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Franscico Gasulla *et al.* (2013) 'The response ofAsterochloris erici(Ahmadjian) Skaloud et Peksa to desiccation: a proteomic approach', *Plant, cell and environment*, 36, pp. 1363–1378.

doi:10.1111/pce.12065

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The response of *Asterochloris erici* (Ahmadjian) Skaloud et Peksa to desiccation: a proteomic approach

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ABSTRACT

The study of desiccation tolerance of lichens, and of their chlorobionts in particular, has frequently focused on the antioxidant system that protects the cell against photo-oxidative stress during dehydration/rehydration cycles. In this study, we used proteomic and transcript analyses to assess the changes associated with desiccation in the isolated phycobiont *Asterochloris erici*. Algae were dried either slowly (5–6 h) or rapidly (<60 min), and rehydrated after 24 h in the desiccated state. To identify proteins that accumulated during the drying or rehydration processes, we employed two-dimensional (2D) difference gel electrophoresis (DIGE) coupled with individual protein identification using trypsin digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Proteomic analyses revealed that desiccation caused an increase in relative abundance of only 11–13 proteins, regardless of drying rate, involved in glycolysis, cellular protection, cytoskeleton, cell cycle, and targeting and degradation. Transcripts of five *Hsp90* and two *β-tubulin* genes accumulated primarily at the end of the dehydration process. In addition, transmission electron microscopy (TEM) images indicate that ultrastructural cell injuries, perhaps resulting from physical or mechanical stress rather than metabolic damage, were more intense after rapid dehydration. This occurred with no major change in the proteome. These results suggest that desiccation tolerance of *A. erici* is achieved by constitutive mechanisms.

Key-words: *Asterochloris*; desiccation tolerance; drying rate; Hsp90; lichen; protome; β-tubulin.

INTRODUCTION

Lichens are individualized symbiotic composites (holobionts) of photobionts (oxygenic photoautotrophs, such as cyanobacteria or green algae) that are morphologically and metabolically integrated with mycobionts (heterotrophic fungi) (Margulis & Barreno 2003). Lichens are among the large number of poikilohydric organisms, capable of surviving long periods in a desiccated state. This desiccated state is defined by attaining equilibrium with the water potential of the air, which is generally dry, and would be fatal for most vascular plants and organisms (Bewley 1979; Rundel 1988; Kranner 2002). The ability of cells to survive from the air-dry state is referred to as desiccation tolerance (Bewley 1979) or anhydrobiosis (Crowe, Heokstra & Crowe 1992). In practical terms, desiccation tolerance is defined as being able to survive drying to equilibrium with air at a relative humidity (RH) of 50% (to approximately −100 MPa), or a water content of between 0.5 and 0.3 g g dm^{-1} (Alpert & Oliver 2002).

Studies of drought and desiccation tolerance of lichens and their phycobionts have focused on the antioxidant system, and many of the antioxidant mechanisms that are commonly expressed in vascular plants have been analysed in lichens: enzyme responses, such as superoxide dismutase (SOD) (Deltoro *et al*. 1999; Weissman, Garty & Hochman 2005), peroxidases (POX) (Silberstein *et al*. 1996; Kranner *et al*. 2005), glutathione reductase (GRX) and ascorbate POX (Kranner 2002; Kranner *et al*. 2005); and low molecular weight substances such as glutathione, α-tocoferol, ascorbic acid (Calatayud *et al*. 1999; Kranner 2002), carotenoids, and specially the components of the xanthophyll cycle (Deltoro *et al*. 1998; Kranner *et al*. 2005; Fernández-Marín, Becerril & García-Plazaola 2010) or nitric oxide (Catalá *et al*. 2010) have all been investigated. However, in most lichens, it has not been possible to establish a clear relationship between the different levels of desiccation tolerance and antioxidant mechanisms. In some cases, antioxidant activities are even reduced after desiccation in isolated tolerant phycobionts (Kranner *et al*. 2005; Gasulla *et al*. 2009). Thus, antioxidant activity alone cannot fully explain levels of desiccation tolerance, and other protective and reparative mechanisms must be involved.

Plants, as sessile organisms, depend on proteomic plasticity to remodel themselves during periods of developmental change, to endure varying environmental conditions and to respond to biotic and abiotic stresses. When subjected to dehydration stress, plants exhibit a change in gene expression (either via transcription or translation or both) and metabolism. In order to identify genes that may be important in desiccation tolerance mechanisms, several studies based on RNA expression have examined changes that occur during dehydration and/or rehydration in the transcriptome of several resurrection plants: *Xerophyta humillis* (Collett *et al*. 2003, 2004), *Craterostigma plantagineum* (Bartels *et al*. 1990;

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Bockel, Salamini & Bartels 1998), *Sporobolus stapfianus* (Blomstedt *et al*. 1998; Neale *et al*. 2000), *Selaginella moelendorfii* (Weng, Tanurdzic & Chapple 2005; Iturriaga, Cushman & Chushman 2006), and in the desiccation-tolerant moss *Tortula ruralis* (Oliver *et al*. 2004, 2009). However, mRNA abundance may only represent a putative function because there is still a questionable correlation between mRNA and protein levels. Transcripts may not be translated, or changes in protein level or enzyme activity may occur without any detectable change in transcript abundance due to translational or other levels of control (Gygi *et al*. 1999). A further consideration in the case of certain resurrection plants and mosses is that mRNAs appear to be stored during drying and only translated during dehydration (Wood & Oliver 1999; Collett *et al*. 2003). By contrast, proteomics can reveal the actively translated portion of the genome that performs the enzymatic, regulatory and structural functions of the cell at a particular moment. Comparative proteomic investigation of plants before and after specific and interactive stresses provides information about which and how defensive mechanisms are activated. Proteomic studies carried out in resurrection plants have shown that during dehydration there may be an increase in reactive oxygen species (ROS) scavenging enzymes (Ingle *et al*. 2007; Jiang *et al*. 2007), chaperone proteins (Oliver *et al*. 2010), energy metabolism enzymes (Ingle *et al*. 2007; Jiang *et al*. 2007; Oliver *et al*. 2010), targeting and proteins involved in protein turnover (Ingle *et al*. 2007; Deeba *et al*. 2009), and proteins involved in signalling (Wang *et al*. 2009). In contrast, as the photosynthetic system is blocked during desiccation, there is generally a decrease in proteins related with photosynthetic activity to avoid ROS formation (Ingle *et al*. 2007; Deeba *et al*. 2009; Oliver *et al*. 2010).

Most free-living algae and cyanobacteria develop in aquatic or at least highly moistened terrestrial habitats, but they occur abundantly in habitats that are frequently dried as when symbiotized in lichen thalli (Nash III, 2008). Although lichen thalli cannot actively control physical water relations, some of their structures can partially isolate them from the surrounding dry air and decrease the rate of water loss (Rundel 1988; Sancho & Kappen 1989; Schroeter & Scheidegger 1995; Gauslaa & Solhaug 2004). In dry habitats, the phycobiont layer is generally excluded from the surface of the lichen thallus and is covered by a cortex structure. The cortex layer protects the algae from extreme dehydration rates when in the lichen symbiosis, and is thus able to colonize dry habitats that would perhaps be unavailable in free-living state.

Nevertheless, the isolated phycobiont *Asterochloris erici* (Ahmadjian) Skaloud et Peksa is able to survive rapid drying (less than 1 h), but the recovery is faster if the drying rate is slower, which suggests that, like other poikilohydric plants, desiccation tolerance in *A. erici* depends both on a constitutively expressed mechanism and other mechanisms that are induced during rehydration (Gasulla *et al*. 2009). Although isolated phycobionts are not afforded the synergistic benefits of their association with the fungal component of the lichen symbiosis, which can increase tolerance to dehydration-induced oxidative damage (Kranner *et al*. 2005) and photoinhibition (Kosugi *et al*. 2009), their study in isolation can still provide insights into the desiccation tolerance strategy employed by the alga. As far as we know, few studies have dealt with the regulation of the responses during dehydration and/or rehydration in lichen chlorobionts at a molecular level. Accordingly, we decided to carry out proteomic and gene expression analyses in the isolated phycobiont *A. erici* to gain insight into its desiccation tolerance mechanism.

MATERIALS AND METHODS

Biological material

An axenic strain of the lichen alga *A. erici* (formally *Trebouxia erici*, SAG 32.85 = UTEX 911; collection of algae, University of Texas at Austin, TX, USA) was used in this study. Stock cultures of the alga were maintained on 3 N Bold's Basal Medium (BBM3 N) supplemented with 10 g casein and 20 g glucose per litre (Ahmadjian 1973) at 20 °C under a 12 h photoperiod with 30 μmol $m^{-2} s^{-1}$ white-light illumination. Culture inoculation was achieved by placing 50 μL of the quantified cell suspension onto sterile cellulose-acetate discs placed on *Asterochloris* agar medium as described by Goldsmith, Thomas & Gries (1997). All experiments were performed 21 d after inoculation.

