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New features of desiccation tolerance in the lichen photobiont *Trebouxia gelatinosa* are revealed by a transcriptomic approach

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Abstract *Trebouxia* is the most common lichen-forming genus of aero-terrestrial green algae and all its species are desiccation tolerant (DT). The molecular bases of this remarkable adaptation are, however, still largely unknown. We applied a transcriptomic approach to a common member of the genus, *T. gelatinosa*, to investigate the alteration of gene expression occurring after dehydration and subsequent rehydration in comparison to cells kept constantly hydrated. We sequenced, de novo assembled and annotated the transcriptome of axenically cultured *T. gelatinosa* by using Illumina sequencing technology. We tracked the expression profiles of over 13,000 protein-coding transcripts. During the dehydration/rehydration cycle c. 92 % of the total protein-coding transcripts displayed a stable expression, suggesting that the desiccation tolerance of *T. gelatinosa* mostly relies on constitutive mechanisms. Dehydration and rehydration affected mainly the gene expression for components of the photosynthetic apparatus, the ROS-scavenging system, Heat Shock Proteins, aquaporins, expansins, and desiccation related proteins (DRPs), which are highly diversified in *T. gelatinosa*, whereas Late Embryogenesis Abundant Proteins were not affected. Only some of these phenomena were previously observed in other DT green algae, bryophytes and resurrection plants, other traits being distinctive of *T. gelatinosa*, and perhaps related to its symbiotic lifestyle. Finally, the phylogenetic inference extended to DRPs of other chlorophytes, embryophytes and bacteria clearly pointed out that DRPs of chlorophytes are not orthologous to those of embryophytes: some of them were likely acquired through horizontal gene transfer from extremophile bacteria which live in symbiosis within the lichen thallus.

Keywords Aero-terrestrial microalgae · Desiccation related proteins · Gene expression · Illumina · Lichenization · Trebouxiophyceae

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Introduction

Poikilohydric organisms are able to colonize very harsh environments, such as hot and cold deserts, rock surfaces or tree barks, thanks to their ability to survive extreme desiccation states and to recover full metabolic activity within minutes to hours upon rewetting (Lidén et al. 2010). This ability is commonly known as desiccation tolerance. It is documented in cyanobacteria (Büdel 2011), aeroterrestrial micro-algae (Trainor and Gladych 1995; Holzinger and Karsten 2013), intertidal algae (Büdel 2011), bryophytes (Richardson and Richardson 1981; Proctor 1990; Proctor et al. 2007), lichens (Mazur 1968; Kranner et al. 2008), and a few vascular plants, the so-called resurrection plants (Proctor and Tuba 2002). It also occurs among heterotrophs, such as tardigrades (Wright 2001), nematodes (Treonis and Wall 2005), and arthropods (Kikawada et al. 2005). This capability may be extended to the whole life cycle of the organism, or it may involve just some stages, as it happens in flowering plants, whose pollen grains and

seeds are frequently desiccation tolerant (DT) (Hoekstra et al. 2001).

The desiccation tolerance in photo-autotrophic organisms involves several essential adaptations to withstand the anatomical, physiological and biochemical alterations caused by water loss. The recent comparative studies on DT versus desiccation sensitive species allowed to identify the most important of these adaptations, which are: (1) the accumulation of non-reducing sugars (sucrose, trehalose, etc.) and Late Embryogenesis Abundant proteins (LEAs) which help to preserve the correct protein conformation and to avoid membrane fusion by replacing hydrogen bonds between water and other molecules (Hoekstra et al. 2001; Yobi et al. 2013); (2) the production of molecular chaperones, such as Heat Shock Proteins (HSPs), to aid the correct refolding of proteins upon rehydration (Gechev et al. 2013; Oliver et al. 2009); (3) the production of antioxidant substances (e.g. ascorbic acid, glutathione, etc.) and ROS scavenging enzymes, to maintain the intracellular redox homeostasis (Kranner et al. 2002, 2008); (4) the involvement of mechanisms, such as the activity of expansins, to allow a safe cell shrinkage upon turgor-loss (Jones and McQueen-Mason 2004).

The analysis of transcriptome, proteome and metabolome have brought important advantages in the study of desiccation tolerance in phototrophic organisms, especially resurrection plants (Collett et al. 2003, 2004; Le et al. 2007; Rodriguez et al. 2010; Gechev et al. 2013; Mitra et al. 2013; Lyall et al. 2014; Ma et al. 2015), seeds (Farrant and Moore 2011; Maia et al. 2014), and mosses (Oliver et al. 2004, 2009; Stark and Brinda 2015). The development of the so-called second generation sequencing technologies, which allow to collect a very large amount of data in a single analysis, has extended the transcriptomic approach to non-model vascular plants (Wang et al. 2010; Fu et al. 2011; Garg et al. 2011), non-vascular plants (Xiao et al. 2011; Gao et al. 2015) and terrestrial algae. In particular, for the latter group, Holzinger et al. (2014) analysed the gene expression profile induced by dehydration in *Klebsormidium crenulatum*, a representative of Streptophytes, i.e. the ancestors of the land plants that were among the first organisms to colonize lands from aquatic environments (Becker 2013; Hori et al. 2014). Also Chlorophytes, the sister group of Streptophytes, experienced the transition to land. In some cases, as in some Trebouxiophyceae, this passage led to the adoption of a symbiotic lifestyle. The most successful, in terms of both habitat extension and irradiative speciation, is lichenization, i.e. the capability of forming a stable extracellular symbiosis between one or more autotrophs (in this case green algae) and one heterotroph (fungi, mostly ascomycetes) (Lipnicki 2015). Intimately connected to this success was the acquisition of desiccation tolerance mechanisms, because lichens are de facto the prevailing organisms (in terms of biomass and species diversity) in several terrestrial macro- and micro-habitats where water is available in scarce and unpredictable quantities and/or soil is virtually absent (Nardini et al. 2013). Unfortunately, our knowledge on the desiccation tolerance mechanisms of lichen-forming algae is relatively poor, and they have never been investigated through a transcriptomic approach, notwithstanding some attempts made in the most recent years. Junttila and Rudd (2012), for instance, characterized the transcriptome of a lichen, *Cladonia rangiferina,* and bioinformatically assigned the expressed mRNAs either to the myco- or to the photobiont based on the comparison with sequence datasets obtained from separate axenic cultures. Later Junttila et al. (2013) also studied the effects of dehydration and rehydration on gene expression in the same lichen species. They discovered that these processes affect the expression of hundreds of genes, especially those related to short-chain and alcohol dehydrogenases, molecular chaperones and transporters. On the other hand, in the first and sole proteomic study on the desiccation tolerance of a lichen photobiont, the green alga *Asterochloris erici*, Gasulla et al. (2013) showed that this ability is mostly related to constitutive mechanisms, since only 11 and 13 proteins involved in glycolysis, cellular protection, cytoskeleton, cell-cycle and targeting and degradation were up-regulated after dehydration and rehydration, respectively.

In this study we describe the de novo assembly of the first complete transcriptome of the axenically cultured lichen photobiont *Trebouxia gelatinosa* Archibald (Trebouxiophyceae, Chlorophyta), chosen as a member of the most widespread genus of lichenized algae. *Trebouxia* Puymaly is present in about half of the estimated 15,000 chlorolichens known so far, and therefore it is the most common aeroterrestrial micro-algal genus worldwide (Ahmadjian 2004). As in the lichen symbiosis, free-living *Trebouxia* spp. can withstand dehydrations to water contents below 10 % of their dry weight, long periods in the desiccated conditions and recover metabolic activity within minutes upon rehydration (Kosugi et al. 2009; Candotto Carniel et al. 2015). The variation of gene expression profiles was studied in cultures kept fully hydrated, slowly dehydrated and subsequently rehydrated, with the aim of unravelling the key molecular processes involved in desiccation tolerance of lichen photobionts.

Materials and methods

Culture isolation of *Trebouxia* photobiont

Isolates of *T. gelatinosa* were obtained according to Yamamoto et al. (2002) from thalli of *Flavoparmelia caperata* (L.) Hale, collected in the Classic Karst plateau

(NW Italy; 45°42′24.54″N; 13°45′21.70″E). The isolates were inoculated in sterile plastic tubes filled with c. 5 ml of slanted solid *Trebouxia* medium (TM) (1.5 % agar) (Ahmadjian 1973). The tubes were kept in a thermostatic chamber at 20 °C, under a light regime of 18 ± 2 µmol photons m^{-2} s^{-1} with a photoperiod of 14 h:10 h, light:-dark until the colony reached a sufficient biomass. Cultures were re-inoculated every 30 days and were grown at the same conditions as the original inocula. The identity of the photobiont was checked by sequencing the nuclear ITS fragment (data available upon request) and by analysing the pyrenoid ultrastructure by TEM. Reference algal material was cryo-conserved according to Dahmen et al. (1983) and is available upon request.

Dehydration and rehydration treatments

Algal cultures were grown on hand-cut sterile filter paper discs (Whatman, 60 ± 5 g m^{-2}, diam. 25 mm), laid on solid TM (1.5 % agar) inside Petri dishes. In each Petri dish four discs were inoculated with 100 µl of a water suspension of 3.5×10^6 cells ml^{-1}. The Petri dishes were kept in a thermostatic chamber at the same controlled condition described above. On the 30th day of growth three discs representing the control samples (C) were randomly selected from the starting set of Petri dishes and promptly soaked in liquid nitrogen and stored at −80 °C. Six discs were slowly dehydrated on a thin layer of solid TM in a biological hood under air flow; complete dehydration took 10 h. The time necessary to obtain a water content (WC, see below) of 0.1 g H_2O g^{-1} DW was assessed in a preliminary experiment by following the weight loss of the discs with a precision balance.

