Enhanced Desiccation Tolerance in Mature Cultures of the Streptophytic Green Alga Zygnema circumcarinatum Revealed by Transcriptomics

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Desiccation tolerance is commonly regarded as one of the key features for the colonization of terrestrial habitats by green algae and the evolution of land plants. Extensive studies, focused mostly on physiology, have been carried out assessing the desiccation tolerance and resilience of the streptophytic genera Klebsormidium and Zygnema. Here we present transcriptomic analyses of Zygnema circumcarinatum exposed to desiccation stress. Cultures of Z. circumcarinatum grown in liquid medium or on agar plates were desiccated at ~86% relative air humidity until the effective quantum yield of PSII [Y(II)] ceased. In general, the response to dehydration was much more pronounced in Z. circumcarinatum cultured in liquid medium for 1 month compared with filaments grown on agar plates for 7 and 12 months. Culture on solid medium enables the alga to acclimate to dehydration much better and an increase in desiccation tolerance was clearly correlated to increased culture age. Moreover, gene expression analysis revealed that photosynthesis was strongly repressed upon desiccation treatment in the liquid culture while only minor effects were detected in filaments cultured on agar plates for 7 months. Otherwise, both samples showed induction of stress protection mechanisms such as reactive oxygen species scavenging (early light-induced proteins, glutathione metabolism) and DNA repair as well as the expression of chaperones and aquaporins. Additionally, Z. circumcarinatum cultured in liquid medium upregulated sucrose-synthesizing enzymes and strongly induced membrane modifications in response to desiccation stress. These results corroborate the previously described hardening and associated desiccation tolerance in Zygnema in response to seasonal fluctuations in water availability.

Keywords: Desiccation tolerance • gene expression • streptophytic algae • transcriptomics • Zygnema.

Abbreviations: ANOVA, analysis of variance; AQP, aquaporin; BLAST, basic local alignment search tool; CTAB, cetyl trimethylammonium bromide; DTT, dithiothreitol; ELIP, early light-induced protein; ERD, early-response-to-dehydration protein; FDR, false discovery rate; GO, Gene Ontology; HSD, honestly significant difference; Hsp, heat shock protein; LEA, late embryogenesis abundant; KEGG, Kyoto encyclopedia of genes and genomes; KO, KEGG orthology; RH, relative humidity; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphat-carboxylase/oxygenase; Y(II), effective quantum yield of photosystem II.

Introduction

The colonization of terrestrial habitats by plants is accompanied by a number of abiotic stress factors such as high irradiance and dehydration (Borstlap 2002, Holzinger and Pichrtová 2016). Tolerating water stress is crucial for survival of land plants as well as terrestrial algae and has been addressed extensively (Borstlap 2002, Oliver 2007, Dinakar and Bartels 2013, Holzinger et al. 2014). Most land plants are able to actively regulate their water status (homoiohydry) but have lost the ability to tolerate desiccation. However, the so-called resurrection plants, such as Craterostigma plantagineum, possess desiccation tolerance (poikilohydry; Norwood et al. 2003) and are not able to actively regulate their water content (Holzinger and Karsten 2013). Hence, C. plantagineum and other resurrection plants were used as models for desiccation stress tolerance (Bartels and Salamin 2001, Dinakar and Bartels 2013). Additionally, other higher plants (Oliver et al. 2011, Ma et al. 2015, Basu et al. 2016) and mosses were studied to unravel the mechanisms of desiccation response and resilience (Shinde et al. 2012, Gao et al. 2015).

Over recent years, substantial information about the impact of desiccation stress on streptophytic green algae, including Zygnema spp. and Klebsormidium spp., has become available (for summary see Holzinger and Karsten 2013, Karsten and Holzinger 2014, Holzinger and Pichrtová 2016). Klebsormidium generally occupies moist terrestrial habitats (Lokhorst 1996) while Zygnema occurs in hydro-terrestrial environments, meaning in or in close vicinity to freshwater bodies or streams (Hawes 1990, Davey 1991). With decreasing air humidity water is rapidly lost, but these poikilohydric organisms have the ability to tolerate dehydration in the vegetative state to a certain degree. However, in Zygnema desiccation tolerance is strongly dependent on the physiological state of the cell. Stress tolerance increases during maturation of the algae (Pichrtová et al. 2014, Herburger et al. 2015), which is associated with the transition from vegetative cells to pre-akinetes and akinetes (McLean and Pessoney 1971). The maturation process is...
accompanied by changes in fatty acid composition, which have recently been studied by Pichrtová et al. (2016a).

To shed light on the molecular mechanisms of desiccation response and tolerance in streptophyte green algae, transcriptomic profiling was performed for Klebsormidium crenulatum, revealing reaction patterns similar to those of land plants when exposed to water stress (Holzinger et al. 2014). Dehydration in plants and algae is linked to a number of defense mechanisms, e.g. protection of the photosynthetic apparatus by the expression of early light-induced proteins (ELIPs), synthesis of low-molecular-weight osmolytes to maintain turgor pressure, induction of reactive oxygen species (ROS) scavenging and increase in the chaperone transcript pool [late embryogenesis abundant (LEA) and heat-shock proteins (Hsps)] (Wang et al. 2004, Fernández-Maríñ et al. 2016). The defense systems mentioned above were also induced in the basal streptophyte alga K. crenulatum upon harsh desiccation over silica gel, as demonstrated by Holzinger et al. (2014).

