



Research article

Relation between water status and desiccation-affected genes in the lichen photobiont *Trebouxia gelatinosa*

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ABSTRACT

The relation between water status and expression profiles of desiccation-related genes has been studied in the desiccation tolerant (DT) aeroterrestrial green microalga *Trebouxia gelatinosa*, a common lichen photobiont. Algal colonies were desiccated in controlled conditions and during desiccation water content (WC) and water potential (Ψ) were measured to find the turgor loss point (Ψ_{tlp}). Quantitative real-time PCR was performed to measure the expression of ten genes related to photosynthesis, antioxidant defense, expansins, heat shock proteins (HSPs), and desiccation related proteins in algal colonies collected during desiccation when still at full turgor ($\text{WC} > 6 \text{ g H}_2\text{O g}^{-1}$ dry weight), immediately before and after Ψ_{tlp} (-4 MPa ; $\text{WC} \sim 1 \text{ g H}_2\text{O g}^{-1}$ dry weight) and before and after complete desiccation ($\text{WC} < 0.01 \text{ g H}_2\text{O g}^{-1}$ dry weight), quantifying the HSP70 protein levels by immunodetection. Our analysis showed that the expression of eight out of ten genes changed immediately before and after Ψ_{tlp} . Interestingly, the expression of five out of ten genes changed also before complete desiccation, i.e. between 0.2 and $0.01 \text{ g H}_2\text{O g}^{-1}$ dry weight. However, the HSP70 protein levels were not affected by changes in water status. The study provides new evidences of the link between the loss of turgor and the expression of genes related to the desiccation tolerance of *T. gelatinosa*, suggesting the former as a signal triggering inducible mechanisms.

1. Introduction

Desiccation tolerance is the ability to survive and recover metabolism after drying to $0.1 \text{ g H}_2\text{O g}^{-1}$ of dry mass (Kranmer et al., 2008; Farrant et al., 2012). This value corresponds to a water potential (Ψ) of $\sim -100 \text{ MPa}$ or even lower (Fernández-Marin et al., 2016). Desiccation tolerant (DT) organisms survive in this state for ecologically relevant periods of time and resume a normal metabolic activity in minutes to days, as soon as water becomes available again. Desiccation tolerance can be found in phylogenetically distant taxa, from fungi to nematodes, rotifers, and tardigrades (Alpert, 2006; Gaff and Oliver, 2013) and is typical of organisms which colonize substrates or environments with little and unpredictable water availability (Nardini et al., 2013). DT photoautotrophs include cyanobacteria, micro-algae, lichens, bryophytes, several clubmosses and ferns and a few hundred adult angiosperms plus most of the angiosperms at the embryo stage (as seeds) (Alpert, 2006). Aero-terrestrial microalgae, in particular, have a

global distribution and typically occur in biofilms on soil, rocks, leaves, tree barks, and man-made substrata (Lüttge and Büdel, 2010), being very important for primary production and nutrient cycling (Holzinger and Karsten, 2013). Furthermore, some taxa form long-living, stable symbiosis with DT fungi, i.e. lichens, and *Trebouxia* (Chlorophyta) is the most common and widespread genus of lichen photobionts (Hawksworth et al., 1995). All *Trebouxias* (c. 30 species; (Friedl, 1989)) are DT, and the mechanisms of their desiccation tolerance are not yet understood.

To cope with the effects derived by water loss, DT photoautotrophs apply multiple strategies. In most DT vascular plants, in which desiccation usually occurs in terms of days, the protection/repair mechanisms are activated by desiccation itself (Kranmer et al., 2002; Gasulla et al., 2013). DT non-vascular plants, on the other side, are subjected to frequent cycles of desiccation/rehydration lasting even a few minutes and thus they mostly rely on constitutive protection mechanisms (Fernández-Marin et al., 2016; Alpert, 2006; Candotto Carniel

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et al., 2016).

When desiccation is moderate, DT photoautotrophs accumulate compatible osmolytes that allow osmotic adjustments (Fernández-Marín et al., 2016). An increased expression and accumulation of dehydrins, late embryogenesis abundant proteins, and heat shock proteins (HSPs) is commonly observed (Gechev et al., 2012). Desiccation causes impairment of redox equilibrium, and both vascular and non-vascular DT photoautotrophs must avoid oxidative damage caused by reactive oxygen species (ROS) (Dinakar et al., 2012). An effective antioxidant system based on protective enzymes and non-enzymatic molecules (Kranter et al., 2005) is one of the most important prerequisites for desiccation tolerance (Holzinger et al., 2011).

During desiccation, one of the major threats is the loss of cell turgor, because the cell starts to shrink (Honegger et al., 1996) and is subjected to mechanical and biochemical modifications that could lead to irreversible damages (Fernández-Marín et al., 2016; Dinakar et al., 2016). For these reasons, cell turgor loss is considered one of the best indicators of water stress in plant science (Dinakar et al., 2016).

At physiological level, turgor loss can be monitored by measuring the water potential (Ψ), that assesses the water status in terms of potential energy per unit volume. At the same time, morphological modifications caused by desiccation, including turgor loss, can be monitored at microscopic level with the use of special chambers that allow observations of samples equilibrated at various relative humidity (RH) (Lajos et al., 2016). Ψ measurements are not regularly applied in lichenological studies (Lange et al., 2007; Hartard et al., 2009) as they are more time consuming than weighing to quantify the water content. However, Ψ is definitely more informative, because it allows a description of the water status derived on irreversible thermodynamics, and the discrimination between extra- and intracellular water loss (Nardini et al., 2013). Ψ of distilled water is 0 MPa, and the potential of cells in equilibrium is slightly negative due to the solutes (González and Reigosa Roger, 2001). When the cells lose water during desiccation, the decrease of turgor pressure (P_t , MPa) and/or the increase of solutes concentration cause a drop in the water potential (González and Reigosa Roger, 2001). When P_t reaches 0, cell turgor is lost, Ψ is mostly determined by the osmotic potential (i.e. cell solutes concentration) and known as Ψ at turgor loss point (Ψ_{tlp}). This parameter describes the ability of an organism to maintain cell turgor in the face of fluctuating water availability (Lenz et al., 2006). Below Ψ_{tlp} , important metabolic processes start to slow down or decrease their efficiency, such as photosynthesis (Petruzzelli et al., 2017). It is reasonable to think that this point might be perceived by the cells which then trigger some countermeasures, starting from changes in gene expression and followed by changes of protein and metabolite levels to cope with the modifications induced by water loss.

