollen grains, spores, and seeds) but relatively uncommon in vegetative organs/tissues. Vegetative desiccation-tolerant plants can be found in less complex clades, including algae, lichens, and bryophytes, as well as in the more complex ferns/fern allies and angiosperms.[46] On the basis of phylogenetic analysis, Oliver et al.[46] postulated that the vegetative desiccation tolerance originally present in bryophytes was lost in the evolution of tracheophytes. During that evolution, at least one independent evolution or re-evolution of desiccation tolerance occurred in *Selaginella*.[46] By comparative analysis of desiccation-responsive proteome in various plant groups, we found that metabolism in resurrection fern-ally *S. tarmaricina* was significantly down-regulated upon desiccation. This may constitute a specific mechanism compared with nontolerant species. Our hierarchical clustering results showed that 114 protein identities in *S. tarmaricina* appeared down-regulation in response to desiccation. These down-regulated proteins were involved in nearly all the major metabolic processes, including photosynthesis, carbohydrate and energy metabolism, stress and defense, transcription and protein metabolism, signaling, membrane and transport, cell structure, and cell division. The reduction of metabolic enzyme levels was indicative of a metabolic shift from active to a quiescent state accompanied with dehydration. This specific mechanism of vegetative desiccation tolerance was related to those occurring in the course of pollen and seed maturation.[54,58,59,93] During soybean seed filling, most proteins involved in metabolism, protein synthesis, disease/defense, cell growth/division, and signaling were decreased.[93] The metabolism activity curtails as desiccation of vegetable tissues, seeds, and pollen grains is a positive strategy to maintain energy and to avoid oxidative damage.

In terms of nontolerant species, some plants prefer increased metabolic levels at the beginning to resist desiccation. This phenomenon can be easily observed from changes in photosynthesis in response to desiccation.[22,32,41] There were 17 photosynthesis-related UPs/UPFs identified in *S. tarmaricina* and other species. Among them, 9 UPs/UPFs were down-regulated in *S. tarmaricina*, but 13 UPs/UPFs were up-regulated in desiccation nontolerant species (Table 1, Table 2, Supporting Information, Tables S1, S6, and S7). Photosynthesis and the photosynthetic apparatus are very sensitive to desiccation in various plants. Under desiccation or water-deficit conditions, fragments of RubisCO LSU and RubisCO SSU have been detected in many plants including resurrection and desiccation nontolerant species.[20,23,34,35] This is mainly due to RubisCO being cleaved by ROS that is generated under drought stress. To cope with intercellular CO_2 deficit resulting from stomata closure, plants evolved diverse strategies. Desiccation nontolerant plants attempted to utilize the limited CO_2 by up-regulation of RuBisCO and RuBisCO activase, such as in rice and wheat.[33,35,41] Some plants (e.g., rice,[32−34] wheat,[35] *Physcomitrella patens*,[20] *Picea abies*,[22] *Arachis hypogaea*,[38,39] *Cicer aretinum*,[40] *Vitis vinifera*,[31] and *Elymus elongatum*[37]) initially increase the efficiency of light utilization and the energy production by up-regulation of the light harvesting complex, chlorophyll a/b-binding protein, OEC, OEE, ferredoxin-NADP reductase, Rieske Fe−S precursor protein, carbonate dehydratase, fructose-1,6-bisphosphatase, and sedoheptulose-1,7-bisphosphatase, respectively. But all these processes led to more ROS generation and result in further damage to plants. In contrast, and similar to other PDT resurrection plants,[20,24] *S. tarmaricina* relies on inhibition of photosynthesis through down-regulation of nearly all the photosynthesis-related UPs/UPFs after 3 DAD. This appears to be a positive strategy to cope with stress.

