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## RESEARCH ARTICLE

# Identification of proteins involved in desiccation tolerance in the red seaweed *Pyropia orbicularis* (Rhodophyta, Bangiales)

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Extreme reduction in cellular water content leads to desiccation, which, if persistent, affects the physiology of organisms, mainly through oxidative stress. Some organisms are highly tolerant to desiccation, including resurrection plants and certain intertidal seaweeds. One such species is *Pyropia orbicularis*, a rhodophycean that colonizes upper intertidal zones along the Chilean coast. Despite long, daily periods of air exposure due to tides, this alga is highly tolerant to desiccation. The present study examined the proteome of *P. orbicularis* by 2DE and LC-MS/MS analyses to determine the proteins associated with desiccation tolerance (DT). The results showed that, under natural conditions, there were significant changes in the protein profile during low tide as compared to naturally hydrated plants at high tide. These changes were mainly in newly appeared protein spots such as chaperones, monodehydroascorbate reductase, and manganese superoxide dismutase, among others. Previously undescribed proteins under desiccation conditions included phycobiliproteins, glyoxalase I, and phosphomannomutase. These changes evidenced that several physiological responses involved in DT are activated during low tide, including decreased photosynthetic activity, increased antioxidant capacity, and the preservation of cell physiology by regulating water content, cell wall structure, and cell volume. Similar responses have been observed in resurrection plants and bryophytes exposed to desiccation. Therefore, the coordinated activation of different desiccation tolerance pathways in *P. orbicularis* could explain the successful biological performance of this seaweed in the upper intertidal rocky zones.

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**Abbreviations:** **APC**, allophycocyanin; **APK**, adenosine 5'-phosphosulfate kinase; **ASC**, L-ascorbic acid; **CA**, carbonic an-

hydrase; **DT**, desiccation tolerance; **FNR**, ferredoxin-NADP<sup>+</sup> reductase; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **MDHAR**, monodehydroascorbate reductase; **MG**, methylglyoxal; **OOE1**, oxygen-evolving enhancer 1; **PC**, phycocyanin; **PE**, phycoerythrin; **PMM**, phosphomannomutase; **PPI**, peptidylprolyl isomerase; **ROS**, reactive oxygen species; **SAM**, S-adenosylmethionine synthetase

**Colour Online:** See the article online to view Figs. 1 and 2 in colour.

## 1 Introduction

Water is an essential component for all living organisms, involved in processes of growth and reproduction, and also known for being a key regulator in species distribution [1–3]. Desiccation, the most severe form of water stress, occurs when almost all cell water content is lost, leading to severe physiological and subcellular alterations [4]. Some of these effects include loss of turgor, oxidative damage leading to disruptions in cellular metabolism, destabilization of macromolecules, and the loss of membrane integrity [5, 6]. Some organisms across all kingdoms, have genetically conserved traits for desiccation tolerance (DT) [7], that allow basal state recover during rehydration after being exposed to desiccation [5, 8].

Mechanisms for DT can be grouped into those that (1) limit the damage to a repairable level, (2) maintain physiological integrity under desiccation, and (3) activate the repair mechanisms after desiccation [5, 9, 10]. Several of these mechanisms have been studied in resurrection plants and bryophytes, which are known to withstand long periods of desiccation [7, 10–12]. These desiccation tolerant plants, mostly native of arid environments, are prone to long droughts and high temperatures [11, 13], and their tolerance has been studied using physiological and biochemical approaches coupled with genomics and proteomics [14–19].

Another group of sessile organisms affected by desiccation are intertidal seaweeds. They stand out as key primary producers in aquatic environments and are important constituents of the intertidal community structure [20]. Intertidal seaweeds are organized in distinctive bands parallel to the coast, and, as a result, those species inhabiting the upper intertidal zone are exposed to greater environmental stress than those living in the lower zones [21–22]. Several studies have shown that tolerance to desiccation varies among species, and this may explain the spatial distribution along the intertidal zone [23–25]. Specifically, studies along the Chilean coast have established that algae inhabiting the upper intertidal zone display greater antioxidant responses and recover better after oxidative stress triggered by desiccation, among other stress factors, than those from the lower intertidal zones [22, 25–27].

Red algae (Rhodophyta), the most ancient lineage of photosynthetic eukaryotes [28–30], are distributed globally and include several commercially important species. Among these, species of the genera *Pyropia* and *Porphyra* (Bangiales, Rhodophyta) inhabit the upper intertidal zone and can lose up to 95% of cellular water content during maximum low tide [22, 25, 26, 31–33]. These species have been harvested and consumed since the late Pleistocene by coastal human populations, and are currently important both economically and culturally [33, 34]. Adaptations to desiccation and other environmental stressors in these species involve modifications in the photosynthetic system, changes in morphology, and in the dynamics of macronutrients absorption [23, 25, 26, 31, 33, 35]. A recent study in *Pyropia orbicularis* (formerly *P. columbina* [36]) by Contreras-Porcia et al. [26] also showed a fast

recovery of physiological parameters during rehydration, just as in resurrection plants and bryophytes. This information suggests a coordinated response of tolerance pathways during the dehydration process, which could help explain the ability of this species to colonize and inhabit the upper rocky intertidal zone. In this context, the aim of this work was to identify proteins involved in DT in *P. orbicularis* by means of a proteomic approach.

## 2 Materials and methods

### 2.1 Algal sampling and level of desiccation

Vegetative fronds of *P. orbicularis* (150–180 fronds) from the same population were manually collected as in Contreras-Porcia et al. [26] from intertidal platforms far from and equidistant to the shore, along 150–200 m of coastline in Maitencillo (32° 37' S, 71° 25' W), ca. 180 km north of Santiago, Chile. The fronds were collected while naturally hydrated and naturally desiccated (4 h of exposure to air with no direct water contact or waves splash reaching the plants), rinsed quickly with MilliQ water to eliminate the salt excess, and frozen in situ in liquid nitrogen. All sampled fronds were divided into three pools (containing 10–20 plants each) for each treatment and used for subsequent analyses. The level of desiccation for both conditions was expressed as relative water content (RWC%) according to Flores-Molina et al. [22].

### 2.2 Protein extraction

For protein extraction, the method published by Contreras et al. [37], originally designed for brown algae and adapted for the red macroalga *P. orbicularis*, was used [38].

### 2.3 Two-dimensional gel electrophoresis

The process used for 2DE was adapted from the protocol described in Contreras et al. [37]. Three modifications were made; first, the quantity of extracted proteins used was 600 µg; second, after IEF, strips were stored at –20°C for two days; finally, the electrophoresis was run on an SDS electrophoretic buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% w/v SDS) in a Protean® II xi Cell (Bio-Rad, Hercules, CA, USA) for 8 h at 30–50 mA until the running front reached the end of the gel. A detailed description of 2DE experiments is available in the MIAPE GE sheet in Supporting Information Table 1.

