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Trehalose and (iso)floridoside production under desiccation stress in red alga *Porphyra umbilicalis* Kützing and the genes involved in their synthesis

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Abstract

The marine red alga *Porphyra umbilicalis* Kützing has high tolerance towards various abiotic stresses. In this study, the contents of floridoside, isofloridoside and trehalose were measured using gas chromatography mass spectrometry (GC-MS) in response to desiccation and rehydration treatments; these conditions are similar to the tidal cycles that *P. umbilicalis* experiences in its natural habitats. The GC-MS analysis showed that the concentration of floridoside and isofloridoside didn't change in response to desiccation as expected of compatible solutes. Genes involved in the synthesis of (iso)floridoside and trehalose were screened from the recently completed *Porphyra* genome, including four putative trehalose-6-phosphate synthase (TPS) genes, two putative trehalose-6-phosphate phosphatase (TPP) genes, and one putative trehalose synthase/amylase (TreS) gene. According to the phylogenetic analysis, conserved domain analysis, and gene expression analysis, we suggest that the *Pum4785* and *Pum5014* genes are related to floridoside and isofloridoside synthesis, respectively, and that the *Pum4637* gene is involved in trehalose synthesis. Our study verifies the occurrences of nanomolar concentrations trehalose in *P. umbilicalis* for the first time and identifies additional genes possibly encoding trehalose phosphate synthases.

Introduction

Porphyra umbilicalis Kützing, a macrophytic red alga of the Bangiales family, grows in a physically stressful habitat, the middle to high intertidal zone. This alga is found from the Northeast Atlantic to the Northwest Atlantic, while its global distribution requires further review (Brodie et al. 2008). *Porphyra* is exposed to daily and seasonally variable temperatures, high levels of irradiance (including UV), and severe osmotic stress and desiccation (Brawley et al. 2017). During desiccation, *Porphyra* uses complex mechanisms at morphological, physiological and molecular levels to maintain homeostasis, to sustain the cellular integrity, repair oxidative stress damage, and to survive the extreme loss of water. Some eukaryotic algae, eubacteria, actinomycetes, fungi and yeast maintain osmotic balance by ion or compatible solute accumulation (Wegmann 1986, Brown 1990).

“Compatible solutes” are low-molecular-weight organic molecules (osmolytes) that can accumulate to high levels in order to balance the external osmotic pressure but which do not inhibit cellular function at high intracellular concentration (Brown 1976). The accumulation of

compatible solutes functions in osmotic balance, acting as thermostabilizing chemical chaperones that help to increase the stability of proteins (Roberts 2005), and as antioxidants (Amor et al. 2005). The most common types of compatible solutes include polyols, sugars, amino acids and their respective derivatives, betaines, ectoines and occasionally some peptides (Galinski 1995). Similar compatible solutes are usually not restricted to specific taxonomic groups (Erdmann & Hagemann 2001). There seems to be some functional specialization of the different classes of compatible solutes, which may contribute to their preferential occurrence in organisms that settle in different niches and are tolerant to adverse environment stresses. Polyols (Reed 1990), trehalose (Karsten et al. 2005), digeneaside (Karsten et al. 2005) and heterosides (Kremer & Vogl 1975; Bondu et al. 2009) have been observed in red algae and have been hypothesized to function as compatible solutes for these organisms.

The heterosides are composed of a sugar linked to a polyol; examples include glucosylglycerol (GG) and galactosylglycerols (floridoside and isofloridoside). Glucosylglycerol is mainly found in cyanobacteria, while galactosylglycerols, floridoside and isofloridoside, are widespread in red algae except the *Ceramiales* (Kirst 1980). Floridoside and isofloridoside are found in several *Porphyra* species, including *P. umbilicalis* (Wiencke & Lauchli, 1981; Karsten et al. 1993; 1999, Meng et al. 1987; 1993). Wiencke & Lauchli (1981) used various concentrations of artificial seawater medium to impose osmotic stress on *P. umbilicalis*; they found that the total amount of floridoside and isofloridoside increased at high hyperosmotic stress, but then decreased with more extreme hyperosmotic pressure. Wjencke & Lauchli (1981) measured the total galactosylglycerols concentration (floridoside+ isofloridoside) under salt stress; they did not measure how floridoside levels changed during desiccation and rehydration.

The disaccharide trehalose can also play a major role in osmotic adjustment by stabilizing membranes and replacing water (Jain and Roy, 2009). Protein stabilizing properties of trehalose are much better than many other compatible solutes because of trehalose's ability to alter the water environment surrounding a protein and stabilize the protein in its native conformation (Kaushik and Bhat 2003, Magazù et al. 2005). High levels of trehalose has been found in some desiccation-tolerant plants, such as the moss *Selaginella lepidophylla* and the resurrection plant *Myrothammus flabellifolius* (Pampurova et al. 2014, Goddijn and Van 1999). Trehalose was found to be not sufficient for desiccation tolerance as a compatible solute in other species, such as yeast (Ratnakumar and Tunnacliffe 2006) and green alga *Klebsormidium* (Kaplan et al., 2012). Surprisingly, trehalose was not detected in *Porphyra* species (Karsten et al. 2007; Craigie 1974) despite the notation of a putative trehalose-6-phosphosphate synthase gene in *Pyropia yezoensis* (Deng et al. 2004) and *P. umbilicalis* (Brawley et al. 2017).

Among five known trehalose biosynthetic pathways, the pathway that involves two enzymatic steps catalyzed by trehalose-6-phosphosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) is the most widely distributed and has been found in eubacteria, archaea, fungi, insects, and plants (Avonce et al. 2006). The transfer of glucose from UDP-glucose to glucose 6-phosphate is catalyzed by TPS and forms trehalose 6-phosphate (T6P) and UDP, while TPP dephosphorylates T6P to trehalose and inorganic phosphate (Elbein et al., 2003). Other pathways are only found in eubacteria and archaea (Avonce et al. 2006, De Smet et al. 2000). According to Pade et al. (2015) the biochemical pathways for the two-step synthesis of trehalose and (iso)floridoside are very similar. In the floridoside synthesis pathway, floridoside phosphate synthase (FPS) first catalyzes the formation of floridoside phosphate from glycerol-3-phosphate (G3P) and UDP-galactose. Then floridoside phosphate is dephosphorylated to floridoside by (iso)floridoside phosphate phosphatase. It is possible that the putative trehalose-6-

phosphosphate synthase gene found in *P. yezoensis* (Deng et al. 2004) and *P. umbilicalis* may not be involved in the synthesis of trehalose but rather in the synthesis of floridoside, as in the red alga *Galdieria sulphuraria* (Pade et al., 2015). The *Porphyra umbilicalis* genome has been previously screened for candidate genes involved in trehalose and (iso)floridoside biosynthesis, but only two of the possible TPS/FPS genes had been identified (Brawley et al. 2017).