Desiccation and rehydration treatments

Slow drying (SD) was achieved by placing algal discs into a closed container over a saturated solution of ammonium nitrate (RH 66%), and maintained under the same environmental condition used in culture. Under this regime, samples reached a stable weight within 6 h. Rapid dehydration (RD) was achieved over silica gel (RH below 10%) under the same conditions and the air-dried state (stable weight) was reached within 1 h. Equilibration of the water potential of the algae to that of the atmosphere within the two chambers results in final algal cell water potentials of –53 MPa (RH 66%) and > –300 MPa (silica gel). The algae were kept dry for 24 h, frozen in liquid nitrogen and stored at –80 °C. Recovery after slow and rapid drying (RS and RR, respectively) was studied by complete rehydration of dried samples by the addition of a volume of distilled water equivalent to the water lost during drying. Fully rehydrated samples were kept under the same conditions used to culture the algae for 3 h before freezing in liquid nitrogen and storage at –80 °C.

Protein extraction and two-dimensional (2D) electrophoresis

Protein extracts were obtained from 100 mg of algae, ground to a fine powder in liquid nitrogen, according to the phenol-based method proposed by Hurkman & Tanaka (1986) and modified by Mooney & Thelen (2004). The protein pellet was air dried and resuspended in 100 μL of extraction media [0.1 M Tris-HCl, pH 8.8, 10 mM ethylenediaminetetraacetic

acid (EDTA), 0.4% (v/v) 2-β-mercaptoethanol, 0.9 M sucrose], and stored at –80 °C until isoelectric focusing (IEF). The protein concentration was determined using the Coomassie Brilliant Blue method (Bradford 1976) using bovine gamma globulin as a standard. An internal standard prepared by pooling equal amounts of the control and treated samples was used in the experimental design.

For difference gel electrophoresis (DIGE) analysis the sample preparation and gel electrophoresis were as described by Oliver *et al*. (2010). Protein (50 μg) of each sample was labelled with either Cy3 or Cy5 and the internal standard with Cy2 CyDye DIGE Fluor minimal dye following the manufacturer's recommended protocols (GE Healthcare, Piscataway, NJ, USA). The labelling experiment was independently repeated four times for each treatment alternating the Cy3 and Cy5 dye labels. Equal volumes of 2X sample buffer {8 M urea, 130 mM dithiothreitol (DTT), 4% (w/v) 3-[(3 Cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS]} were added to each of the individually labelled protein samples prior to mixing.

Individual images from the 2D gel separations of proteins labelled Cy2, Cy3 and Cy5 in each gel were obtained by scanning on an Ettan DIGE Imager (GE Healthcare) with 480/530 nm excitation/emission wavelengths for Cy2, 520/590 nm for Cy3, and 620/680 nm for Cy5. After imaging, gels were stained with silver following the protocol described by Shevchenko *et al*. (1996).

Image analysis was performed in an automated mode using DeCyder Differential Image Analysis Software (GE Healthcare). Spot patterns from different gels were matched using the internal standard sample present on every gel to allow comparison and statistical analysis of spot-volume ratios.

Spots were excised from the preparative gels, washed three times with Milli-Q water, and the silver was reduced with 50 mM sodium thiosulphate and 15 mM potassium ferricyanide. Gel pieces were equilibrated with a series of 100 mM ammonium bicarbonate, 50 mM ammonium bicarbonate/50% acetonitrile and 100% acetonitrile prior to dehydration overnight with acetonitrile. Proteins were digested overnight at 37 °C with 0.02 mg mL^{-1} trypsin [Promega-modified porcine L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated] in 50 mM ammonium bicarbonate. Peptides were extracted twice with 0.1% (v/v) formic acid (FA) in 60% acetonitrile. Extracts were combined and frozen at –80 °C and freeze dried. Dried peptides were reconstituted in 40 μL of 0.1% (v/v) FA, from which 18 μL was used for mass spectrometry analysis on an LTQ ProteomeX linear ion trap liquid chromatography-tandem mass spectrometry (LC-MS/MS) (ThermoFisher, San Jose, CA, USA) as described by Oliver *et al*. (2010).

Acquired MS/MS data were searched against a comprehensive database comprising the translated six reading frames of the available sequences for the following species: *Volvox carteri* (v.1) and *Chlamydomonas reinhardtii* (v.8), obtained from the Gene Index Project (http://compbio.dfci.harvard.edu/tgi/); and *Chlamydomonas incerta* (v.1), *C. reinhardtii* (v.2), *Dunaliella salina* (v.2), *Helicosporidium* sp. (v.2), *Polymella parva* (v.1), *Scenedesmus obliquus* (v.1), *Scherffleradubia* (v.2) and *Ulva linza* (v.2), obtained from the TIGR Plant Transcript Assemblies database (http://plantta.jcvi.org/). Protein sequences from *C. reinhardtii* and *V. carteri* obtained from the Phytozome 7.0 database (http://www.phytozome.net/) were concatenated to generate a comprehensive database containing 153 097 entries.

For false discovery rate determination, the rice database was randomized and concatenated to the forward database using 'DecoyDBCreator' available at http://www.p3db.org. The FASTA database utilities and indexer of the BioWorks 3.3.1 SP1 software allowed us to index the database against trypsin enzyme. Cysteine (carboxyamidomethylation; +57 Da) and methionine (oxidation; +16 Da) were selected as static and differential modifications, respectively. Acquired data were searched against the indexed database using the SEQUEST algorithm as part of the BioWorks 3.3.1 SP1 software suite. The search parameters for this database were set as follows: enzyme: trypsin; number of internal cleavage sites: 2; mass range: 200–2000; threshold: 1000; minimum ion count: 10; and peptide mass tolerance: 1 mg g^{-1}. Matching peptides were filtered for non-redundant and non-overlapping peptides, as well as correlation scores (X_{Corr}) at least 1.5, 2.0 and 2.5 for +1, +2 and +3 charged ions, respectively. Proteins were only considered if inferred from two distinct peptides. No decoy proteins were identified using these filtering criteria. For some protein spots, more than one protein met these assignment thresholds. All proteins meeting these assignment criteria are included and represented equally in discussions about differential expression.

In parallel to the SEQUEST-driven database search, sequence similarity searches were also performed. For that, acquired MS/MS spectra were *de novo* interpreted using the software PepNovo (Frank & Pevzner 2005). For each MS/MS spectrum, seven sequence candidates were proposed, all with score equal to 6.0 or above. Sequence candidates were searched separately against the NCBInr and the UniProtKB/Swiss-Prot databases using the MSBLAST tool (Shevchenko *et al*. 2001, 2003; Junqueira *et al*. 2008) available at http://genetics.bwh.harvard.edu/msblast/. Statistical significance of the obtained hits was evaluated according to the scoring scheme proposed by Habermann *et al*. (2004).

mRNA quantification of *β-tubulin* and *H*sp90 genes

RNA extraction was achieved by using QiaGen's Plant RNeasy Plant extraction kit according to the manufacturer's guidelines. RNA was quantified using a NanoDrop ND-1000TM spectrophotometer (Daemyung, Korea). Co-extracted DNA contaminants were degraded using the DNA Free RNA kit (Zymo Research, Irvine, CA, USA) and the complementary DNA (cDNA) was synthesized with the SuperScript^{TM} II RNase-H RT kit (Invitrogen, Carlsbad, CA, USA) using the supplied oligo-dT primer following the manufacturer's guidelines.

A. erici transcript sequences for both *β-tubulins* and *Hsp90 genes* for use in qRT-PCR quantification assays were isolated from the cDNA preparations using a combination of

degenerate primers and rapid amplification of cDNA ends (RACE) technology. The RACE products, which were biased for the 3′ ends of the available transcripts, were cloned and sequenced to determine the number of functional genes of each family and the 3′ end sequence of each gene.

Degenerate forward gene-specific primers (GSPs) were designed from conserved regions within consensus sequences generated from gene sequences for members of these two gene families in plants available from the National Center for Biotechnology Information (NCBI) GenBank (Supporting Information Table S1). RACE-PCR was performed using the GeneRacer™ kit (Invitrogen) as described by the manufacturer. In order to increase the specificity and sensitivity, nested PCR reactions were performed with the RACE products in all the possible combinations of the internal GSPs.

The 12 nested PCR products were ligated into the pCR4-TOPO plasmid vector and transformed into chemically competent TOP 10 *Escherichia coli* cells (TOPO TA Cloning kit; Invitrogen). Isolation of plasmid DNA was performed using the standard following the alkaline lysis method as described by Sambrook *et al*. (1989). Cloned fragments in the pCR4-TOPO plasmid were sequenced, using M13 sense and antisense primers (Invitrogen), using the BigDye® Terminator v.3.1 cycle sequencing kit. DNA sequences were assembled into contigs and checked for sequence similarities to *β-tubulins* and *Hsp90* genes using the Basic Local Alignment Search Tool (BLAST, NCBI, NIH; http://www.ncbi.nlm.nih.gov/BLAST) score system.