In contrast to Klebsormidiophyceae, which are located closer to the basis of the Streptophyta (Becker and Marin 2009), Zygnematophyceae are the sister lineage of the land plants (Wickett et al. 2014). This has been proved through several phylogenetic analyses but is also confirmed by the fact that zygnematophyte algae possess a modified plastid, the ‘embryoplast’, which played a key role in the development of the land plants (Wodniok et al. 2011, Zhong et al. 2013, Ruhfel et al. 2014, de Vries et al. 2016). Thus, the investigation of their water stress tolerance on a molecular level is particularly interesting from an evolutionary point of view. While the dehydration-induced changes in physiology of Zygnema circumcarinatum and K. crenulatum are similar, with a drastic reduction in effective quantum yield of PSII [Y(II)], the kinetics of water loss are different (Herburger and Holzinger 2015, Lajos et al. 2016). Herburger and Holzinger (2015) reported an elevated callose content in the cell walls of K. crenulatum, which enables shrinkage of the whole cell compared with Z. circumcarinatum, which forms rigid cellulosic secondary walls. In terms of water loss, Z. circumcarinatum reduces the protoplast volume more rapidly compared with Klebsormidium at a relative air humidity (RH) <85%; however, this observation was reversed for higher RH (Lajos et al. 2016). To gain deeper insights into the mechanisms of desiccation tolerance in Z. circumcarinatum and study the differences from K. crenulatum, transcriptomic analyses were performed on algal cultures grown either in liquid medium or on agar plates and subsequently subjected to dehydration. Furthermore, differently matured cultures (1, 7 and 12 months) were used to investigate the influence of culture age, which is associated with pre-akinete formation in cultures older than 7 months. In contrast to Klebsormidium, the genome of Zygnema has not been published yet. Thus, transcriptomic data also provide a valuable information resource and give insights into a plethora of molecular mechanisms.

### Results

#### Physiological response to desiccation

All samples were desiccated over KCl and Y(II) was monitored. An experimental overview is given in Fig. 1. For all samples, dehydration stress was applied until Y(II) dropped to zero. Hence, the physiological state of all filaments was comparable. The biomass of the liquid culture (L) and 12-month-old agar plates (P3) maintained photosynthesis the longest, until approximately 390 and 360 min, respectively (Fig. 2a, d). Y(II) of the filaments, cultured for 1 month on solid medium (P1), dropped first at about 90 min (Fig. 2a), while Y(II) of the 7-month-old culture (P2) reached zero at approximately 220 min (Fig. 2c). Table 1 displays the desiccation time and observed water loss for all samples. When comparing the time required for the cells cultured on agar plates to reduce Y(II) to zero, increased age was clearly correlated ($r = 0.98$) with prolonged activity. All three cultures grown on agar (P1, P2, P3) differed significantly in desiccation time ($P < 0.001$). No significant
difference was observed between P3 and L, but P2 and P1 differed significantly from L. Concerning water loss, no significant differences were detected between L, P2 and P3. Sample P1 differs significantly in water content reduction from L ($P < 0.001$), P2 ($P < 0.01$) and P3 ($P < 0.01$).

Sequencing outcome and reference library

The triplicates for P3 were pooled for sequencing due to low RNA quantities. Hence, this group was excluded from analysis of differential gene expression. Furthermore, one replicate of P2D exhibited a low extraction yield and thus was not sequenced. The sequencing results for all libraries are summarized in Supplementary Table S1. For the reference, which was pooled from all samples, 13,241 Mbp were obtained, and for all samples a total of 85,721 Mbp was sequenced.

Fig. 3a gives an overview of the bioinformatic pipeline used for raw read processing, assembly and further analysis. The assembly of the quality-filtered and trimmed reference reads yielded a total of 135,572 contigs with an N50 of 950 bp, and a smallest and largest contig size of 224 and 24,724 bp, respectively. To assess the completeness of the established reference transcriptome, Benchmarking Universal Single-Copy Orthologs (BUSCO) was used. As displayed in Fig. 3b, we found 76% to be complete and 8% to be fragmented, while only 16% were missing. Compared with a variety of other transcriptomes, these values can be regarded as excellent (Simão et al. 2015). Annotating the assembly against the Swiss-Prot database resulted in 28,427 (21%) hits with an E-value smaller than E-10 (Fig. 3c). Moreover, all contigs were tested for homology to amino acid sequences, retrieved from complete streptophyte and chlorophyte genomes, resulting overall in higher annotation rates for streptophytic than for chlorophytic sequences (Fig. 3d, Supplementary Fig. S2). For all examined E-values, ranging from E-3 to E-20, all assembled contigs shared most sequences with Physcomitrella patens and Klebsormidium flaccidum (Supplementary Fig. S2).
The homology comparison of the assembly to all streptophytic and all chlorophytic sequences showed that our assembly shared 21,262 sequences with both groups, while 10,672 and 615 sequences were exclusively aligned to streptophytic and chlorophytic proteins, respectively (Fig. 3d). In order to evaluate the coverage of metabolic networks, the assigned Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) numbers (5.9% of all contigs) were mapped onto the KEGG metabolic pathways map (ko01100, Supplementary Fig. S3). In general, we observed very good coverage, with the most important pathways (e.g. carbohydrate metabolism, amino acid metabolism, fatty acid metabolism, nucleotide metabolism, respiration) being complete. The annotation rate for Gene Ontology (GO) terms was 28% of all contigs.

rRNA analysis

The Z. circumcarinatum culture used in this study was only recently established (Herburger et al. 2015). It is unialgal but neither axenic nor characterized with respect to possible contamination by heterotrophic eukaryotes. The rRNA reads, which were filtered out during quality control, were used to investigate the presence of putative contaminants in the algal culture (Supplementary Fig. S4). The largest fraction of all rRNA reads could be annotated as Zygnema sp. However, a large number of different bacterial rRNA species as well as a few eukaryotic rRNAs, with most of them mapping to the genus Naegleria sp., were detected. We also observed a large number of putative rRNAs that could not be annotated at all (up to 40% in P3C). For these samples much contamination with bacterial (up to 40%) and eukaryotic (Naegleria sp., up to 17%, among others) sequences was observed. Hence, P1C and P1D were excluded from analyses if not indicated otherwise.