To date, few studies have been conducted on the turgor loss response in DT non-vascular plants (Holzinger and Karsten, 2013), and an estimation of Ψ in relation to water status would be important to understand if and how this process is involved in desiccation tolerance of these organisms.

Up to date, a single work was performed considering both Ψ and RWC in the lichen photobiont *Trebouxia sp.* TR9 (Centeno et al., 2016), showing that, at metabolomic level, water status affects mainly cell wall, extracellular polysaccharides, polyols and antioxidant protection. Candoito Carniel et al. (2016) showed that desiccation tolerance of *Trebouxia gelatinosa* Archibald mostly relies on constitutive mechanisms, but desiccation and rehydration affect also the gene expression of components of the photosynthetic apparatus, the ROS-scavenging system, HSPs, expansins, and desiccation related proteins (DRPs). The latter, firstly described in the resurrection plant *Craterostigma plantagineum*, have been linked to desiccation tolerance (Bartels et al., 1990). One of these DRPs, predicted to exist as small gene family (pcC13-62, (Piatkowski et al., 1990)), share similarities with *T. gelatinosa* DRPs (Candoito Carniel et al., 2016).

Here, we aimed at understanding how the water status of *T.*

gelatinosa causes changes in the expression of stress- and desiccation-related genes. We monitored the expression of selected genes related to desiccation tolerance in colonies of *T. gelatinosa* during desiccation in order to verify whether the loss of turgor can trigger inducible tolerance mechanisms.

2. Materials and methods

2.1. Cultures of *Trebouxia photobiont*

Trebouxia gelatinosa was isolated following Yamamoto et al. (2002) from thalli of *Flavoparmelia caperata* (L.) Hale collected in the Classical Karst (NW Italy). The algal cultures were subcultured on solid *Trebouxia* Medium (TM; 1.5% agar) (Ahmadjian et al., 1973) every 30–45 days and kept in a thermostatic chamber at $18 \pm 1^\circ\text{C}$ and $20 \pm 2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a light/dark regime of 14/10 h and $53 \pm 2\%$ RH.

Axenic cultures of *T. gelatinosa* were inoculated using 100 μl of a water suspension of approximately 3.5×10^6 cells mL^{-1} on cellulose acetate membranes (25 mm diameter, pore size 0.45 μm , Sartorius Lab Holding GmbH), which were laid on 25 mL of solid TM (1.5% agar) (Ahmadjian et al., 1973) at the bottom of Microbox Junior 40 vessels (Duchefa Biochemie), equipped with a micro-filter strip on the cover which allows gas exchange while keeping the internal volume in sterile conditions. The vessels, each containing six membranes, were kept in the thermostatic chamber at the same conditions reported above. On the 30th day of growth, three groups of colonies (weight: 0.22 ± 0.01 g) were used for Ψ_{tlp} assessment, desiccation treatment and chlorophyll *a* fluorescence measurements.

2.2. Desiccation treatment

Sixty-three colonies were randomly (Lehmer Pseudo random number generator) selected from the vessels and placed in groups of seven along the border of single filter paper discs (Whatman, $60 \pm 5 \text{ g m}^{-2}$, 25 mm diameter) wetted with 100 μl of distilled water inside plate lids, and left to desiccate at the air of the thermostatic chamber, at the same conditions described above.

During desiccation, 9 sampling points were selected as defined in a preliminary experiment performed at the same conditions, in which the decreasing weight of fully-hydrated *T. gelatinosa* colonies was followed over time with a precision balance. In the final experiment, at each sampling point (T0-T8), one plate lid with seven colonies was randomly selected and one of its colonies was placed in a dew point water potential meter (WP4, Decagon Devices, Inc.) to measure the water potential (Ψ). Then this colony was gently transferred from the membrane to a pre-weighed labeled 1.5 mL tube, soaked in liquid nitrogen and freeze-dried for 48 h. After freeze-drying, the tube was weighed on a precision balance to obtain the dry weight (DW) of the colony.

Just before finishing the Ψ measurement, the other six colonies were transferred on the plate lid from the membrane to pre-weighed labeled 1.5 mL tubes, weighed on a precision balance for water content (WC) estimation, soaked in liquid nitrogen and stored at -80°C . For the last time point (T8), colonies were kept in silica-gel for 24 h. The six colonies of each plate lid represent six replicates of the same sampling point (sample), which were referred to the specific Ψ measured on the seventh replicate.

The water content (WC) was expressed as $\text{g H}_2\text{O g}^{-1}$ dry weight and calculated as $\text{WC} = [(\text{FW} - \text{DW})/\text{DW}]$, where the fresh weight (FW) was the weight at the sampling point, and the dry weight (DW) was the average weight of the freeze-dried colonies. The relative water content was calculated as $\text{RWC} = [(\text{FW} - \text{DW})/(\text{IFW} - \text{DW})] \times 100$ following Nardini et al. (2013), where the water lost until the decline of Ψ was interpreted as extracellular, and subtracted from the T0 fresh weight to get the initial fresh weight (IFW), which was considered the weight at full turgor (Nardini et al., 2013).

Subsequent analyses, *i.e.* quantitative real-time PCR (qRT-PCR) of ten genes and immunodetection of HSP70, were performed on a subset of samples, selected on the basis of their Ψ , Ψ_{tip} and/or RWC. This subset consisted of samples in the fully-hydrated state (T0), after loss of most extracellular water (T1), before (T4) and after (T5) turgor loss, and immediately before (T7) and after (T8) reaching the desiccated state (RWC < 10%).