To analyse gene expression, RNA was extracted from algal cultures at each hour during SD and during recovery. cDNA was synthesized with random primers using the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) following the manufacturer's guidelines. Specific primers were designed for each gene (Supporting Information Table S2) for transcript quantification by a real-time PCR (qRT-PCR). PCR amplification was accomplished in a 10 μL total volume containing 1 μL of 10× diluted cDNA, 0.5 μM each primer, and 5 μL of 2× FluoCycle™ SYBR-Green (Euroclone, Pero, Italy) hot-start Taq ready mix using an ABI PRISM® 7700 instrument (Applied Biosystems, Carlsbad, CA, USA). Absence of non-specific PCR products and primer dimmers was verified by dissociation curves and by agarose gel electrophoresis. Transcript quantifications were normalized to the level of 18S rRNA, made possible by the use of random primer-directed cDNA synthesis, using primers specific to the 18S rRNA for *A. erici* (AB080310).

Statistical analysis

Four protein extractions and CyDye labelling were analysed for each dehydration/rehydration treatment. One-way analysis of variance (ANOVA) in combination with Fisher's least significant difference (LSD) procedure was performed with the standardized log abundance ratios (R_i) to identify significantly differential expression. Principal component analysis (PCA) was performed with the R_i data for those spots for which significant differences were determined.

Electron microscopy

Approximately 20 mg of hydrated and rehydrated samples, both slowly and rapidly dried, were fixed in 2% (v/v) glutaraldehyde and 2% (v/v) formaldehyde for 4 h at 4 °C. The specimens were washed with 0.01 M phosphate buffered saline (PBS), pH 7.4, and fixed with 2% (w/v) OsO4 for 2 h at 4 °C. Thereafter, samples were washed in 0.01 M PBS, pH 7.4, and dehydrated at room temperature by passage through a graded ethanol series. Fixed and dehydrated samples were washed in propylene oxide prior to sequential infiltration by rotation in propylene oxide:Spurr resin (2:1 v/v), propylene oxide:Spurr resin (1:1), and propylene oxide:Spurr resin (1:2) for 24 h. Samples were embedded in 100% Spurr epoxy resin (Taab, Aldermaston, UK) polymerized at 65 °C for 2 d.

For transmission electron microscopy (TEM), 90 nm sections were cut with a diamond knife (DIATOME Ultra 45°; Diatome, Hatfield, PA, USA) using an ultramicrotome (Ultratome Nova, LKB, Bromma, Sweden), mounted on copper 100 mesh grids, and post-stained with 2% (w/v) aqueous uranyl acetate and 2% (w/v) lead citrate. The prepared sections were examined with a JEOL JEM-1010 (80 kV – Joel Korea, Seoul, South Korea) electron microscope, equipped with a MegaView III Olympus digital camera 1 and 'AnalySIS' image acquisition software.

Similar cellular organization disruption was observed in desiccated (data not shown) and rehydrated samples, which infers that a partial rehydration of the desiccated samples may have occurred during the fixation process. To avoid the complication of this possible artefact we only present the results for cells fixed after rehydration. The ultrastructural changes observed in the rehydrated samples accurately reflect the extent of cellular disruption that occurred during the previous drying step.

RESULTS

2D electrophoretic analysis of total proteins

To investigate the changes in protein profiles during dehydration and recovery, and to elucidate the timing of the response, we applied two different drying rates to cultures of *A. erici*. When the rapid drying method was employed (equilibration with dry air over silica gel), approximately 10% of the original fresh weight was achieved in around 60 min. Using the SD method, equilibration with the dry air (67% RH) was achieved in approximately 300 min. We conducted a 2D-DIGE analysis of the total proteins from 4 replicates per treatment to attain a sufficient level of reproducibility. A representative gel is shown in Supporting Information Fig. S1. More than 2000 protein spots were detected by DeCyder 2D 6.3 Software, and more than 500 were confirmed manually. Quantitative image analysis revealed a total of 62 protein spots with significantly altered intensities ($Pv < 0.05$) in at least one of the four treatments (SD, RD, RS, RR) compared with the control (Table 1). A total of 11 proteins increase in abundance and 51 decline in abundance in response to SD, the same number of proteins increase and

Table 1. Changes in protein abundance with respect to the hydrated control: s^C, s^{SD}, s^{RD}, s^{RS} and s^{RR} are statistical groupings for the mean standard log abundance ratio (R_i) value from the control, slow-dried, rapid-dried, rehydrated slow-dried, and rehydrated rapid-dried samples, respectively

Spot	s^C	SD	s^{SD}	RD	s^{RD}	RS	s^{RS}	RR	s^{RR}
239	a	0.77	b	1.50	b	0.96	b	0.98	B
300	a	3.36	b	3.46	b	2.65	b	2.81	B
307	a	4.48	c	1.08	bc	3.96	ab	5.11	C
308	a	4.84	b	3.05	b	4.80	ab	5.59	B
364	a	3.33	b	1.86	b	4.10	ab	3.77	B
369	ab	0.49	ab	–2.42	b	2.68	a	2.67	B
458	a	4.97	b	3.51	b	3.78	b	2.21	Ab
462	a	6.71	b	5.52	b	6.46	b	5.98	B
463	a	4.88	b	4.94	b	4.87	b	5.04	B
523	b	–4.99	a	–1.61	a	–4.67	b	–1.76	b
544	b	–1.93	a	–1.18	a	–2.26	a	–1.98	ab
570	c	–0.62	abc	–0.22	a	–1.00	bc	–0.83	ab
662	b	–1.06	a	–0.90	ab	–0.27	a	–0.82	ab
682	b	–1.32	a	–1.08	ab	–0.02	a	–0.42	a
721	b	–2.54	a	0.11	a	–2.56	b	–2.67	a
860	a	–0.12	a	0.88	a	–0.61	b	–0.56	a
887	a	2.72	b	3.24	b	2.33	b	2.28	b
891	a	3.03	b	3.15	b	2.91	b	2.69	b
1021	b	–4.25	a	–2.66	b	0.68	a	0.06	b
1137	c	–1.29	abc	–0.34	ab	–2.26	bc	–3.17	a
1138	b	–1.55	a	–0.70	a	–2.04	b	–1.99	a
1245	b	–1.39	b	–0.73	b	–1.63	ab	–1.81	b
1268	c	–1.23	ab	–0.23	a	–1.58	bc	–1.25	ab
1274	b	–1.19	b	–0.71	b	–1.12	ab	–1.01	b
1301	b	–1.33	a	–1.35	a	–0.99	a	–1.06	a
1314	b	–1.02	a	–1.10	a	–1.22	a	–1.06	a
1353	b	–1.54	a	–1.64	a	–1.33	a	–1.53	a
1360	bc	–2.40	ab	1.61	a	–3.02	c	–0.22	abc
1376	b	–2.47	a	–4.30	a	–2.80	a	–3.05	a
1383	b	–0.59	ab	–1.27	a	–1.27	a	–1.41	a
1429	b	–1.59	ab	–2.42	b	–0.29	ab	–3.90	a
1447	c	–0.52	bc	–3.32	bc	–0.59	ab	–5.38	a
1450	c	–1.20	ab	–0.64	ab	–1.26	bc	–1.77	a
1451	b	–1.39	a	–0.95	a	–1.69	ab	–1.81	a
1455	c	–1.72	bc	–1.89	b	–2.79	bc	–5.22	a
1462	c	–0.97	ab	–0.43	ab	–1.20	bc	–1.36	a
1464	d	–5.11	ab	–0.86	a	–6.65	cd	–2.97	bc
1470	c	–1.68	bc	–0.95	a	–4.40	c	–3.71	ab
1471	c	–1.45	abc	–1.11	ab	–1.56	bc	–2.63	a
1472	b	–2.80	a	–4.47	a	–2.61	a	–2.62	a
1479	b	–0.04	b	–0.19	a	–4.36	b	–1.58	b
1536	b	–4.32	a	–3.34	a	–2.77	a	–3.68	a
1554	b	–2.71	a	–2.70	ab	–0.97	a	–0.76	ab
1580	b	–1.41	a	–1.44	a	–1.26	a	–1.48	a
1604	b	–0.82	a	–0.23	a	–0.81	ab	–0.81	a
1689	b	0.45	b	–2.91	b	–0.15	a	–0.13	b
1692	b	–0.96	ab	–1.19	a	–1.40	a	–1.84	a
1693	b	–2.43	ab	–0.99	a	–4.55	b	–4.14	a
1694	ab	1.08	b	1.23	a	–1.85	b	–1.60	a
1695	c	–2.41	ab	–1.82	ab	–2.84	b	–3.55	a
1699	c	–3.68	a	–3.27	a	–3.73	ab	–2.49	b
1729	a	0.07	a	–1.00	a	–0.01	a	1.28	b
1744	b	–1.18	ab	–1.76	a	–7.06	ab	–5.26	a
1755	b	–3.78	a	–2.43	a	–2.65	a	–1.29	ab
1796	b	–4.69	a	–2.52	a	–5.02	ab	–4.17	a
1799	b	–2.75	a	–2.31	a	–3.07	a	–2.10	a
1826	c	–0.90	ab	–1.41	bc	–0.53	a	–0.27	bc
1841	b	–3.96	a	–1.89	a	–4.38	ab	–5.32	a
1854	c	–4.69	a	–0.88	ab	–3.77	bc	–3.51	ab
1857	b	–4.72	a	0.27	a	–3.50	b	–0.41	b
1931	b	–4.22	a	–0.49	a	–3.31	b	–3.71	a
1950	c	–1.45	bc	–1.97	ab	–3.26	bc	–4.92	a