After dehydration, three discs representing dehydrated samples (D), were soaked in liquid nitrogen and stored at −80 °C. The remaining three discs were wetted with a water drop and laid on solid TM inside a Petri dish for 12 h to allow the full rehydration of the algae at the same, original growth conditions. After rehydration, the three discs representing rehydrated samples (R) were frozen in liquid nitrogen and stored at −80 °C.

The water content (WC) was measured on a different set of samples grown over acetate-cellulose discs at the same conditions. The cultures were harvested from the discs, weighed, freeze-dried and then weighed again. The WC of cultures was calculated as $(FW_t - DW)/DW \times 100$, where FW_t is the sample weight after each treatment (t: C, D, R) and DW is the sample weight after a freeze-drying of 72 h. The WC of the samples was 4.92 (C), 0.10 (D), 4.35 (R) g H_2O g^{-1} DW, respectively.

RNA sequencing and de novo transcriptome assembly

Total RNA was extracted from the frozen culture discs, each one comprising c. 40 mg DW algal colonies, accounting for several millions *T. gelatinosa* individuals, using the PowerPlant® RNA Isolation Kit (MO BIO Laboratories, Inc.). RNA quality was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies). All samples achieved a RNA Integrity Number > 8 and the absence of RNA degradation in dehydrated samples was further evaluated, in a later stage, by an analysis of the 3′–5′ sequencing coverage drop on a subset of transcripts longer than 10 Kb (Online Resource 1). The RNA extracted from the three replicates prepared for each sample (C, D and R) were pooled together in equimolar quantities. The preparation of cDNA libraries and RNA-sequencing were carried out at the Institute of Applied Genomics (IGA) in Udine, Italy. Sequencing was performed on a single lane of an Illumina HiSeq 2000 instrument, with a 100 cycles paired-end sequencing protocol. Raw sequencing reads were trimmed according to their base calling quality before proceeding with further analyses. Trimmed reads shorter than 60 bp were discarded.

Trimmed reads were used for a de novo transcriptome assembly by Trinity (Grabherr et al. 2011), selecting the Jaccard-clip option to allow the splitting of chimeric contigs resulting from overlapping genes. Minimum allowed contig length was set at 201 bp. The complete assembly obtained, containing the transcript variants produced from each of the predicted gene models, was processed as follows, prior to the annotation and gene expression analysis steps. Only the longest transcript of each gene was selected in order to reduce sequence redundancy and to obtain a set of transcripts suitable for a gene expression analysis. A minimum threshold of the mean coverage was set to discard all low quality transcripts which cumulatively contributed to the back mapping of just the 2 % of the reads (including poorly expressed and highly fragmented transcripts).

Contigs resulting from mitochondrial and plastidial mRNAs or from ribosomal RNA were detected by BLASTn search (Altschul et al. 1990) based on the *Trebouxiophyceae* sp. MX-AZ01 plastidial (GenBank: NC_018569) and mitochondrial (GenBank: NC_018568) genomes, and on the *Trebouxia arboricola* 5.8S, 18S and 26S rRNA genes (GenBank: Z68705.1) available at public databases. Matching sequences (e-value cut off = 1×10^{-30}) were discarded prior to further analyses.

The entire RNA-seq experiment was deposited at the NCBI Sequence Read Archive database (SRA accessions: SRX330016 (C), SRX330011 (D) and SRX330015 (R); Bioproject: PRJNA213702).

Transcripts annotation and ORFeome definition

To overcome the technical issues linked with the high compactness of *T. gelatinosa* genome (see *infra*), in the non-redundant reference transcriptome we identified all the regions corresponding to Open Reading Frames (ORFs). ORFs were predicted based on their protein-coding potential, assessed either by a significant BLASTx similarity (e-value cutoff 1×10^{-5}) with the available complete proteomes of other Trebouxiophyceae, namely *Coccomyxa subellipsoidea* C-169 (v2.0, http://genome.jgi.doe.gov/Coc_C169_1/), *Chlorella variabilis* (v1.0, http://genome.jgi.doe.gov/ChlNC64A_1/) or *Asterochloris* sp. Cgr/Dha1pho (v.1.0, http://genome.jgi.doe.gov/Astpho1/), by the presence of PFAM domains (e-value cutoff 1×10^{-5}) or by a length of at least 300 codons (if none of the previous criteria were met).

The resulting ORFs sequences were extracted from the assembled contigs and annotated with the Trinotate pipeline. Sequence similarities were identified by BLASTx (Altschul et al. 1990) performed against the UniProtKB/Swiss-Prot database; functional domains were detected by a HMMER (Finn et al. 2011) search against the PFAM domain database (Punta et al. 2012). ORFeome were also annotated based on eggNOG (Powell et al. 2012) and Gene Ontology (Ashburner et al. 2000) 'biological process', 'molecular function' and 'cellular component' functional categories. These annotations were automatically extracted from BLASTx matches and linked to the corresponding assembled transcripts by Trinotate.

The presence of orthologous sequences in the genomes of other representative Viridiplantae was assessed by reciprocal best BLASTp matches, comparing the proteins predicted from the *T. gelatinosa* transcriptome to the proteins encoded by those genomes. The e-value and identity cut-off used to consider two sequences as orthologous were set to 1×10^{-5} and 35 %, respectively. The selected species comprised the three Trebouxiophyceae mentioned above (*C. subellipsoidea*, *C. variabilis* and *Asterochloris* sp.), *Chlamydomonas reinhardtii* (v.4.0, http://genome.jgi.doe.gov/chlamy/), *Volvox carteri* (v.1.0, http://genome.jgi.doe.gov/Volca1/), *Klebsormidium flaccidum* (ASM70883v1), *Selaginella moellendorffii* (v.1.0, http://genome.jgi.doe.gov/Selmo1/), *Micromonas pusilla* (v.2.0, http://genome.jgi.doe.gov/MicpuC2/), *Ostreococcus tauri* (v.2.0, http://genome.jgi.doe.gov/Ostta4/), *Zea mays* (B73 RefGen_v3) and *Arabidopsis thaliana* (TAIR10).

Gene expression analysis

Trimmed reads obtained from the sequencing of the three samples (C, D and R *T. gelatinosa* cultures) were mapped on the annotated ORFeome with the RNA-seq tool included in the CLC Genomics Workbench v.8.0. Length and similarity fractions parameters were set to 0.75 and 0.95, respectively; the maximum number of matching contigs was set to 10. Paired reads distance was assumed, based on fragment length data, to be comprised between 100 and 500 bp.

We calculated gene expression levels as Transcript Per Million (TPM), a measure of RNA abundance which takes into account both transcript length and sequencing depth for normalization, thus being proportional to the relative molar RNA concentration (Wagner et al. 2012). TPM values were used for the differential expression analysis using a Kal's Z-test on proportions (Kal et al. 1999) in the following comparisons: (a) D versus C samples; (b) R versus D samples; (c) R versus C samples. Differentially expressed genes (DEGs) were identified with a False Discovery Rate-corrected p value lower than 0.01, applied according to the Benjamini and Hochberg procedure (1995), and a proportions fold change (FC) value higher than 2 for up-regulated genes, or lower than −2 for down-regulated genes. TPM gene expression values were transformed by $\log_2$ for the graphical representation in the scatter plot.

Gene Ontology terms, PFAM domains and eggNOG functional categories over-represented in the subsets of differentially expressed genes were detected with a hypergeometric test on annotations. The sets of up-regulated and down-regulated genes were analysed separately. Significant over-representation was detected at p value < 0.01 and observed—expected >3. The over-representation of annotations displaying p values lower than 1×10^{-5} was considered highly significant.

qRT-PCR analysis

To further validate the expression profiles obtained with RNA-seq and to take into account biological variation across replicates, a qRT-PCR analysis was carried out on the three non-pooled samples for each experimental condition (C, D and R), targeting six representative differentially expressed transcripts. Primers were designed with Primer3Plus (Untergasser et al. 2007) (Online Resource 2). For this analysis, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad).

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′ and 40 cycles at 95 °C for 10′ and 60 °C for 20′. A melting curve analysis (65–95 °C, increment 0.5 °C each 5′) was performed to verify the absence of non-specific

Table 1 Sequencing and trimming statistics of *Trebouxia gelatinosa* transcriptomic analysis

	Reads number	Average length (bp)	Sequenced data (Gbp)
Raw data			
Control	60,409,470	100	6.0
Dehydrated	92,783,850	100	9.3
Rehydrated	90,569,258	100	9.1
Total	243,762,578	100	24.4
After trimming			
Control	58,604,069	97.6	5.7
Dehydrated	90,464,518	97.6	8.8
Rehydrated	88,336,044	97.6	8.6
Total	237,404,631	97.6	23.0

amplification products. Transcript levels were calculated with Bio-Rad CFX Manager software, based on the comparative Ct method ($2^{-\Delta\Delta Ct}$ method) (Livak and Schmittgen 2001) and normalizing gene expression data using as housekeeping genes two transcripts showing steady expression levels in our RNA-seq experiment and frequently used for this purpose in literature: the ribosomal protein L6 (RPL6) and the translation elongation factor 1 beta (EF1b).

Phylogenetic analysis of desiccation related proteins (DRPs)

Proteins pertaining to the desiccation related proteins family (DRPs) predicted from *T. gelatinosa* and other 45 representative species of green algae, embryophytes and bacteria (Online Resource 3) were aligned with MUSCLE (Edgar 2004) and the resulting alignment was trimmed to remove highly divergent and poorly informative regions with Gblocks v.0.91b (Castresana 2000). The Bayesian inference phylogenetic analysis was performed with MrBayes 3.2 (Ronquist et al. 2012) under a GTR + G + I model, identified by ProtTest 3.1 (Abascal et al. 2005) as the best-fitting our data, with two parallel runs with four chains each, for 1 million generations. Trees were sampled each 1000 generations; a 25 % burnin was adopted and the convergence of the analysis was reassured by a standard deviation of split frequencies lower than 0.01. Nodes with low statistical support (posterior probability < 0.5) were collapsed in the graphical tree representation.