### 2.4 Staining and gel analysis

The 2DE gels were stained and scanned as described in Contreras et al. [37]. The 2DE gel images and protein spots were analyzed using the Melanie v5.0 software (Swiss Institute of

Bioinformatics, Switzerland) according to the manufacturer's instructions. An automatic process was used for both spot detection and matching between both conditions (hydrated and desiccated fronds). Both automatic processes were followed by a manual-editing step. Spot quantification was based on the density of each spot (which consisted in the integration of the highest calibrated pixel intensity in the spot) over the spot area in  $\text{mm}^2$  (Melanie v5.0 User Manual). Afterward, each spot was normalized with the software by using the total volume of all spots in the gel to eliminate possible differences between staining and/or protein charges in the gel analyzed. Variation in the normalized volumes of protein spots was estimated and analyzed only when spots were visible in the preparations of the two natural conditions (hydrated and desiccated fronds), where six gels (biological replicates with two technical replicates) were used for each pI range (i.e. 3–10 and 4–7). The terms up- and downregulated were used to describe variations in protein spot volumes between conditions, with the naturally hydrated condition representing the basal control status, and the proteins in the naturally desiccated condition being up- or downregulated in relation to the control. Importantly, for proteins detected in naturally desiccated fronds but not in naturally hydrated fronds, the term “newly appeared” was used. The term de novo was not used since this could not be inferred from the gels due to limitations in the dynamic range of staining and to possible overestimations of molecular processes, since the proteins might have existed in undetectable quantities.

## 2.5 Protein identification and bioinformatics analysis

Selected spots, which were those that had differential volumes between the treatment conditions (hydration and desiccation), were excised from the gels and sent for sequencing to the Mass Spectrometry Core Facility of the “Station Biologique de Roscoff, CNRS-UPMC” (<http://www.sb-roscoff.fr/technological-core-facilities/mass-spectrometry-core-facility.html>). Gel pieces were washed with different ACN acetonitrile/100 mM  $\text{NH}_4\text{HCO}_3$  solutions. Digestion was performed overnight at 37°C with 12.5 ng/ $\mu\text{L}$  of trypsin. Proteolytic peptides were then extracted from the gel by sequential incubation in the following solutions: 70% ACN/0.1% TFA, 100% ACN, and 70% ACN/0.1% TFA. Extracts were concentrated by evaporation to a final volume of 20  $\mu\text{L}$ . Nano-LC-MS/MS experiments were performed using a Proxeon nano-LC system (Odense, Denmark) coupled on-line to a LTQ Orbitrap Discovery system mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Methods for MS/MS data acquisition and processing are reported in a MIAPE MS sheet in Supporting Information Table 2.

Sequences were deduced from the resulting fragment ion spectra using the PEAKS Studio software (Peaks Studio 5.3, Bioinformatics Solutions Inc., Waterloo, ON, Canada). For protein identification, the resulting peptide sequences were

searched for in the PEAKS database using the parameters specified by the manufacturer. This database included *P. orbicularis* (Rhodophyta) ESTs (54 998 sequences) [35], *Chondrus crispus* (Rhodophyta) genes (39 395 sequences) [39], and *Ectocarpus siliculosus* (Ochrophyta) genes (16 461 sequences) [40]. Methods for protein processing and identification are reported in the MIAPE GE sheet in Supporting Information Table 3. The threshold selection for the protein sequences was a PEAKS protein score greater than 20 (the sum of the supporting peptide scores for each distinct sequence that are a representation of the *p*-value in PEAKS as a proxy of the LDF score, which measures the quality of the peptide-spectrum match) [41]. Only proteins showing at least one peptide with individual score confidence > 20 and/or 4 amino acids with confidence > 90% were considered as valid candidates. For these proteins, MS/MS spectra were also manually validated by the presence of a series of at least four  $\gamma$ -ions. After PEAKS identification, the protein sequences were analyzed using BLAST-P to determine similarity with known proteins in the NCBI database. The threshold was set to a minimal significance of  $1\text{e}^{-3}$  and an identity percentage of greater than 25%. The theoretical pI and molecular weight of the blast hit was calculated using the Expasy Compute pI/MW tool ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)). Subsequently, proteins were categorized into functional groups according to their metabolic function by using the KO database (KEGG Orthology, <http://www.genome.jp/kegg/ko.html>) [42].

## 2.6 Statistical analyses

Prior to statistical analyses, data were checked for homogeneity using the Levene test and for normal distribution using the Kolmogorov–Smirnov test [43]. Then, a one-way ANOVA followed by Tukey's multiple comparisons tests (*T*), considering treatments as fixed factor (natural hydration and desiccation, using three biological replicates for each condition), were used to test whether protein spots were significantly different in terms of volume variation between both conditions. Differences between mean values were considered to be significant at a probability of 5% ( $p \leq 0.05$ ). After this analysis, the size effect of each condition was determined estimating Cohen's *d* [44], where a size effect between  $0.2 \leq 0.4$  was considered small, between  $0.4 \leq 0.7$  medium, and greater than 0.7 was large.

## 3 Results

For assessing the desiccation level of fronds exposed to air due to low tide, RWC% was measured in desiccated fronds, and showed a  $10 \pm 2\%$  RWC as compared to 100% for hydrated fronds (see Flores-Molina et al. [22] for calculation details). At a proteomic level, the results indicated that this variation in RWC% during the natural hydration-desiccation cycle affects the protein profile of *P. orbicularis*. The total

number of spots detectable on gels stained with Coomassie was 550 and 650 for naturally hydrated and desiccated tissues, respectively. Comparative analysis of the proteomes visualized in 2DE gels (pI 3–10 and 4–7) revealed that a total of 129 spots underwent changes in volume variation due to desiccation (Fig. 1), which suggests differential gene expression during the hydration-desiccation cycle. Of these spots, 18 were downregulated (14%), 9 were upregulated (7%) and 102 (79%) newly appeared as compared to the naturally hydrated controls. All proteins were characterized by nano-LC-MS/MS, and 56 of them (43.4% total) were identified by bioinformatics analysis (Table 1, Fig. 1). Of the total proteins identified, 92% were related to proteins reported for Rhodophyta followed by those known for Ochrophyta (2%), Magnoliophyta (2%), Tracheophyta (2%), and Dinophyta (2%) (Fig. 2A). In addition to this, within Rhodophyta, 60% of the identified proteins matched those of the genera *Pyropia* (e.g. *P. yezoensis* and *P. haitanensis*) and *Porphyra* (e.g. *P. purpurea*), both of which are phylogenetically close to *P. orbicularis*.