Conclusions and Perspective Remarks

Desiccation tolerance is conserved in reproductive structures, but uncommon in vegetative organs of vascular plants. Fern/fern-allies are primitive vascular plants and include 60−70 species of resurrection plants, represented by species of *Selaginella*. In this study, the samples of *S. tamariscina* were water-deprived for up to seven days followed by 12 h of rewatering. We found that endogenous ABA, the contents of osmolytes (soluble sugars and proline) and the activities of four antioxidant enzymes (SOD, POD, CAT, and GR) were all increased in response to dehydration stress, but photosynthesis decreased and plasma membrane integrity was lost. Moreover, our comparative proteomics analysis revealed 103 desiccation-responsive unique proteins, and 83% of the proteins were down-regulated upon dehydration. On the basis of the dynamic physiological data and the expression changes of the desiccation-responsive proteins, we defined *S. tamariscina* as a poikilochlorophyllous desiccation tolerant species. Its desiccation tolerance relies on ABA-induced tissue morphological modification, photosynthesis reduction, osmolyte accumulation, and activation of antioxidant systems. We also set up a database of drought/dehydration-responsive proteins in plants by integrating our proteomic results with existing data obtained from various resurrection/nontolerant plants. This analysis revealed that the metabolism in resurrection plants includes metabolic quiescence upon desiccation, which is specific when compared with nontolerance species.

Our study has laid the foundation for further investigation of independent evolution of desiccation tolerance in *Selaginella*. Because of the complexity of vegetative desiccation tolerance in plants, further studies are necessary using advanced proteomics approaches (e.g., isobaric tags for relative and absolute quantitation (iTRAQ) and label free quantification MS) to capture less abundant regulatory proteins involved in the dehydration-tolerant process. Detailed functional characterization of the proteins/genes revealed by proteomic studies may lead to improved understanding of the underlying molecular mechanisms and genetically modified crops with enhanced drought tolerance.

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; BHT, butylated hydroxytoluene; CAT, catalase; DAD, days after dehydration; GR, glutathione reductase; HAR, hours after rehydration; IAA, indoleacetic acid; IDs, identities; MALDI TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PBS, phosphate buffer solution; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; 2-DE, two-dimensional gel electrophoresis.

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Supporting Information Available: Detailed dehydration-responsive proteins in the aerial parts of *Selaginella tamariscina* identified using 2-DE and mass spectrometry, and drought/dehydration-responsive proteins in different evolutionary plant groups identified by previous proteomics studies. Supplemental Tables S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Chaves, M. M.; Maroco, J. P.; Pereira, J. Understanding plant response to drought - from genes to the whole plant. *Funct. Plant Biol.* **2003**, *30* (3), 239–264.
(2) Ingram, J.; Bartels, D. The molecular basis of dehydration tolerance in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 377–403.
(3) Ramanjulu, S.; Bartels, D. Drought- and desiccation-induced modulation of gene expression in plants. *Plant Cell Environ.* **2002**, *25* (2), 141–151.
(4) Proctor, M. C. F.; Tuba, Z. Poikilohydry and homoihydry: antithesis or spectrum of possibilities. *New Phytol.* **2002**, *156*, 327–349.
(5) Farrant, J. M. In *Plant Desiccation Tolerance*; Jenks, M. A.; Wood, A. J., Eds.; Blackwell Publishing: Oxford, 2007; pp 3−90.
(6) Wood, A. J. The nature and distribution of vegetative desiccation tolerance in hornworts, liverworts and mosses. *Bryologist* **2007**, *110* (2), 163–177.
(7) Bewley, J. D.; Krochko, J. E. In *Encyclopedia of Plant Physiology, Vol 12B, Physiological Ecology II*; Lange, O. L.; Nobel, P. S.; Osmond, C. B.; Ziegler, H., Eds.; Springer-Verlag: Berlin, 1982; pp 325−378.
(8) Bartels, D.; Salamini, F. Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at the molecular level. *Plant Physiol.* **2001**, *127* (4), 1346–1353.
(9) Mundree, S. G.; Baker, B.; Mowla, S.; Peters, S.; Marais, S.; Willigen, C. V.; Govender, K.; Maredza, A.; Muyanga, S.; Farrant, J. M.; Thomson, J. A. Physiological and molecular insights into drought tolerance. *Afr. J. Biotechnol.* **2002**, *1*, 28–38.
(10) Vicré, M.; Farrant, J. M.; Driouich, A. Insights into the cellular mechanisms of desiccation tolerance among angiosperm resurrection plant species. *Plant Cell Environ.* **2004**, *27* (11), 1329–1340.
(11) Farrant, J. M.; Brandt, W.; Lindsey, G. G. An Overview of Mechanisms of desiccation tolerance in selected angiosperm resurrection plants. *Plant Stress* **2007**, *1* (1), 72–84.
(12) Furini, A.; Koncz, C.; Salamini, F.; Bartels, D. High level transcription of a member of a repeated gene family confers dehydration tolerance to callus tissues of *Craterostigma plantagineum*. *EMBO J.* **1997**, *16* (12), 3599–3608.