Letters indicate significant differences in the means using Fisher's least significant difference (LSD). The SD, RD, RS and RR columns report the $\log^2$ fold change in abundance from the control as the ratio of the exponentials of the R_i means of spots that had significantly different expression in at least one treatment by analysis of variance (ANOVA) ($Pv < 0.05$). $n = 4$.
SD, slow drying; RD, rapid drying; RS, recovery after slow drying; RR, recovery after rapid drying.

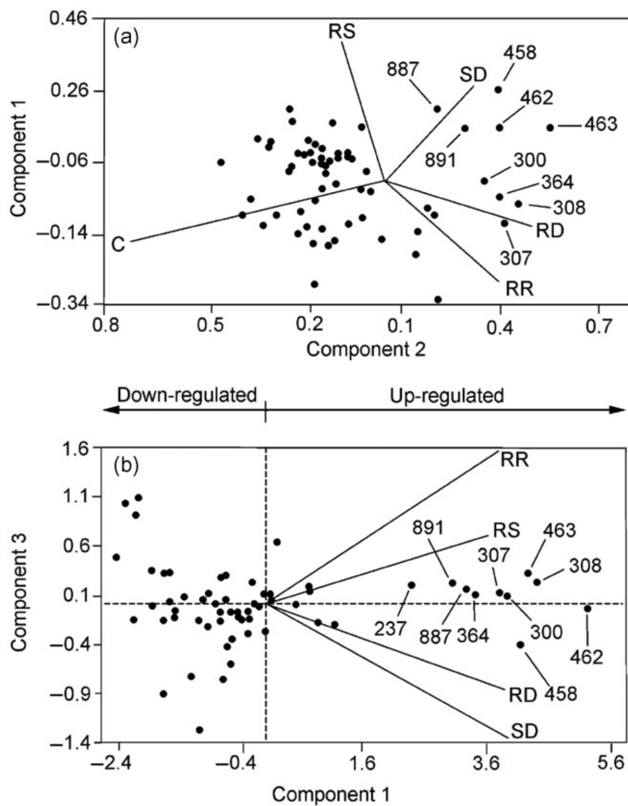

Figure 1. (a) Principal component analysis (PCA) with the standard log abundant ratio (R_i) of the spots that had significant different expression in at least one treatment ($Pv < 0.05$). C, control; SD, slow drying; RS, recovery after slow drying; RD, rapid drying; RR, recovery after rapid drying. (b) PCA analysis with treated R_i with respect to control R_i values. Positive and negative values indicate up-regulation and down-regulation, respectively. Annotated spots numbered in (b) correspond to highly up-regulated proteins.

decline in abundance during rapid drying, however not exactly the same proteins. Spot 369 declines during rapid drying but increases during SD, and spot 721 that declines during SD exhibits a slight increase during rapid desiccation. Upon rehydration of both slow and rapidly dried algae, an identical group of 12 proteins increase in abundance in both treatments but an additional protein, spot 1729, accumulates above control levels in the samples that were rapidly dried and rehydrated. The group of 12 proteins that increase in abundance upon rehydration include all of the proteins that also increase in abundance during drying with the exception of spot 1021, which is reduced by desiccation, and 1729 that only increases in abundance in response to rehydration following rapid desiccation. Figure 1a represents a PCA performed with the R_i values of these spots. The first component of the PCA is not represented in the graphic because, although it accounted the 65.42% of the total variance in data set, this variable can be simply explained by the differences among the relative abundance of the proteins in the five treatments. On the contrary, the second and third principal components, which accounted the 22.50 and 6.89% of the total variance, respectively, better represent the changes between the different proteomes after each treatment. In this way, the PCA depicts the eigenvectors of the control and the dehydrated/rehydrated samples in opposite directions, delineating a clear difference between the proteome of the non-dehydrated samples and those that were dehydrated or dehydrated and rehydrated. The spots with a negative value indicate those proteins that are more abundant in control samples, whereas a positive value indicates a higher expression after dehydration. Those spots that accumulate after dehydration formed two groups: one with the proteins that were more abundant after a slow dehydration: spots 458, 462, 887 and 891; and the other group with proteins that were more abundant after a rapid drying: spots 300, 307, 308 and 364. Only the treatment variable RS, rehydrated after SD, had a negative value in the second principal component similar to that of the control, to indicate that the slowly dried samples had begun to recover the initial hydrated (control) proteome, but this was not completed within 3 h of rehydration as the RS and C groups were still separate in the PCA. Figure 1b depicts the results of a PCA performed with the R_i values of the treated samples computed with respect to those of the non-dehydrated samples. In this diagram, the first component absorbs the 86.98% of the total variance, and allows easy differentiation of those proteins that either accumulated or decreased in abundance in the treated samples with respect to the controls. The majority of the 62 protein spots had a negative value on the component 1 axis, indicating a down-regulation of the protein abundance. Only 19 proteins had a positive value for component 1 and among these, the 10 most up-regulated proteins formed a defined group in a region of the PCA. The surprisingly homogeneity of this change in the proteome suggests that these proteins may play a central and perhaps co-ordinate role in the response to dehydration and may help to elucidate the mechanism of desiccation tolerance in *A. erici*.

Differentially expressed proteins were excised from the gel to be analysed by LS-MS/MS (Supporting Information Fig. S1). The results of the LS-MS/MS analysis and subsequent bioinformatic inquiries to generate putative annotations for the proteins represented by the sequenced tryptic peptide fragments are presented in Table 2. Three sequential inquiries were completed in order to generate the most robust annotations possible. Of the selected proteins, peptides from only 16 of these could be identified with acceptable MSBLAST scores. Of those that increase in abundance in all of the samples regardless of the drying rate (spots 239, 300, 307, 308, 364, 458, 462, 463, 887, 891), both as a result of dehydration and either retained or elevated further upon rehydration, all of the 10 could be annotated as proteins with known functions. These proteins were actin, enolase, Hsp90 (three spots), beta-tubulin (two spots), beta-subunit of ATP synthase, a cell division cycle protein 48, and a protease. Half of the highly up-regulated proteins spots could be attributed to just two classes of protein families: the beta-tubulins (spots 887 and 891) and Hsp90 proteins (spots 458, 462, 463). The DIGE-generated data for spots 458 and 887 are presented as examples of these analyses in Fig. 2. In general, the

Table 2. Proteins of interest identified from a 450 μg system preparative gel using BioWorks Rev 3.3 software and the MASCOT system

	SEQUEST			*de novo* + Error TolBlast versus SWISSPROT			*de novo* + Error TolBlast versus nrNCBI		
Spot	Protein description	P	XC-δCn	Protein description	MSBLAST	HSP-P	Protein description	MSBLAST	HSP-P
239	NA			Actin	155	2	Actin	155	2
300	NA			NA			Hypothetical protein	63	1
307	NA			Enolase	82	1	Enolase	82	1
							Presequence protease	69	1
308	NA			NA			Presequence protease	69	1
364*				Cell division cell cycle protein 48	159	1			
458	Similar to Hsp90-alpha	2	2.99-0.38	Heat shock cognate protein 80	534	8	Hsp90A	349	5
462	Similar to Hsp90-alpha	2	2.57-0.26	Hsp 80	871	13	Hsp90-2	863	13
463	Similar to Hsp90-alpha	2	2.70-0.42	Heat shock cognate protein 80	531	8	Hsp90-2-like	531	8
523	NA			Elongation factor-2	275	4	Elongation factor-2	397	6
860	NA			NA			Hypothetical protein	70	1
887	Tubulin beta chain	6	3.40-0.30	Tubulin	374	5	Beta-tubulin	286	4
	Tubulin beta chain	7	4.38-0.39	Tubulin beta chain	575	8	Beta-tubulin	287	4
891	ATP synthase subunit beta	2	3.84-0.43	ATP synthase subunit beta-2	354	5	ATP synthase subunit beta-2	284	4
1138	NA			NA			Peptidyl-prolyl cis-trans isomerase	80	1
1274	Plastidic aldolase	2	4.48-0.62	NA			Fructose-1,6-bisphosphate aldolase	296	5
1353	NA			Sedoheptulose-1,7-bisphosphatase	67	1	Sedoheptulose-1,7-bisphosphatase	67	1
1450	NA			L-ascorbate peroxidase	75	1	Fructose-1,6-bisphosphate aldolase	116	2