In this study, we analyzed the accumulation of floridoside, isofloridoside and trehalose under desiccation and rehydration conditions. Phylogenetic and conserved domain analyses of the putative TPS and FPS genes in *P. umbilicalis* were conducted in order to distinguish the functions of genes that are involved in trehalose, floridoside, and isofloridoside synthesis. Quantitative real-time PCR (qPCR) was used to determine the expression profile of the four TPS/FPS genes at different times before, during and after desiccation in *P. umbilicalis*. Our study represents the first successful attempt to measure trehalose in *P. umbilicalis*. It is also the first examination of how the expression profiles of the TPS/FPS genes change relative to the levels of compatible solutes (trehalose and (iso)floridoside) in *P. umbilicalis* under desiccation stress, which is the stress that the alga encounters twice daily in its intertidal habitat. Furthermore, our results identify probable genes in *P. umbilicalis* that are involved in trehalose and (iso)floridoside synthesis.

Material and Methods

Sample preparation

Porphyra umbilicalis samples were collected from an open coastal population at Fort Stark, NH (43°03'30.3"N 70°42'43.0"W) and brought to the lab for species confirmation by comparison to rbcL-rbcS intergenic spacer using methods described in Teasdale et al. (2002). Tissues that were confirmed to be *P. umbilicalis* were cultured under lab conditions at 15°C according to Redmond et al. (2014) for more than 6 hours before exposure to different treatments. The six treatments, each with four replications, were: hydrated, air-dried for half an hour, air-dried for one hour, air-dried for two hours, air-dried for three hours, and rehydrated with autoclaved seawater from Fort Stark, NH for half hour after air-drying for three hours. Each “individual” (each defined as thalli connected to a single holdfast) was used as one replication for a treatment. Wet tissues were blotted with a soft paper towel to remove any free surface moisture. They were then weighed immediately to determine the initial fresh weight in order to calculate trehalose and (iso)floridoside contents after various treatments. The initial fresh weight for each sample was around 0.5g.

Analysis of low-molecular-mass organic solutes

After samples were subjected to their various treatments, they were put into 5 ml 80% ethanol. Internal standards, sorbitol and inositol, were added into ethanol solution for subsequent gas chromatography mass spectrometry (GC-MS) analysis. The samples were then ultrasonic treated at 70°C in 80% ethanol for two hours in order to extract low-molecular-mass organic solutes. The extracts were centrifuged at 10,000 RMP for 10 min and the supernatants were collected to dry in a Speedvac Concentrator at -4°C. The concentrated material was then re-suspended in deionized water (HPLC grade), and centrifuged at 10,000 RMP for 10 min. A total of 200 µl of the supernatants were collected and then dried for further derivatization. The derivatization of samples was performed by Tri-Sil HTP (HDMS:TMCS:Pyridine) Reagent (ThermoFisher Scientific, USA) according to the manufacturer’s protocol. After

dying the samples in nitrogen gas, they were dissolved in 2ml chloroform and injected into GC-MS for further analysis.

Gas chromatography mass spectrometry analysis was performed using a Trace GC Ultra system (FINNIGAN) comprising a Tri Plus auto-sampler. For GC-MS detection, helium gas was used as a carrier gas at a constant flow rate of 1.0 ml min⁻¹, and an injection volume of 1 µl was employed (splitless injection). The injector and ion source temperature were maintained at 260°C and 200°C, respectively. The oven temperature was programmed for 100°C with an increase of 30°C min⁻¹ to 160°C, isothermal for 2 min, and then increased to 280°C with 10°C min⁻¹, ending with a 9-min isothermal step at 280°C. Mass spectra were taken in a full scan mode and for fragments from 50 to 650 amu. The mass detector used was a PolarisQ (FINNIGAN), Xcalibur™ software (ThermoFisher Scientific, USA) was applied to handle mass spectra and chromatogram results.

Phylogenetic analysis and conserved domain analysis

Representative protein sequences involved in trehalose synthesis in eubacteria, plants, fungi and animals (Avonce et al. 2006) and (iso)floridoside synthesis in *Galdieria sulphuraria* (Pade et al. 2015) were downloaded from the NCBI database. The protein sequences were used to identify putative homologous genes in comparison of all versions of the *Porphyra umbilicalis* genome using TBLASTN with a E-value threshold of 10⁻¹⁰. Nucleotide sequences predicted to encode TPS/TPP and FPS proteins were used as queries for BLASTX searches against protein sequences from the *P. umbilicalis* genome. The final version of *P. umbilicalis* genome can be accessed in NCBI (P_umbilicalis_V1) and all other draft versions of the *P. umbilicalis* genome can be accessed at <http://porphyra.rutgers.edu/bindex.php>.

A phylogenetic tree was built using functionally characterized protein sequences for TPS enzymes from a wide range of species (Avonce et al. 2006), FPS enzymes from *Galdieria sulphuraria* (Pade et al. 2015) and glucosylglycerol-phosphate synthase (GgpS) from cyanobacteria. The complete list of sequences used is provided in supplemental material 1. A total of 63 amino acid sequences were aligned using MUSCLE (Edgar 2004) and were edited with Jalview (Waterhouse et al., 2009). The best-fitting evolutionary model for the alignment was evaluated in ProtTest (Darriba et al. 2011). Maximum-likelihood trees were constructed in RAxML (Stamatakis, 2014) using WAG model with 1000 bootstraps. Graphical representation and editing of the phylogenetic tree were performed with Dendroscope 3 (Huson and Scornavacca, 2012).

The protein sequences of the four putative TPS/FPS enzymes and two putative TPP enzymes were analyzed in Pfam to identify protein domain families. The protein sequences were subsequently aligned with the trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB) protein sequences of *Escherichia coli* (Gibson et al. 2002, Rao et al. 2006) using MUSCLE (Edgar, 2004) in order to identify if the amino acids important for the binding of substrates and to determine whether the catalytic residues were conserved.

RNA isolation, cDNA synthesis and quantitative RT-PCR

Tissues were subjected to different treatments and then were ground in liquid nitrogen to a fine powder. Total RNAs were extracted according to Eriksen (2015). To remove DNA, the extracts were treated with RNase-free ezDNase™ Enzyme (ThermoFisher Scientific, USA). The quality and quantity of the RNA were checked on 1% agarose gel and with a Nanodrop (DeNovix DS-11). The amount of total RNA was adjusted to be 1 ng per sample.