The identified proteins were classified into the following six categories according to their function: (i) energy and biomolecule metabolism, (ii) genetic and environmental information processing, (iii) transport and catabolism of cellular processes, (iv) antioxidant and defense function, (v) cytoskeleton-related, and (vi) unknown (Fig. 2B, Table 1). Most proteins were classified within the categories of energy and biomolecule metabolism (50%), antioxidant and defense function (23%) and genetic and environmental information processing (11%) (Fig. 2B). Within the six categories, down- and upregulated proteins were significantly different between desiccated and control hydration conditions ( $p < 0.01$ ), and Cohen's  $d$  absolute values ranged from 2.9 to 12.9 (Fig. 3). Specifically, within the energy and biomolecule metabolism functional group under desiccation, the ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) large subunit (e.g. spot 56, Fig. 1) was significantly downregulated (36%) as compared to the control condition ( $F = 19.30$ ,  $p = 0.012$ , Cohen's  $d = -3.857$ ; Fig. 3). Other proteins associated with the process of photosynthesis were downregulated during desiccation, such as those for ferredoxin-NADP<sup>+</sup> reductase (FNR) (spots 7–9) and carbonic anhydrase (CA) (spot 12) (Fig. 1, Table 1). On the other hand, 20 of the 28 proteins (71%) within this group were newly appeared in desiccated fronds, several of which were involved in sugar metabolism, including triosephosphate isomerase (spot 2 and 39), 3-dehydroquinate synthase/O-methyltransferase fusion (spot 14), aconitate hydratase (spot 15), phosphoglycerate kinase (PGK) (spots 20 and 23), monodehydroascorbate reductase (MDHAR) (spot 21), lactoylglutathione lyase (spot 30), GDP-D-mannose-3',5'-epimerase (spot 31), cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (spot 35), and phosphomannomutase (PMM) (spot 37) (Fig. 1; Table 1). The remaining enzymes within this group appeared involved in energy metabolism, such as the plastid oxygen-evolving enhancer 1 (OEE1) (spots 1, 27, and 34) and adenosine 5'-phosphosulfate kinase (APK) (spot 33), involved in sulfate

activation; S-adenosylmethionine synthetase (SAM) (spot 17) and cysteine synthase (spot 25, 28 and 29), involved in amino acid metabolism; and, finally, the stress-inducible pyridoxine biosynthesis protein SOR (spot 32).

Within the genetic and environmental information processing group, five were newly appeared proteins and one was upregulated in the proteome of *P. orbicularis* exposed to desiccation (Fig. 1; Table 1). These proteins were elongation factor 2 (spot 13), the RNP domain-containing protein (spot 26), and voltage-dependent anion channels (spots 36, 38, and 40). The translation initiation factor (eIF) 5A (spot 46) was upregulated 4.3-fold during desiccation in relation to hydration (Fig. 3).

Four proteins were classified within the transport and catabolism of cellular processes group, of which the Sec 7 domain (spot 11) and Rab GTPase family 11 (spot 47) were downregulated during desiccation. Two other newly appeared proteins were present during the stress condition and corresponded to Gbp1 (spot 24) and the small GTP-binding protein Sar1 (spot 42) (Fig. 1, Table 1).

In the antioxidant and defense function group, the alpha subunit of the phycobiliprotein allophycocyanin (APC) (spot 54) was significantly upregulated (3.9-fold) in desiccated fronds ( $F = 35.54$ ,  $p = 0.004$ , Cohen's  $d = 4.868$ ) (Figs. 1 and 3, Table 1). Moreover, 12 additional proteins were upregulated or newly appeared during desiccation, and the majority corresponded to various phycobiliproteins, such as the phycoerythrin (PE) beta subunit (spots 43 and 45) and the phycocyanin (PC) beta subunit (spots 49–53) (Fig. 1; Table 1). The four remaining proteins within this group were identified as peptidylprolyl isomerase (PPI) (spot 16), chaperonin 60 (spot 18 and 19), HSP 22 (spot 44), and enzyme manganese superoxide dismutase (spot 41).

In the group of cytoskeleton-related proteins, spots 5 and 6 were identified as actin and were downregulated in desiccation, whereas spot 55, which was upregulated in desiccated fronds, was identified as cofilin/actin depolymerizing factor 3. Finally, in the unknown functional group, only two spots were identified. These were both ferritin (spots 22 and 48), which were upregulated and newly appeared during desiccation. These results indicate that variation in cellular RWC during the natural hydration-desiccation cycle in *P. orbicularis* strongly affects its protein profile.

## 4 Discussion

*Pyropia orbicularis* displays efficient, specific physiological, and transcriptional responses that permit for high DT [25, 26, 35]. The present results showed that desiccation induces major changes in the protein profile of *P. orbicularis*, mainly regarding newly appeared proteins. These data support the physiological adaptation to desiccation of *P. orbicularis*. Moreover, the mechanisms employed by *P. orbicularis* seem similar to those that resurrection plants and bryophytes activate during desiccation stress [15–19, 45]. Changes

**Table 1.** Identification of proteins with varied normalized volumes between naturally hydrated and desiccated fronds of *Pyropia orbicularis*

Functional groups	Spot N <sup>oa)</sup>	Protein	Species	Access N <sup>o</sup>	pI (Obs/Theo)	MW (Obs/Theo) (kD)	Variation changes <sup>b)</sup>	Score (PEAKS)	SC	NMP
Energy and biomolecules metabolism	3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast)	<i>Porphyra rosengurttii</i>	ACZ57927.1	4.64 / 6.10	66.67 / 52.99	DR	205.97	12	4
	4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	<i>Porphyra rosengurttii</i>	ACZ57927.1	4.20 / 6.10	24.33 / 52.99	DR	190.03	24	7
	10	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	<i>Porphyra rosengurttii</i>	ACZ57927.1	6.23 / 6.10	28.67 / 52.99	DR	153.98	14	6
	56	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) (partial)	<i>Pyropia yezoensis</i>	ABI99377.1	4.26 / 4.89	9.79 / 12.37	DR (-2.94)	90.75	23	2
	7	Ferredoxin-NADP <sup>+</sup> reductase	<i>Pyropia yezoensis</i>	ADM64306.2	6.66 / 8.32	38.00 / 33.33	DR	191.18	27	6
	8	Ferredoxin-NADP <sup>+</sup> reductase	<i>Pyropia yezoensis</i>	ADM64306.2	6.63 / 8.32	37.00 / 33.33	DR	190.75	27	6
	9	Ferredoxin-NADP <sup>+</sup> reductase	<i>Pyropia yezoensis</i>	ADM64306.2	6.56 / 5.90	37.00 / 33.33	DR	229.59	39	7
	12	Carbonic anhydrase	<i>Pyropia yezoensis</i>	ACI01451.1	6.36 / 8.51	25.00 / 29.80	DR	107.14	30	3
	2	Triose-phosphate isomerase	<i>Chondrus crispus</i>	XP_005716285.1	6.09 / 4.84	26.29 / 27.03	NA	121.76	10	2
	39	Triose-phosphate isomerase	<i>Chondrus crispus</i>	XP_005716285.1	5.64 / 4.84	29.00 / 27.03	NA	91.93	10	2
	14	3-dehydroquininate synthase/O-methyltransferase fusion	<i>Oxyrrhis marina</i>	ABF61767.1	5.88 / 5.38	100.00 / 94.20	NA	61.61	3	1
	15	Aconitate hydratase	<i>Gracilaria gracilis</i>	P49609.1	5.30 / 5.20	83.67 / 83.73	NA	161.91	23	4
	17	S-adenosylmethionine synthetase	<i>Pyropia yezoensis</i>	ACJ38094.1	5.40 / 5.52	71.00 / 41.83	NA	120.14	11	3
	20	Phosphoglycerate kinase	<i>Pyropia yezoensis</i>	BAG09537.1	4.70 / 5.40	47.00 / 47.09	NA	202.86	19	9
	23	Phosphoglycerate kinase	<i>Pyropia yezoensis</i>	BAG09537.1	6.50 / 5.40	46.00 / 47.09	NA	21.34	5	1
	21	Monodehydroascorbate reductase	<i>Medicago sativa</i>	AEX20344.1	4.80 / 6.62	47.00 / 47.12	NA	131.58	17	3
	25	Cysteine synthase	<i>Porphyra purpurea</i>	AAP97124.1	5.30 / 6.05	41.00 / 38.69	NA	142.26	20	2
28	Cysteine synthase	<i>Pyropia yezoensis</i>	ACI47323.1	5.30 / 5.81	40.00 / 39.25	NA	263.40	31	8	
29	Cysteine synthase	<i>Pyropia yezoensis</i>	ACI47323.1	5.40 / 5.81	38.00 / 39.25	NA	48.60	7	2	
1	Plastid oxygen-evolving enhancer 1	<i>Chondrus crispus</i>	XP_005713751.1	5.31 / 4.96	24.76 / 26.13	NA	203.13	38	7	