Sequence candidates were searched separately against the SEQUEST, the UniProtKB/Swiss-Prot and NCBInr databases. Statistically significant identifications require minimum MSBLAST scores of 64 (1 HSP), 104 (2 HSP) and 134 (3 HSP) according to Habermann *et al*. (2004).
364*: This protein was identified through the ProteoRed platform, a service of the Carlos III Networked Proteomics Platform of the Ministry of Science and Innovation of the Government of Spain (http://www.proteored.org/). XC-δCn Cross Correlation Score from SEQUEST. NA, not applicable; P, numbers of peptides; HSP-P, high-scoring segment pair – peptides (Habermann *et al*. 2004).

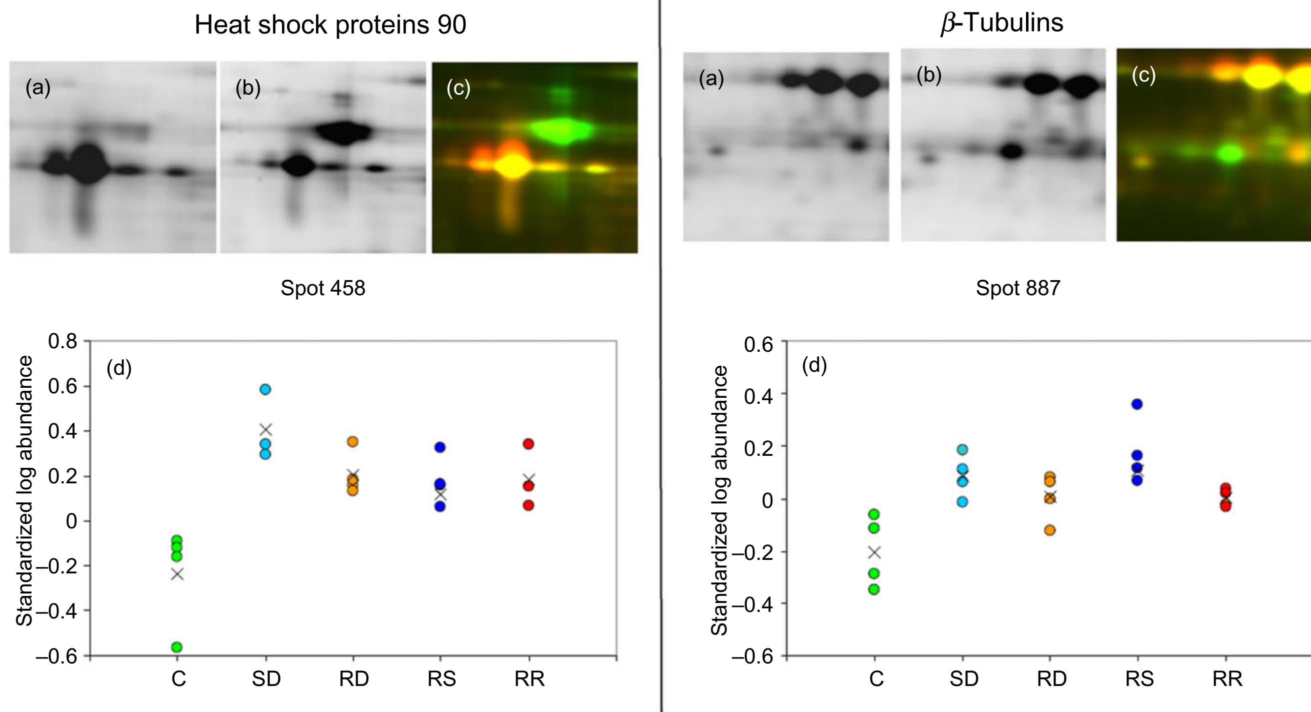

Figure 2. Example of a two-dimensional difference gel electrophoresis (2D-DIGE) gel image of heat shock protein 90 and β-tubulin proteins: (a) Cy5 image, control. (b) Cy3 image, recovery after rapid drying. (c) Two-colour-merged 2D-DIGE gel image, the green spots show that the protein expression during the recovery after rapid drying was greater than that of non-dehydrated samples; the yellow spots show no differences. (d) Standard log abundance of each sample used in each treatment, × symbol represents the average value: C, control; SD, slow drying; RS, recovery after slow drying; RD, rapid drying; RR, recovery after rapid drying.

abundance of these proteins was greater in the slow-dried samples (SD) than in the rapid-dried (RD) samples. Upon rehydration, the abundance in samples that were slow dried (RS) tends to decrease in all cases but increase in abundance if the initial drying event was rapid (RR) (Table 1).

Of those annotated proteins that decrease in abundance in response to dehydration (spots 523, 860, 1138, 1274, 1353, 1450), the majority were diminished more by SD than by rapid drying. However, following rehydration these proteins either exhibited a further small decrease in abundance or remained depressed at approximately the same level of abundance in the slow-dried samples. Rehydration of the rapid-dried samples resulted in a continued significant decrease in abundance of the majority of these proteins.

mRNA quantification

Cloning of the products of the nested and RACE-PCR allowed us to find five functional forms of the *Hsp90* gene and two of the *β-tubulin* gene.

To investigate the changes of gene expression at the mRNA level during a slow dehydration and rehydration, we performed qRT-PCR analysis (Fig. 3). All the genes analysed exhibited a similar pattern: an initial decrease in transcript abundance in the first hour of the drying treatment with respect to the control transcript accumulation levels (value 1 in Fig. 3), followed by a significant elevation in abundance as the drying process progresses. The maximum abundance for all genes occurs when the samples are completely dry to approximately 1.5- to 4-fold greater than the control level. Upon rehydration, the transcript levels for all genes decreased rapidly to levels below that seen for the hydrated control before a gradual recovery to normal levels.

Electron microscopy

Non-dehydrated *A. erici* cells exhibited a large, central and lobate chloroplast (Fig. 4a1), and as in most organisms of the chlorophytes lineage the thylakoid structure remains quite simple (Fig. 4a2). *A. erici* thylakoid membranes were arranged in a parallel fashion with small starch grains situated among them (Fig. 4a2). The nucleus and mitochondria have a peripheral location (Fig. 4a3) and there are numerous 0.1–2 μm diameter lipid bodies and vesicular or multivesicular (lysosome-like) bodies (Fig. 4a1). The pyrenoid matrix was usually absent or not readily clearly recognizable and pyrenoglobules were not observed.

Slow dehydration resulted in some structural changes that can be seen in the rehydrated samples. The most visible of which is an increase in the number of lipid bodies, and of a smaller size, compared with hydrated cells (compare Fig. 4a1 and 4b1). The quantity of starch deposits located within the thylakoids also increased along with the number and size of electrodense deposits (Fig. 4b1 and 4b3). Thylakoids were