(13) Frank, W.; Phillips, J.; Salamini, F.; Bartels, D. Two dehydration-inducible transcripts from the resurrection plant *Craterostigma plantagineum* encode interacting homeodomain leucine zipper proteins. *Plant J.* **1998**, *15* (3), 413–421.
(14) Frank, W.; Munnik, T.; Kerkmann, K.; Salamini, F.; Bartels, D. Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. *Plant Cell* **2000**, *12* (1), 111–124.
(15) Oliver, M. J.; Dowd, S. E.; Zaragoza, J.; Mauget, S. A.; Payton, P. R. The rehydration transcriptome of the desiccation-tolerant bryophyte *Tortula ruralis*: transcript classification and analysis. *BMC Genomics* **2004**, *5*, 1–19.
(16) Bockel, C.; Salamini, F.; Bartels, D. Isolation and characterization of genes expressed during early events of the dehydration process in the resurrection plant *Craterostigma plantagineum*. *J. Plant Physiol.* **1998**, *152*, 158–166.
(17) Collett, H.; Shen, A.; Gardner, M.; Farrant, J. M.; Denby, K. J.; Illing, N. Towards transcript profiling of desiccation tolerance in *Xerophyta humilis*: Construction of a normalized 11 k *X. humilis* cDNA set and microarray expression analysis of 424 cDNAs in response to dehydration. *Physiol. Plantarum* **2004**, *122* (1), 39–53.
(18) Iturriaga, G.; Cushman, M. A. F.; Cushman, J. C. An EST catalogue from the resurrection plant *Selaginella lepidophylla* reveals abiotic stress-adaptive genes. *Plant Sci.* **2006**, *170*, 1173–1184.
(19) Weng, J. K.; Tanurdzic, M.; Chapple, C. Functional analysis and comparative genomics of expressed sequence tags from the Lycophyte *Selaginella moellendorffii*. *BMC Genomics* **2005**, *6*, 85.
(20) Wang, X. Q.; Yang, P. F.; Liu, Z.; Liu, W. Z.; Hu, Y.; Chen, H.; Kuang, T. Y.; Pei, Z. M.; Shen, S. H.; He, Y. K. Exploring the mechanism of *Physcomitrella patens* desiccation tolerance through a proteomic strategy. *Plant Physiol.* **2009**, *149* (4), 1739–1750.
(21) Costa, P.; Bahrman, N.; Jean-Marc, F.; Kremer, A.; Plomion, C. Water-deficit-responsive proteins in maritime pine. *Plant Mol. Biol.* **1998**, *38* (4), 587–596.
(22) Blödner, C.; Majcherczyk, A.; Kües, U.; Polle, A. Early drought-induced changes to the needle proteome of *Norway spruce*. *Tree Physiol.* **2007**, *27* (10), 1423–1431.
(23) Jiang, G. Q.; Wang, Z.; Shang, H. H.; Yang, W. L.; Hu, Z. A.; Phillips, J.; Deng, X. Proteome analysis of leaves from the resurrection plant *Boea hygrometrica* in response to dehydration and rehydration. *Planta* **2007**, *225* (6), 1405–1420.
(24) Ingle, R. A.; Schmidt, U. G.; Farrant, J. M.; Thomson, J. A.; Mundree, S. G. Proteomic analysis of leaf proteins during dehydration of the resurrection plant *Xerophyta viscosa*. *Plant Cell Environ.* **2007**, *30* (4), 435–446.
(25) Abdalla, K.; Baker, B.; Rafudeen, M. S. Proteomic analysis of nuclear proteins during dehydration of the resurrection plant *Xerophyta viscosa*. *Plant Growth Regul.* **2010**. DOI: 10.1007/s10725-010-9497-2.
(26) Bonhomme, L.; Monclus, R.; Vincent, D.; Carpin, S.; Lomenech, A. M.; Plomion, C.; Brignolas, F.; Morabito, D. Leaf proteome analysis of eight *Populus* × *euramericana* genotypes: Genetic variation in drought response and in water-use efficiency involves photosynthesis-related proteins. *Proteomics* **2009**, *9* (17), 4121–4142.