2.3. *Trebouxia gelatinosa* Ψ_{tip} assessment

The water potential at the turgor loss point (Ψ_{tip}) were derived from PV curves, according to Tyree and Hammel (1972). Four colonies were taken from the vessels and used for the PV-curve measurements. Fresh colonies were measured to obtain turgid weight (TW) and then progressively desiccated on a laboratory bench while maintained in the dark, at 21 ± 1 °C and with RH ranging between 40% and 55%. Water potential (Ψ) measurements were performed using a dew point water potential meter (WP4, Decagon Devices, Inc.) as the average of three subsequent values in the range below the error of the instrument (0.1 MPa) and coupled with measurements of sample fresh weight (FW). The cumulative water loss of colonies ($W_l = TW - FW$) was plotted versus $-1/\Psi$, and experiments were concluded when this relationship became linear ($R^2 > 0.98$) (Savi et al., 2015). Ψ_{tip} was estimated as the flex point transition between the curvilinear and linear parts of the relationship (Tyree and Hammel, 1972; Bartlett et al., 2012).

2.4. Cell morphological analysis

To evaluate the morphological modifications caused by water loss, photomicrographs of *T. gelatinosa* cells (a) fully hydrated, (b) at incipient plasmolysis, and (c) after desiccation were taken with a Zeiss Axiovert 200 M microscope using an adapted version of the microscopy chamber described by Lajos et al. (2016). This chamber allows to maintain a constant RH for desiccation experiments while observing the samples at the light microscope. In the lower part of the chamber, few drops (~20 μL) of algal suspension are transferred and spread carefully with a spatula. After the water surrounding cells is evaporated, the upper part of the chamber is filled either with dH_2O , a salt solution, or silica gel, to reach different RHs.

Three *T. gelatinosa* liquid cultures were prepared in Bold's Basal Medium (BBM) (Deason and Bold, 1960) and grown for 30 days in a Percival® growth chamber at 20 °C, 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a light/dark regime of 14/10 h.

For our study, the upper part of the microscopy chamber was filled with: (a) 15 mL of dH_2O to observe fully hydrated cells at 100% RH; (b) 15 mL of a LiCl solution (3 g/100 ml dH_2O) to reach 94.6% RH, corresponding to -3.38 ± 0.01 MPa, just before Ψ_{tip} ; (c) 25 g of silica gel to reach 15.2% RH to observe complete desiccation. The RH values were measured with a Rotronic® humidity sensor at 20 °C. The cells were then allowed to completely equilibrate for up to 12 h using the same light and temperature conditions required for growth before at least 10 microscopic images for each treatment were taken.

2.5. Chlorophyll a fluorescence (Chl_aF) measurements

To assess cell viability, Chl_aF measurements were taken on three colonies (a) fully hydrated, (b) after desiccation in silica gel for 24 h and successive fast rehydration (2 min) with few drops of water, and (c) after further 24 h of full hydration in the thermostatic chamber. Chl_aF emission was measured with a fluorimeter Handy-PEA (Hansatech, UK). A modified clip was positioned right over the sample on the membrane. A saturating red-light pulse of 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 1 s was emitted to obtain the Kautsky induction and thus F_m (transient maximum Chl_aF level). F_0 (minimum Chl_aF level), which is needed to calculate F_v (variable Chl_aF level, *i.e.* $F_m - F_0$) and thus F_v/F_m (maximum

quantum efficiency of PSII photochemistry), was calculated *a posteriori* by an algorithm that determines a line of best fit through the data points recorded immediately after the start of illumination.

2.6. RNA isolation and cDNA synthesis

RNA was extracted with PowerPlant® RNA Isolation Kit (MO BIO Laboratories Inc.) from three out of six random chosen replicates of all the selected samples. RNA quality was verified with NanoDrop® 2000 (Thermo Fisher Scientific), followed by a denaturing 1% agarose gel. cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). 2.6 Quantitative real-time PCR (qRT-PCR).

qRT-PCR was performed to measure the expression of ten different transcripts, five encoding stress-related proteins and five desiccation-related proteins. The former were ascorbate peroxidase (APX), expansin 1 (EXP1), manganese superoxide dismutase (MnSOD), heat shock protein 70 1 (HSP70-1) and the chlorophyll a-b binding protein of the light harvesting complex II (LHCII); the latter were the desiccation related proteins 1 (DRP1), 2 (DRP2), 6 (DRP6), 11 (DRP11) and 13 (DRP13). The transcripts were selected to provide a wide gene expression profile that included the most important mechanisms possibly linked to *T. gelatinosa* desiccation tolerance. APX and MnSOD were chosen among other anti-oxidant enzymes as they presented the highest gene expression change in *T. gelatinosa* colonies subjected to desiccation and oxidative stress (Candotto Carniel et al., 2016; Montagner, 2017). EXP1 and LHCII were analyzed to monitor cell wall and photosynthetic apparatus response. HSP70-1 transcript was chosen as representative of the HSP70s found in *T. gelatinosa* transcriptome, as it presented the common behavior to be downregulated during desiccation (Candotto Carniel et al., 2016). A phylogenetic analysis of *T. gelatinosa* HSP70 members was conducted (see paragraph 2.7) to better characterize this family in this species. Finally, the five most responsive DRPs among the 13 found in *T. gelatinosa* transcriptome (Candotto Carniel et al., 2016) were selected.

Primers for each transcript (Table A.1) were chosen following Candotto Carniel et al. (2016), Montagner (2017), or custom-designed with Primer3Plus (Untergasser et al., 2007). Each reaction was performed in three technical replicates in a mix containing 1 μL cDNA (1:10 template dilution), 8 μL SSOAdvanced™ SYBR® Green Supermix (Bio-Rad) and 200 nM of each primer. The PCR amplifications were performed with CFX 96™ real-time PCR System (Bio-Rad) using the following cycle: 98 °C for 30 s and 40 cycles at 95 °C for 10 s and 60 °C for 20 s. A melting curve analysis (65 °C–95 °C increment 0.5 °C for 5 s) was performed to verify the absence of non-specific amplification products. Transcript levels were calculated with Bio-Rad CFX Manager software (Bio-Rad), based on the comparative Ct method ($2^{-\Delta\Delta C_t}$ method) (Livak and Schmittgen, 2001) and gene expression data were normalized using as housekeeping gene the ribosomal protein L6 (RPL6) (Candotto Carniel et al., 2016; Montagner, 2017). Gene expression data were normalized on the average of the T0 samples for each transcript to provide fold change expression values.