Results

High throughput sequencing, de novo assembly and annotation of *T. gelatinosa* transcriptome

The output of the Illumina paired-end sequencing of the *T. gelatinosa* samples is summarized in Table 1. The overall de novo transcriptome assembly generated 19,601 contigs, with almost null sequence redundancy (Table 2). The genomes of Trebouxiophyceae are highly compact with a relevant number of overlapping genes potentially leading to the de novo assembly of chimeric contigs (see "Discussion" section for details). This genomic peculiarity contributed to the generation of a high number of chimeric contigs in the assembly process of *T. gelatinosa* transcriptome (Online Resource 4). Non-directional sequencing reads originating from overlapping exons of different transcripts, either encoded by genes on the same or on the opposite DNA strand, were assembled de facto as belonging to a common transcript, generating chimeric contigs with multiple Open Reading Frames (ORFs). Despite the use of the Jaccard-clip option of the Trinity assembler, we estimate that almost 2000 assembled contigs included multiple transcripts originated from genes spatially close to each other (Online Resource 4). We therefore relied on predicted ORFs (see "Materials and methods") for the downstream gene expression analysis. Overall, we identified and annotated 13,648 ORFs, which likely correspond to c. 10,000 protein-coding genes. This set of protein-coding sequences is available as a multiFASTA file in Online Resource 5. This number is not far from those observed in the complete genome of other Trebouxiophyceae (Table 3), suggesting that the coverage applied in our RNA-sequencing was adequate to obtain a nearly-complete collection of *T. gelatinosa* transcripts. Overall, the de novo assembled transcriptome potentially provides a good reference for large-scale comparative analyses within Viridiplantae, as we could detect over 3000 *bona fide* orthologous sequences even in largely divergent model vascular plants (Online Resource 6).

The annotation process permitted to assign a putative function to about 60 % of the ORFs, due to the presence of conserved functional domains or significant BLAST similarity to proteins with known function deposited in the UniProtKB sequence database (Table 2).

Table 2 De novo assembly and annotation statistics of *Trebouxia gelatinosa* transcriptomic analysis

Number of non-redundant assembled contigs	19,601
Total number of ORFs identified	13,648
Average contig length	1605 nt
Contigs N50	3594
Contigs longer than 5 Kb	1261
Longest assembled contig	31,749 nt
Number of residual chimeric contigs*	3509
Mapping rate** (contigs)	82.49 %
Average ORF length	1261 nt
Longest ORF	26,346 nt
Mapping rate** (ORFs)	47.17 %
Sequence redundancy (non-specific matches)	0.57 %
ORFs with BLAST matches (UniProtKB/SwissProt)	7311 (53.6 %)
ORFs with PFAM annotation	7976 (58.4 %)
ORFs with eggNOG annotation	5941 (45.5 %)
ORFs with Gene Ontology Cellular Component annotation	5725 (41.9 %)
ORFs with Gene Ontology Biological Process annotation	5497 (40.3 %)
ORFs with Gene Ontology Molecular Function annotation	5576 (40.9 %)

* Chimeric contigs are identified as those comprising more than one ORF (Open Reading Frame). ** Mapping rate is defined as the percentage of reads that match contigs or ORFs with the CLC Genomic Workbench RNA-seq tool, based on 0.75 and 0.95 length and similarity fraction parameters. N50: this value is calculated by summing the lengths of the longest contigs until 50 % of the total assembly length is reached. The minimum contig length in this set of contigs is the number that is usually used to report the N50 value of a de novo assembly

Table 3 Number of three *Trebouxiophyceae* predicted protein models showing significant similarity (tBLASTn vs genome, cut-off = $1E^{-5}$) with *Trebouxia gelatinosa* and percentage

	Total	Similar to *T. gelatinosa*	Ratio (%)
Asterochloris sp.	7159	6233	87.1
Chlorella variabilis	9791	7426	75.8
Coccomyxa subellipsoidea	9629	7173	75.1

Global effect of the dehydration/rehydration cycle on the transcriptional profiles

During the dehydration/rehydration cycle 12,533 protein-coding transcripts (91.83 % of the total) displayed a stable expression level (Fig. 1, group 5 in Online Resource 7), whereas 112 genes (0.82 % of the total) were perturbed in both processes (groups 1, 3, 7 and 9 in Online Resources 7, 8). Both dehydration and rehydration modified the gene expression of *T. gelatinosa*, with a perturbation of 7.01 and 2.22 % of all genes respectively, with a more prominent effect triggered by dehydration (Fig. 1).

The Kal's Z-test revealed 957 DEGs following dehydration: 530 DEGs were up-regulated and 427 down-regulated (Fig. 1 and Online Resource 9) versus the C sample. In R samples, 270 DEGs were identified, 150 genes being up-regulated and 120 down-regulated (Fig. 1 and Online Resource 9) versus the D sample. A significant overlap of gene expression profiles between D and R samples was observed (Fig. 2). Compared to the control, 1208 protein-coding genes were differentially expressed upon rehydration (Online Resource 9). Some genes, in particular during dehydration, experienced a highly significant induction by up to 100 folds (Online Resource 10).

qRT-PCR data validation

We confirmed the gene expression changes observed in the RNA-seq experiment by qRT-PCR, which was performed on the three non-pooled samples for each experimental condition (C, D and R). The trends of expression of all the genes analysed were consistent, with a Pearson correlation index of 0.77 (*F* test of linear regression p value = 0.01) (Fig. 3), although the FC values varied to some extent between qRT-PCR and RNA-Seq and among replicates. This slight discrepancy is compatible with the technical limitations and the differences of the two methodological approaches (SEQC/MAQC-III Consortium 2014).

Gene set enrichment analysis

The information gathered from the annotation of the predicted gene models was used for the identification of the

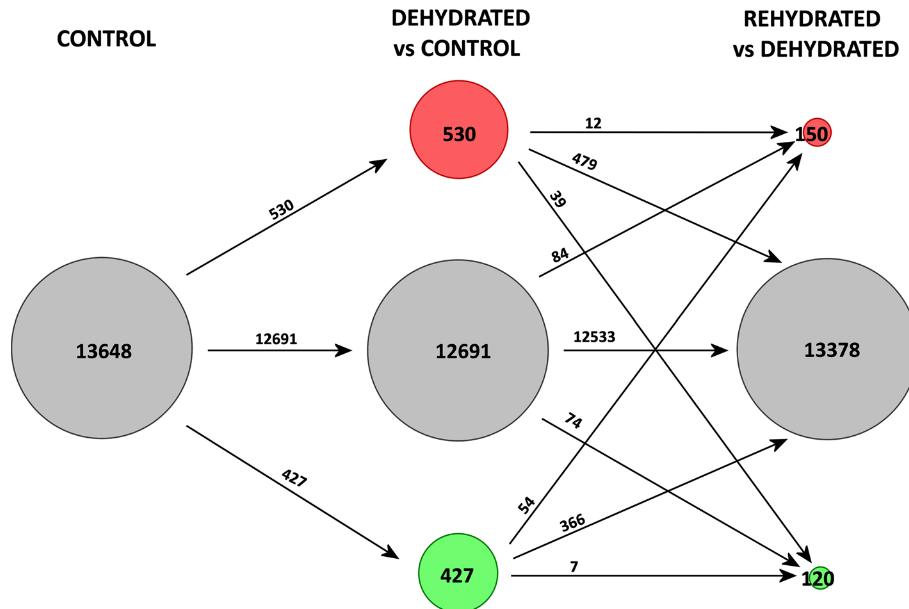


Fig. 1 Diagrams displaying the number of stable (*grey*), up-regulated (*red*) and down-regulated (*green*) transcripts in the dehydrated versus control and rehydrated versus dehydrated comparisons. The numbers displayed on the *arrows* indicate the number of transcripts. See Online Resources 7 and 8 for details

main gene categories influenced by dehydration and by the following rehydration. The eggNOG assignments, Gene Ontology (GO) terms and PFAM domains over-represented in each gene set are shown in Table 4 (D vs C and R vs D comparisons) and Online Resource 11 (R vs C comparison).

The GO terms related to the photosynthetic apparatus stand out as the largest group of up-regulated genes in response to dehydration. The GO terms with the best *p* value score in the hypergeometric test were light-harvesting complex (GO:0009765), chlorophyll binding (GO:0016168), PSI (GO:0009522), and PSII (GO:0009523). The 70 and 52 % of the genes encoding for structural components of PSI and PSII, respectively, were strongly over-expressed, together with five out of the five detected genes encoding for components of the PSI reaction centre, chlorophyll a/b binding proteins and their transcriptional regulator Tbc2. The massive up-regulation of the photosynthetic machinery is graphically summarized in Fig. 4, according to the "photosynthesis" and "photosynthesis—antenna proteins" KEGG reference pathways (Kanehisa and Goto 2000).

The Major Intrinsic Protein family (PF00230 and COG0580) was also significantly up-regulated upon dehydration; the five over expressed members of this protein family were all TIP (Tonoplast Intrinsic Proteins) or PIP (Plasma membrane Intrinsic Proteins) aquaporins. A remarkable protein family whose expression was induced by dehydration was MAPEG (Membrane-associated, eicosanoid/glutathione metabolism, PF01124), with three out of its four members being glutathione S-transferases. Another large class of enzymes induced by dehydration was represented by a series of short chain dehydrogenases with various functions (PF00106 and COG1029) with their associated domains (PF01370, PF08659) which include, among the others, two dihydroflavonol-4-reductases (DFRs).