Expression analysis

Differential expression analysis was carried out for control versus desiccated samples of the liquid culture (LC to LD) and the 7-month-old solid culture (P2C to P2D). The analysis was performed without P1C and P1D as well as P3C and P3D
shared between the two groups.

The Venn diagram displays the number of regulated genes under desiccation in groups L and P2. Only contigs with a FDR of 0.001 were considered. All contigs were annotated against the Swiss-Prot database using BLASTx with an E-value of 0.001. A total of 2,886 (2.1%) transcripts exhibited differential expression upon desiccation in groups L and P2. Only contigs with a FDR of 0.001 were considered. The Venn diagram displays the number of regulated genes shared between the two groups.

due to high levels of contamination and low RNA extraction yields, respectively. A total of 2,886 (2.1%) transcripts exhibited differential expression (false discovery rate (FDR) ≤ 0.001) upon desiccation treatment compared with the corresponding control in at least one of the two group comparisons (L, P2). For the liquid culture (L) we observed the strongest reaction, with 2,494 contigs regulated, while only 849 were differentially expressed in group P2 (Fig. 4a, Supplementary Table S5). Between 30% and 54% of the upregulated and downregulated contigs in these groups were successfully annotated. The largest proportion of annotated sequences showed similarities to proteins of Viridiplantae. Regarding the overlap of genes responsive to desiccation in both groups, a total of 457 contigs were regulated; 317 were induced while 140 were repressed. Lists of all enriched GO terms are included in the Supplementary Table S7.

**Individual analysis of desiccation-responsive genes**

Based on gene set enrichment analyses, we studied individual differentially expressed transcripts responsive to the applied desiccation treatment (Supplementary Table S5). Our main focus lies on photosynthesis, carbohydrate and lipid metabolism, transporter proteins and signaling as well as stress protection. Selected genes, exhibiting differential expression, and the detected fold changes (all given in log2 below) are displayed in Table 4.

**Gene set enrichment analyses**

In order to identify desiccation-related metabolic pathways, a KEGG pathway enrichment analysis based on KO annotations was performed for the up- and downregulated transcripts in both analyzed groups. A total of 16,910 contigs could be annotated with KO terms. Significantly enriched pathways were found for the liquid culture dehydration treatment (L). Among upregulated transcripts, we found ‘starch and sucrose metabolism’ to be enriched, while ‘photosynthesis’ and ‘glyoxylate and dicarboxylate metabolism’ were enriched in the downregulated contigs (Table 2).

The concept of GO categorization enables the comparison of homologous genes in different organisms (Ashburner et al. 2000). One or multiple GO terms, belonging to one of the three root categories, are assigned to each protein, and similar parent categories are grouped and subsequently tested for enrichment in one sample compared with another (Ashburner et al. 2000, Young et al. 2010). For example, the large subunit of RuBisCo was assigned the ‘biological process’ photosynthesis, the ‘molecular function’ ribulose-bisphosphate carboxylase activity and the ‘cellular component’ chloroplast. In our study, GO terms could be assigned to 26,879 sequences, forming the basis for a GO enrichment analysis. Enriched root categories for each group and directed regulation are summarized in Table 3, featuring more enriched GO terms for group L, with 80 and 107 up- and downregulated, respectively, than for group P2, with 55 and 6 terms up- and downregulated, respectively. Most enriched GO terms belonged to the root category ‘biological process’, while ‘molecular function’ and ‘cellular component’ were represented to a lesser extent. In Fig. 5 a network of enriched non-redundant GO terms in up- and downregulated gene sets is displayed with major categories highlighted in light gray. Both ‘photosynthesis’ and ‘stress response’ were enriched in up- and downregulated contigs, while ‘carbohydrate metabolism’, ‘lipid metabolism’ and ‘transport’ appeared upregulated and ‘signaling’ downregulated. Lists of all enriched GO terms are included in the Supplementary Table S7.