DNA-free RNA was reverse-transcribed into cDNA by SuperScript™ IV Reverse Transcriptase (ThermoFisher Scientific, USA) according to the manufacturer's protocol. One of the actin genes was previously demonstrated as a good endogenous standard for relative RT-PCR under stress conditions in red algae *Pyropia* (Li et al. 2014, Kong et al. 2015), so it was used in our study as an endogenous standard in *Porphyra umbilicalis* under desiccation stress. Gene-specific primers for actin and the *TPS/FPS* genes are shown in Table 1. The expression levels were determined with QuantStudio™ 3 Real-Time system (ThermoFisher Scientific, USA) and PowerUP™ SYBR® Green Master Mix (ThermoFisher Scientific, USA). Quantitative PCR was performed in a total reaction volume of 10 μ L using PCR cycling of 50°C for 2 min and 95 °C for 2 min; followed by 40 cycles of 95 °C for 1 sec and 60 °C 30 sec. The melt curve was generated at 95°C for 15 secs with a ramp rate of 1.6°C/sec, followed by 60°C for 1 min with ramp rate of 16°C/sec, and then ended with 95°C for 15 secs with ramp rate of 0.15°C/sec. Six biological replications for each time point were measured and reactions for each gene at each time point was performed in triplicate. Relative quantifications were performed following $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Cycle threshold values generated from the reactions were averaged. The expressions levels for different genes were normalized by subtraction of their average cycle threshold values from the mean of the control housekeeping gene. The relative level of expression changes at different time-point were normalized to that of the fresh hydrated time point.

Table 1. Primers used for the expression analysis of four putative *TPS/FPS* genes

Name	Sequence (from 5' to 3')	fragment length (bp)
Pum4785_qPCR_fw	CCGTTACCCTCCCTTCGTC	96
Pum4785_qPCR_rev	TACCAAGCGAGCCGTAATCA	
Pum5014_qPCR_fw	GTGAACGACGGGGAGTTTG	106
Pum5014_qPCR_rev	TGACCAAGCTCTCGATGGAA	
Pum8501_qPCR_fw	CCATGTGGTGCTCGTACAGA	139
Pum8501_qPCR_rev	GTAGTGGATGGGCATGTTCGT	
Pum4637_qPCR_fw	CAATGCGGAACAAGGTGCTG	62
Pum4637_qPCR_rev	GTACGTGTGGAAGCCGATCA	
Actin_qPCR_fw	GGGTACAGCTTCACGACGT	135
Actin_qPCR_rev	TTCGTA CTCTTCTCCAGCG	

Results

Floridoside, isofloridoside and trehalose accumulation under desiccation and rehydration conditions

The changes of floridoside, isofloridoside and trehalose content during desiccation and rehydration treatments are shown in Figure 1. Before desiccation treatment, the levels of floridoside and isofloridoside in *Porphyra umbilicalis* were similar (40-50 μ mol/g F.W.), with isofloridoside content being slightly higher than that of floridoside. However, the floridoside and isofloridoside contents were more than five thousand times higher than trehalose content (5-8 nmol/g F.W.) before desiccation. For floridoside content, there was a slight decrease from hydrated control to half hour dehydration treatment ($P=0.086$), and from 2-hour dehydration to

3-hour dehydration treatment ($P=0.079$). Also a sudden increase occurred from 3-hour dehydration to rehydration treatment ($P=0.098$). Isofloridoside content did not change significantly for all treatment groups ($P>0.1$; Figure 2b). Trehalose content continued to increase during drying, and then its level dropped a little bit after long-term desiccation treatment and rehydration treatment.

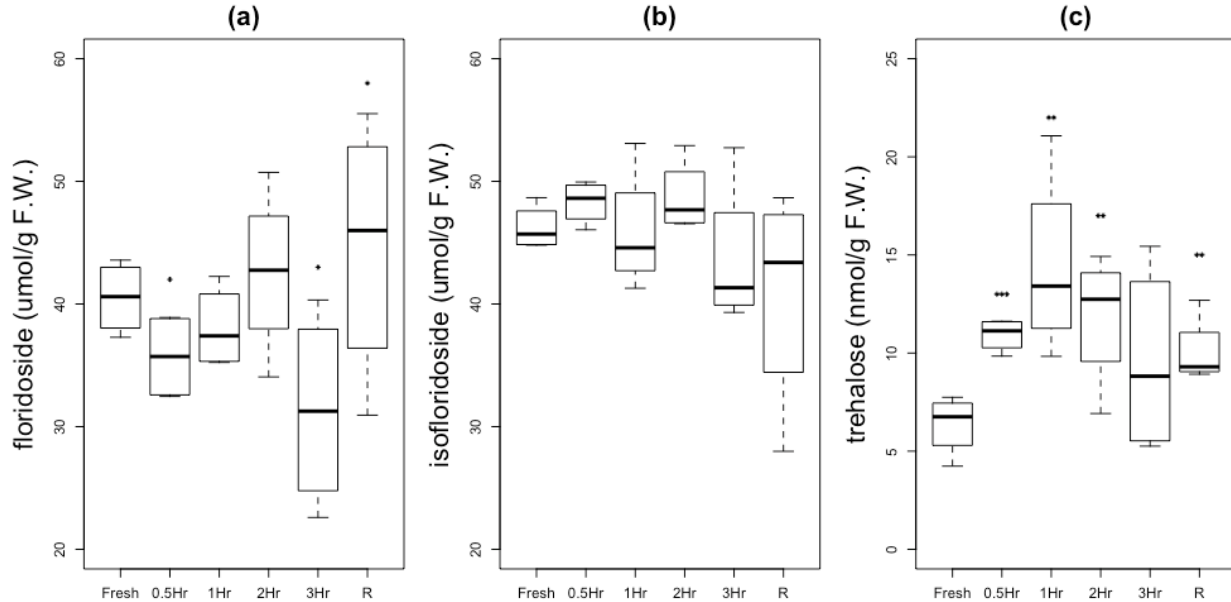


Figure 1. Floridoside, isofloridoside and trehalose content under drying or rehydration. The six time points are as follows: (1) fresh hydrated; (2) half-hour drying effect; (3) one-hour drying effect; (4) two-hour drying effect; (5) three-hour desiccation state; (6) half hour rehydration after three hours of desiccation. (* $P<0.1$ ** $P<0.05$ *** <0.01)

Identification of proteins involved in trehalose and (iso)floridoside synthesis in *P. umbilicalis*

The TBLASTN search detected four proteins that were homologous to TPS, FPS or GgpS proteins from a wide range of species with significant E-values ($<10^{-10}$), one protein that has high similarity to trehalose synthase/amylase (TreS) in eubacteria with E-value of $2e^{-16}$, and two proteins that had significant similarity to TPP proteins. The gene information is given in Table 2 and the predicted protein sequences for each putative gene are provided in the supplement material 2. Protein Pum4785 and Pum5014 correspond to protein ID Bu14_0021s0026 and BU14_0082s0055 from GenBank. Gene *Pum8501* was present in the final version of the *Porphyra* genome; however, its protein prediction was missing from the final genome model.