Table 1. Continued

Functional groups	Spot N <sup>oa)</sup>	Protein	Species	Access N <sup>o</sup>	p/(Obs/Theo)	MW (Obs/Theo) (kD)	Variation changes <sup>b)</sup>	Score (PEAKS)	SC	NMP
	27	Plastid oxygen-evolving enhancer 1	<i>Chondrus crispus</i>	XP_005713751.1	4.53 / 4.96	38.00 / 26.13	NA	195.53	33	4
	34	Plastid oxygen-evolving enhancer 1	<i>Chondrus crispus</i>	XP_005713751.1	5.40 / 4.96	35.00 / 26.13	NA	143.99	23	3
	30	Lactoylglutathione lyase	<i>Selaginella moellendorffii</i>	XP_002960285.1	5.40 / 5.34	37.00 / 32.58	NA	141.21	19	5
	31	GDP-D-mannose-3,5'-epimerase	<i>Galdieria sulphuraria</i>	XP_005703429.1	5.60 / 6.23	37.00 / 40.56	NA	185.47	45	7
	32	Stress-inducible pyridoxine biosynthesis protein SOR	<i>Chondrus crispus</i>	XP_005713413.1	6.20 / 6.46	36.00 / 29.77	NA	142.20	34	4
	33	Adenosine 5'-phosphosulfate kinase	<i>Porphyra purpurea</i>	AAP97123.1	5.54 / 5.65	36.33 / 25.36	NA	150.94	21	6
	35	Cytosolic glyceraldehyde 3-phosphate dehydrogenase	<i>Pyropia zeoensis</i>	AAP32470.1	6.30 / 6.00	35.00 / 35.91	NA	64.88	14	1
	37	Phosphomannomutase	<i>Chondrus crispus</i>	XP_005713596.1	5.61 / 5.56	30.00 / 28.15	NA	103.41	7	2
Genetic and environmental information processing	13	Elongation factor 2	<i>Porphyra purpurea</i>	AAG40108.1	6.07 / 6.02	100.00 / 85.69	NA	131.98	9	4
	26	RNP domain-containing protein	<i>Chondrus crispus</i>	XP_005718172.1	5.00 / 5.36	41.00 / 23.51	NA	179.66	42	5
	36	Voltage-dependent anion channel, MPP family	<i>Galdieria sulphuraria</i>	XP_005706832.1	6.10 / 8.56	32.00 / 37.53	NA	146.2	30	5
	38	Voltage-dependent anion channel, MPP family	<i>Galdieria sulphuraria</i>	XP_005706832.1	5.56 / 8.56	31.33 / 37.53	NA	141.94	26	3
	40	Voltage-dependent anion channel, MPP family	<i>Galdieria sulphuraria</i>	XP_005706832.1	6.65 / 8.56	25.00 / 37.53	NA	154.66	31	6
	46	Translation initiation factor eIF5A	<i>Chondrus crispus</i>	XP_005717992.1	6.13 / 6.89	19.00 / 17.26	UR (4.3)	78.3	12	1
Transport and catabolism of cellular processes	11	Sec 7 domain	<i>Ectocarpus siliculosus</i>	CBN76083.1	6.09 / 8.56	31.00 / 139.66	DR	41.30	2	1
	24	Gbp1	<i>Griffithsia japonica</i>	AAM93968.1	4.90 / 5.81	42.00 / 17.38	NA	60.67	6	1
	42	Small GTP-binding protein Sar1	<i>Chondrus crispus</i>	XP_005715707.1	6.20 / 6.83	24.00 / 21.84	NA	127.9	22	4
	47	f GTPase family 11	<i>Galdieria sulphuraria</i>	XP_005708023.1	4.20 / 5.66	24.33 / 23.84	DR (-2.94)	271.04	81	15