**Photosynthesis and photorespiration.** Transcriptomic analysis of photosynthetic processes revealed a strong downregulation of components of both PSs in group L. Repression of transcripts encoding parts of PSI and II with fold changes of 2.3 to 3.4 was observed. The strongest downregulated transcript was the PSII 22 kDa protein (PsbS), with a fold change of 4.6. In contrast, group P2 exhibits only a repression...
of PSI subunit IV and PSII 22 kDa protein with fold changes of −4.7 and −4.1, respectively. Furthermore, several contigs coding for light-harvesting complexes as well as some proton-transporting ATPase subunits and plastocyanin, which is part of the electron transport chain, were downregulated in group L. Detected fold changes lay in the range of −2.3 to −3.8 while the PSII light-harvesting complex protein 2 displayed rather strong repression of −6.2. In addition, the putative chlorophyllide a oxygenase and the magnesium chelatase subunit, which are both part of the chlorophyll metabolism, were repressed 2.1- and 4.5-fold, respectively. In contrast, group P2 exhibited weaker downregulation. Upon desiccation, the light-harvesting complex PSIAtPase subunit b′ and magnesium chelatase subunit were repressed 3.9-, 4.3- and 4.4-fold, respectively. Rather striking is the plethora of ELIPs that showed differential expression during dehydration. Desiccation of the liquid culture caused the transcription level of ELIP1 and the chloroplastic high-molecular-mass ELIP to increase while the chloroplast low-molecular-mass ELIP was repressed. The solid culture showed upregulation of both LEA4 and LEA5 proteins. The LEA5 gene exhibited a 9.8-fold increase in expression. BLAST analysis of several Chlorophyta and Streptophyta revealed LEA group distribution among Viridiplantae species.

Lipid metabolism. Investigating both glycerolipid and glycerophospholipid metabolism, enhanced gene expression of certain enzymes was found. Group L showed an increased transcript level for the lysophospholipid acyltransferase, diacylglycerol kinase, ω-galactosidase, sulfoquinovosyltransferase, phosphoethanolamine N-methyltransferase and phospholipase D1/2. The response of group P2 was less pronounced, with upregulation of ω-galactosidase, phosphoethanolamine N-methyltransferase, phosphatidyl-dyserine synthase 2 and 2-acetylgluteral 0-acetyltransferase 1. Transcript levels were increased between 2- and 3.8-fold.

Transcript and signaling. The upregulation of the contig TR43432|c0_g2_i2, which was annotated to be an aquaporin (AQP) of type TIP (tonoplast intrinsic protein), was most pronounced considering all differentially expressed transcripts. Additionally, TIP2–1 in both group L and group P2 was enhanced and TIP2–3 only in group L. Moreover, the expression of various putative sugar transporters (plastidic glucose transporter 2, sucrose transport protein 3, glucose-6-phosphate/phosphate translocator 1, sugar transport protein 13, sugar transport protein ERD6-like 16) was strongly induced in the liquid sample and to a lesser extent also in group P2. The sugar-transport protein ERD6-like 16 exhibited a fold-change of 9, while the induction of other sugar transport proteins lay in the range of 2.1- to 5.8-fold.

A complex regulation of signaling pathways in group L was identified. Mainly transcripts that showed similarities to the family of serine/threonine-protein kinases, but also other kinases and transcription factors, were differentially expressed. Leucine-rich repeat receptor and receptor-like serine/threonine-protein kinases calcium-dependent protein kinases 17 and 20, were the most prominent transcripts. Overall, more signaling-related contigs were repressed than upregulated.

Response. Uregulation of other stress protection associated genes was discovered. Chaperone- and Hsp-encoding genes, such as the chaperone protein ClpB1, proteasome assembly chaperone 2, the chaperone protein DnaJ and the molecular chaperone Hsp31, appeared to be highly transcribed during desiccation, primarily in group L, while genes involved in ROS scavenging, such as glutathione-S-transferase, peroxisomal catalase, peroxiredoxin, peptide methionine sulfoxide reductase and (chloroquine-resistance transporter)-like transporter 3, were induced in both groups. For the desiccated liquid culture, proteasome assembly chaperone 2 exhibited the largest change, of 11.2-fold. Transcripts similar to the Nijmegen breakage syndrome 1 protein and DNA-damage-repair/tolerance protein, both involved in DNA repair, also showed enhanced expression. Astonishingly, Nijmegen breakage syndrome 1 protein was upregulated 9.5- and 9.7-fold in group L and P2, respectively. Furthermore, a large number of LEA proteins showed high induction; these consisted only of LEA4 proteins in group L while group P2 showed upregulation of both LEA4 and LEAS proteins. The LEAS gene exhibited a 9.8-fold increase in expression. BLAST analysis of several Chlorophyta and Streptophyta revealed LEA group distribution among Viridiplantae species.
We observed that all studied streptophyte genomes contained at least LEA proteins from groups LEA2, LEA4 and LEA5, while chlorophytes mostly only possessed LEA2 proteins with some exceptions (in our example, *Dunaliella salina*).

Overall, photosynthesis appeared to be repressed in group L while several ELIPs were upregulated in both groups. Desiccation stress also caused upregulation of transcripts involved in lipid metabolism and transport in L and P2. Furthermore, group L exhibited induction of carbohydrate metabolism. Both groups increased the transcript pool of ROS scavenging and chaperone proteins.

**Discussion**

**Photosynthesis and photorespiration**

Dehydration has an extremely negative influence on photosynthesis as water is crucial for structural integrity and functionality of the algal cell and also acts as an electron donor in the electron transport chain (Fernández-Marín et al. 2016). Herburger et al. (2015) observed a complete loss of photosynthetic activity in *Z. circumcarinatum* during prolonged desiccation. However, older cultures appeared to be more tolerant of dehydration as photosynthesis was maintained longer compared with younger cells (Herburger et al. 2015). These findings are in agreement with our observations for *Z. circumcarinatum* cultured on agar (Figure 2b–d). Culture P1 abandoned photosynthetic activity first and then the effective quantum yield of P2, dropped while P3 resisted the longest. According to Herburger et al. (2015), this increased tolerance is likely caused by the formation of pre-akinetes, which can be regarded as a stress-tolerant resting stage (Pichrtová et al. 2016b). Typical features of pre-akinetes are hardened cell walls and accumulation of starch and lipid bodies in the cytoplasm, as well as reduced growth and physiological activity (Herburger et al. 2015, Pichrtová et al. 2016b). Compared with the agar cultures, filaments grown in liquid medium appeared to maintain photosynthesis as long as P3. However, this effect is clearly linked to the clinging water, which could not be completely removed by blotting. During desiccation treatment, the excess water had to evaporate before the algal biomass could be desiccated effectively.