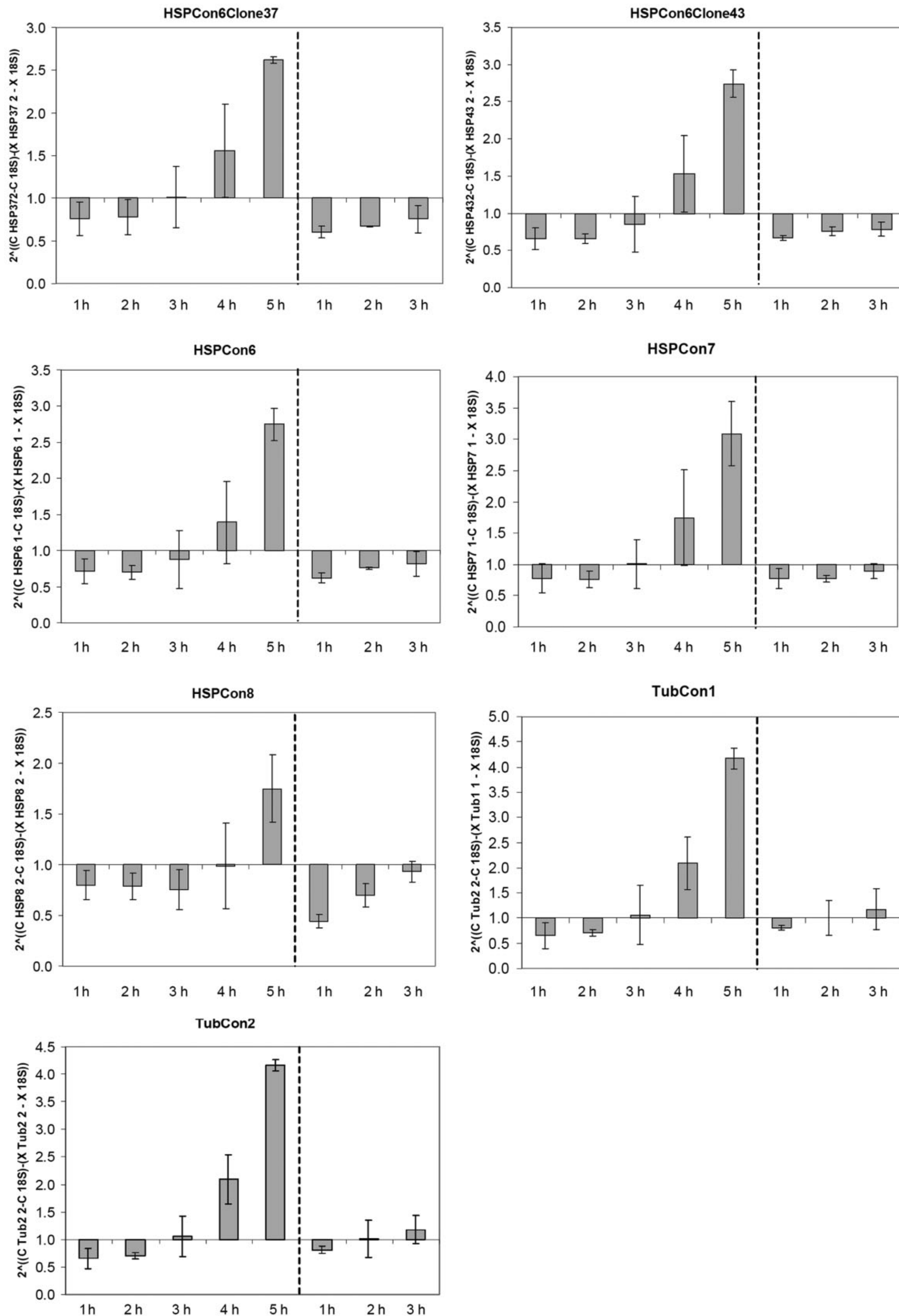

Figure 3. *Hsp90* and *β-tubulin* gene expression analysis by RT-qPCR during dehydration (h, hours), and during recovery after rehydration (dotted lines). Quantitative real-time PCR was performed using gene-specific primers (Table 2) and SYBR-Green Real-time Master Mix. The relative gene expression was evaluated using the comparative cycle threshold method with 18S rRNA as the reference transcript. Control value = 1, $n = 3$.

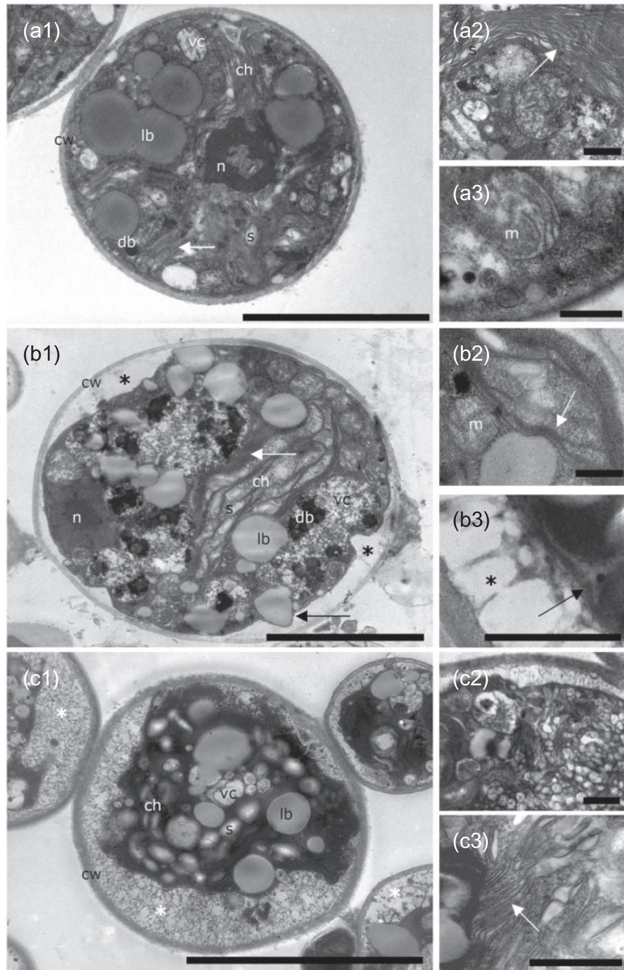

Figure 4. Transmission electron microscopy images of *Asterochloris erici* cells: (a) non-dehydrated, (b) rehydrated for 3 h after a slow dehydration, (c) rehydrated for 3 h after a rapid dehydration. Abbreviations: ch, chloroplast; cw, cell wall, db, dark body; lb, lipid body; m, mitochondria; n, nucleus; s, starch; vc, vesicular complex; black star, plasmolysis; white star, cytorrhysis; black arrow, intact plasma membrane; white arrow, parallel thylakoid organization. Bar in a1, b1 and c1: 5 μm. Bar in a2, a3, b2, b3, c2 and c3: 0.5 μm.

more packed but the parallel organization of the membranes was maintained (Fig. 4b2). The most suggestive sign of injury in slow-dried cells (following rehydration) was a slight retraction of the plasma membrane from the cell wall in several dispersed sites (black star, Fig. 4b1 and 4b3). However, in general, the integrity of the plasma membrane, and membranes from all other organelles, was apparently intact and most of the cells did not show cytoplasm leaking (cytorrhysis) (Figs 4b1 & 4b3).

Rapid-dried cells following rehydration clearly exhibited degenerate ultrastructure, indicative of serious injury (Fig. 4c1). The cytoplasm was highly vacuolated and filled with small lipid bodies (compare Fig. 4b and 4c). The cytoplasm and the chloroplast were shrunken and appeared severely disrupted. Thylakoids were swollen by large and numerous starch deposits and most of the thylakoid membranes were apparently fused or tightly stacked (Fig. 4c), although it was possible to find some non-fused thylakoid membranes parallel organized (detail in Fig. 4c3). Unlike slowly dried cells, the majority of the rapid-dried cells exhibited (Fig. 4c1) extensive plasmolysis and cytorrhysis. As a consequence, it was difficult to find other cellular organelles, such as mitochondria or nucleus, in rapid-dried cells. Nevertheless, *A. erici* recovered and survived this treatment.

DISCUSSION

Changes in proteome during dehydration and rehydration

The analysis of protein expression changes to understand mechanisms of desiccation tolerance in plants is not new (Eickmeier 1982), but recent advances in protein identification strategies using high-throughput technologies have resulted in a renewed interest in this area (Cushman & Oliver 2011). Most of these studies have focused on resurrection plants, the desiccation-tolerant angiosperms, with only minimal interest in the less complex plants such as ferns, lycophytes and mosses, and so an evolutionary perspective is at best extremely limited. Until now, there have been no reported investigations that have included desiccation-tolerant algae, a key taxon for an evolutionary perspective. In choosing *A. erici*, a desiccation-tolerant phycobiont, this study was conducted in order to gain an insight into what processes and cellular activities are important in the response to desiccation and rehydration in a plant that may reflect a more primitive and basal mechanism in the evolution of desiccation tolerance within the land plants. Although there is not enough information or studies for realistic comparisons that might shed light on which responses to desiccation and rehydration might be adaptive across species and taxa, there are some patterns emerging from those proteomic-based investigations that have been accomplished.

In *A. erici*, this study has documented that significant alterations in a relatively small percentage of total proteins observable using 2D gel electrophoresis, approximately 3% (62 of approximately 2000) or 12.4% of those that can be manually confirmed, are differentially expressed during desiccation and rehydration as measured by changes in abundance. The majority of differentially expressed proteins, almost 80%, appear to decrease in abundance in response to desiccation and fail to regain control levels within the 3 h rehydration period employed in this study (which was not designed to sample fully recovered algae). The remainder, 11 proteins (12–13 upon rehydration) or approximately 18%, increase in response to desiccation and also fail to regain control levels of abundance after 3 h of rehydration. The relative abundance data suggest that in general SD allows for more accumulation of those proteins that respond positively to desiccation than does rapid desiccation, but there is too great a spot-to-spot variation in the comparative accumulation patterns among those proteins that decrease in abundance to suggest any drying rate-related effects on protein stability. The effect of rehydration on protein abundance

levels is again too varied for any general conclusions to be drawn. To date, this is the only study in which a comparison has been made with regard to drying rate and the effect on protein accumulation.