The ferritin-like domain (PF13668) was the most up-regulated Protein Family term and a highly significant *p* value in the hypergeometric test, with seven out of thirteen genes up-regulated by the dehydration process (Table 4). These transcripts clearly belong to the same multigenic family, which displays remarkable sequence similarity with the desiccation related proteins (DRPs) family previously described in some DT seed plants (Piatkowski et al. 1990; Zha et al. 2013). In total, 9 out of these 13 sequences were significantly responsive to at least one of the two treatments, either being up- or down-regulated. Most of them displayed a trend of expression that involved an increase during dehydration and a repression after rehydration. DRP1 and DRP2, however, followed a completely opposite trend, whereas the expression of DRP5 progressively increased during the experiment (Table 5). Other categories significantly up-regulated by dehydration were cytokinin-mediated signaling pathway (GO:0009736), carbohydrate metabolic process (GO:0005975), cyclins (PF00134), omega peptidase activity (GO:0008242) and C2 domain-containing proteins (PF00168).

Due to the high similarity of the transcriptional profiles between rehydrated and dehydrated samples, just a few gene categories affected by the rehydration process were identified. However, their involvement remains questionable due to the low number of over-expressed genes compared to the total and to the poorly significant *p* values (Table 4).

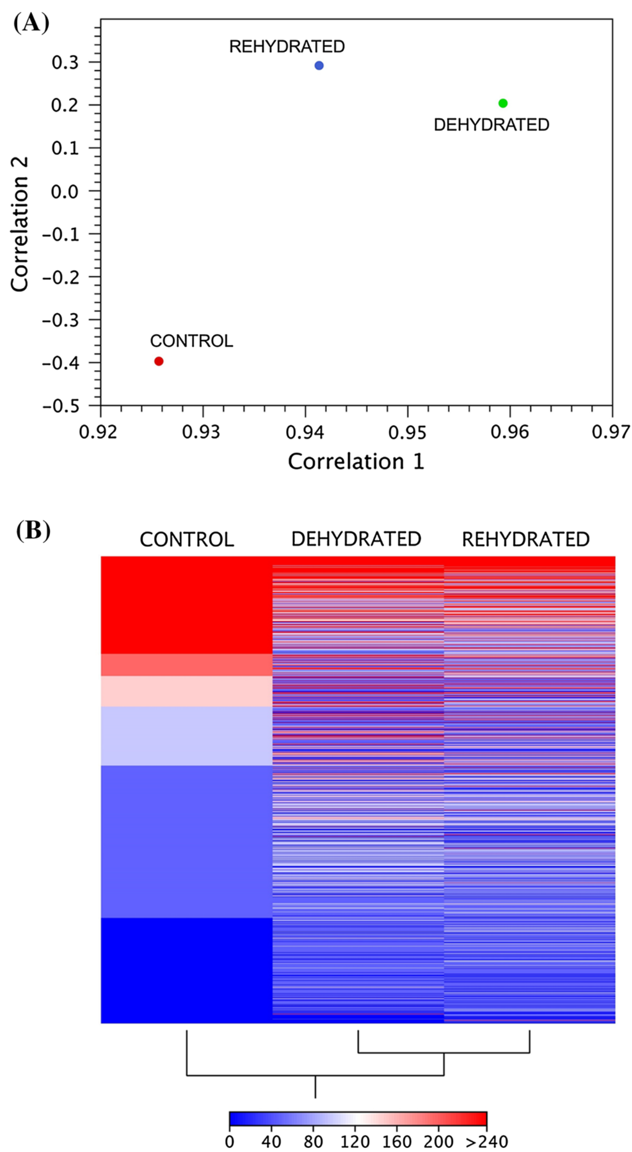


Fig. 2 Principal component analysis and Hierarchical Clustering of control, dehydrated and rehydrated samples of *Trebouxia gelatinosa*. Principal component analysis based on TPM expression values (**a**), Hierarchical Clustering based on Euclidean distance and average linkage on TPM, with the gradient colour scheme where *blue* and *red* stand for *high* or *low* expression levels respectively (**b**)

Desiccation related proteins (DRPs): structure and phylogeny

The ferritin-like domain denotes a taxonomically widespread structural fold, which characterizes different protein families including, among the others, ferritins, bacterioferritins and bacterial DNA-binding proteins from starved cells (DPS). However, DRPs show low sequence homology to *bona fide* ferritins and other ferritin-like protein families available in GenBank. Furthermore, they all present a ~100 aa long conserved C-terminal region, here named DUF1 (Domain of Unknown Function 1), which is absent in other ferritin-like proteins. The structural architecture of green algae DRPs is not uniform: they all present a ferritin-like domain followed by DUF1, but additional long N-terminal or C-terminal regions with unknown function are present in several cases (Fig. 5a).

A comparative genomic analysis revealed that DRPs are absent in most Chlorophyta genomes: no DRPs could be found in Ulvophyceae and they are present in a few Chlorophyceae (*Monoraphidium* spp. and *Chlamydomonas* spp.) as single-copy genes. However, the DRP gene family appears to have undergone expansion in some Trebouxiophyceae including, besides *T. gelatinosa*, other lichen photobionts, such as *Asterochloris* spp. and *Coccomyxa* spp. On the other hand, DRPs are absent in other genera of the same class (*Chlorella*, *Helicosporidium* and *Picochlorum*). The spread of DRPs within Charophyta, Chlorophyta's sister group within which the Embryophyta emerged, is difficult to address due to the limited sequence resources available; only a single DRP gene was actually found in *Klebsormidium flaccidum*. Among Embryophyta, DRPs are present in the genomes of all the species analysed so far, from those belonging to basal groups such as bryophytes (e.g. *Physcomitrella patens*) and lycophytes (e.g. *Selaginella moellendorffii*), to seed plants. Differently to what observed for Chlorophyta, Embryophyta DRPs share a similar length (~300 aa) and a more uniform protein architecture, with a N-terminal leader peptide, followed by the central ferritin-like domain and always ending with the C-terminal DUF1 domain (Fig. 5a).

The taxonomic distribution of DRPs, however, is not limited to Viridiplantae, since this protein family is also present in several phylogenetically distant groups of Bacteria, but not in heterotrophic Eukaryota. All bacterial DRPs display a similar length and a protein architecture identical to that of most DRPs of green algae and higher plants.

The Bayesian phylogenetic analysis divided higher plants and bacterial DRPs in two well distinct clades, but it surprisingly revealed that the DRPs of Chlorophyta are more closely related to bacterial proteins than they are to those of vascular plants. Although the remarkable sequence diversity did not permit to fully resolve the exact phylogenetic relationship among green algae DRPs, two major distinct subgroups could be identified. The first one is more closely related to bacterial DRPs and includes *T. gelatinosa* DRP1-DRP8 and DRP11, proteins with heterogeneous architecture and subcellular localization (Fig. 5b; Table 5). The second subgroup, which includes DRP9, DRP10, DRP12 and DRP13, is more distantly related to bacteria and comprises proteins characterized by a conserved C-terminal ~100 aa extension with unknown function (DUF2) located after DUF1 (Fig. 5a, b). While basal land plants (bryophytes and lycophytes) DRPs are clearly

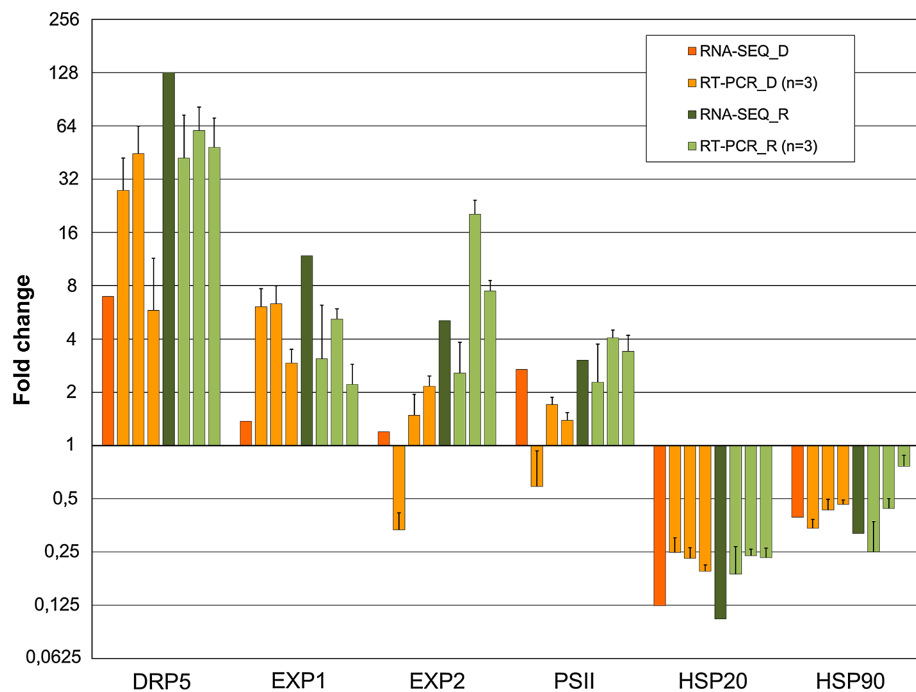


Fig. 3 Comparison between the fold change of six differently expressed transcripts: Desiccation Related Protein 5 (DRP 5), Expansin 1 (EXP1), Expansin 2 (EXP2), Photosystem II (PSII), Heat Shock Protein 20 (HSP20) and Heat Shock Protein 90 (HSP90) obtained with RNA-seq (on three pooled biological replicates) and with qRT-PCR (on the three non-pooled biological replicates) in dehydrated (D) and rehydrated (R) samples of *Trebouxia gelatinosa*. Results of RT-PCR are given as the mean ± SD of three technical replicates

clustered within the same clade of vascular plants, the placement of the *K. flaccidum* DRP is enigmatic, as it shows marked similarity with bacterial and green algae DRPs.

Expression of genes and gene families related to stress response

We analysed in detail the expression profiles of specific genes or genes families which could be potentially involved in the responses to dehydration stress and rehydration, based on the recent literature concerning desiccation tolerance in Streptophyta and Embryophyta. This permitted us to comparatively investigate the regulation of molecular adaptive strategies to water stress. The comparison revealed that in *T. gelatinosa* there is a different regulation of genes included in the categories, "Aquaporins", "Cell wall modifications", "HSPs and other chaperones", "Late Embryogenesis Abundant proteins", "Oxidative stress response" and "Photosynthetic apparatus" (Table 6).