**Fig. 5** GO network displaying all enriched categories in both groups L and P2 as well as (a) upregulation and (b) downregulation. The root categories are 'biological process' (BP; blue or red), 'molecular function' (MF; violet or orange) and 'cellular component' (CC; green or yellow). Edges depict shared terms. Highlighted in gray are selected groups, such as photosynthesis, lipid metabolism, transport, carbohydrate metabolism, stress response and signaling.
Table 4 Selection of contigs showing differential expression in response to desiccation stress (complete list can be found in Supplementary Table S5)

| Contig ID | Annotation | E-value | L  | P2 |
|-----------|------------|---------|----|----|
| TR1438/c0_g2_i1 | Photosystem I subunit II | 2.18E-85 | -3.2 | - |
| TR18990/c0_g9_i1 | Photosystem I subunit IV | 5.94E-22 | -2.7 | -4.7 |
| TR1369/c1_g1_i1 | Photosystem I subunit III | 8.25E-76 | -2.5 | - |
| TR59163/c0_g5_i2 | Photosystem I subunit V | 1.36E-28 | -3.0 | - |
| TR21504/c0_g2_i1 | Photosystem I subunit VI | 8.11E-40 | -2.3 | - |
| TR16905/c0_g2_i1 | Photosystem I subunit X | 1.42E-34 | -2.3 | - |
| TR33976/c1_g1_i1 | Photosystem II oxygen-evolving enhancer protein 1 | 3.57E-135 | -3.0 | - |
| TR48275/c1_g1_i1 | Photosystem II oxygen-evolving enhancer protein 3 | 3.9E-49 | -3.4 | - |
| TR20185/c2_g1_i1 | Photosystem II 22 kDa protein (PsbS) | 1.08E-83 | -4.6 | -4.1 |
| TR24382/c0_g1_i1 | Photosystem II protein (PsbY) | 4.85E-13 | -3.4 | - |
| TR71655/c0_g1_i1 | Photosystem II protein (Psb27) | 2.46E-36 | -2.5 | - |
| TR64030/c0_g1_i1 | Light-harvesting chlorophyll-protein complex I subunit A4 | 4.67E-116 | -2.9 | - |
| TR37377/c0_g2_i1 | Photosystem I light harvesting complex protein 5 | 2E-93 | -3.0 | - |
| TR62754/c5_g48_i1 | Photosystem II light harvesting complex protein 2.2 | 5.38E-82 | -6.2 | - |
| TR12320/c6_g1_i1 | Light-harvesting chlorophyll b-binding protein 3 | 1.83E-96 | -3.5 | - |
| TR37376/c0_g1_i1 | Light harvesting complex photosystem II | 5.2E-121 | -3.8 | -3.9 |
| TR25593/c4_g1_i1 | Light harvesting complex of photosystem II 5 | 1.57E-117 | -2.7 | - |
| TR1329/c0_g2_i1 | Light harvesting complex photosystem II subunit 6 | 3.79E-92 | -2.8 | - |
| TR75181/c0_g1_i1 | ATPase delta chain | 6.28E-52 | -2.3 | - |
| TR3752/c0_g5_i1 | ATPase subunit b' | 2E-37 | -2.7 | -4.3 |
| TR4441/c0_g1_i2 | Plastocyanin | -3.75E-37 | -2.5 | - |
| TR31328/c0_g1_i1 | Chlorophyllide a oxygenase | 0 | -2.1 | - |
| TR68443/c0_g1_i3 | Magnesium chelatase subunit | 0 | -4.5 | -4.4 |
| TR8034/c1_g29_i1 | Early light-induced protein, chloroplastic (ELI) | 1.83E-15 | -12.2 | - |
| TR58021/c0_g5_i1 | Early light-induced protein 1, chloroplastic (ELI1P1) | 1.43E-21 | 3.5 | 5.2 |
| TR4192/c1_g15_i1 | High molecular mass early light-inducible protein, chloroplastic (HV58) | 1.86E-21 | 5.2 | 4.3 |
| TR4440/c0_g2_i1 | Low molecular mass early light-inducible protein, chloroplastic (HV60) | 4.1E-16 | -3.4 | - |
| TR73556/c0_g9_i2 | (S)-2-Hydroxy-acid oxidase | 0 | -2.5 | -3.5 |
| TR29652/c0_g1_i1 | Serine-glyoxylate transaminase | 0 | -3.4 | -3.6 |
| TR31318/c0_g1_i1 | Phospholipase D1/2 | 0 | -3.7 | -4.2 |
| TR42973/c1_g1_i1 | Sucrose-phosphatase | 6.91E-108 | 3.6 | - |
| TR48225/c1_g1_i1 | Glycerate dehydrogenase | 0 | -3.5 | - |
| TR61067/c0_g1_i2 | Glycerate dehydrogenase | 0 | -3.5 | - |
| TR39622/c0_g1_i1 | Lysophospholipid acyltransferase | 4.12E-110 | 2.0 | - |
| TR3082/c0_g1_i1 | Dicacylglycerol kinase | 2.68E-160 | 2.0 | - |
| TR16611/c0_g1_i2 | alpha-Galactosidase | 1.91E-154 | 2.3 | 3.5 |
| TR45454/c0_g2_i1 | Sulfoquinovosyltransferase | 4.73E-12 | 3.7 | - |
| TR31318/c0_g1_i1 | Phospholipase D1/2 | 0 | 3.7 | - |
| TR28615/c0_g1_i1 | Phosphoethanolamine N-methyltransferase | 5.8E-102 | 2.9 | 3.2 |