2.7. HSP70 phylogenetic analysis

To characterize more in detail the *T. gelatinosa* HSP70 members in terms of subfamily and cellular localization, a phylogenetic analysis was performed. Eleven open reading frames coding for HSP70 were found in *T. gelatinosa* transcriptome (Candotto Carniel et al., 2016). These protein sequences were compared with 17 *Arabidopsis thaliana* HSP70 protein sequences from Lin et al. (Lin et al., 2001), downloaded from the NCBI database. Proteins were aligned with MUSCLE (Edgar, 2004) and the resulting alignment was trimmed to remove highly divergent and poorly informative regions with GBlocks 0.91b (Castresana, 2000). The phylogenetic tree construction was conducted with MEGA7 (Tamura et al., 2007) using the Maximum Likelihood method at default parameters. The bootstrap consensus tree was

inferred from 10000 replicates. Cellular localization was predicted with TargetP (Emanuelsson et al., 2000) and PSORT (Nakai and Kanehisa, 1992).

2.8. Proteins isolation

Three out of six random chosen replicates of all the selected samples were grinded in liquid nitrogen, transferred to 1.5 mL tubes and resuspended in 100 μ L of $1 \times$ Laemmli buffer [62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.2M dithiothreitol (DTT) and 0.1% (w/v) bromophenol blue (Laemmli, 1970)]. Samples were then vortexed and incubated at 95 °C for 5 min. After a 3 min centrifugation at 14000 r.p.m., protein extracts were recovered from the upper phase of the tube and transferred to a new tube. Samples were stored at -20 °C and incubated 5 min at 95 °C before loading on the gel, when not immediately used for the analysis. To check the quality and quantity of the total proteins extracted, 12% sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970) and the gel was stained with Coomassie brilliant blue R250 (Zehr et al., 1989).

2.9. HSP70 immunodetection

To perform 12% SDS-PAGE, 15 μ g of proteins were used. Proteins were then transferred on a Hybond™ nitrocellulose membrane (Amersham) using the Criterion™ blotter apparatus (Bio-Rad, USA) (Dinakar and Bartels, 2012). The transfer of the proteins was obtained after 1 h at 70 V with pre-chilled buffer. Before immunodetection, the membrane was stained for 30 min with Ponceau S red to check equal protein transfer. The membrane was incubated at 4 °C overnight in blocking solution [3% (w/v) skimmed milk in Tris-buffered saline] to prevent unspecific binding of antibodies. The membranes were incubated for 1 h with HSP70/HSC70 primary antibody ((Ulbricht et al., 2013), 1:1000 dilution), and for 45 min with secondary antibody (anti-rabbit IgG-peroxidase, 1:5000 dilution, Sigma-Aldrich). Antigen-antibody complexes were detected with the ECL kit (Amersham) and a lumi-imager (LAS 1000, Fujifilm). Densitometry of protein bands was done with Image J software 1.37 V (National Institute of Health).

2.10. Statistics

Statistics were performed with R version 3.2.0 (R Development Core Team, 2015). The non-parametric Kruskal-Wallis test and Wilcoxon non-paired test were applied to verify the significance of differences for Chl_aF measurements. A one-way Anova followed by a Fisher's LSD post-hoc test was applied to verify significant differences between the relative abundancy of transcripts and HSP70 protein content among samples. Figures were produced with Sigmaplot 10.0 (Systat Software).

3. Results

3.1. Water relations

Complete desiccation of the *T. gelatinosa* colonies (RWC ~ 1%) occurred in approximately 10 h (Fig. 1, Table A.2). Water loss was faster at the beginning of the desiccation: after the first 4 h (T1), WC was halved (Fig. 1, Table A.2) and reached 0.01 g H₂O g⁻¹ DW in T8.

Water potential decreased slowly from T0 to T4, and strongly decreased between T4 and T5 (Fig. 1, Table A.2). Ψ_{tp} assessed through PV-curves measurement was ~ -3.62 \pm 0.62 MPa (see Fig. A.1 for a representative curve), corresponding to a point between T4 and T5 of the desiccation process shown in Fig. 1. After turgor loss point, Ψ continued to strongly decrease (i.e. between T6 and T7), without a corresponding change in the water content. As after the turgor loss Ψ mostly depends on the osmotic potential, at that point even small water volume decrease (and the respective solutes concentration increase)

corresponds to fast decrease in the osmotic potential, that is reflected in a fast decrease in Ψ .

The lower part of the figure shows light microscopic representative images corresponding to the A, B, C points marked in the upper graph. A: fully hydrated cells (100% RH); B: before turgor loss (94.6% RH); C: after complete desiccation (15.2% RH). Arrows indicates cells at the beginning of morphological modifications (i.e. plasmolysis and loss of sub-spherical shape caused by contraction of the protoplast). Scale bar 20 μ m.

3.2. Microscopic images

Fully hydrated *T. gelatinosa* cells are sub-spherical, fully turgid (Fig. 1A) while at incipient plasmolysis (Fig. 1B) some cells show a slightly contracted shape, with protoplasts detached from the cell wall in some portions. When deep-desiccated (WC < 0.01 g H₂O g⁻¹ DW), cells are shrunk, with the walls also strongly collapsed (Fig. 1C), due to the almost complete water loss from the protoplasts.

3.3. Effects of desiccation on the quantum yield of primary photochemistry (F_v/F_m)

No significant differences were observed among the F_v/F_m values of control (0.650 \pm 0.016, n = 4), deep-desiccated samples immediately after a 2-min-long rehydration (0.630 \pm 0.005, n = 4) and in deep-desiccated samples after a 24-h-long rehydration (0.630 \pm 0.002, n = 4), showing that neither the desiccation nor the modality of rehydration affect cells viability.