Discussion

Features of *T. gelatinosa* transcriptome

The genomes of Trebouxiophyceae are generally small in size, with a 2C nuclear DNA content estimated between 0.01 and 1.06 pg (Kapraun 2007). For instance, the genome of *Asterochloris* sp. has 56.1 Mbp (~0.06 pg, http://genome.jgi.doe.gov/Astpho1/), that of *Coccomyxa subellipsoidea* 49.0 Mbp (~0.05 pg) (Blanc et al. 2012), and that of *Helicosporidium* sp. only 10.5 Mbp (~0.01 pg), being one of the smallest genomes among free-living eukariotes (Pombert et al. 2014). The small genome size, however, is not due to a functional reduction (e.g. through massive loss of gene families) and therefore results in high genomic compactness (Pombert et al. 2014). *Asterochloris* sp. shows 128 gene models per Mbp, i.e. approximately 58 % of its genome consists of coding genes, for the shrinkage of introns and non-coding intergenic regions. This is frequently associated to a transcriptional overlap (Williams et al. 2005), that potentially represents a problem in the de novo transcriptome assembly process. Unfortunately, the recently sequenced genome of a *T. gelatinosa* strain (ASM81890v1, see Bioproject PRJNA263654), with its 60.1 Mbp size, is not yet annotated. Furthermore, that strain differs genetically, on average, by 6.56 % within ORFs from the isolate of this study, although the two strains have been given the same species name. This fact reflects the unsatisfactory taxonomy of the genus *Trebouxia* and the actual underestimation of genetic divergence within infrageneric taxa (Friedl 1989). The analysis of the nuclear ITS region have indeed already detected a high heterogeneity within other *Trebouxia* species (Piercey-Normore 2006; Leavitt et al. 2013; Muggia et al. 2014), and in the near future new infrageneric taxa will likely be recognized for lineages now gathered under the same name (O'Brien 2013; Muggia et al. unpublished

Table 4 Summary of the hypergeometric tests on annotations performed on the *Trebouxia gelatinosa* sets of differentially expressed genes in the dehydrated versus control and rehydrated versus dehydrated comparisons

Category	ID	Description	*p* value	Proportion
Dehydrated versus control				
Up-regulated				
eggNOG	COG0580	Glycerol uptake facilitator and related permeases	3.91E−4*	4/9
eggNOG	COG1028	Dehydrogenases with different specificities	1.38E−3*	8/50
GO_BP	GO:0009765	Photosynthesis, light harvesting	2.72E−14**	14/21
GO_BP	GO:0018298	Protein-chromophore linkage	3.67E−12**	14/27
GO_BP	GO:0015979	Photosynthesis	1.13E−5*	11/47
GO_BP	GO:0009736	Cytokinin mediated signaling pathway	9.32E−4*	4/10
GO_BP	GO:0005975	Carbohydrate metabolic process	1.09E−3*	10/65
GO_BP	GO:0006950	Response to stress	4.39E−3*	10/78
GO_CC	GO:0009522	Photosystem I	2.22E−16**	16/23
GO_CC	GO:0009523	Photosystem II	3.87E−12**	14/27
GO_CC	GO:0009535	Chloroplast thylakoid membrane	2.66E−10**	28/146
GO_CC	GO:0009538	Photosystem I reaction center	2.75E−7**	5/5
GO_CC	GO:0016021	Integral to membrane	8.95E−6**	96/1319
GO_CC	GO:0009543	Chloroplast thylakoid lumen	5.31E−5*	8/29
GO_CC	GO:0009505	Plant-type cell wall	9.95E−5*	5/11
GO_CC	GO:0005615	Extracellular space	4.46E−3*	5/23
GO_MF	GO:0016168	Chlorophyll binding	1.55E−15**	15/23
GO_MF	GO:0008242	Omega peptidase activity	2.51E−4*	4/8
GO_MF	GO:0043169	Cation binding	4.27E−3*	7/46
PFAM	PF00504	Chlorophyll A–B binding protein	7.48E−11**	14/32
PFAM	PF13668	Ferritin-like domain	1.61E−6**	7/14
PFAM	PF01124	MAPEG family	5.63E−6**	4/4
PFAM	PF00134	Cyclin, N-terminal domain	5.60E−5*	5/10
PFAM	PF00230	Major intrinsic protein	1.62E−4*	5/12
PFAM	PF01370	NAD dependent epimerase/dehydratase family	3.20E−4*	9/46
PFAM	PF00106	Short chain dehydrogenase	3.55E−4*	13/89
PFAM	PF00168	C2 domain	3.81E−4*	6/21
PFAM	PF13561	Enoyl-(Acyl carrier protein) reductase	4.41E−4*	10/58
PFAM	PF08659	KR domain	2.47E−3*	10/72
Down-regulated				
GO_BP	GO:0006950	Response to stress	1.14E−5*	12/78
GO_BP	GO:0009408	Response to heat	1.38E−5*	10/55
GO_BP	GO:0016485	Protein processing	2.42E−4*	4/10
GO_CC	GO:0005886	Plasma membrane	6.14E−4*	33/575
GO_CC	GO:0000502	Proteasome complex	2.41E−3*	5/30
GO_CC	GO:0009706	Chloroplast inner membrane	7.70E−3*	5/39
GO_MF	GO:0017111	Nucleoside-triphosphatase activity	3.35E−3*	7/61
GO_MF	GO:0043565	Sequence-specific DNA binding	6.40E−3*	5/37
PFAM	PF00012	Hsp70 protein	4.56E−6**	6/14
PFAM	PF00320	GATA zinc finger	6.21E−4*	4/12
PFAM	PF00004	ATPase associated with various cellular activities (AAA)	3.88E−3*	8/72
Rehydrated versus dehydrated				
Up-regulated				
GO_CC	GO:0005886	Plasma membrane	7.99E−3*	11/575

Table 4 continued

Category	ID	Description	p value	Proportion
Down-regulated				
GO_CC	GO:0005788	Endoplasmic reticulum lumen	3.80E−4*	4/41

* Significant; ** highly significant

Up- and down-regulated genes were analyzed separately. *eggNOG* evolutionary genealogy of genes: Non-supervised Orthologous Groups; *GO_BP* Gene Ontology Biological Process; *GO_CC* Gene Ontology Cellular Component; *GO_MF* Gene Ontology Molecular Function; *PFAM* Protein Family. The proportion column indicates the number of differentially expressed genes with respect to the total number of genes annotated with the same term in the entire *Trebouxia gelatinosa* transcriptome

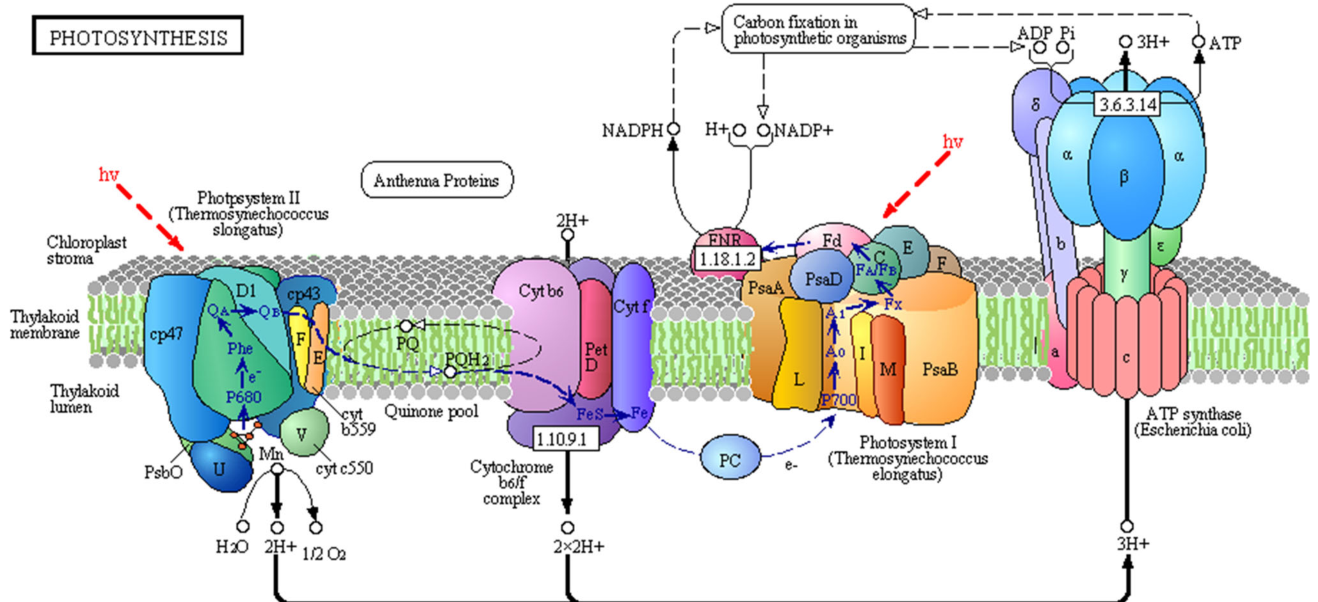
data). Also *T. gelatinosa* is evidently heterogeneous and certainly deserves further study from this point of view.

Dehydration- and rehydration-induced gene expression changes in *T. gelatinosa* and comparison with other desiccation-tolerant (DT) photosynthetically active organisms

RNA-seq analyses can only provide information concerning the relative abundance of different mRNAs in a sample, so one should take into account that the TPM values we used do not represent an absolute measure of the actual abundance of mRNAs in a sample, but they are rather proportional to the ratio of the their molar concentration over the sum of those of all transcripts in the pool of mRNAs (Musser and Wagner 2015).