(continued)
encoding components of PSI and II as well as light-harvesting proteins, indicating photoinhibition. In contrast, *K. crenulatum* (Holzinger et al. 2014) showed upregulation of genes related to photosynthesis in response to desiccation. Holzinger et al. (2014) suggest that this mechanism serves as a preparation for rapid resumption of photosynthesis upon rehydration. Similar results were obtained for *Trebouxia gelatinosa* (Carniel et al. 2016). However, Carniel et al. (2016) compared 10 different transcriptomic studies on desiccation of Viridiplantae, detecting repression of photosynthetic transcripts in *Syntrichya ruralis* (desiccation tolerant moss), *Haberlea rhodopensis* (resurrection plant) and *Xerophyta humilis* (resurrection plant). Moreover, the resurrection plant *Myrothamnus flabellifolia* also exhibited downregulation of photosynthesis genes when desiccated (Ma et al. 2015). Surprisingly, mainly genes encoding parts of PSI and II, the electron transport chain and ATP synthase were repressed in *M. flabellifolia* (Ma et al. 2015). This is also true for *Z. circumcarinatum*, as demonstrated in the present study. Ma et al. (2015) argue that it is likely in this way that excitation energy and, thus, ROS production are reduced. Moreover, the expression of ELIPs was enhanced in groups L and P2. ELIPs are photoprotectors and belong to the chlorophyll a/b-binding (CAB) superfamily, respond to abiotic stress, mainly to high light and ultraviolet radiation, and are located in the thylakoid membrane (Hutin et al. 2003, Noreň et al. 2003, Hayami et al. 2015). Similar reactions were observed for other

### Table 4 Continued

| Contig ID       | Annotation                            | E-value     | L   | P2   |
|-----------------|---------------------------------------|-------------|-----|------|
| TR41908|c1_g1_i4 | Phosphatidylserine synthase 2 | 1.62E-177   | –   | 3.8  |
| TR13652|c0_g1_i13 | 2-Acylglycerol O-acyltransferase 1 | 2.54E-89    | –   | 3.5  |
| Transporter proteins |
| TR43432|c0_g2_i2 | Probable aquaporin TIP1–2 | 8.15E-17    | 13.3| 13.5 |
| TR43432|c0_g3_i1 | Aquaporin TIP2–1 | 2.25E-32     | 2.9  | 2.7  |
| TR61568|c0_g2_i1 | Aquaporin TIP2-3 | 2.22E-33    | 4.1  | –    |
| TR34049|c0_g1_i2 | Plastidic glucose transporter 2 | 2.02E-138    | 4.2  | 3.6  |
| TR23238|c0_g1_i2 | Sucrose transport protein 3 | 2.09E-147   | 2.7  | –    |
| TR31|c0_g1_i1 | Glucose-6-phosphate/phosphate translocator 1 | 1.22E-164 | 2.1  | –    |
| TR40733|c1_g1_i1 | sugar transport protein 13 | 8.24E-177    | 5.8  | 5.5  |
| TR41946|c0_g1_i8 | sugar-transport protein ERD6-like 16 | 3.33E-71    | 9.0  | –    |
| Signaling |
| TR52105|c7_g5_i3 | Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM2 | 2.56E-18 | 3.4 | – |
| TR58701|c1_g2_i3 | Leucine-rich repeat receptor-like serine/threonine-protein kinase FLS2 | 7.92E-65 | –5.4 | –6.7 |
| TR34848|c0_g2_i1 | Calcium-dependent protein kinase 17 | 1.25E-81 | 3.4 | – |
| TR6882|c0_g1_i1 | Calcium-dependent protein kinase 20 | 5.18E-76 | –6.1 | – |
| TR10757|c0_g3_i1 | Chaperone protein CipB1 | 7.43E-16 | 3.4 | – |
| TR35960|c0_g2_i2 | Proteasome assembly chaperone 2 | 4.89E-28 | 11.2 | – |
| TR14947|c0_g1_i3 | Chaperone protein DnaJ | 1.06E-10 | 3.9 | – |
| TR52105|c0_g1_i2 | Molecular chaperone Hsp31 | 1.57E-10 | 2.5 | 4.2 |
| TR39621|c0_g1_i2 | Glutathione S-transferase | 4.85E-53 | 2.9 | 5.0 |
| TR14048|c0_g1_i1 | Peroxosomal catalase | 0 | 3.7 | 6.7 |
| TR58823|c0_g2_i1 | Peroxiredoxin | 6.92E-54 | 2.3 | 3.4 |
| TR57779|c0_g1_i1 | Peptide methionine sulf oxide reductase | 4.35E-79 | 2.5 | 3.0 |
| TR35953|c0_g2_i4 | (Chloroquine-resistance transporter)-like transporter 3 | 7.29E-81 | 9.0 | – |
| TR50557|c0_g2_i12 | Nijmegen breakage syndrome 1 protein | 3.14E-13 | 9.5 | 9.7 |
| TR35997|c1_g1_i8 | DNA-damage-repair/toleration protein | 1.42E-42 | 3.0 | – |
| TR49464|c0_g1_i1 | Late embryogenesis abundant protein 4 (LEA4; AT3G53040) | 8.1E-19 | 5.1 | 3.6 |
| TR39628|c0_g2_i1 | Late embryogenesis abundant protein 4 (LEA4; AT2G18340) | 4.6E-24 | 5.0 | – |
| TR6744|c2_g3_i1 | Late embryogenesis abundant protein 4 (LEA4; AT4G36600) | 1.1E-14 | 5.3 | 3.4 |
| TR60896|c0_g1_i1 | Late embryogenesis abundant protein 5 (LEA5; AT2G40170) | 6.5E-27 | – | 9.8 |