Drying rates have long been documented to have profound effects on protein synthesis in desiccation-tolerant plants and most extensively described for desiccation-tolerant bryophytes (Bewley 1979; Alpert & Oliver 2002). In *T. ruralis*, a desiccation-tolerant moss, protein synthesis declines almost immediately during dehydration, whether slow (within 6 h) or rapid (within 1 h), and ceases within minutes if dehydration is rapid (see Bewley 1979 for a review). During rapid desiccation, the rate of water loss from the *T. ruralis* cells is such that polysomes are trapped on the transcripts while still in the act of nascent polypeptide synthesis. Using radiolabelling techniques, Oliver (1991) was able to demonstrate that drying did reduce the capability of *T. ruralis* to direct the synthesis of certain proteins (hydrins) when the plants were rehydrated, indicating that desiccation induced a reduction in the transcripts that encode them. However, the synthesis of proteins during dehydration was not reported and addition of labelled amino acids 1 min prior to dehydration, either rapid or slow, did not result in detectable incorporation into the protein fraction (M. J. Oliver, unpublished data) presumably because protein synthesis was severely inhibited by water loss from the cells. The rates of drying used in the aforementioned bryophyte studies are identical to those used to dry *A. erici*, and so it is reasonable to assume that the increase in abundance of proteins during drying in this species is primarily the product of an increase in the stability of individual proteins rather than their *de novo* synthesis, certainly with regard to the rapidly dried samples. This possibility is also supported by the demonstration that the transcripts of two of the protein families identified in the proteomic analysis as increasing in abundance only exhibit significant accumulation to levels over that of the hydrated controls late in the drying process (4–6 h) when actual protein synthesis is unlikely. The increase in abundance of transcripts during dehydration when protein synthesis is negligible is also observed for *T. ruralis* where it was demonstrated that select transcripts, presumably required during rehydration, are stabilized by sequestration in messenger ribonucleoprotein complexes (mRNPs) during SD (Wood & Oliver 1999). The increase in transcript abundance is thus the result of stabilization and not *de novo* synthesis in this moss. The evidence suggests that this might also be the case for *A. erici*. Both selective sequestration of transcripts combined with reduced protein turnover, presumably also selective, could explain the observed increase in abundance of certain proteins during drying and their continued elevation in synthesis upon rehydration. At this juncture, further work is required, especially regarding the ability of the algae to actually synthesize proteins during the SD process (it is relatively certain that rapid drying is too fast to allow for any significant protein synthesis to occur), before this hypothesis can be strengthened and conclusions are drawn.

Protein expression studies in the more complex plants, including the resurrection fern *Polypodium virginianum* (Reynolds & Bewley 1993), the lycophytes *Selaginella lepidophylla* (Eickmeier 1982) and *Selaginella bryopteris* (Deeba *et al*. 2009), and the angiosperms (resurrection plants) *Craterostigma plantagineum* (Bernacchia, Salamini & Bartels 1996; Alamillo & Bartels 2001; Röhrig *et al*. 2008), *Haberlea rhodopensis* (Georgieva, Röding & Büchel 2009), *Boea hygrometrica* (Jiang *et al*. 2007), *Xerophyta viscosa* (Ingle *et al*. 2007), *Sporobolus stapfianus* (Kuang *et al*. 1995; Oliver *et al*. 2011), and *Sporobolus elongatus* (Ghasempour & Kianian 2007), also report changes in the accumulation of proteins in response to desiccation and/or rehydration. However, in all of these cases drying rates are by necessity much slower, ranging from 12 to 24 h for the lycophytes and fern to several days in the angiosperms. All of these desiccation-tolerant plant species cannot survive rapid desiccation and several actively retard water losses in order to slow down the drying process, as has been demonstrated for *S. stapfianus* (Oliver *et al*. 2011). In these plants, it is much more likely that increases in abundance for specific proteins are the result of increased transcription and subsequent translation while cellular water levels are sufficiently high to support protein synthesis even as the plant as a whole is dehydrating.

The decrease in the abundance of a protein during desiccation and/or rehydration is likely the result of a decrease in *de novo* synthesis coupled to increased or targeted turnover rates. Protein degradation during desiccation, and also upon rehydration, is thought to involve both the ubiquitination of targeted proteins and recruitment into proteasomes (O'Mahony & Oliver 1999; Chen & Wood 2003) and autophagy via the endosome pathway (Chakrabortee *et al*. 2007).

Historically, there has been a strong focus on understanding the relationship between lichen thalli hydration and photosynthesis and respiration. In most terrestrial habitats, lichens undergo frequent drying and wetting cycles produced by nocturnal dewfall or fog (Lange 1970; Kershaw 1985; Lange *et al*. 2006; del Prado & Sancho 2007). Although rehydration/desiccation occurs reasonably slowly during these daily cycles, over hours rather than minutes, lichens can also experience natural (relative) rapid rehydration/desiccation cycles as seen during a summer storm. The results obtained in the proteomic analysis suggest that desiccation tolerance of photobionts is primarily constitutive. The permanent protection of the photosynthetic machinery reduces the time of repair and recovery to a minimum and enables the photobiont to fix CO_2 at high rates during the periods when thalli are hydrated, even when such times are short. A rapid recovery of photosynthesis after rehydration is critical to reach a net positive carbon gain within the lichen symbiosis because the photobionts have to share the photosynthetically derived products with the mycobiont partner.

Differentially regulated proteins

During dehydration and rehydration over 40 proteins were observed to decrease in abundance. Degradation of proteins and an increase in proteolytic enzymes are a common plant

response to dehydration (Ingle *et al.* 2007; Deeba *et al.* 2009) or other environmental stresses (Yan *et al.* 2006). It is possible that induction of proteolytic enzymes may play a role in the reallocation of resources for biosynthesis of novel proteins involved in dehydration tolerance mechanisms, such as an increase in nitrogenous osmolytes as seen in *S. stapfianus* (Oliver *et al.* 2011); however, in this instance, where dehydration occurs relatively fast (even though slow for alga), this seems unlikely as the osmolytes would be produced when cellular dehydration has significantly progressed. The loss of proteins during dehydration is more likely a reflection of the sensitivity of the protein to the rigors of dehydration and the inactivation of metabolic pathways during dehydration. An example of the latter would be proteins involved in photosynthesis which, if left in place, could render the plant susceptible to photo-oxidative damage in high light when in the dried state (Ingle *et al.* 2007; Deeba *et al.* 2009; Wang *et al.* 2009). Although we were unable to identify proteins involved in the light reactions of photosynthesis, it is of note that the proteins we did identify that decreased in abundance during drying were associated with the Calvin cycle, indicating that the carbon fixation aspects of photosynthesis are also degraded during dehydration. However, *A. erici* is able to maintain photosynthesis at below 10% relative water content (RWC) under experimental conditions (Gasulla *et al.* 2009), so presumably the loss of certain proteins does not preclude the possibility that carbon fixation could occur during drying.

Those identified proteins that accumulated during dehydration and rehydration, either by a reduction in the turnover rate (during drying) or by *de novo* synthesis (upon rehydration), in the alga offer insights into what processes and cellular functions are important in the response to desiccation and rehydration. In this study, we were able to identify 10 such proteins.

The protein spot 364, identified as cell division cycle 48 protein (AtCdcd48 in *Arabidopsis thaliana*, Cdc48 in yeast, CDC-48 in *Caenorhabditis elegans*, p97 in mammal), is a highly abundant type II AAA-ATPase (Peters, Walsh & Franke 1990) involved in cell cycle control (Moir *et al.* 1982) and cell proliferation (Egerton & Samelson 1994). CDC48/97 is essential for cytokinesis, cell expansion and cellular differentiation in plants (Park, Rancour & Bednarek 2008). At the molecular level, CDC48/p97 is involved in many different cellular processes and its activity is modulated by alternative adaptor proteins that determine recruitment and processing of specific substrates (Kondo *et al.* 1997; Yuang *et al.* 2001). CDC48/p97 is also a central factor for mobilizing and targeting ubiquitylated substrates to the 26S proteasome when it forms a complex with the cofactors Ufd and Npl4 (Meusser *et al.* 2005). This latter function would, perhaps along with the protease indicated for spot 308 (and a component of spot 307), be consistent with the idea that proteins are targeted for degradation and removal during dehydration and during the initial phases following rehydration (as discussed above).