Table 2: Gene and protein information for the putative TPS/FPS, TreS and TPP enzymes in *P. umbilicalis*

	Gene name	Protein name	Genome assembly Version	Scaffold
	<i>Pum4637</i>	Pum4637	Assembly V_0.77	None

<i>TPS</i> gene family	<i>Pum8501</i>	Pum8501	Final version	483
	<i>Pum4785</i>	Pum4785 (Bu14_0021s0026)	Final version	21
	<i>Pum5014</i>	Pum5014 (BU14_0082s0055)	Final version	82
	<i>TreS</i>	TreS (BU14_0165s0020)	Final version	165
	<i>TPP1</i>	TPP1 (BU14_0615S0010)	Final version	615
	<i>TPP2</i>	TPP2 (BU14_0615S0014)	Final version	615

* There is no scaffold information for an earlier version of the *P. umbilicalis* genome.

* The nomenclatures inside the parentheses correspond to the protein IDs listed in GenBank.

* The genes *Pum4637*, *Pum8501*, *Pum4785*, *Pum5014* are all putative TPS family genes.

The protein family search using Pfam showed that all four putative TPS proteins contain a glucosyltransferase family 20 (GT20) and phosphatase domain(s), and the two putative TPP proteins possess phosphate phosphatase (PPase) family domains with predicted trehalose-phosphatase functions (Figure 2). However, the alignment of the four predicted TPS proteins of *Porphyra umbilicalis* with the trehalose-6-phosphate synthase OtsA of *Escherichia coli* revealed that only protein *Pum4637* contains the conserved motif important for binding of substrates and for catalysis among the four putative TPS proteins (Figure 2). The TPS and TPP proteins were also aligned with the trehalose-6-phosphate phosphatase OtsB protein sequences of *Escherichia coli*. Although all TPS and TPP protein sequences contains complete or partial trehalose PPase family according to Pfam, only *Pum5014*, *Pum4738* and BU14_0615S0010 contained the highly conserved residues of the trehalose PPase family active site in their three respective TPP conserved regions (Figure 2) (Rao et al. 2006). The protein alignment of the four *P. umbilicalis* TPS proteins with OtsA of *E. coli* (UniProtKB/Swiss-Prot: P31677.3) are provided in Supplement 3, Figure 1 and the protein alignment of the four *P. umbilicalis* TPS proteins and two TPP proteins with OtsB of *Escherichia coli* (UniProtKB/Swiss-Prot: P31678.2) are provided in Supplement 3, Figure 2 and Figure 3.

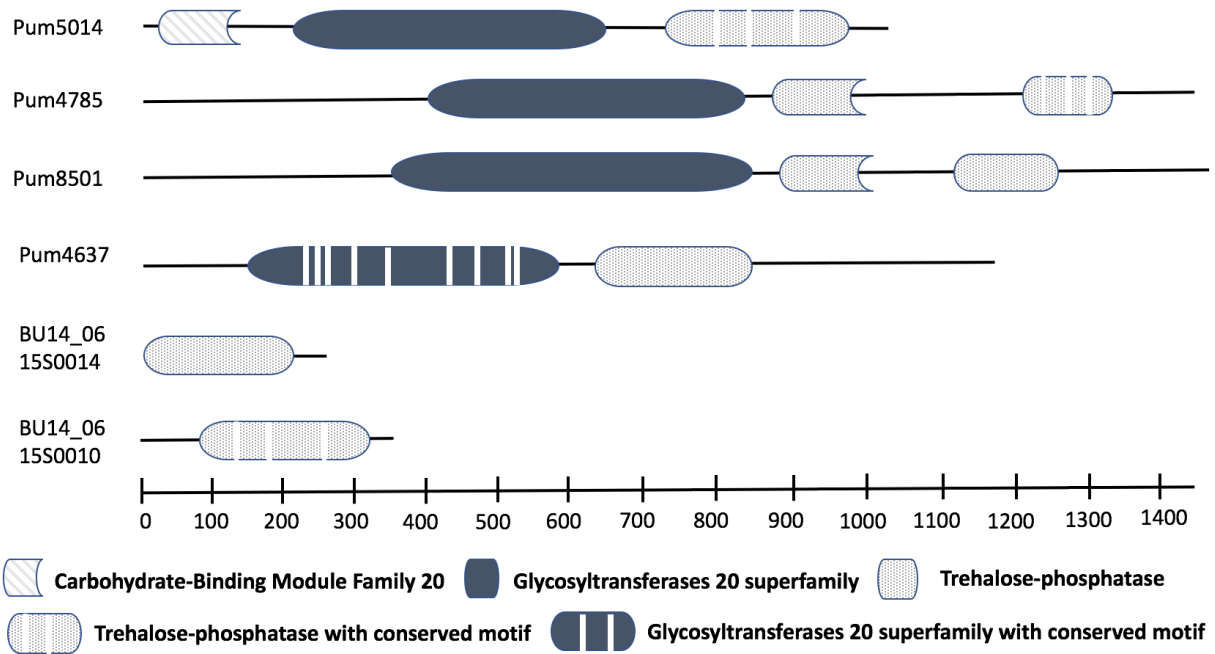


Figure 2. Results from the conserved domain search (CD-Search) for the TPP/FPS and TPP genes in *P. umbilicalis*. The domains with conserved catalytic amino acids required for the TPS or TPP activity contained white stripes.

We constructed the phylogenetic tree of 63 TPS proteins from a wide range of species, plus FPS proteins from *Galdieria sulphuraria* and GgpS proteins from cyanobacteria (Figure 3). In red algae, there were six, four and three putative TPS/FPS proteins in *Pyropia haitanensis*, in *Chondrus crispus*, and in *Cyanidioschyzon merolae*, respectively. The red alga *Pyropia yezoensis* and the brown algae *Undaria pinnatifida* and *Sargassum henslowianum* only had one putative TPS/FPS protein.