Selected contigs are displayed with ID, annotation, E-value and fold change (log2 transformed) for groups L and P2.

This is also true for *Z. circumcarinatum*, as demonstrated in the present study. Ma et al. (2015) argue that it is likely in this way that excitation energy and, thus, ROS production are reduced. Moreover, the level of transcripts involved in chlorophyll biosynthesis decreased. Similar findings were obtained for desiccated *Vitis vinifera* (grapevine) leaves (Salman et al. 2016) and salt-stressed *O. sativa* (rice) seedlings (Turan and Tripathy 2015). The observed impairment of chlorophyll biosynthesis probably occurs to avoid ROS formation (Farrant et al. 2003). As an additional protection, the expression of ELIPs was enhanced in groups L and P2. ELIPs are photoprotectors and belong to the chlorophyll a/b-binding (CAB) superfamily, respond to abiotic stress, mainly to high light and ultraviolet radiation, and are located in the thylakoid membrane (Hutin et al. 2003, Noreň et al. 2003, Hayami et al. 2015). Similar reactions were observed for other
green algae, e.g. C. reinhardtii and D. bardawil, both of which increased the ELIP transcript pool in response to high light stress, which is also common for various higher plants, such as Arabidopsis (Lers et al. 1991, Teramoto et al. 2004). Fewer studies have been dedicated to the relationship of cold stress and ELIP expression in algae. Kröl et al. (1997) reported induction of ELIPs in D. salina when exposed to low temperatures. Spirogyra varians (Zygnematales) also exhibits accumulation of ELIP-like transcripts when cultured at 4°C (Han and Kim 2013). Nevertheless, the accumulation of ELIPs is also commonly associated with desiccation stress (Zeng et al. 2002, Dinakar and Bartels 2013, Ma et al. 2015). These proteins protect the thylakoid membranes against photooxidative damage by scavenging free chlorophyll molecules and act as sinks for excitation energy (Zeng et al. 2002, Heddad et al. 2012). Paradoxically, some contigs of Zygnema, exhibiting similarities to ELIPs, are negatively regulated. However, Holzinger et al. (2014) also found a complex regulation of ELIP-related transcripts for K. crenulatum, hinting at a multigenetic family with several ELIPs being responsive to desiccation stress (Zeng et al. 2002, Hutin et al. 2003, Marraccini et al. 2012).

A decrease in photosynthetic transcripts upon desiccation was detected in both groups of Z. circumcarinatum, suggesting repression of photosynthesis. In contrast, photosynthesis can function as a protection of the photosynthetic apparatus against photoinhibition (Wingler et al. 1999), especially during drought stress, when carbon dioxide fixation and thus the consumption of electrons is reduced (Wingler et al. 2000). Furthermore, dehydration is reported to reduce photosynthetic activity in plants, which may be caused by decreased photosynthetic activity (Levitt 1980).

### Carbohydrate metabolism

A common protective mechanism against water stress is the accumulation of low-molecular-weight osmolytes, which can be

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**Fig. 6** LEA proteins found in A. thaliana and described by Hundermark and Hincha (2008). Annotations were retrieved from Phytozome v11.0 for A. thaliana, C. reinhardtii, C. subellipsoidea, D. salina, Marchantia polymorpha, P. patens and Selaginella moellendorfii. The same is true for the transcripts of O. sativa, which were annotated using diamond BLASTx with E-9. The transcripts of Chlorella sp. NC64A, K. crenulatum and K. flaccidum were downloaded from the JGI Genome Portal, Holzinger et al. (2014) and the K. flaccidum genome project, respectively, and processed accordingly. Points indicate streptophytes while squares stand for chlorophytes. Solid and hollow symbols represent sequences derived from genomes or only transcriptomes, respectively.

| Arabidopsis thaliana | Oryza sativa | Selaginella moellendorfii | Marchantia polymorpha | Zygnema circumcarinatum | Keaksooidea scutatum | Chlorella sp. NC64A | Dunaliella salina | Chlamydomonas reinhardtii | Coccomyxa subellipsoidea |
|----------------------|-------------|--------------------------|-----------------------|------------------------|---------------------|-------------------|-----------------|-------------------------|------------------------|
| AT5G53270             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G53270             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G58082             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G58082             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G53270             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G58082             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G53270             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G58082             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G53270             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |
| AT5G58082             |             | AT5G58082                | AT5G22890             | AT5G22890              | AT5G22890           | AT5G22890         | AT5G22890       | AT5G22890                | AT5G22890              |