3.4. Effects of desiccation on genes expression at transcript level

Significant changes in the expression of all tested genes were observed during desiccation.

Regarding anti-oxidant enzymes, both APX and MnSOD increased their expression at T1 (~40% and 100%, respectively), then decreased (from ~110% to ~180%) from T4 to T6 and increased again at T8 around initial levels (Fig. 2). EXP1 had an opposite expression pattern; it increased six times from T0 to T4, remaining high at T5 and then it decreased to less than one tenth of the T0 level at T7 and T8 (Fig. 2). HSP70-1 and LHCI had a different expression pattern as well: they remained stable until T2, to significantly decrease between T4 and T7 (~90%) and increase again to the T0 levels between T7 and T8 (Fig. 2).

The expression of DRP1 significantly increased to 40% between T0 and T4, then it decreased back to T0 levels between T4 and T5 and increased again between T7 and T8 (Fig. 2). DRP2 and DRP6 shared a similar pattern, with the major increase (~50%) of their expression at T4 and then a decrease of ~20% from T5 to T8 (Fig. 2). DRP11 had the major increase at T4 (~40%), then the expression decreased to the initial level from T5 to T8 (Fig. 2). DRP13 had also a major increase at T4 (~60%), then it returned to initial levels at T5, decreased ~30% at T7 and then it returned to initial levels at T8 (Fig. 2).

3.5. HSP70 phylogenetic analysis

The phylogenetic analysis revealed that most *T. gelatinosa* HSP70 proteins find correspondence in both sequence homology and sub-cellular localization with those from *A. thaliana*. *T. gelatinosa* HSP70-1, that corresponds to the transcript analyzed with qRT-PCR, is likely orthologous to chloroplastidial *A. thaliana* HSP70s (Fig. A.2) and belongs to DnaK subfamily, highly supported by a bootstrap value of 76. The other *T. gelatinosa* HSP70s belong to DnaK subfamily as well, apart from HSP-7 and HSP-8 that cluster to the other subfamily, Hsp110/SSE (Fig. A.2).

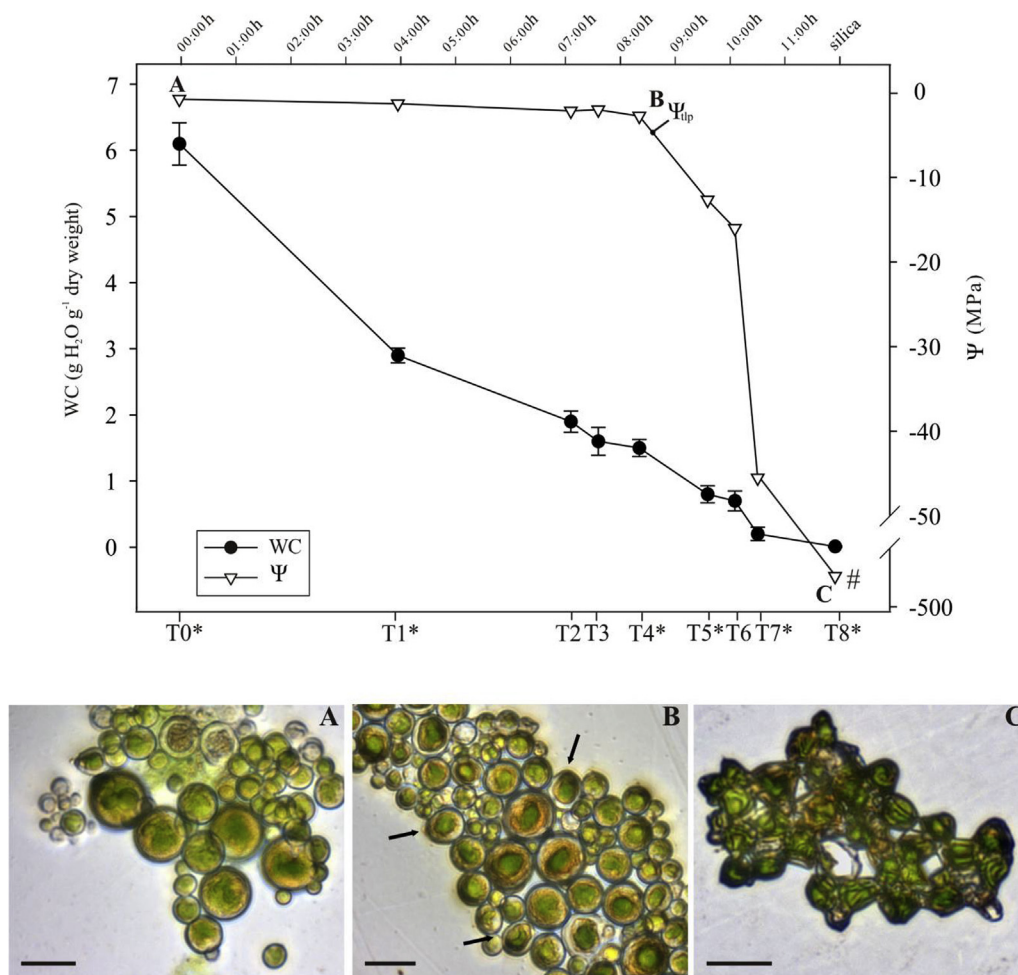


Fig. 1. The upper part of the figure represents water content (WC, $n = 6$) and water potential (Ψ) during desiccation in *Trebouxia gelatinosa* colonies (T0-T8). Samples used in the subsequent analyses were sampled at the measuring points marked with an asterisk. Ψ_{tjp} according to PV-curves. #according to Candotto Carniel et al. (Candotto Carniel et al., 2016).

3.6. Effects of desiccation on the HSP70 protein level

Desiccation did not affect the HSP70 protein level: no significant differences were found among the sampling points (Fig. 3).