The gene expression analysis showed that the D and R transcriptomes were unexpectedly very similar. This is an important feature which cannot be interpreted as a failure of the algae to recover from desiccation, which typically occurs within minutes or a few hours (Gasulla et al. 2009). The same conditions applied in this experiment should not be considered as particularly stressful for *T. gelatinosa,* for two reasons. First, *Trebouxia* species can withstand harsher conditions than those applied in this study, such as very long periods in the desiccated state (up to 45 days) under photo-oxidative conditions (Candotto Carniel et al. 2015, 2016). Second, we could not detect any significant degradation of mRNAs caused by dehydration (D samples) in comparison to the hydrated situations (C and R samples). Maintenance of RNA stability has been previously demonstrated to be a key protective strategy in DT plants (Dinakar and Bartels 2012). Interestingly, the transcripts obtained from D displayed a lower drop in the 3′–5′ end sequencing coverage, which suggests the presence of an unknown mRNA protection mechanism activated in response to dehydration (Online Resource 1). On the other side, the choice of keeping cultures at full hydration for a long period of time was essential to single out those genes which are expressed under the best conditions, although these rarely occur in nature (Lange and Green 2008), and usually only in a limited period of the year (Tretiach et al. 2013).

In *T. gelatinosa*, one of the major differences between C, D and R is the highly significant up-regulation of most genes related to the photosynthetic apparatus. Its maintenance is a priority in homoiochlorophyllous DT organisms because photosynthesis must fully recover as soon as possible upon rehydration to gain a positive CO_2 balance even when rehydration events are short and erratic. Several aeroterrestrial algae, all lichens and DT mosses can actually restore photosynthesis in a few minutes after rehydration (Veerman et al. 2007; Kosugi et al. 2009; Lüttge and Büdel 2010; Cruz de Carvalho et al. 2014; Holzinger and Karsten 2013; Candotto Carniel et al. 2015). The presence of abundant mRNA coding for components of the photosynthetic apparatus upon rehydration, such as photosystems (see Results session), may contribute to the fast re-establishment of photosynthesis. This pattern was also observed in the streptophyte *Klebsormidium crenulatum* by Holzinger et al. (2014), who argued that the up-regulation of genes related to the photosynthetic apparatus is aimed at preparing the organism for the next rehydration event. This hypothesis is supported by the findings of Aubert et al. (2007), who observed the increase of ribulose 1,5-diphospate to control levels after a few minutes upon rehydration of *Xanthoria elegans* (a lichen which has a species of *Trebouxia* as photobiont), meaning that the whole photosynthetic machinery is steadily maintained functional. Contrasting results have been collected in homoiochlorophyllous resurrection plants, displaying either a reduced (Rodriguez et al. 2010) or enhanced (Ma et al. 2015) production of photosynthesis-related mRNAs (Table 6). The immediate synthesis of structural components of the photosynthetic apparatus upon rehydration could also be interpreted as a strategy to reduce the damage caused by prolonged periods in the desiccated status under high light. At these conditions PSI and PSII are, in fact, potential sources of ROS (Veerman et al. 2007) which may damage the photosystems themselves and also membranes and nucleic acids (Smirnoff 1993; Kranner et al. 2008). This damaging action is usually countered by non-enzymatic



Photosystem II

D1	D2	cp43	cp47	cyt b559	
PsbA	PsbD	PsbC	PsbB	PsbE	PsbF

						MSP	OEC
PsbL	PsbJ	PsbK	PsbM	PsbH	PsbI	PsbO	PsbP
PsbQ	PsbR	PsbS	PsbT	PsbU	PsbV	PsbW	PsbX
PsbY	PsbZ	Psb27	Psb28	Psb28-2			

Photosystem I

PsaA	PsaB	PsaC	PsaD	PsaE	PsaF	PsaG	PsaH
PsaI	PsaJ	PsaK	PsaL	PsaM	PsaN	PsaO	PsaX

Cytochrome b6/f complex

PetB	PetD	PetA	PetC	PetL	PetM	PetN	PetG

Photosynthethic electron transport

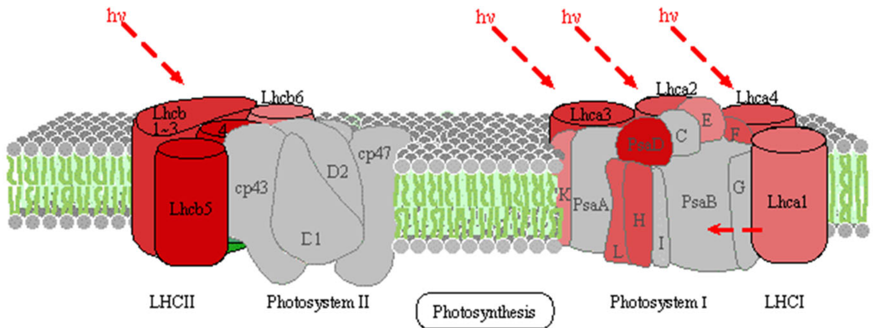
PC	Fd	FNR	cyt c6
PetE	PetF	PetH	PetJ

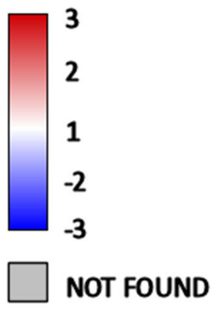
F-type ATPase

beta	alpha	gamma	delta	epsilon	c	a	b

PHOTOSYNTHESIS - ANTENNA PROTEINS

Light-harvesting chlorophyll protein complex (Plant, Green alga)





Light-harvesting chlorophyll protein complex(LHC)

Lhca1	Lhca2	Lhca3	Lhca4	Lhca5		
Lhcb1	Lhcb2	Lhcb3	Lhcb4	Lhcb5	Lhcb6	Lhcb7

◀**Fig. 4** Summary of the gene expression alterations which concern the photosynthetic machinery observed during dehydration. The KEGG reference pathway maps from "photosynthesis" and "photosynthesis-antenna proteins" are displayed. The genes involved are grouped in *boxes* below the figures and are coloured based on up- or down-regulation. Due to their very small size (<100 aa) and high sequence divergence between the reference organism (*Arabidopsis thaliana*) and *T. gelatinosa*, several orthologous components, indicated by a *gray colour*, could not be identified

antioxidant molecules coupled with the activity of ROS scavenging enzymes (Kranner and Birtić 2005). The expression of genes coding for these mechanisms is usually up-regulated in DT plants (Mowla et al. 2002; Iturriaga et al. 2006; Rodriguez et al. 2010; Gechev et al. 2013) (Table 6). In our case, the expression of most of the primary ROS scavenging enzymes remained steady but two DFR homologs were over-expressed (with a FC ~ 2 times) in dehydrated samples. DFR is a crucial enzyme for the production of the flavonoid-like anthocyanins and proanthocyanidins, which is up-regulated in response to dehydration in drought-tolerant cowpea as well as in loblolly pine seedlings (Iuchi et al. 1996; Watkinson et al. 2003). Interestingly, flavonoids are thought to act as a secondary ROS-scavenging system in plants subject to high light stress, especially when the chloroplast antioxidants are depleted and the ROS are free to diffuse in the cytosol (Fini et al. 2011). Considering that the synthesis of flavonoids and the up-regulation of related enzymes have been reported in resurrection plants (Ma et al. 2015; Moore et al. 2007) subjected to water stress we cannot exclude that this secondary ROS-scavenging system might play a role in the intracellular redox homeostasis in *Trebouxia*, especially when dehydration occurs under light regimes/levels that can induce ROS production.

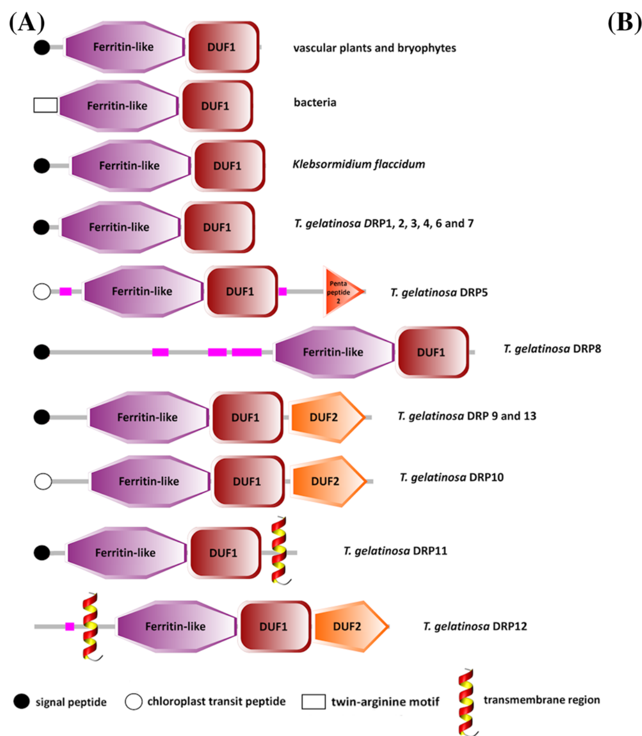
Another important ROS source, especially upon metabolism reactivation, is the mitochondrion. We detected the over-expression of one mitochondrial manganese superoxide dismutase (MnSOD), an enzyme catalyzing the dismutation of superoxide anion to hydrogen peroxide. The up-regulation of this enzyme upon dehydration may be a way to build-up MnSOD mRNAs in preparation for rehydration, to replace the enzymes inactivated by ROS (Weissman et al. 2005). In addition, the up-regulation of three microsomal glutathione S-transferases pertaining to the MAPEG family could also be part of a mechanism to keep the intracellular redox state under control. Although the role of these trans-membrane enzymes has not been elucidated yet in plants, their role in cellular protection against ROS damage in animals has been clearly documented (Shi et al. 2012).