(27) Xiao, X. W.; Yang, F.; Zhang, S.; Korpelainen, H.; Li, C. Y. Physiological and proteomic responses of two contrasting *Populus cathayana* populations to drought stress. *Physiol. Plantarum* **2009**, *136* (2), 150–168.
(28) Bogeat-Triboulot, M. B.; Brosché, M.; Renaut, J.; Jouve, L.; Le, T. D.; Fayyaz, P.; Vinocur, B.; Witters, E.; Laukens, K.; Teichmann, T.; Altman, A.; Hausman, J. F.; Polle, A.; Kangasjärvi, J.; Dreyer, E. Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in *Populus euphratica*, a poplar growing in arid regions. *Plant Physiol.* **2007**, *143* (2), 876–892.
(29) Jorge, I.; Navarro, R. M.; Lenz, C.; Ariza, D.; Jorrín, J. Variation in the holm oak leaf proteome at different plant developmental stages, between provenances and in response to drought stress. *Proteomics* **2006**, *6* (Suppl 1), S207–S214.
(30) Echevarría-Zomeño, S.; Ariza, D.; Jorge, I.; Lenz, C.; Campo, A. D.; Jorrín Jesùs, V.; Navarro, R. M. Changes in the protein profile of *Quercus ilex* leaves in response to drought stress and recovery. *J. Plant Physiol.* **2009**, *166* (3), 233–245.
(31) Vincent, D.; Ergül, A.; Bohlman, M. C.; Tattersall, E. A.; Tillett, R. L.; Wheatley, M. D.; Woolsey, R.; Quilici, D R.; Joets, J.; Schlauch, K.; Schooley, D A.; Cushman, J C.; Cramer, G. R. Proteomic analysis reveals differences between *Vitis vinifera* L. *cv. Chardonnay* and *cv. Cabernet Sauvignon* and their responses to water deficit and salinity. *J. Exp. Bot.* **2007**, *58* (7), 1873–1892.
(32) Salekdeh, G. H.; Siopongco, J.; Wade, L. J.; Ghareyazie, B.; Bennett, J. A peoteomic approach to analyzing drought- and salt-responsiveness in rice. *Field Crops Res.* **2002**, *76* (2−3), 199–219.
(33) Salekdeh, G. H.; Siopongco, J.; Wade, L. J.; Ghareyazie, B.; Bennett, J. Proteomic analysis of rice leaves during drought stress and recovery. *Proteomics* **2002**, *2* (9), 1131–1145.
(34) Ali, G. M.; Komatsu, S. Proteomic analysis of rice leaf sheath during drought stress. *J. Proteome Res.* **2006**, *5* (2), 396–403.
(35) Peng, Z.; Wang, M.; Li, F.; Lv, H.; Li, C.; Xia, G. A proteomic study of the response to salinity and drought stress in an introgression strain of bread wheat. *Mol. Cell. Proteomics* **2009**, *8* (12), 2676–2686.
(36) Frédérique, R.; Riccardi, F.; Gazeau, P.; de Vienne, D.; Zivy, M. Protein changes in response to progressive water deficit in maize. *Plant Physiol.* **1998**, *117* (4), 1253–1263.
(37) Gazanchian, A.; Hajheidari, M.; Sima, N. K.; Salekdeh, G. H. Proteome response of *Elymus elongatum* to severe water stress and recovery. *J. Exp. Bot.* **2007**, *58* (2), 291–300.
(38) Akkasaeng, C.; Tantisuwichwong, N.; Chairam, I.; Prakrongrak, N.; Jogloy, S.; Pathanothai, A. Isolation and identification of peanut leaf proteins regulated by water stress. *Pak. J. Biol. Sci.* **2007**, *10* (10), 1611–1617.
(39) Kottapalli, K. R.; Rakwal, R.; Shibato, J.; Burow, G.; Tissue, D.; Burke, J.; Puppala, N.; Burow, M.; Payton, P. Physiology and

proteomics of the water-deficit stress response in three contrasting peanut genotypes. *Plant Cell Environ.* **2009**, *32* (4), 380–407.
(40) Pandey, A.; Chakraborty, S.; Datta, A.; Chakraborty, N. Proteomics approach to identify dehydration responsive nuclear proteins from chickpea (*Cicer arietinum* L.). *Mol. Cell. Proteomics.* **2008**, *7* (1), 88–107.