The phylogenetic tree (Figure 3) shows two distinct clades. The first clade comprises enzymes for glucosylglycerol synthesis from cyanobacteria, whereas the other clade harbors enzymes for trehalose and floridoside synthesis from prokaryotes and eukaryotes. The second clade further divides into two subclades and *Mycobacterium tuberculosis* outlier. The first subclade contains TPS proteins from nematodes and bacteria, and the second subclade contains all four putative TPS proteins from *Porphyra umbilicalis*. The four putative TPS proteins in *P. umbilicalis* cluster into 4 different groups. Pum4785 clusters with three other red algal TPS/FPS proteins, including *Galdieria sulphuraria*_Gasu26940 (bootstrap 100); found adjacently, with very low bootstraps, are TPS family proteins from Amoebozoa and Fungi. Pum5014 clustered with red and brown algae (bootstrap 100) adjacent to a cluster containing *G. sulphuraria*_10960 and unicellular red alga *Cyanidioschyzon* (bootstrap 100). Enzymes Gasu_10960 and Gasu_26940 have been biochemically characterized and are responsible for isofloridoside phosphate synthase/phosphatase and the floridoside phosphate synthase/phosphatase in *Galdieria sulphuraria*. Pum8501 and Pum4637 each clustered separately with red algal TPS proteins (bootstrap 99). The putative TPS/FPS proteins in *Chondrus crispus* and in *Porphyra haitanensis* were also separated into the same 4 clusters as the *P. umbilicalis* proteins. ~~TPS/FPS proteins~~

from yeast, cyanobacteria, nematodes and bacteria clustered with those from plants and algae with low bootstrap value, suggesting their related relationship.

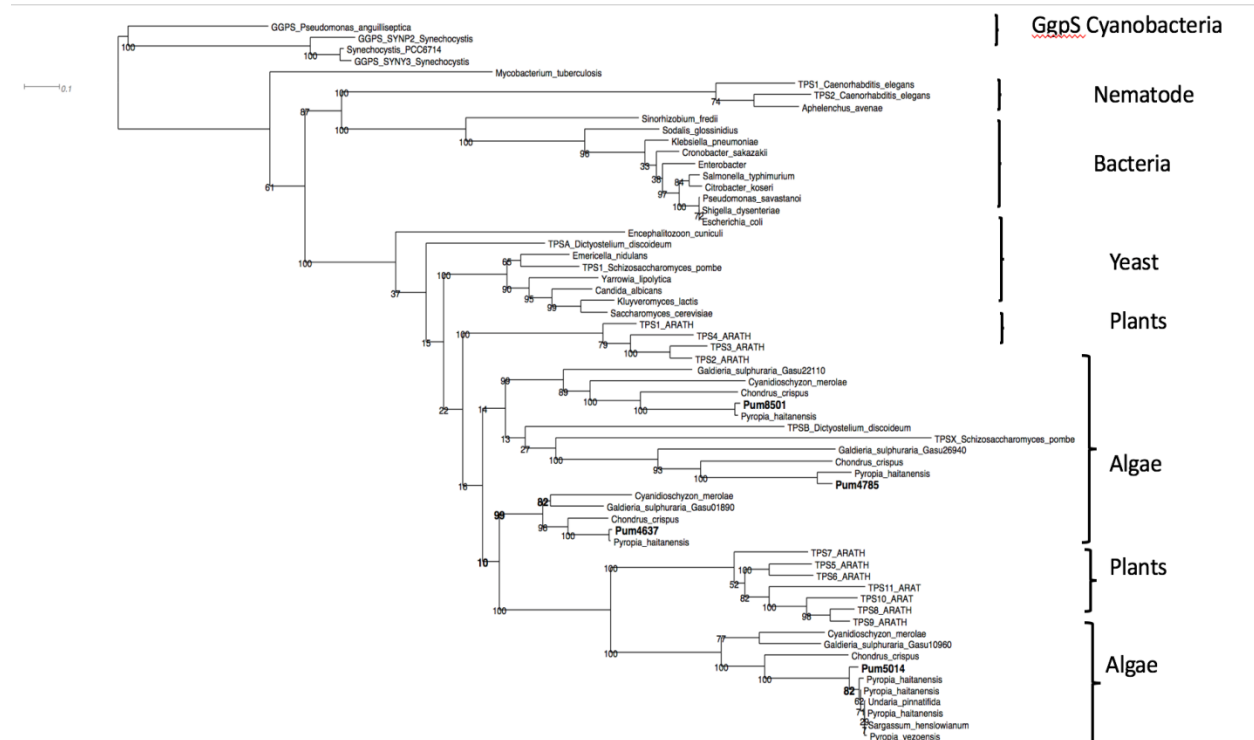


Figure 3. Unrooted phylogenetic tree of amino acid sequences from putative trehalose 6 phosphate synthases and putative (iso)floridoside phosphate synthases using maximum likelihood algorithm. The scale is shown in the upper left-hand corner and bootstrap values are shown on the nodes. The proteins of interest from *Porphyra umbilicalis* are in bold. The cutoff for the bootstraps is 70.

The expression of the four *TPS/FPS* genes were analyzed in *Porphyra umbilicalis* under different levels of desiccation and rehydration by qRT-PCR (Figure 4). Similar expression patterns were found for *Pum4785* and *Pum8501*, their expression levels did not change significantly during the gradual desiccation up to two hours; however, the expression levels of *Pum4785* and *Pum8501* dropped significantly after long-term desiccation and rehydration. The most significant increase in expression was found in *Pum4637* that reached a peak of about 3.5 fold at 2h drying in comparison with the fresh control. Its expression level decreased during long-term desiccation (3Hr) and after rehydration. Expression levels of *Pum5014* did not correlate with either desiccation or rehydration.