4. Discussion

Part of the WC of fully hydrated *T. gelatinosa* is due to extracellular/apoplasmic water (Proctor et al., 2008); a similar water storage is commonly found in other species of *Trebouxia* (Candotto Carniel et al., 2015) and green micro-algae (Holzinger and Karsten, 2013). In these organisms, a considerable amount of water can be lost before the water potential falls sufficiently to affect metabolism. For these reasons, fresh weight and RWC measurements alone are poorly informative about the actual cell water status, whose correct assessment can be ensured only through water potential measurements. Therefore, the correct assessment of Ψ should be included in all lichenological and phycological studies regarding the biological significance of the water relations between cells and surrounding environment.

In plant cells, transcriptional activation or repression induced by a stress factor involve sensors located inside the cells, at the plasma membrane or at the cell wall, the generation and release of second messengers (including calcium ions, Ca^{2+}) and the activation/inhibition of protein kinases (PKs) and phosphatase (PPs) (Neill and Burnett, 1999). A possible signal leading to gene expression changes could be the modification of the environment surrounding the cells and/or

morphological modifications of the cell wall-plasma membrane system. In *T. gelatinosa* such stimuli might be the trigger for the inducible mechanisms activated during desiccation described so far (Candotto Carniel et al., 2016; Gasulla et al., 2009).

Morphological modifications occur when the cells lose their turgor as a consequence of water loss (Lang et al., 2014). Generally, Ψ_{tjp} of vascular plants is between -1 MPa and -3 MPa (Lenz et al., 2006; Nardini et al., 2012; Maréchaux et al., 2015). Lower Ψ_{tjp} values have been linked to higher water deficit tolerance in organisms adapted to arid environments, as seen in vascular plants (Bartlett et al., 2012) and lichens (Nardini et al., 2013). Here, *T. gelatinosa* lost cell turgor at Ψ_{tjp} of ~ -4 MPa, i.e. the same value recorded by Petruzzellis et al. (2017) in a study on intact thalli of the lichen *Flavoparmelia caperata* and in its axenically grown *T. gelatinosa* photobiont, which showed that the turgor state influences the functionality of photosynthesis in both lichenized and non-lichenized photobionts. Thus, the loss of turgor with the correlated morphological modifications of the cell wall-plasma membrane system can be a promising candidate as threshold signal for dangerous water losses.

In this work, ten and eight out of ten genes showed significant expression changes respect to the control (T0) right before and after the turgor loss, respectively.

The connection between water loss and cell wall has been investigated both in higher plants (see (Moore et al., 2008) and reference within) and in green algae (Holzinger and Pichrtová, 2016). When a plant cell loses water, the most evident result is a progressive reduction

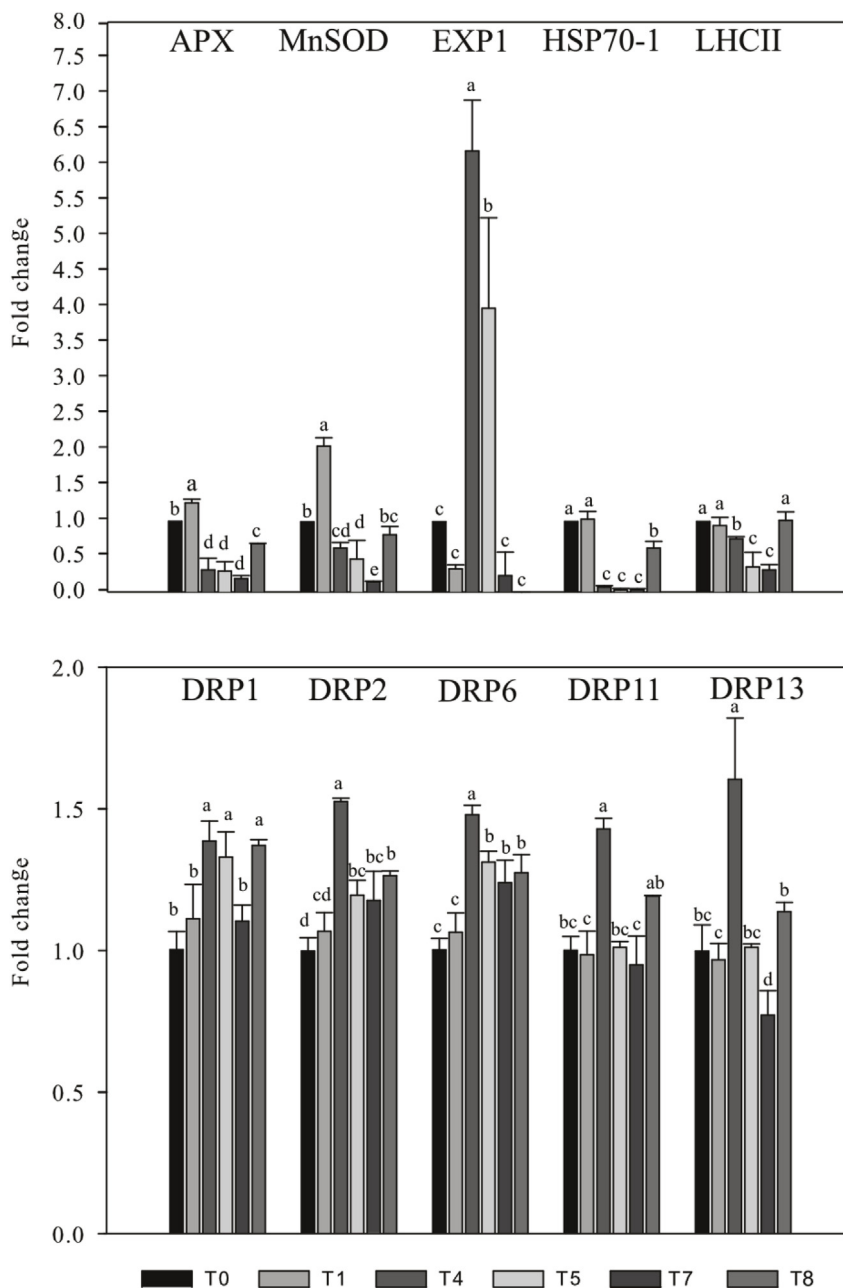


Fig. 2. Fold change in the expression of the 10 selected transcripts. Gene expression (mean ± SD) was determined using qRT-PCR for RNA of colonies of *Trebouxia gelatinosa* during desiccation. Different letters on the top of the bars indicate significant differences among samples ($p < 0.05$, $n = 3$). APX: ascorbate peroxidase; EXP1: expansin 1; MnSOD: manganese superoxide dismutase; HSP70-1: heat shock protein 70-1; LHCII: chlorophyll a-b binding protein of the light harvesting complex II; DRP: desiccation related protein.