(41) Hajheidari, M.; Abdollahian-Noghabi, M.; Askari, H.; Heidai, M.; Sadeghian, S. Y.; Ober, E. S.; Salekdeh, G. H. Proteome analysis of sugar beet leaves under drought stress. *Proteomics* **2005**, *5* (4), 950–960.
(42) Xu, G.; Li, C. Y.; Yao, Y. N. Proteomics analysis of drought stress-responsive proteins in *Hippophae rhamnoides* L. *Plant Mol. Biol. Rep.* **2009**, *27* (2), 153–161.
(43) Abdalla, K.; Thomson, J. A.; Rafudeen, M. S. Protocols for nuclei isolation and nuclear protein extraction from the resurrection plant *Xerophyta viscosa* for proteomic studies. *Anal. Biochem.* **2009**, *384* (2), 365–367.
(44) Garwe, D.; Thomson, J. A.; Mundree, S. G. Molecular characterization of XvSAP1, a stress-responsive gene from the resurrection plant *Xerophyta viscosa* Baker. *J. Exp. Bot.* **2003**, *54*, 191–201.
(45) Kendrick, P.; Crane, P. R. The origin and early evolution of plants on land. *Nature* **2003**, *389*, 33–39.
(46) Oliver, M. J.; Tuba, Z.; Mishler, B. D. The evolution of vegetative desiccation tolerance in land plants. *Plant Ecol.* **2000**, *151*, 85–100.
(47) Liu, M. S.; Chien, C. T.; Lin, T. P. Constitutive components and induced gene expression are Involved in the desiccation tolerance of *Selaginella tamariscina*. *Plant Cell Physiol.* **2008**, *49* (4), 653–663.
(48) Yang, Y. M.; Xu, C. N.; Wang, B. M.; Jia, J. Z. Effects of plant growth regulators on secondary wall thickening of *Cotton fibres*. *Plant Growth Regul.* **2001**, *35* (3), 233–237.
(49) Li, H. S.; Sun, Q.; Zhao, S. J.; Zhang, W. H., Ed. In *Principles and Techniques of Plant Physiological Biochemical Experiment*; Higher Education Press: Beijing, 2000.
(50) Lichtenthaler, H. K.; Wellburn, A. R. Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* **1983**, *11*, 591–592.
(51) Change, B.; Maehly, A. C. Assay of catalases and peroxidase. *Methods Enzymol.* **1955**, *2*, 764–775.
(52) Rao, M. V.; Hale, B. A.; Ormrod, D. P. Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. *Plant Physiol.* **1995**, *109* (2), 421–432.
(53) Halliwell, B.; Foyer, C. H. Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. *Planta* **1978**, *139* (1), 9–17.
(54) Dai, S. J.; Li, L.; Chen, T. T.; Chong, K.; Xue, Y. B.; Wang, T. Proteomic analyses of *Oryza sativa* mature pollen reveal novel proteins associated with pollen germination and tube growth. *Proteomics* **2006**, *6* (8), 2504–2529.
(55) Wang, X. C.; Fan, P. X.; Song, H. M.; Chen, X. Y.; Li, X. F.; Li, Y. X. Comparative proteomic analysis of differentially expressed proteins in shoots of *Salicornia europaea* under different salinity. *J. Proteome Res.* **2009**, *8* (7), 3331–3345.
(56) Phillips, J. R.; Hilbricht, T.; Salamini, F.; Bartels, D. A novel abscisic acid and dehydration- responsive gene family from the resurrection plant *Craterostigma Plantagineum* encodes a plastid-targeted protein with DNA-binding activity. *Planta* **2002**, *215* (2), 258–266.
(57) Hoekstra, F. A.; Golovina, E. A.; Buitink, J. Mechanisms of plant desiccation tolerance. *Trends Plant Sci.* **2001**, *6* (9), 431–438.
(58) Dai, S. J.; Chen, T. T.; Chong, K.; Xue, Y. B,; Liu, S. q.; Wang, T. Proteomic identification of differentially expressed proteins associated with pollen germination and tube growth reveals characteristics of germinated *Oryza sativa* pollen. *Mol. Cell. Proteomics* **2007**, *6*, 207–230.
(59) Dai, S. J.; Wang, T.; Yan, X. F.; Chen, S. X. Proteomics of pollen development and germination. *J. Proteome Res.* **2007**, *6*, 4556–4563.