In *Trebouxia* spp., loss and gain of water cause gross morphological modifications (Honegger 1995), with important effects on cell ultrastructure. Whenever cells experience dehydration, certain mechanisms need to be adopted to follow the shrinkage of the cell wall, avoiding the detachment of the plasma membrane. The regulation of expansins may possibly explain how this is achieved in *T. gelatinosa*. Expansins disrupt non-covalent polysaccharides bonds and are involved in cell wall flexibility (Cosgrove 2000). Their over-expression upon rehydration can

Table 5 *Trebouxia gelatinosa* desiccation related proteins (DRPs) with the respective expression values shown as normalized read counts, proportion fold change values in the dehydrated versus control and rehydrated versus dehydrated comparisons and predicted cellular localization according to TargetP

ID	Normalized expression values			Fold change		Cellular localization
	Control	Dehydrated	Rehydrated	Dehydrated versus control	Rehydrated versus dehydrated	
*Trebouxia*_DRP1	4805.87	346.73	1647.46	−13.86*	4.75*	S
*Trebouxia*_DRP2	1893.78	79.53	420.01	−23.81*	5.28*	S
*Trebouxia*_DRP3	23.64	34.88	34.67	1.48	−1.01	S
*Trebouxia*_DRP4	69.03	257.4	333.66	3.73*	1.3	S
*Trebouxia*_DRP5	2.19	15.22	280.94	6.96*	18.45*	P
*Trebouxia*_DRP6	7.97	160.74	38.88	20.17*	−4.13*	S
*Trebouxia*_DRP7	483.62	1889.74	971.5	3.91*	−1.95	S
*Trebouxia*_DRP8	20.86	39.42	45.78	1.89	1.16	S
*Trebouxia*_DRP9	55.3	100.32	152.08	1.81	1.52	S
*Trebouxia*_DRP10	179.13	568.66	351.74	3.17*	−1.62	P
*Trebouxia*_DRP11	286.36	1325.71	667.34	4.63*	−1.99	T
*Trebouxia*_DRP12	145.44	208.69	210.09	1.43	1.01	S
*Trebouxia*_DRP13	2.02	17.73	4.77	8.78*	−3.71	M or S

* Significant difference in the statistical expression analysis; *S* secreted, *P* plastidial, *T* transmenbrane, *M* mitochondrial



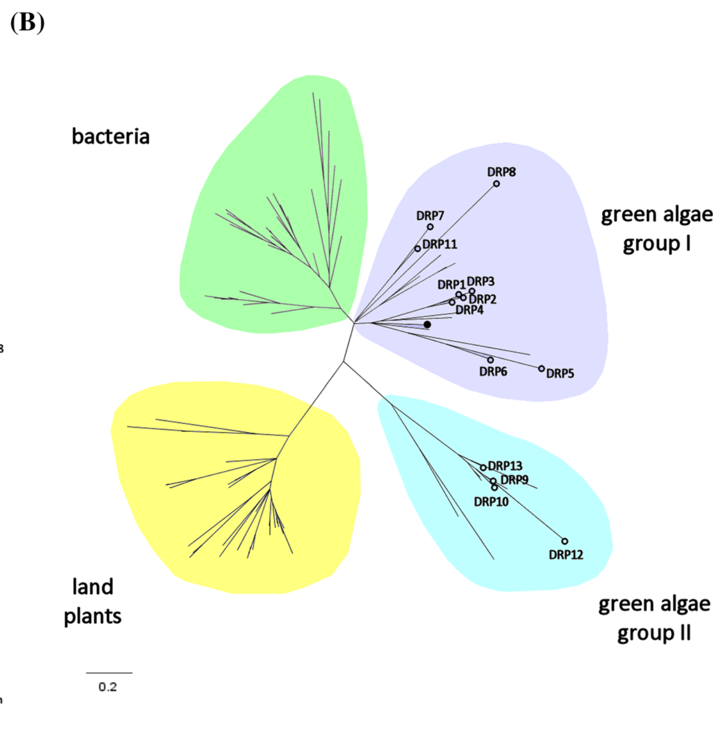


Fig. 5 Structure characteristics of the 13 desiccation related proteins (DRP1-13) found in the transcriptome of *Trebouxia gelatinosa* in comparison to those found in bacteria, other green microalgae and embryophytes. DUF1 and 2, Domains of Unknown Function 1 and 2. Proteins length not in scale (**a**). Bayesian phylogenetic tree of Desiccation-Related Proteins (DRPs). *Trebouxia gelatinosa* DRPs are indicated by *empty circles*, whereas the *full circle* indicates the DRP belonging to the streptophyte *Klebsormidium crenulatum* (**b**). The full list of the DRP sequences used for the phylogenetic analysis and their accession IDs is reported in Online Resource 3

Table 6 Expression profiles during dehydration and rehydration of gene categories commonly associated to desiccation tolerance in the green alga *Trebouxia gelatinosa* (*Tg*), the Streptophyte *Klebsormidium crenulatum* (*Kc*) (Holzinger et al. 2014), the moss *Syntrichya ruralis* (*Sr*) (Oliver et al. 2004, 2009), the club moss *Selaginella lepidophylla* (*Sl*) (Iturriaga et al. 2006), the dicots *Craterostigma plantagineum* (*Cp*) (Rodriguez et al. 2010; Mariaux et al. 1998; Jones and McQueen-Mason 2004), *Myrothamnus flabellifolia* (*Mf*) (Ma et al. 2015) and *Haberlea rhodopensis* (*Hr*) (Gechev et al. 2013), and in the monocot *Xerophyta humilis* (*Xh*) (Collett et al. 2004)

	Dehydration								Rehydration				
	Tg	*Kc*	*Sr*	*Sl*	*Cp*	*Mf*	*Hr*	*Xh*	*Tg*	*Sr*	*Cp*	*Mf*	*Hr*
Aquaporins	↑	=	=	↑	↑↓	=	=	↓	=	↑	↑	=	=
Cell wall modifications	=	=	=	=	↓↑	=	↓	=	↑	=	↑	=	↑
HSPs and other chaperones	↓	=	↑	↑	=	=	↑	=	↓	=	=	=	=
Late embryogenesis abundant proteins	=	↑	↑	↑	↑	↑	↑	↑	=	↑	↓	↓	↓
Oxidative stress response	↑*	↑	↑	↑	↓	↑	↑↓	↑	=	↑	↑	↓	↑↓
Photosynthetic apparatus	↑	↑	↓	↑	↓	↑	↓	↓	=	↑	↑	=	↑

↑ up-regulated; ↓ down-regulated; ↑↓ contrasting results; = not affected. * The expression of most ROS-scavenging enzymes was not affected

thus be read as a preparation for the next dehydration event. Although their role in desiccation tolerance of the resurrection plant *C. plantagineum* has been recognized for a long time (Jones and McQueen-Mason 2004), this is the first evidence of their possible involvement in desiccation tolerance in an aero-terrestrial Chlorophyte.

Gain of water can also be harmful since tearing of the membranes due to the sudden inrush of water is thought to be a cause of irreparable damage in many desiccation sensitive plants (Pammenter and Berjak 1999). The increase of membrane permeability to water can be seen as an adaptation to avoid damage to the membranes upon rehydration, especially if rehydration events are frequent. In *T. gelatinosa*, five TIP and PIP aquaporins were significantly up-regulated in dehydrated samples, but their expression remained steady with rehydration. Plant aquaporins have been implicated in

drought stress tolerance in several vascular plants, although their expression patterns largely vary depending on the isoform and tissue (Šurbanovski et al. 2013). For example, an enhanced expression of aquaporins has been observed in the desiccated resurrection plants *C. plantagineum*, *Reaumuria soongorica* and *Sporobolus stapfianus*, suggesting that they may have a regulatory role in maintaining cell turgor (Liu et al. 2014; Mariaux et al. 1998; Neale et al. 2000). More recent studies identified a Tonoplast Intrinsic Protein (TIP) in the small vacuoles of the bundle sheath cells of the resurrection grass *Eragrostis nindensis*, indicating that it may also cover an important role in the mobilization of solutes from the small vacuoles upon rehydration (Vander Willigen et al. 2004). On the other hand, aquaporins were strongly down-regulated in the dehydrated monocot resurrection plant *Xerophyta humilis* (Collett et al. 2004) (Table 6).

Beside morphological and ultrastructural modifications, loss and sudden gain of water cause the alteration of the structure and interactions of molecules. Interestingly HSPs, which prevent the denaturation of proteins and avoid their aggregation (Bartels and Sunkar 2005; Prieto-Dapena et al. 2008; Sun et al. 2001), seem not to be involved in the desiccation tolerance of *T. gelatinosa*. Indeed, the high expression level of several HSPs in the control samples (Online resource 12) was followed by a highly significant reduction during dehydration, which in many cases further decreased upon rehydration. On the contrary the *A. erici* HSP90 expression increased during dehydration, remaining higher than controls also during rehydration (Gasulla et al. 2013). The same trend, but for other HSPs, was observed in mosses (e.g. *Fontinalis antipyretica*, Wang et al. 2004; *Syntrichya ruralis,* Cruz de Carvalho et al. 2014), lycophytes (e.g. *Selaginella lepidophylla*, Iturriaga et al. 2006) and resurrection plants as well (e.g. *C. plantagineum*, Alamillo 1995) (Table 6). We are still unable to explain this peculiar behaviour, and certainly further analyses are needed to clarify this pattern and to understand the role of chaperones in the desiccation tolerance of *T. gelatinosa*.