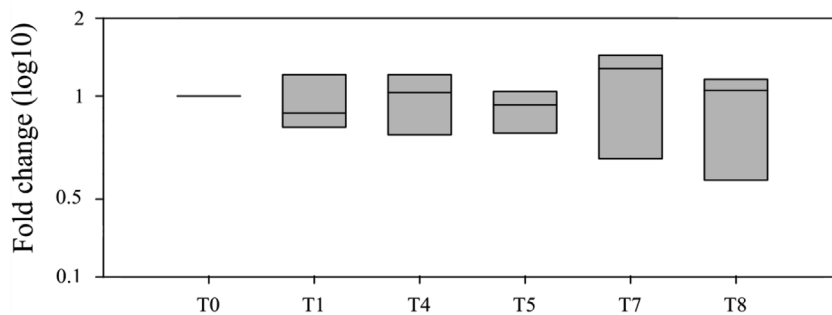


Fig. 3. Fold change in the level of HSP70 protein. The level was assessed using immunodetection on 15 µg of proteins extracted from colonies of *Trebouxia gelatinosa* during desiccation. No significant differences were found among samples ($p < 0.05$, $n = 3$).

of cell volume which, below a certain threshold, can lead to mechanical stress, followed by cell wall collapse (Farrant, 2000). The shrinkage process is particularly spectacular in the sub-spherical cells of *Trebouxia*, with important effects on cell ultrastructure (Honegger et al., 1996). When a cell changes its volume during desiccation or rehydration, cell wall plasticity has a crucial role for the maintenance of its integrity. Cell wall plasticity depends on the underlying structure and on the activity of wall-modifying enzymes, such as expansins (McQueen-Mason et al., 1992). Not surprisingly, in the studied alga the major change in terms of up-regulation at turgor loss was recorded in the expression of the gene coding for expansin 1. These enzymes have been found to be up-regulated in response to several water-related stresses, such as desiccation and salinity (Tenhaken, 2015). Candotto Carniel et al. (2016) proposed that they might play a key role also in desiccation tolerance of *T. gelatinosa*. Assuming that the cells change their gene expression profile in coincidence to the cell turgor loss, the upregulation observed immediately before Ψ_{tip} , can be explained by the fraction of the cell population at incipient plasmolysis (see Fig. 1B).

After expansin 1, the major change in gene expression regarded HSP70-1, which had a strong down-regulation immediately before Ψ_{tip} . In higher eukaryotes, including plants, HSP70s are stress-inducible proteins (Wang et al., 2004). They have a crucial role in protecting living organisms from environmental stresses and support correct protein folding under stress conditions (Yu et al., 2015). Nine out of the eleven *T. gelatinosa* HSPs members, including HSP70-1, were found to pertain to *A. thaliana* HSP70 DnaK subfamily members, each localized in different cellular compartments, including chloroplast, cytoplasm and mitochondrion, similarly to the ones from *A. thaliana*. This suggests distinct functional roles, as proposed by (Lin et al., 2001), and makes their down-regulation even more interesting. In fact, in the alga investigated here, the major down-regulation before turgor loss occurred for the HSP70-1 gene. Then, the expression of HSP70-1 significantly increased when RWC diminished under the threshold of 10%. The return to control level possibly permits to *T. gelatinosa* to recover some functions right after rehydration, when metabolism and protein synthesis recover.

A down-regulation of HSP70 gene expression was already documented in desiccated *T. gelatinosa* (Candotto Carniel et al., 2016) and the moss *Physcomitrella patens* (Tang et al., 2016), and in *Trebouxia sp.* exposed to oxidative stress and heavy metals (Montagner, 2017; Bačkor et al., 2006; del Hoyo et al., 2010). The up-regulation of HSP70 genes generally determines increased tolerance to abiotic stress in plants (Wang et al., 2004; Bartels and Sunkar, 2005): for this reason, the HSP70 down-regulation observed in *T. gelatinosa* still needs to be clarified. Although the function mechanisms of HSP70 are not fully understood (Wang et al., 2004), a change in plant HSP70 expression is generally linked to the induction of Ca^{2+} /calmodulin (CaM) genes (Wu et al., 2006) or mitogen activated protein kinases (MAPK) that are involved in signal transduction and in signaling of plant abiotic stress (Sinha et al., 2011).

The HSP70 protein family is one of the most conserved in evolution, with all eukaryotes containing multiple members (Murphy, 2013). This implies the anti-HSP70 antibodies are cross-reactive (i.e. can recognize proteins of one organism even if they are produced against the protein of another organism) (Bačkor et al., 2006), and therefore we were allowed to use them in *Trebouxia*, as already did by other authors (Bačkor et al., 2006). The low correlation we found between HSP70 mRNA abundance and protein level is common (Day and Tuite, 1998). In fact, the accumulation/reduction of mRNA during water deficit may indicate gene induction or repression, but further regulatory mechanisms, such as translational and post-translational modifications, could be necessary (Bray, 1997). Further investigations are needed to clarify the role of molecular chaperones in *T. gelatinosa*; however, the constitutive protein expression of HSP70 seems to be required during water stress.