(60) Röhrig, H.; Schmidt, J.; Colby, T.; Brautigam, A.; Hufnagel, P.; Bartels, D. Desiccation of the resurrection plant *Craterostigma plantagineum* induces dynamic changes in protein phosphorylation. *Plant Cell Environ.* **2006**, *29*, 1606–1617.
(61) Brini, F.; Hanin, M.; Lumbreras, V.; Irar, S.; Pages, M.; Masmoudi, K. Functional characterization of DHN-5, a dehydrin showing a differential phosphorylation pattern in two Tunisian durum wheat (Triticum durum Desf.) varieties with marked differences in salt and drought tolerance. *Plant Sci.* **2007**, *172* (1), 20–28.
(62) Chini, A.; Grant, J. J.; Se, K. M.; Shinozaki, K.; Loake, G. J. Drought tolerance established by enhanced express ion of the CC-NBS-LRR gene, ADR1, requires salicylic acid, EDS1 and ABI1. *Plant J.* **2004**, *38* (5), 810–822.
(63) Li, R.; Rimmer, R.; Yu, M.; Sharpe, A. G.; Séguin-Swartz, G.; Lydiate, D.; Hegedus, D. D. Two *Brassica napus* polygalacturonase inhibitory protein genes are expressed at different levels in response to biotic and abiotic stresses. *Planta* **2003**, *217* (2), 299–308.
(64) van Ooijen, G.; van den Burg, H. A.; Cornelissen, B. J.; Takken, F. L. Structure and function of resistance proteins in solanaceous plants. *Ann. Rev. Phytopath.* **2007**, *45*, 43–72.
(65) Komis, G.; Galatis, B.; Quader, H.; Galanopoulou, D.; Apostolakos, P. Phospholipase C signaling involvement in macrotubule assembly and activation of the mechanism regulating protoplast volume in plasmolyzed root cells of *Triticum turgidum*. *New Phytol.* **2008**, *178* (2), 267–282.
(66) Wang, X.; Yang, P.; Gao, Q.; Liu, X.; Kuang, T.; Shen, S.; He, Y. Proteomic analysis of the response to high-salinity stress in *Physcomitrella patens*. *Planta* **2008**, *228* (1), 167–177.
(67) Tuba, Z.; Lichtenthaler, H. K.; Csintalan, Z.; Pócs, T. Regreening of desiccated leaves of the poikilochlorophyllous *Xerophyta scabrida* upon rehydration. *J. Plant Physiol.* **1993**, *142*, 103–108.
(68) Hallam, N. D.; Luff, S. E. Fine structural changes in the mesophyll tissue of the leaves of *Xerophyta villosa* during *desiccation*. *Bot. Gaz.* **1980**, *141*, 173–179.
(69) Sherwin, H. W.; Farrant, J. M. Protection mechanism against excess light in the resurrection plants *Craterosigma wilmsii* and *Xerophyta viscosa*. *Plant Growth Regul.* **1998**, *24*, 203–210.
(70) Hetherington, S. E.; Smillie, R. M. Humidity-sensitive degreening and regreening of leaves of *Borya nitida* Labill. as followed by changes in chlorophyll fluorescence. *Aust. J. Plant Physiol.* **1982**, *9*, 587–599.
(71) Smirnoff, N. The role of active oxygen in the response of plants to water deficits and desiccation. *New Phytol.* **1993**, *125* (1), 27–58.
(72) Farrant, J. M.; Bartsch, S.; Loffell, D.; van der Willigen, C.; Whittaker, A. An investigation into the effects of light on the desiccation of three resurrection plants species. *Plant Cell Environ.* **2003**, *26* (8), 1275–1286.
(73) Kranner, I.; Beckett, R. P.; Wornik, S.; Zorn, M.; Pfeifhofer, H. W. Revival of a resurrection plant correlates with its antioxidant status. *Plant J.* **2002**, *31* (1), 13–24.
(74) Illing, N.; Denby, K.; Collett, H.; Shen, A.; Farrant, J. M. The signature of seeds in resurrection plants: a molecular and physiological comparison of desiccation tolerance in seeds and vegetative tissues. *Integr. Comp. Biol.* **2005**, *45* (5), 771–787.
(75) Vitu, E.; Bentzur, M.; Lisowsky, T.; Kaiser, C. A.; Fass, D. Gain of function in an ERV/ALR sulfhydryl oxidase by molecular engineering of the shuttle disulfide. *J. Mol. Biol.* **2006**, *362* (1), 89–101.