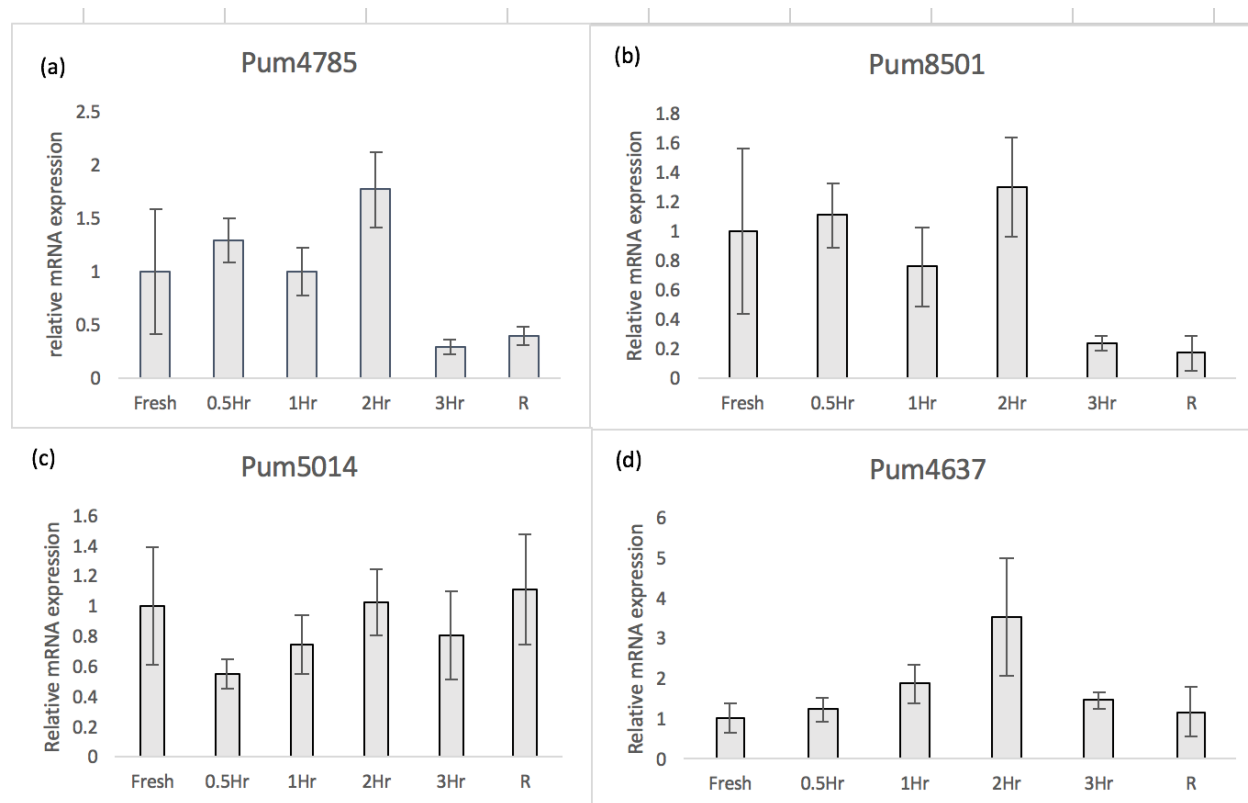


Figure 4. Relative expression of the putative trehalose-6-phosphate synthase genes in *Porphyra umbilicalis* under drying or rehydration: (a) Pum4785; (b) Pum8501; (c) Pum5014; (d) Pum4637. The six time points are as follows: (1) fresh hydrated; (2) half-hour drying; (3) one-hour drying; (4) two-hour drying; (5) three-hour desiccation state; (6) half hour rehydration after three hours of desiccation. The constitutively expressed actin gene served as a control gene and the expression level of each gene was normalized by reference to the constitutively expressed actin gene; relative level of expression changes were normalized to that of the fresh hydrated time point. Error bars represent standard deviation.

Discussion

Porphyra umbilicalis is a middle to high intertidal marine red alga, which is exposed to desiccation stress twice a day with the changing of tides, and simultaneously to many other abiotic stresses. The mechanism(s) of high tolerance to water loss in *P. umbilicalis* is still unclear. Desiccation and salt stress are different types of water deprivation regarding the physiological process of ion uptake and ion ratios (Kumari et al., 2014, Munns, 2002). Under hypersaline conditions, seaweed cells are still in full contact with liquid water of decreased water potential, while desiccation leads to more intense cellular dehydration (Holzinger and Karsten, 2013). So it is not easy to distinguish between the effects of osmotic pressure and toxicity due to increased concentrations of Na^+ and Cl^- ions (Hirasawa et al. 2006). In this study, we measured the levels of (iso)floridoside and trehalose in *P. umbilicalis* under desiccation and rehydration conditions.

(Iso)floridoside and trehalose content

The contents of floridoside and isofloridoside were both quantifiable before *Porphyra umbilicalis* was osmotically stressed, which agrees with the conclusions from previous studies that these heterosides function as the alga's major carbon reservoir (Li et al. 2001). The content of floridoside and isofloridoside are around 40~50 $\mu\text{mol/g}$ of fresh weight (fully hydrated). Our measurements of combined floridoside and isofloridoside are in the same order of magnitude as those measured by Wiencke and Lauchli (1981), who showed that the combined floridoside and isofloridoside are at around 100 $\mu\text{mol/g}$ fresh wt^{-1} . However, the level of floridoside in *P. umbilicalis* was almost ten times higher than that reported for *Pyropia haitanensis* (1140 +70 $\mu\text{g/g}$ fresh $\text{wt}^{-1} \approx 4.5 \mu\text{mol/g}$ fresh wt^{-1}), while the level of isofloridoside in *P. umbilicalis* was similar to that in *Pyropia haitanensis* (9260 + 1190 $\mu\text{g/g}$ fresh $\text{wt}^{-1} \approx 36 \mu\text{mol/g}$ fresh wt^{-1} , measured by Chen et al. 2014). The different amounts of floridoside may be due to species or treatments, as there was no mention of how *P. haitanensis* was treated before the measurement of floridoside.

Our experiments showed that the floridoside content in *Porphyra umbilicalis* decreased slightly after short-term dehydration treatment and then dropped again after long-term desiccation, while floridoside levels increased significantly after rehydration treatment. The observed decline of floridoside content after dehydration was consistent with a previous study of *Pyropia perforata* (another high intertidal red alga) which showed that the highest floridoside content was observed at noon when *P. perforata* was submerged for many hours (Meng and Srivastava, 1993) and dropped gradually after exposed to air. In *P. umbilicalis*, the combined floridoside and isofloridoside levels almost doubled under salinity stress (Wiencke and Lauchli, 1981). Similar patterns of increasing floridoside content under salinity stress has also been reported for *Galdieria sulphuraria* (Martinez-Garcia and Maarel, 2016), *Pyropia columbina* (Karsten et al. 1993) and *Porphyra purpurea* (Reed et al. 1980). The different amounts of floridoside and isofloridoside produced in *P. umbilicalis* during desiccation and salt stress suggests that *P. umbilicalis* may have different strategies for coping with desiccation versus salt stress. Lippert and Galinski (1992) suggest that the degree of protection against heating, freezing and drying by dissimilar compatible solutes is different, perhaps because (iso)floridoside has a less protective effect towards desiccation than salt stress, as demonstrated for other compatible solutes like glycine betaine (Sakamoto and Murata, 2002). Reed et al. (1980) and Martinez-Garcia and Maarel (2016) showed that increased CaCl_2 did not change floridoside content in *P. purpurea* and in *G. sulphuraria*, respectively. However, the results from Reed et al. (1980) and Martinez-Garcia and Maarel (2016) contrasted on the floridoside content responses to KCl. Based on the consistent levels of floridoside under desiccation and CaCl_2 stress, and the increase of floridoside levels under salt stress, we postulate that floridoside content maybe related to specific ion(s) or to ionic ratios, possibly NaCl-induced K^+ efflux, as desiccation stress does not induce massive K^+ efflux from the cell (Shabala et al. 2006). Floridoside is hypothesized to be the main soluble photosynthesis product (Bondu et al. 2009) and acts as a dynamic carbon pool (Li et al. 2001) in red algae, which can be converted to polysaccharides and other end products. In this study, floridoside behaved like a typical reserve compound that was accumulated when exogenous resources and conditions were good (upon rehydration) and was consumed when water availability was low, unlike a typical stress metabolite (Baud et al. 2008).