During water stress, another important mechanism involved in response to desiccation is photosynthesis. In plants and green algae

photosynthesis is generally suppressed by desiccation as a protective mechanism in order to avoid further generation of ROS (Holzinger and Karsten, 2013). A suppression in photosynthesis was recorded in *Trebouxia sp.* at low (15–3%) RWC (Candotto Carniel et al., 2015). Furthermore, Petruzzellis et al. (2017) found that in *T. gelatinosa*, photosynthesis decreased markedly only after Ψ_{tip} . At transcriptomic level, however, an increase of photosynthesis-related transcripts (including LHCI) at the end of desiccation process was detected in *T. gelatinosa* (Candotto Carniel et al., 2016). In our study, LHCI was down-regulated before turgor loss, and in the desiccated state it returned to the T0 level (Fig. 2). This behavior could be due to a faster desiccation rate in this study than in Candotto Carniel et al. (2016), especially before the complete desiccation, resulting in a shorter metabolic activity needed to build up transcripts. Accordingly, desiccation rate was important for photosynthesis recovery upon rehydration in another taxonomically-near photobiont species, *Asterochloris erici* (Gasulla et al., 2009). These organisms recover photosynthesis as soon as water returns available; LHCI transcription returning to initial level (T0, when photosynthesis was active) in the desiccated state might put the alga in the condition of being prepared for the rehydration event, as already discussed by Holzinger et al. (2014) and Candotto Carniel et al. (2016).

In both vascular and non-vascular plants including green algae and lichen photobionts, one of the most dangerous effects of desiccation is the oxidative burst related to ROS production during desiccation and upon rehydration (Kranner et al., 2008; Holzinger and Karsten, 2013; Candotto Carniel et al., 2015; Weissman et al., 2005). In lichens and their photobionts, the oxidative burst is faced by an efficient anti-oxidant system, which is constitutive in these organisms (Kranner et al., 2008; Candotto Carniel et al., 2016). Here, we aimed at understanding if water loss induces changes in gene expression of antioxidant enzymes. At transcript level, both MnSOD and APX were up-regulated at the beginning of desiccation, when extracellular water was still present, but then they were down-regulated before and after turgor loss.

The last group of transcripts which members had their expression changed before and/or after Ψ_{tip} is DRPs. Among the numerous and highly diversified DRPs found in *T. gelatinosa* (Candotto Carniel et al., 2016), here we analyzed the most desiccation-responsive ones in terms of fold change. The response of these genes showed all the same trend. DRP1 and DRP2 expression profiles differ from the one found in Candotto Carniel et al. (Candotto Carniel et al., 2016), as their expression was not down- but up-regulated in the desiccated state. It could be possible that in Candotto Carniel et al. (2016) these genes were early and transiently up-regulated; DRPs and other stress-induced genes can show highest expression in partial desiccation stages. Furthermore, as the predicted cellular localization of these proteins is “secreted” (Candotto Carniel et al., 2016), the secretory pathways involved could have a role in the different regulation of these DRPs. DRPs in plants are involved in numerous processes, including drought tolerance (Bartels et al., 1990; Piatkowski et al., 1990), and their transcripts are frequently up-regulated during desiccation (Zha et al., 2013). Although a clear explanation for the role of DRPs during desiccation is still missing, the presence of the ferritin-like domain suggests a relation with oxidative stress protection (Liang et al., 2012). DRPs have been proposed also to work for the detoxification of the ROS produced during rehydration, when metabolic activity restart (Liang et al., 2012), but in *T. gelatinosa* the mechanisms seems more complex. In fact, expression of DRPs in *T. gelatinosa* was not responsive to oxidative stress caused by hydrogen peroxide (H_2O_2 , (Montagner, 2017)); the hypothesis of a stress specific response is intriguing and deserves more investigations. If the role of the *T. gelatinosa* DRPs is at the cell wall or apoplast level, early activation of gene expression should permit the protein to accumulate in the apoplastic space to carry out biochemical functions, such as the protection of cell wall from mechanical stress generated during desiccation and, similarly, upon rehydration. This hypothesis is further supported by the fact that the modification of cells, observed at microscopic level, starts before turgor loss (when DRPs show the higher

expression change).

In this study, seven genes showed significant expression changes right before (RWC ~ 13%; WC ~ 0.2 g H₂O g⁻¹ DW) the desiccated state. It has been shown that in orthodox seeds low WCs do not totally inhibit transcription (Fernández-Marín et al., 2013) thanks to the remaining of tightly bound water pockets into the cells. Therefore, as the final phase of desiccation process represents a key point in *T. gelatinosa* response, a further step in understanding what happens in these moments will be important. To characterize these moments, an experimental plan in which low WCs are assessed with closer time points, starting from WC ~ 0.3 g H₂O g⁻¹ DW, when normally no bulk water remains in the cytoplasm (Hoekstra et al., 2001), to WC = 0.1 g H₂O g⁻¹ DW, when vitrification process starts (Green et al., 2011) up to complete desiccation, should be performed.

It has been shown that different lichen chlorobionts apply contrasting strategies to cope with desiccation and rehydration, in correspondence with the water regime of their habitats (Centeno et al., 2016). The development of an experimental method based on the approach applied here could open a field study in which the heterogeneous ecological strategies of these organisms can be compared and discussed in an integrated view of physiology and molecular data.

5. Conclusions

Plants respond to stress with specific changes in gene expression, metabolism, and physiology, and this indicates the ability to sense environmental stress conditions (Zhu, 2016). From our analysis, it is clear that the moments just before and after the turgor loss and the final part of desiccation process are key points in terms of gene expression in the response of *T. gelatinosa*, triggering inducible tolerance mechanisms. Each key point induced a response in terms of up- or down-regulation of specific genes, which implies a finely regulated perception of water stress. Furthermore, this study provides new evidences that DRPs are involved in desiccation tolerance of *T. gelatinosa*.

Contribution

E.B., F.C.C., A.M., and M.T. conceived and planned the experiments. E.B., A.M., F.P. and G.P. carried out the experiments. E.B. performed the analyses. E.B. wrote the manuscript in consultation with F.C.C., A.M., D.B., V.G., F.P., G.P., A.P., and M.T. M.T. supervised the project. All the authors critically contributed to the discussion of the manuscript and approved the final version.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2018.06.004>.

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