The trypsin digest of protein spot 307 contained at least one peptide sequence that suggests the enzyme enolase accumulates during dehydration and subsequent rehydration of the alga. Enolase is a glycolytic enzyme that converts 2-phosphoglycerate to phosphoenolpyruvate and is present in many isoforms both cytoplasmic and plastidic (Gottlieb 1982). Enolase, although of unknown location, was identified as a protein that accumulated above control levels during desiccation of leaf tissues of the desiccation-tolerant angiosperm, *X. viscosa* (Ingle *et al.* 2007), and it was suggested that the increase in the abundance of this enzyme was related to the build-up of hexose sugars and sucrose in this plant as it dried. An increase in enolase abundance, associated with the induction of hypoxia prior to an anoxia treatment, occurs in rice and is thought to play a role in the maintenance of the energy status of the cell through regeneration of NADH, such that subsequent anoxia is better tolerated (Blokhina, Virolainen & Fagerstedt 2003). The need to maintain an energy balance may also be indicated by the accumulation of the beta-subunit of ATP synthase (indicated by a peptide associated with spot 891), which has previously been observed to accumulate during desiccation of vegetative cells in the grass *S. stapfianus* (Oliver *et al.* 2010).

Several of the proteins that accumulate in response to dehydration and rehydration, spots 458, 462 and 463, were identified as belonging to the 90 kDa heat shock family of proteins: the Hsp90s. Heat shock proteins are induced by a broad spectrum of stresses such as low and high temperatures, strong light intensity, heavy metals, salt or dehydration (Timperio, Egidi & Zolla 2008). Most Hsps have strong cytoprotective effects, maintaining proteins in their functional conformation, preventing aggregation of non-native proteins, refolding of denaturated proteins to regain their functional conformation, and removal of non-functional but potentially harmful polypeptides (arising from misfolding, denaturation or aggregation). Thereby, Hsps ensure maintenance of homeostasis, protect cells and help them to return to equilibrium during stress recovery (Vierling 1991; Timperio *et al.* 2008). In *A. erici*, only Hsp90 (83–99 kDa) proteins and transcripts were identified as accumulating in response to dehydration and rehydration, but it is quite likely that a more extensive study would reveal other members of the Hsps responding. The Hsp90s are the most abundant and evolutionarily conserved molecular chaperones (Young, Moarefi & Hartl 2001; Picard 2002; Wegele, Muller & Buchner 2004) and are actively involved in protecting the secondary structural aspects of key structural proteins, and perhaps key enzymes. In fully desiccation-tolerant plants, like bryophytes, recovery of photosynthesis after periods of dryness requires only limited chloroplast protein synthesis and is substantially independent of protein synthesis in the cytoplasm (Proctor & Smirnoff 2000). Therefore, we presume that Hsp90s are involved in the protection of chloroplast proteins which could explain the fast recovery of photosynthetic activity after rehydration of lichen photobionts.

The remainder of the proteins that accumulate in response to desiccation and rehydration in the alga are the major cytoskeletal proteins actin and beta-tubulin. Actin microfilaments depolymerize during dehydration, more so if water loss is slow (Pressel & Duckett 2010), but reform upon rehydration. The observed increase in actin during both desiccation and rehydration in *A. erici* probably reflects an increase

in stability during dehydration as the microfilaments disassemble, and perhaps an increase in synthesis related to the need to reconstitute the microfilaments as water returns to the cell. Beta-tubulins, as one of the main structural components of cellular microtubules, are involved in many essential processes, including cell division, ciliary and flagellar motility, and intracellular transport (Hyams & Lloyd 1993). Microtubules play a key role in the response of cells to dehydration and rehydration (Pressel, Ligrone & Duckett 2006; Wang *et al*. 2009). In the desiccation-sensitive moss *Physcomitrella patens* (Koster *et al*. 2010), microtubules are disassembled during dehydration coincidently with a decrease in both alpha and beta-tubulins (Wang *et al*. 2009). Pressel & Duckett (2010), following a comprehensive microscopic examination of the effects of drying and rehydration on protonemal structure in several bryophytes, concluded that 'the controlled disassembly of microtubules during drying, and their subsequent reassembly following rewetting, are a prerequisite for the survival of protonemal cells.'

Ultrastructural changes upon desiccation

Elucidation of the effects of drying and rehydration on cellular organization and ultrastructure is pivotal for understanding the mechanisms involved in the tolerance of desiccation. The rate of water loss is well documented as a major factor that can alter the type, pattern and extent of ultrastructural modifications to cells during desiccation of tolerant plants (Oliver & Bewley 1984; Sherwin 1995; Farrant *et al*. 1999). Under field conditions, slow dehydration results in responses in the cells of lichen phycobionts that are commonly seen in both mosses and resurrection plants (angiosperms). These common responses include the shrinkage of cytoplasm and chloroplasts, accumulation of starch in thylakoids, vacuolar fragmentation, and slight withdrawal of plasmalemma from cell wall but maintenance of membrane integrity (Oliver & Bewley 1984; Valladares & Ascaso 1992; Farrant *et al*. 1999). RD results in a greater disruption of cellular structure, for example, a marked withdrawal of plasma membrane from the cell wall, frequent membrane ruptures, cytorrhysis and degeneration of cytoplasm (Oliver & Bewley 1984; Sherwin 1995; Farrant *et al*. 1999). The results with desiccated samples of *A. erici* (Fig. 4) are consistent with these general observations. Ultrastructural changes were clearly different between slow-dried (Fig. 4b) and rapid-dried (Fig. 4c) rehydrated samples accentuating the importance of the drying rate on the ability to conserve cellular integrity in the alga. Regardless of the amount of cellular disruption observed by the ultrastructural examination of the dried algae it ultimately survives both drying regimes (Gasulla *et al*. 2009).

The clear ultrastructural changes associated with dehydration, in particular when dehydration is rapid, are not accompanied by a large alteration in the proteome of the alga: the levels of 62 proteins visibly change, only 11 of which increase in abundance. In addition, the differences in the extent of the ultrastructural changes resulting from RD compared with SD are not fully reflected in the quantitative differences seen between the corresponding proteome alterations, which although different are generally small (Table 1). This is consistent with previous studies (Gasulla *et al*. 2009) where it was observed that processes involved in cellular protection, for example, antioxidant pathways, the xanthophyll cycle, or late embryogenesis abundant (LEA) protein expression, were also similar in their response to both drying regimes. The most parsimonious explanation for this is that most of the proteins needed for desiccation tolerance are constitutively present in hydrated cells, and only a few proteins, primarily those involved in maintaining structural integrity, require some strategy for an increase in accumulation during dehydration, most likely for immediate use upon rehydration.

CONCLUSIONS

The results presented in this study suggest that desiccation tolerance of *A. erici* is achieved by constitutive mechanisms as suggested by Gasulla *et al*. (2009), and similar to that seen for desiccation-tolerant bryophytes (Oliver, Velten & Mishler 2005). This mechanism allows the alga to survive the rigors of rapid desiccation when protein synthesis is rapidly inhibited. The constitutive mechanism that operates in *A. erici* appears to involve an effective and perhaps selective control of protein degradative activities. Although the alga employs a mechanism that enables it to survive rapid drying rates, it can also recover biosynthetic activity quickly upon rehydration which indicates that, like desiccation-tolerant bryophytes, it may employ a rehydration-induced change in gene expression. The constitutive mechanism appears to involve protein stability, structural cell recovery, protein targeting and degradation. This complex system of protection/repair mechanisms may allow *A. erici* to cope very well with the oxidative and metabolic damages produced in the field during a natural-slow dehydration, but it can also survive the greater mechanical or physical stress caused by a perhaps less natural RD. These results offer new hypotheses concerning the mechanisms and processes that lichens may use to survive continuous cycles of desiccation/rehydration, opening new windows for the study of desiccation tolerance in lichens.

ACKNOWLEDGMENTS

This study was supported in part by the Spanish Ministry of Science and Innovation (CGL2009-13429-C02-01/02, FEDER) and the Generalitat Valenciana (PROMETEO 174/2008 and CGL2012-40058-C02-01/02) and the USDA-APRS (CRIS Project 3622-21000-034, USA). We would like to thank Drs F. Gracía-Breijo and J. Reig-Armiñana (Jardí Botànic, University of Valencia) for their help in TEM analysis of structural changes. We are grateful to the Central Support Service in Experimental Research (SCSIE), University of Valencia and UPV (Spain) for providing TEM, and to Jim Elder (USDA-ARS-PGRU, MO, USA) for his technical assistance in the genomic work. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

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Received 6 April 2012; received in revised form 21 December 2012; accepted for publication 26 December 2012

SUPPORTING INFORMATION

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Figure S1. Example output from DeCyder showing the Cy2-labelled standard gel image. The relative molecular weight (kDa) is given on the left-hand side and the pH gradient is shown above the gel. Annotated spots numbered correspond to those identified (Table 1).

Table S1. List of primers used for the nested and RACE-PCR (forward/reverse; 5′-sequence-3′).

Table S2. List of primers used for qRT-PCR (forward/reverse; 5′-sequence-3′).

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