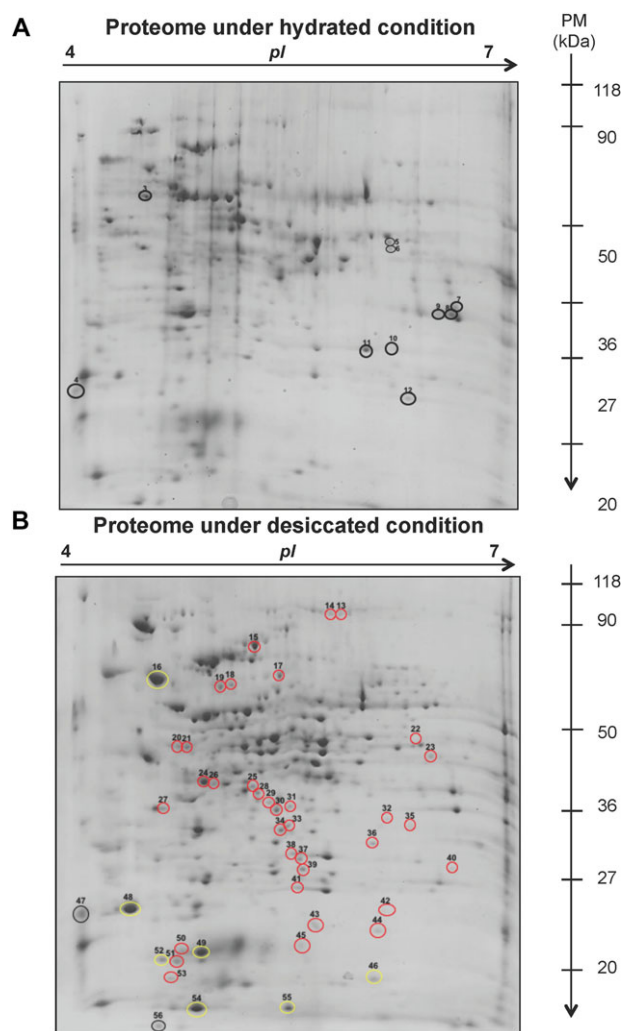
Table 1. Continued

Functional groups	Spot N <sup>(a)</sup>	Protein	Species	Access N <sup>o</sup>	pI (Obs/Theo)	MW (Obs/Theo) (kD)	Variation changes <sup>b)</sup>	Score (PEAKS)	SC	NMP
Antioxidant and defense functions	16	Peptidylprolyl isomerase	<i>Galdieria sulphuraria</i>	XP_005705635.1	4.67 / 4.67	69.67 / 59.06	UR (3.65)	189.64	56	6
	18	Chaperonine 60	<i>Chondrus crispus</i>	XP_005715325.1	5.18 / 6.54	66.00 / 59.95	NA	82.49	16	2
	19	Chaperonine 60	<i>Chondrus crispus</i>	XP_005715325.1	5.00 / 6.54	66.00 / 59.95	NA	229.59	46	10
	44	Heat shock protein 22	<i>Pyropia haitanensis</i>	ALS25017.1	6.15 / 5.24	22.67 / 19.07	NA	41.19	3	2
	41	Manganese superoxide dismutase	<i>Pyropia yezoensis</i>	AAZ75664.1	5.61 / 5.75	27.00 / 24.47	NA	165	14	3
	43	Phycocerythrin beta subunit (chloroplast)	<i>Pyropia haitanensis</i>	YP_007947897.1	5.75 / 6.23	22.67 / 18.42	NA	104.6	21	3
	45	Phycocerythrin beta subunit (chloroplast)	<i>Pyropia haitanensis</i>	YP_007947897.1	5.63 / 6.23	21.67 / 18.42	NA	156.76	21	3
	49	Phycocyanin beta subunit	<i>Pyropia yezoensis</i>	YP_537058.1	4.97 / 4.94	21.33 / 18.20	UR (1.74)	191.21	64	7
	50	Phycocyanin beta subunit	<i>Pyropia yezoensis</i>	YP_537058.1	4.80 / 4.94	21.00 / 18.20	NA	176.49	64	7
	51	Phycocyanin beta subunit	<i>Pyropia yezoensis</i>	YP_537058.1	4.70 / 4.94	21.00 / 18.20	NA	217.25	71	9
Cytoskeleton-related proteins	52	Phycocyanin beta subunit	<i>Pyropia yezoensis</i>	YP_537058.1	4.71 / 4.94	21.00 / 18.20	UR (4.04)	201.14	64	7
	53	Phycocyanin beta subunit	<i>Pyropia yezoensis</i>	YP_537058.1	4.67 / 4.94	19.33 / 18.20	NA	176.22	71	7
	54	Allophycocyanin alpha subunit	<i>Porphyra purpurea</i>	NP_053872.1	4.94 / 5.06	17.67 / 17.51	UR (3.92)	98.77	19	2
	5	Actin	<i>Pyropia yezoensis</i>	BAB64309.1	6.27 / 5.83	48.33 / 41.22	DR	347.99	48	17
	6	Actin	<i>Pyropia yezoensis</i>	BAB64309.1	6.28 / 5.83	47.00 / 41.22	DR	288.12	41	14
	55	Cofilin /actin depolymerizing factor	<i>Galdieria sulphuraria</i>	XP_005702926.1	5.55 / 5.47	17.67 / 15.91	UR (1.30)	193.72	22	4
	22	Ferritin	<i>Pyropia yezoensis</i>	AFR78246.1	6.30 / 4.52	51.00 / 28.14	NA	196.46	23	6
	48	Ferritin	<i>Pyropia yezoensis</i>	AFR78246.1	4.40 / 4.52	25.33 / 28.14	UR (3.39)	200.14	23	6

a) Spots not visible in Fig. 1 were identified on gels of p/3–10 (see Supporting Information Fig. 1).

b) Variation volume of protein spots according to desiccation condition, fold-change is only given for those visible on gels from hydrated and desiccated tissue (detailed information on staining and gel analysis in the Material and methods).  
 pI, isoelectric point; Obs, observed; Theo, theoretical; MW, molecular weight; UR, upregulated; DR, downregulated, and NA, newly appeared during desiccation. SC, sequence coverage; NMP, number of match peptides.



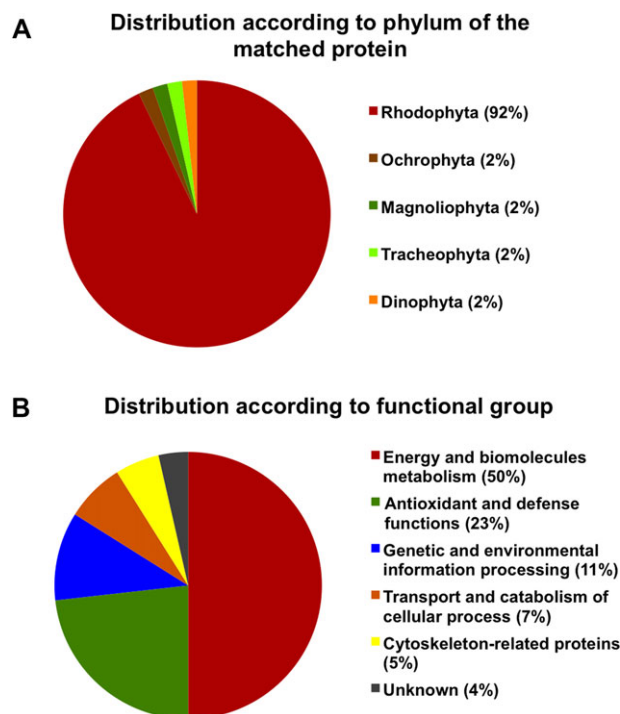


**Figure 1.** Representative 2DE ( $pI$  range 4–7) of the *Pyropia orbicularis* proteome. (A) Hydrated plants ( $n = 3$ ) and (B) desiccated plants ( $n = 3$ ). Spots circled in black were downregulated, in yellow were upregulated, and in red were newly appeared during desiccation as compared to the naturally hydrated condition. Only identified proteins are shown and encircled according to volume variation. Gels were stained with ProtoBlue Safe Coomassie Stain G-250. See 2DE gels of  $pI$  3–10 in the Supporting Information.

observed in the *P. orbicularis* proteome will be discussed according to DT mechanism, as based on current knowledge in resurrection plants and bryophytes and on previous genetic and biochemical studies in *P. orbicularis* (Fig. 4).