(76) Majewski, J.; Stec, B. X-ray scattering studies of model lipid membrane interacting with purothionin provide support for a previously proposed mechanism of membrane lysis. *Eur. Biophys. J.* **2010**, *39* (8), 1155–1165.
(77) Hare, P. D.; Cress, W. A.; Van Staden, J. Dissecting the roles of osmolyte accumulation during stress. *Plant Cell Environ.* **1998**, *21* (6), 535–553.
(78) Carvalho, M. H. C. Drought stress and reactive oxygen species. *Plant Signal. Behav.* **2008**, *3* (3), 156–165.
(79) Scott, P. Resurrection plants and the secrets of eternal leaf. *Annals Bot.-London* **2000**, *85*, 159–166.
(80) Shinozaki, K.; Yamaguchi-Shinozaki, K. Gene expression and signal transduction in water-stress response. *Plant Physiol.* **1997**, *115* (2), 327–334.
(81) Schwall, G.; Elster, R.; Ingram, J.; Bernacchia, G.; Bianchi, G.; Gallagher, L.; Salamini, F.; Bartels, D. In *Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology. Current Topics in Plant Physiology: 14*; Pontis, H., Salerno, G., Echeverria, E., Eds.; American Society of Plant Physiologists: Rockville, MD, 1995; pp 245−253.
(82) Wang, P. T.; Song, C. P. Guard-cell signaling for hydrogen peroxide and abscisic acid. *New Phytol.* **2008**, *178* (4), 703–718.
(83) Norwood, M.; Toldi, O.; Richter, A.; Scott, P. Investigation into the ability of roots of the poikilohydric plant *Craterostigma plantagineum* to survive dehydration stress. *J. Exp. Bot.* **2003**, *54* (391), 2313–2327.
(84) Kaiser, K.; Gaff, D.; Outlaw, W. H. Sugar contents of leaves of desiccation sensitive and desiccation-tolerant plants. *Naturwissenschaften* **1985**, *72*, 608–609.
(85) Drennan, P. M.; Smith, M. T.; Goldsworthy, D.; van Staden, J. The occurrence of trehalose in the leaves of the desiccation-tolerant angiosperm *Myrothamnus flabellifolius* Welw. *J. Plant Physiol.* **1993**, *142* (2), 493–496.
(86) Bianchi, G.; Murelli, C.; Bochicchio, A.; Vazzana, C. Changes in low- molecular weight substances in *Boea hygroscopica* in response to desiccation and rehydration. *Phytochemistry* **1991**, *30* (2), 461–466.

(87) Gaff, D. In *Structural and Functional Responses to Environmental Stresses*; Krebb, K. H.; Richter, H.; Hinkley, T. M., Eds.; SPB Academic Publishers: Hague, Netherlands, 1989; pp 255−268.
(88) Couée, I.; Sulmon, C.; Gouesbet, G.; Amrani, E. A. Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *J. Exp. Bot.* **2006**, *57* (3), 449–459.
(89) Sulmon, C.; Gouesbet, G.; Amrani, E. A.; Couée, I. Sugar-induced tolerance to the herbicide atrazine in Arabidopsis seedlings involves activation of oxidative and xenobiotic stress responses. *Plant Cell Rep.* **2006**, *25* (5), 489–498.
(90) Crowe, J. H.; Carpenter, J. F.; Crowe, L. M. The role of vitrification in anhydrobiosis. *Annu. Rev. Physiol.* **1998**, *60*, 73–103.
(91) Reiter, W. D. The molecular analysis of cell wall components. *Trends Plant Sci.* **1998**, *3*, 27–32.
(92) Moore, J. P.; Nguema-Ona, E.; Chevalier, L. M.; Lindsey, G. G.; Brandt, W.; Lerouge, P.; Farrant, J. M.; Driouich, A. The response of the leaf cell wall to desiccation in the resurrection plant *Myrothamnus flabellifolia*. *Plant Physiol.* **2006**, *141* (2), 651–662.
(93) Hajduch, M.; Ganapathy, A.; Stein, J. W.; Thelen, J. J. A systematic proteomic study of seed filling in soybean. Establishment of high-resolution two-dimensional reference maps, expression profiles, and an interactive proteome database. *Plant Physiol.* **2005**, *137* (4), 1397–1419.

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