The ability for the photosynthetic apparatus to recover from water loss after rehydration, not the ability to avoid desiccation, is the key factor for desiccation-tolerant species (Kumari et al., 2014). The floridoside level increased significantly after rehydration, which suggested that floridoside may either play an important role in the recovery process from desiccation or may be

a by-product of the rehydration process. Hagemann (2016) pointed out the function of high amounts of compatible solutes is to make the cytoplasm hyperosmotic toward the external medium ensuring water uptake and positive turgor. Increased floridoside after rehydration may help the cell uptake water and recover from desiccation.

Isofloridoside is considered as one of the predominant heterosides involved in osmotic acclimation. Its content remained constant in all treatments in our study, which agrees with previous research on *Porphyra purpurea* that was exposed to salt stress (Reed et al. 1980), but differs with the increased isofloridoside levels in *Pyropia columbina* under salt stress (Karsten et al. 1993). The different changes in isofloridoside content reacting to salt stress in different *Porphyra* species (sensu lato) suggest that the mechanisms that control isofloridoside levels may vary between species.

Our work is the first time that trehalose has been quantified in *Porphyra*. Trehalose was not detected previously in *Porphyra* species using Nuclear Magnetic Resonance, regardless of the high desiccation tolerance of most of the *Porphyra* species (Karsten et al. 2007). However, we were successful in using the more sensitive Gas Chromatography Mass Spectrometry (GC-MS) to detect trehalose in *Porphyra umbilicalis*. In a large range of organisms such as bacteria, fungi, nematodes and invertebrates, trehalose accumulates to mM levels, serving as a reserve carbohydrate and stress protectant; in contrast, in most higher plants, only minute amounts can be detected (Wiemken 1990; Goddijn *et al.* 1997; Crowe, Carpenter & Crowe 1998). It has been shown that disaccharides preserve the structural integrity of membranes in the dried state by substituting for the structural water hydrogen bonded to the phospholipid headgroups (Welsh, 2000). The small amount of trehalose found in *P. umbilicalis* (5-20 nmol/g fresh weight) is similar to its level in a lot of algae and Cyanobacteria (Kaplan et al., 2012, Reed et al. 1984). Its content remained too low both before and after desiccation treatment to function as compatible solute. However, the amount of trehalose almost doubled after drying; this suggests that trehalose or its precursor (trehalose-6-phosphate) may be involved in osmotic regulation and might function as a signaling molecule under dehydration stress as in yeast and plants (Elbein et al. 2003; Argüelles, 2000). The increase of trehalose corresponded to the decrease of floridoside, which is similar to the relationship between glucosylglycerol (GG) and trehalose in cyanobacteria. Mikkat et al. (1996) showed that synthesis of GG was repressed by trehalose and that trehalose uptake caused a decrease in the content of previously synthesized GG in cyanobacteria, which is due to trehalose and GG being transported by the same active transport system.

Identifying putative functions of four TPS like genes in *P. umbilicalis*

Avonce et al. (2006) proposed that eukaryotes have only the TPS/TPP pathway while several eubacterial species have multiple pathways. Using TBLASTN search of the *Porphyra umbilicalis* genome, a total of four putative TPSs, two putative TPPs and one putative TreS genes were identified in the genome of *P. umbilicalis* (Brawley et al. 2017). The putative trehalose synthase/amylase protein (TreS, BU14_0165s0020) found in the *P. umbilicalis* genome has previously only been found in eubacteria and archaea (Avonce et al. 2006), suggesting that this trehalose synthase/amylase gene may have resulted from horizontal gene transfer from eubacteria or archaea. Since the putative trehalose synthase/amylase protein (BU14_0165s0020) was found on scaffold 165 in *P. umbilicalis* genome and has RNA support, it is unlikely that this putative trehalose synthase/amylase gene resulted from contamination of the *P. umbilicalis* genome.

A total of four putative TPS/FPS proteins were identified in *Porphyra umbilicalis*, which was the same as the number of putative TPS/FPS proteins identified in *Chondrus crispus* (Collén et al. 2013). These four putative TPS/FPS found in *P. umbilicalis* and *C. crispus* were separated into four clusters (Figure 3). Although there were six putative TPS/FPS proteins found in *P. haitanensis*, based on our phylogenetic tree, they are found in the same four clusters as in *P. umbilicalis* and in *C. crispus*. The two additional TPS/FPS proteins may be due to recent gene duplication events in *P. haitanensis*. The four putative TPS proteins in *P. umbilicalis* were annotated based on their sequence similarity to TPS protein sequences in eubacteria, archaea, fungi, metazoan and plants (Avonce et al. 2006), which all possess glucosyltransferase (GT20) and phosphatase domain(s) (Figure 2). However, some of these proteins may be involved in (iso)floridoside metabolism in red algae because of the similarity of biochemical synthetic pathways and the physical structure similarities between trehalose and (iso)floridoside (Hagemann and Pade 2015, Brawley et al. 2017). The amino acid sequence similarity between TPS and FPS and lack of experimental biochemical evidence of TPS/FPS activities could explain the automatic annotation of only TPS rather than TPS or FPS proteins in red algae.

The ¹⁴C labelling experiments conducted by Craigie et al (1968) suggested floridoside and isofloridoside are non-equivalent as metabolites in the Rhodophyta despite their structural similarities. Pade et al. (2015) demonstrated experimentally that the *Gasu_10960* gene codes for isofloridoside phosphate synthase/phosphatase enzyme and the *Gasu_26940* gene codes for floridoside phosphate synthase/phosphatase enzyme in *Galdieria sulphuraria*. Among the four putative TPS/FPS proteins, *Pum5014*, which clusters with *Gasu_10960*, is probably involved in isofloridoside synthesis; and *Pum4785*, which clusters with *Gasu_26940*, is related to floridoside synthesis (see Figure 3). According to the results from the gene expression analysis (Figure 4), the mostly stable expression of the *Pum5014* gene corresponded to the unchanged isofloridoside content under desiccation and rehydration status, except for the minor down-regulation after short-term desiccation. The expression of the *Pum4785* gene had a minor increase right after short-term desiccation, while expression dropped after long-term desiccation and did not increase after rehydration. The decline of floridoside under long-term desiccation corresponded to the reduced expression of the *Pum4785* gene. However, the minor drop of floridoside content after short-term desiccation and the significant increase after rehydration treatment cannot be explained by the expression of *Pum4785* alone. An inconsistency between transcriptional expression and floridoside content has also been found in *G. sulphuraria* under high salt condition. Pade et al. (2015) suggested that biochemical activation of the synthetic enzyme(s) probably regulates stress-proportional floridoside accumulation rather than transcriptional regulation.