#### 4.1 Limiting damage to a repairable level

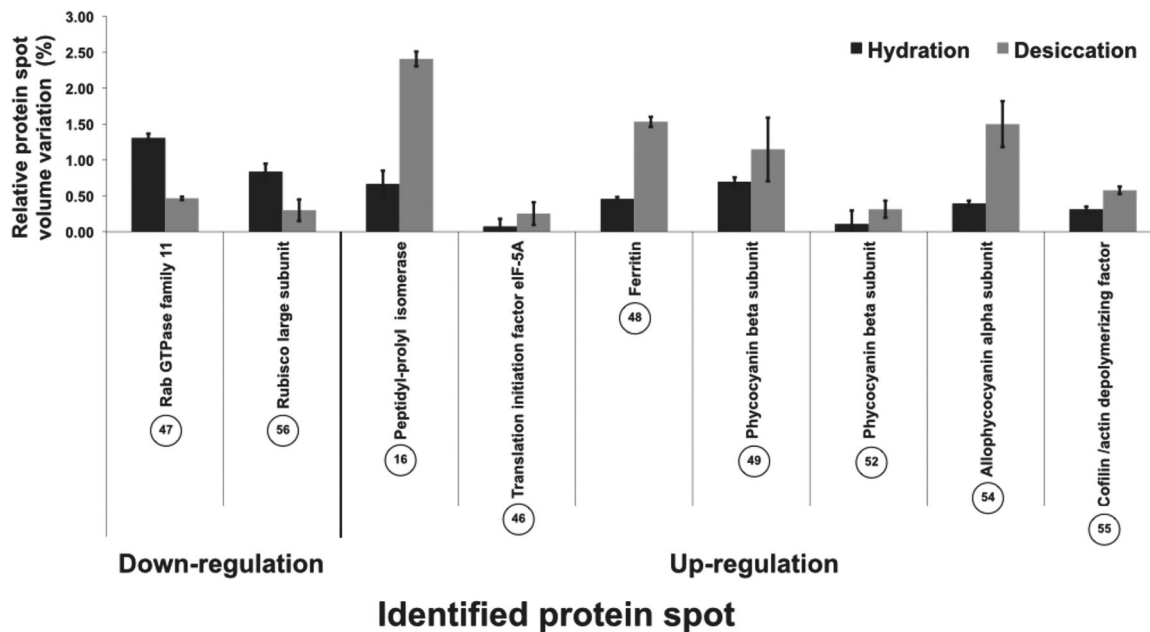
One mechanism of desiccation-tolerant plants is declining photosynthetic activity in order to reduce damage associated with photosynthetic by-products, such as ROS [46, 47]. This phenomenon has been reported in various intertidal species [23, 24, 48–50], including *P. orbicularis* [26]. The diminished



**Figure 2.** Protein distribution according to (A) the phylum of the matched proteins and to (B) functional group classification.

photosynthesis of *P. orbicularis* is likely due to dismantlement instead of degradation, suggesting it is a homoiochlorophyllous rather than poikilochlorophyllous resurrection plant [47]. This was reported by Contreras-Porcia et al. [26], who showed that the optimum quantum yield of photosynthesis ( $F_v/F_m$ ) in desiccated *P. orbicularis* plants was recovered just five minutes after rehydration, such as also occurred at the level of chlorophyll. In this context, three enzymes involved in photosynthesis RubisCO, FNR, and CA, were identified in the present study, and all were downregulated in naturally desiccated individuals compared to naturally hydrated controls. Related to this, three newly appeared spots were identified, including plastid OEE1. It is possible that the extended structure of these is needed to protect the D1 protein reaction center from ROS formed during light exposure, probably through ligating the MSN cluster, in addition to providing protection as a chaperone [50–52]. Other studies in resurrection plants and bryophytes have also reported lower RubisCO and higher plastid OEE expression concomitant with declined photosynthesis during desiccation [5, 14, 15, 17, 19, 52–55].

An elevated production of phycobiliproteins (PE, PC, and APC) was also detected during desiccation in *P. orbicularis*. This could lead to channeling light energy and, consequently, further reduce ROS production, similar to what anthocyanins do in resurrection plants [5]. Increased phycobiliproteins are in agreement with Contreras-Porcia et al. (2011), a study at the transcriptome level that found increased PE and PC transcripts in desiccated *P. orbicularis* individuals [35].



**Figure 3.** Relative volume variation of *Pyropia orbicularis* protein spots between naturally hydrated and desiccated conditions. Numbers in circle correspond to the spot number of each identified protein. The relative protein spot volume variation in all differential spots between both conditions was significantly different (ANOVA;  $p < 0.05$ ).

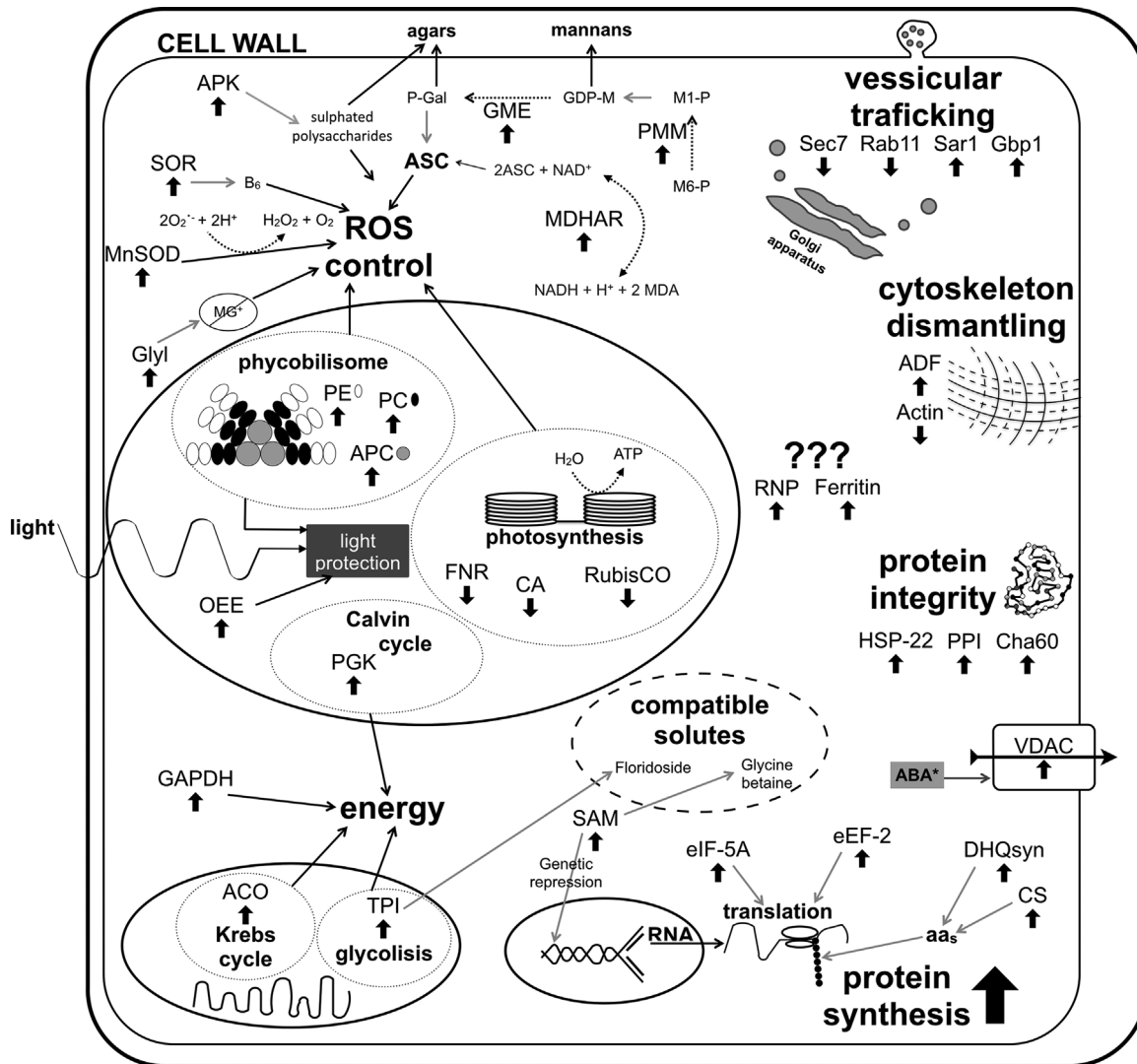
#### 4.2 Maintaining physiological integrity under desiccation