Another unexpected result regards Late Embryogenesis Abundant proteins (LEAs). We found only few genes encoding for LEAs sensu stricto in *T. gelatinosa*: one dehydrin (group 2 LEAs) and two group 3 LEAs. More interestingly, the expression of these genes was constitutive and did not vary in relation to hydration. This expression pattern differs from those observed in other DT organisms (Table 6), where LEA proteins are commonly up-regulated during dehydration and/or rehydration. A constitutive expression of these proteins (i.e. in fully hydrated samples) was observed in the resurrection plant *H. rhodopensis* (Gechev et al. 2013), but in this case these genes were further up-regulated during dehydration. In vascular plants LEAs form a heterogeneous class that can be subdivided into at least five major groups (Cuming 1999), with several dozen genes (Hundertmark and Hincha 2008; Du et al. 2013; Lan et al. 2013), whose precise mechanism of action is still unclear (Goyal et al. 2005; Tunnacliffe and Wise 2007). The low number of LEAs in *T. gelatinosa*, consistent with the situation observed in the genomes of other Trebouxiophyceae, suggests that the gene family expansion observed in vascular plants did not occur in this lineage. However, we found seven genes coding for proteins similar to the cold regulated LEA-like proteins of the Antarctic strain NJ-7 of *Chlorella vulgaris* (Liu et al. 2011). While it is certainly noteworthy that four out of these seven genes were constitutively expressed at very high levels and that the remaining three were over-expressed upon dehydration, the involvement of this poorly known gene family in desiccation-tolerance in *Trebouxia* remains to be investigated. Overall, LEAs activation does not seem to play a relevant role in response to dehydration in *T. gelatinosa*, further corroborating the idea that this organism mostly relies on alternative strategies to deal with the effects of dehydration.

The over-expression of cyclins (specifically of G2/mitotic-specific cyclins A) is a puzzling feature of the dehydration trascriptome of *T. gelatinosa*. Since the persistence of cyclin A prevents the stabilization of the kinetochore/microtubules complex (Kabeche and Compton 2013), this suggests that cell cycle progression has been blocked in dehydrated *T. gelatinosa* cells, similarly to what occurs in *K. crenulatum* (Holzinger et al. 2014).

The gene expression patterns described here for *T. gelatinosa* subjected to dehydration and a subsequent rehydration supports the idea that desiccation tolerance in *T. gelatinosa* is mostly achieved through: (1) constitutive mechanisms, which confer a background protection (Oliver et al. 2005), as already hypothesized by Junttila and Rudd (2012) for the lichen *C. rangiferina* and by Gasulla et al. (2013) for the alga *A. erici*; (2) inducible mechanisms similar to those observed in mosses and resurrection plants, e.g. cell wall modifications and aquaporins; (3) inducible mechanisms typical of *T. gelatinosa*, i.e. those mediated by DRPs, that will be further discussed in the next section.

DRPs: a peculiarity of *Trebouxia gelatinosa* transcriptome

The most intriguing feature of the *T. gelatinosa* trascriptome probably regards the gene family encoding for desiccation related proteins (DRPs), whose diversification finds no parallelism in other DT organisms investigated so far. DRPs were firstly described in *C. plantagineum* (Bartels et al. 1990; Piatkowski et al. 1990) and later in other resurrection (Iturriaga et al. 1992; Collett et al. 2004; Ingle et al. 2007) and non-resurrection plants (Zha et al. 2013). However, although DRPs have been frequently implicated in desiccation tolerance (Bartels et al. 1990; Piatkowski

et al. 1990; Battista et al. 2001), their mechanism of action is still unknown and several authors have pointed out that they may cover additional functions in higher plants (Zuo et al. 2005; Guo et al. 2008, 2011; Zha et al. 2013). As we have reported in the results section, DRPs are present also in several unrelated bacterial groups, including the *Deinococcus/Thermus* phylum, some sporigenous Actinobacteria, Acidobacteria, some Betaproteobacteria (Burkholderiales) and some Alphaproteobacteria (mainly pertaining to Sphingomonadales and Rizhobiales). The function of DRPs has been unequivocally linked to DT in *Deinococcus radiodurans*, since the deletion of the locus DRB0118 determines a 75 % loss in viability of desiccated cultures (Battista et al. 2001), and its expression is regulated by the crucial stress response regulator drRRA (Wang et al. 2008). Overall, it is certainly noteworthy that bacterial DRPs are mostly associated with taxa adapted to extreme environments or even tolerant to extreme desiccation (Mattimore and Battista 1996; Hiraishi et al. 2000; Farias et al. 2011; Quintana et al. 2013; Tatar et al. 2013).

Despite the lack of functional evidence, the presence of the ferritin-like domain suggests that DRPs might be involved in the protection mechanisms against oxidative stress, since other protein families containing the ferritin-like domain have been implicated in this function. For example DPS prevent oxidative damage in bacteria, by either oxidizing iron to avoid the formation of oxidative radicals or by physically protecting DNA chains. This behaviour has also been specifically linked to desiccation tolerance in *Rhodococcus* sp. (Haikarainen and Papageorgiou 2010) and to photo-oxidative stress related to rehydration in the cyanobacterium *Nostoc flagelliforme* (Liang et al. 2012). There is clear evidence that classical ferritins themselves have a fundamental role in oxidative stress protection in various vascular plants, due to their potential to detoxify excess iron and dioxygen in mitochondria (Ravet et al. 2009; Briat et al. 2010). Moreover, the up-regulation of ferritins has previously been described in response to dehydration in mosses and red algae (Wang et al. 2009; López-Cristoffanini et al. 2015). The possibility that *Trebouxia* DRPs are similarly involved in protection from oxidative stress is particularly intriguing, considering that several ROS-scavenging enzymes were expressed at a steady level during the dehydration/rehydration experiment.

However, the sequence peculiarities of DPRs, including the presence of the DUF1 domain, leave their functional role still open to speculation. The 13 DRPs genes found in the *T. gelatinosa* transcriptome display a very different behavior in response to the water status of the alga (Table 5). This, together with their structural diversification (Fig. 5a) and different predicted subcellular localization, makes the understanding of the possible functions even more puzzling.

It is evident that the DRPs gene family has undergone relevant expansion in *T. gelatinosa.* Two other lichen chlorobionts belonging to Trebouxiophyceae, *Asterochloris* sp. and *C. subellipsoidea*, have only 5 and 7 DRPs, respectively. Alternatively, in most other green algae DRPs are usually absent or, as in few cases, they are present in just single copy. Bayesian phylogenetic inference clearly pointed out that DRPs of green algae are unlikely to be orthologous to those found in Embryophytes, as they share a surprising sequence similarity to those found in Bacteria (Fig. 5b). In particular, bacterial DRPs and those belonging to green algae group I are virtually indistinguishable, whereas those belonging to group II are well recognizable due to the presence of the C-terminal DUF2 domain. This unexpected result suggests a bacterial origin for *Trebouxia* DRP genes, which may have been ancestrally acquired by horizontal gene transfer (HGT) from bacterial species associated with lichens.

Indeed, the recognition of bacterial communities as a "third partner" in lichen symbioses shows how the traditional concept of lichens needs to be expanded (Grube et al. 2009; Erlacher et al. 2015), as originally proposed by Farrar (1976). Several metagenomic studies have identified Alphaproteobacteria (Rizhobiales, Rhodospirillales and Sphingomonadales in particular) as the dominant group in lichen-associated bacterial communities, usually followed by extremophile Acidobacteria (Bates et al. 2011; Printzen et al. 2012; Erlacher et al. 2015). The overlap between lichen-associated bacteria and those which have DRPs genes is striking and suggests that the possession of DRPs represents an evolutionary advantage for bacteria associated with lichens. On the other hand, beneficial genes could be theoretically passed by HGT between any of the three components of the lichen symbiosis (the bacterial community, the photobiont and the mycobiont), enabling long-term adaptation to specific environmental conditions or stresses (Tunjić and Korac 2013), possibly including water stress tolerance. HGT events between photobionts (including *Trebouxia* spp.) and fungi are well documented (Beck et al. 2014), as well as HGT from bacteria to fungi (Schmitt and Lumbsch 2009; McDonald et al. 2012). To the best of our knowledge, the acquisition of DRPs from symbiotic extremophile bacteria would be one of the first documented instances of HGT from bacteria to lichen photobionts.

Regardless of the evolutionary origin of DRPs, the investigation of their function and diversification in other species of the genus *Trebouxia* will be the next challenge.

Conclusions

Our transcriptomic analysis of the green aero-terrestrial alga/lichen photobiont *T. gelatinosa* shed some light on the molecular mechanisms which are activated or repressed by

changes in the water status. Some of them are similar to those observed in other DT organisms, including the regulation of aquaporins and genes involved in the photosynthetic apparatus or the steady expression of most genes related to the primary antioxidant defenses. In particular, the latter behaviour highlights how constitutive mechanisms are used by *T. gelatinosa* to cope with unpredictable, sudden water loss. This could be an additional explanation on how *Trebouxia*-bearing lichens can face photo-oxidative conditions (Kranner et al. 2005; Candotto Carniel et al. 2015) and specific photochemical pollutants, such as ozone (Bertuzzi et al. 2013). On the other hand, the differences observed with DT vascular plants (Table 6) might be attributed to the remarkable differences existing between these organisms. *Trebouxia gelatinosa* and vascular plants are indeed phylogenetically very distant (they diverged approximately 700 mya). This results in pronounced morphological differences, which in particular determine the different speed of dehydration these organisms are subjected to: in the pluricellular, histologically complex DT vascular plants dehydration is completed in days, whereas in the unicellular lichen photobiont dehydration can occur even in less than 1 h. Constitutively expressed physiological mechanisms could thus help the organism to better cope with frequent and fast dehydration/rehydration cycles.

Our study also revealed novel, potentially important features of *T. gelatinosa* adaptation to water stress: the most relevant one is the expansion and diversification of the DRPs gene family, which finds no parallelism in other DT organisms investigated so far. Although the function of these proteins is currently unknown, their responsiveness to changes in the water status, and their similarity to proteins mostly found in extremophile bacteria offer important cues for future studies.

The high completeness of the assembled *T. gelatinosa* transcriptome here presented, represents now a valuable standard reference for RNA-seq based gene expression studies and a new tool to investigate in more detail the mechanisms at the base of desiccation tolerance of aero-terrestrial microalgae and lichens.

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