The other two putative TPS proteins (Figure 3), *Pum4637* and *Pum8501*, are candidates to have TPS functions involved in trehalose synthesis. Unicellular organisms and vertebrates typically possess single copy of gene encoding trehalose biosynthesis enzymes, while terrestrial plant genomes contain a remarkably larger number of homologs putatively involved in trehalose metabolism (Vandesteene et al., 2010). However, usually only one protein among the numbers of homologs possess TPS function. In *Arabidopsis* (to date), only *TPS1* among the 11 putative *TPS* genes has been shown to have TPS activity (Blazquez et al. 1998). In *Arabidopsis*, the *TPS1* protein contains a TPS domain at its amino-terminus and a fused putative TPP domain at its carboxyterminus (Eastmond and Graham, 2003); however, the TPP domain lacks consensus sequences that are conserved in phosphatases and has no demonstrated function (Zentella et al. 1999). Comparison with the reported 3D structure of the *Escherichia coli* TPS enzyme (PDB:

1GZ5) allowed identification of the predicted amino acid residues functioning in substrate binding and catalysis (Gibson et al. 2002). All residues that are involved in the binding of glucose 6-phosphate (Arg9, Trp40, Tyr76, Trp85 and Arg300) and in the binding of UDP-glucose (Gly22, Asp130, His154, Arg262, Asp361 and Glu369) (Gibson et al. 2002) are conserved only in *Pum4637* among the four putative *P. umbilicalis* TPS proteins. Avonce et al. (2006) showed that all the residues mentioned above are conserved in proteins displaying TPS activity, or in proteins from organisms that are known to produce trehalose. Proteins without conserved residues from *Arabidopsis* and yeast did not complement a $\Delta TPS1$ mutant of *Saccharomyces cerevisiae* (Vogel et al. 2001, Bell et al. 1998), and do not possess TPS activity. The expression of *Pum4637* was up-regulated in response to desiccation, which also corresponded to the increase of trehalose content under desiccation, thus we propose that *Pum4637* is the most promising candidate for TPS enzyme catalyzing the trehalose synthesis. The up-regulation of *Pum4637* was consistent with TPS up-regulation in wide range of species, such as bacteria *Bradyrhizobium japonicum* (Cytryn et al. 2007) and higher plants (Junior et al. 2013, Xu et al. 2017, Jiang et al. 2010; Nepomuceno et al. 2002) under different stresses. The up-regulation of the *Pum4637* gene reached a peak of about 3.5-fold at 2h drying, which did not correspond to the changes of trehalose that peaked at half-hour drying and stayed almost stable during desiccation process and rehydration. This may be explained by the regulation of the intracellular concentration of trehalose through hydrolysis by trehalase (Brawley et al. 2017).

Trehalose-6-phosphate phosphatase, which belongs to the HAD (L-2-haloacid dehalogenase) superfamily of magnesium-dependent phosphatases/phosphotransferases, has three highly conserved motifs (Avonce et al. 2006, Farelli et al. 2014). TPP domains in TPS proteins usually lack consensus sequences that are conserved in phosphatases and are unknown for function (Zentella et al. 1999). This is partially consistent with what we found in our study. The two putative TPS-like proteins (*Pum8501* and *Pum4637*; Figure 2) in *P. umbilicalis* lack conserved TPP domains, while TPP domains are conserved in the two putative FPS like proteins (*Pum5014* and *Pum4785*; Figure 2). In higher plants, such as *Arabidopsis* (Vogel et al. 1998), TPS domains are usually independent from TPP proteins. Although Pfam identified TPP signatures in *P. umbilicalis*; the sequences of BU14_0615S0014, *Pum8501* and *Pum4637* proteins lack some of the conserved motifs, suggesting that it is unlikely that these proteins have TPP activity. The lack of conserved TPP domains in *Pum8501* and *Pum4637* protein sequences is similar to those of the AtTPS1-4 subfamily in *Arabidopsis*, where only the TPS or TPS-similar domains are present and TPP domains are absent (Leyman et al., 2001). Protein BU14_0615S0014 sequence was conserved in all 3 regions except for missing the key amino acids near the N-terminal end (DXDX sequence). The first aspartate residue forms a phosphorylated intermediate with the substrate, while the second aspartate residue plays an important role in catalysis (Farelli et al. 2014). They showed that replacement of the catalytic DXDX resulted in the loss of all detectable activity of TPP. This leaves BU14_0615S0014 as the most probable candidate with TPP activity to convert trehalose-6-phosphate to trehalose.

In conclusion, a total of seven genes that are involved in (iso)floridoside and trehalose synthesis have been found in the *P. umbilicalis* genome. Based on phylogenetic analysis, *Pum4785* and *Pum5014* genes are probably involved in floridoside and isofloridoside synthesis. Gene *Pum4637* is probably related to trehalose-6-phosphatase synthase as it has all the conserved amino acid residues important for the binding of glucose 6-phosphate and in the binding of UDP-glucose for TPS. The enzyme function for *Pum8501* gene is still unclear. The trehalose synthase/amylase gene (*BU14_0165s0020*) in the *P. umbilicalis* genome may have resulted from

horizontal gene transfer from eubacteria or archaea. Among the two predicted TPP proteins found in *P. umbilicalis*, only BU14_0615S0010 is likely to function as a TPP while the other TPP-like protein BU14_0615S0014 did not possess all the conserved motifs important for TPP activity.

This is the first time that trehalose has been verified to occur in *P. umbilicalis*. However, the trehalose concentration was too low to be considered as a compatible solute in *P. umbilicalis*. Floridoside and isofloridoside concentrations were measured in *P. umbilicalis* undergoing desiccation and rehydration. The content of isofloridoside did not change dramatically during desiccation and rehydration processes, while the level of floridoside decreased during desiccation and then increased after rehydration, which suggests that neither floridoside nor isofloridoside act as compatible solutes in *P. umbilicalis* under desiccation stress.

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