Chaperonin 60, HSP 22, and PPI, which maintain protein structure, were either newly appeared or upregulated under desiccation. Chaperones maintain functional protein conformation by preventing nonnative protein aggregation and by refolding proteins into natural conformations under stress [56]. The HSPs act as chaperonins, and one of these, HSP-70, is upregulated during desiccation in *Physcomitrella patens* and *Fontinalis antipyretica* (Bryophyta), and *Sporobolus stapfianus* (Tracheophyta) [14, 16, 18]. Also involved in maintaining protein structure, PPI catalyzes the rotation of the peptide bond preceding proline residues to induce protein folding, thus preventing damage [57]. This enzyme intervenes in protein folding in rice exposed to cold and heat stress, thus supporting its protective role [57, 58]. Recently, an EST corresponding to PPI was identified under desiccation conditions in *P. orbicularis* [35]. The above suggests an active participation of these proteins in *P. orbicularis* under oxidative stress.

As with other algae, when *P. orbicularis* is desiccated, cell wall folding and a loss in cell volume occur [22, 26, 59]. A cofilin/actin depolymerizing factor was upregulated in association with reduced actin quantity, with both belonging to cytoskeleton-related protein groups. The cofilin/actin depolymerizing factor disassembles actin filaments and prevents denaturation, thereby maintaining an actin pool in the cell [60]. Similar mechanisms have also been observed in tolerant plants such as *Polytrichum formosum*, *P. patens*, and *F. antipyretica*, which showed downregulation of microtubules and

microfilaments proteins such as actin and  $\beta$ -tubulin, among others [14, 18, 61, 63]. Suggestions made by these authors appear correct, in that cytoskeleton dismantling or deconstruction must occur during desiccation within the cells to withstand variation in cell volume during a desiccated state. Actin repolymerization during *P. orbicularis* rehydration might be correlated with the transcriptional activation of actin genes, as suggested by previous EST analyses [35]. Furthermore, desiccation also induces newly appeared voltage-dependent anion channels, and this protein has been identified in other *P. orbicularis* transcriptomic studies [35]. These channels are activated during desiccation and saline stress through overstimulation by the ABA hormone [62, 63], which was found overexpressed in desiccated *P. orbicularis* [64]. These channels are associated with stomata closing in guard cells to reduce water loss from transpiration in vascular plants, which in addition to anionic release through ABA-triggering, diminishes osmotic pressure [62]. Considering this, enzymes of the cytoskeleton-related proteins (actin and cofilin/actin depolymerizing factor) and those involved in processing genetic and environmental information could explain cell wall folding and cell volume decrease in *P. orbicularis* cells during desiccation [26].

Another mechanism employed by resurrection plants to maintain physiological integrity is compatible solute synthesis [65–67]. In *P. orbicularis*, four enzymes associated with compatible solutes were newly appeared, including: triose-phosphate isomerase, SAM, GDP-D-mannose-3',5'-epimerase, and PMM. Triose-phosphate isomerase is involved in glycolysis and the metabolic pathway of sucrose



**Figure 4.** Cell diagram of *Pyropia orbicularis* mechanisms involved in the tolerance to desiccation stress induced during natural low tide periods. Proteins that were downregulated are followed by ↓, whereas those that were upregulated or newly appeared during desiccation are followed by ↑. Organelles within the cell are depicted by a circle with a continuous line, whereas cycles or structures within these are denoted by a circle with a dotted line. The direct action of an enzyme/protein/molecule on a mechanism is indicated with black arrows. An enzyme/protein/molecule is indicated with a gray arrow when part of an enzymatic pathway. Dotted arrows indicate the enzyme's mechanism. Protein abbreviation is as followed: ABA: abscisic acid; ACO: aconitate hydratase; ADF: cofilin/actin depolymerizing factor; APC: allophycocyanin alpha subunit; APK: adenosine 5'-phosphosulfate kinase; ASC: ascorbic acid; B6: vitamin B6; CA: carbonic anhydrase; Cha 60: chaperonine 60; CS: cysteine synthase; DHQsyn: 3-dehydroquinone synthase/O-methyltransferase fusion; eEF-2: elongation factor 2; eIF-5A: translation initiation factor eIF5A; FNR: ferredoxin-NADP<sup>+</sup> reductase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GDP-M: GDP-d-mannose; Glyl: lactoylglutathione lyase; GME: GDP-D-mannose-3'-5'-epimerase; HSP-22: Heat shock protein 22; K<sup>+</sup><sub>in</sub>: inward rectifying K<sup>+</sup> channels; M1-P: mannose 1-fosfato; M6-P: mannose 6-fosfato; MDHAR: monodehydroascorbate reductase; MG: methylglyoxal; MnSOD: manganese superoxide dismutase; OEE1: plastid oxygen-evolving enhancer 1; P-Gal: GDP-L-galactose; PC: phycocyanin beta subunit; PE: phycoerythrin beta subunit; PGK: phosphoglycerate kinase; PMM: phosphomannomutase; PPI: peptidylprolyl isomerase; Rab11: rab GTPase family 11; RNP: RNP domain-containing protein; RubisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast); SAM: S-adenosylmethionine synthetase; Sar1: small GTP-binding protein Sar1; Sec 7: Sec 7 domain; SOR: stress-inducible pyridoxine biosynthesis protein SOR; TPI: triose-phosphate isomerase; VDAC: voltage-dependent anion channel. MPP family. †, MG detoxification. \*, according to Guajardo 2013.

in land plants and bryophytes, acting as a compatible solute in diminishing damage associated with water loss [18,65–67]. This enzyme might also be important in the synthesis of glycerol in *P. orbicularis*, which couples with D-galactose to form floridoside and isofloridoside, the main photosynthate in Bangiales [68] as sucrose cannot be produced by red algae. GDP-D-mannose-3',5'-epimerase and PMM are probably involved in the early metabolism of mannose, and GDP-mannose plays a crucial role in the polysaccharide biosynthesis of *Pyropia* gametophytes, whose cell walls are rich in mannans [69,70]. Finally, SAM, which is upregulated in plants exposed to stress, synthesizes adenosylmethionine, a cofactor in the synthesis of compatible solutes such as glycine betaine [65,66]. Additionally, SAM may also be involved in gene repression through the methylation of nucleic acids by its product adenosylmethionine, which is a methyl donor in several reactions [71–73].

### 4.3 Increasing antioxidant control

Besides reducing photosynthetic activity, resurrection plants increase antioxidant enzyme expression and the production of antioxidant compounds to diminish damage associated with ROS. Analysis identified the newly appeared superoxide dismutase (MnSOD), which catalyzes the conversion of the superoxide radical ( $O_2^{\cdot-}$ ) to oxygen and hydrogen peroxide [74], and its role has been documented in other seaweeds [75]. In addition to channeling light, upregulated phycobiliproteins (PE, PC, and APC) act as antioxidant compounds. For example, PC is able to eliminate peroxyxynitrite, a powerful oxidant capable of causing alterations in the DNA [76], while PE and APC can reduce oxidative stress in kidney cells exposed to mercury [77].

Data also showed that APK was newly appeared during desiccation. This enzyme catalyzes the formation of the 3'-phosphoadenosin 5'-phosphosulfate [78], which, in algae, is involved in the biosynthesis of sulfated polysaccharides [79]. Sulfate-containing molecules have high antioxidant potential since they reduce ROS levels in several cellular types, such as lymphocytes and fibroblasts [80,81]. Furthermore, two enzymes (PMM and GME; GDP-D-mannose-3',5'-epimerase) involved in the biosynthesis of l-ascorbic acid (ASC) in plants and algae [82–84] were upregulated during desiccation in *P. orbicularis*. Where PMM catalyzes the conversion of mannose 6-phosphate to mannose 1-phosphate, GME uses the product of PMM to form GDP-L-galactose (P-GAL) and, subsequently, ASC [85,86]. P-GAL is an essential precursor in agar biosynthesis [68], and ASC is an important antioxidant compound present in many organisms. In *Arabidopsis thaliana* and *Nicotiana benthamiana*, when the PMM gene changes or is silenced, ASC production decreases [86,87]. Moreover, the present results showed an upregulation of MDHAR, which uses NAD(P)H to regenerate oxidized ASC [88]. The stress-inducible pyridoxine biosynthesis protein (SOR) was newly appeared during desiccation, which is involved in the

synthesis of pyridoxal 5'-phosphate, a form of vitamin B6 with potent antioxidant functions [89,90].

Lactoylglutathione lyase, also known as glyoxalase I (GlyI), was another newly appeared enzyme during desiccation. This is involved in detoxifying methylglyoxal (MG), a byproduct of glycolysis [91] that is harmful at high concentrations because it reacts with macromolecules such as DNA, RNA, and proteins [92]. In pumpkin, MG is induced by osmotic stress, generating superoxide radicals [93]. Thus, the newly appeared PMM and MDHAR could be directly linked to greater antioxidant capacity through increased ASC, while GlyI prevents superoxide radical formation by detoxifying the cell of MG. Therefore, the combined upregulation of enzymes such as phycobiliproteins, vitamin B6, GlyI, and MnSOD could explain part of the ROS scavenging capacity of *P. orbicularis* in comparison to sensitive seaweeds.

### 4.4 Proteins with nonspecific roles during desiccation

Among the upregulated and newly appeared proteins, several had no desiccation-specific functions. However, many were classified according to functions of energy or biomolecule metabolism. Enzymes such as PGK and GAPDH are involved in the Calvin cycle and, as in *P. patens* [14], *S. stapfianus* [16], and *F. antipyretica* [18], these were newly appeared during desiccation in *P. orbicularis*, thus implying that this process could play an important role in DT. The enzymes triose phosphate isomerase and aconitate hydratase catalyze the reversible interconversion of the triosephosphate isomer and citrate to isocitrate, respectively [94,95]. This could explain the energy supply needed for protein synthesis. Other upregulated enzymes within this group were cysteine synthase, 3-dehydroquinate synthase, eIF5A, and eukaryotic elongation factor 2 (eEF-2), all of which are involved in amino acid biosynthesis. The 3-dehydroquinate synthase enzyme is involved in the shikimate pathway that is implicated in the biosynthesis of aromatic amino acids [96]. While eIF5A is involved in the recognition of the AUG codon [97], eEF-2 mediates the translocation step of peptide-chain elongation in eukaryotic cells [98]. The upregulation of these enzymes is not surprising since other factors involved in protein synthesis are upregulated during abiotic stress in plants [99–100].

Three enzymes linked to the vesicular network were also upregulated; Sar1, a GTP-binding protein; Gbp1, a small GTP-binding protein; and a Rab GTPase family 11. Interestingly, the ESTs of GTPase and the small GTP-binding protein were previously identified under desiccation conditions in *P. orbicularis* [35]. Moreover, O'Mahony and Oliver (1999) extensively studied the Rab2 small GTP-binding protein, which accumulates in *S. stapfianus* when exposed to desiccation [101]. Thus, the endomembrane system in *P. orbicularis* may be an important part of a protection-based mechanism for desiccation, and the vesicular trafficking system in cells could also be involved in the ROS detoxifying system [101–102].

Moreover, this system is essential in de novo cell wall assembly of new polysaccharides, which are crucial for regulating ion exchange and water movement [103].

Finally, ferritin was upregulated under desiccation, as also reported in the desiccation tolerant bryophyte *Physcomitrella patens* [14] and also in *Zea mays* [104]. This protein is involved in iron storage [105] and has been associated with a cyto-protective antioxidant function [106]. Two ferritin ESTs were previously identified in *P. orbicularis* under desiccation [35]; however, the definite involvement of this protein in seaweeds under environmental stress must be evaluated.

#### 4.5 Conclusion

The present results show that there is a clear response in *P. orbicularis* to desiccation that is expressed by a drastic change in the protein profile. The large number of up-regulated and newly appeared proteins suggests a notable genetic plasticity of this alga to desiccation stress. The mechanisms proposed in this work for DT by *P. orbicularis* are diverse, and Fig. 4 integrally summarizes these mechanisms. A large number of similar and upregulated proteins have also been observed in other land plants and bryophytes, supporting the idea that the presently proposed mechanisms are giving this red alga its DT. Furthermore, this is the first study demonstrating changes at the proteomic level of an algal species under desiccation stress, and, consequently, this data can serve as the basis for future molecular ecophysiology research aimed at fully understanding other physiological processes scarcely studied in macroalgae.

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