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**Table 1** LEA proteins found in A. thaliana and described by Hundermark and Hincha (2008). Annotations were retrieved from Phytozome v11.0 for A. thaliana, C. reinhardtii, C. subellipsoidea, D. salina, Marchantia polymorpha, P. patens and Selaginella moellendorfii. The same is true for the transcripts of O. sativa, which were annotated using diamond BLASTx with E-9. The transcripts of Chlorella sp. NC64A, K. crenulatum and K. flaccidum were downloaded from the JGI Genome Portal, Holzinger et al. (2014) and the K. flaccidum genome project, respectively, and processed accordingly. Points indicate streptophytes while squares stand for chlorophytes. Solid and hollow symbols represent sequences derived from genomes or only transcriptomes, respectively.
sugars, polyols and proteins (Hincha et al. 1996, Dinakar and Bartels 2013, Ma et al. 2015, Fernández-Marin et al. 2016, Holzinger and Pichrtová 2016). By increasing the amount of osmoprotectants in the cell, a negative osmotic potential is achieved, membranes are stabilized and protein protection is enhanced (Bisson and Kirst 1995). Nagao et al. (2008) found that *K. flaccidum* accumulates the osmolyte sucrose during cold acclimation, which contributes to greater freezing tolerance. Moreover, the alga *Chlorella vulgaris* exhibits an increase in sucrose and raffinose content in response to cold shock treatment (Salerno and Pontis 1989). However, sucrose is also typically formed upon desiccation stress in plants and algae (Ramanjulu and Bartels 2002, Dinakar and Bartels 2013, Cruz de Carvalho et al. 2014, Holzinger and Pichrtová 2016, Sadowsky et al. 2016). Sadowsky et al. (2016) reported increased sucrose levels in an Antarctic Trebouxia strain to counteract desiccation. Our data indicate a metabolic shift towards sucrose as starch-degrading as well as sucrose biosynthetic enzymes were upregulated in dehydrated filaments. The *KEGG* enrichment analysis also clearly indicated enhancement of ‘starch and sucrose metabolism’. A similar strategy is pursued by *K. crenulatum*, inducing transcripts encoding sucrose synthase and sucrose phosphate synthase (Holzinger et al. 2014). The authors suggest that raffinose family oligosaccharides also function as osmoprotectants because several enzymes belonging to galactolinal/raffinose metabolism exhibited higher expression in desiccated cells (Holzinger et al. 2014). *Zygnema circumcarinatum* did not show this expression pattern in response to water stress. However, the sucrose phosphate synthase of *Z. circumcarinatum* contains a conserved phosphorylation site (results not shown), which is typically found in angiosperms and known to become modified upon osmotic stress (Winter and Huber 2000). Hence, sucrose metabolism in *Z. circumcarinatum* is most likely not only regulated by transcription but also by posttranslational modifications.

Callose is an important polysaccharide found to be involved in responses to different abiotic stress factors, e.g. in drought stress in plants such as *Gossypium hirsutum* L. (cotton; McNairn 1972), but also algae such as *K. crenulatum* (Herberger and Holzinger 2015). The desiccation transcriptome of *Klebsormidium* revealed upregulation of the callose synthase complex, confirming the significance of this carbohydrate during dehydration events (Holzinger et al. 2014). Although this enzyme occurs in the *Zygnema* transcriptome, no differential expression was detected. These results are in agreement with Herberger and Holzinger (2015), who reported a stable callose content throughout desiccation for 2.5 h. As callose synthase is located in the plasma membrane and the protoplast retracts from the cell wall upon dehydration, callose incorporation is prevented (Herberger and Holzinger 2015).

**Lipid metabolism and membranes**

The fact that low temperatures, dehydration etc. initially target biomembranes highlights the importance of membrane modification upon water stress to preserve integrity and fluidity (Dinakar and Bartels 2013, Valledor et al. 2013, Holzinger et al. 2014,Perliskowski et al. 2016). For example, decreased temperatures cause membrane modifications in the green alga *C. reinhardtii* (Valledor et al. 2013, Wang et al. 2016). Gasulla et al. (2013) observed similar tendencies in *C. plantagineum* induced by desiccation treatment. Monogalactosyldiacylglycerol was removed from the thylakoid membranes and either transformed to digalactosyldiacylglycerol or hydrolyzed to form diacylglycerol (Gasulla et al. 2013). In contrast, our results indicate the conversion from digalactosyldiacylglycerol to monogalactosyldiacylglycerol to be amplified as the putative α-galactosidase was upregulated in group L. However, parts of glycerol- and glycerophospholipid metabolism are enhanced during desiccation, suggesting other membrane modifications. Similarly, the lichen phycobiont *Asterochloris erici* exhibited elevated levels of phosphatidic acid upon desiccation, indicating that phospholipase D is involved in stress protection mechanisms (Gasulla et al. 2016). *Zygnema circumcarinatum* induced phospholipase D1/2 during dehydration stress, confirming that phospholipase D1/2 is part of the stress response.

**Transporter proteins and signaling**

Major intrinsic proteins (MIPs), or AQPs, establish channels for passive transportation of small uncharged substances, such as water or glycerol, across the membrane (Barkla et al. 1999, Anderberg et al. 2011, Anderberg et al. 2012). AQPs in embrophytes comprise seven groups: GlpF-like intrinsic proteins (GIPs), hybrid intrinsic proteins (HIPs), X intrinsic proteins (XIPs), small basic intrinsic proteins (SIPs), nodulin-26 like intrinsic proteins (NIPs), plasma membrane intrinsic proteins (PIP) and TIPs (Danielson et al. 2008). Anderberg et al. (2011) studied different chlorophylls and found AQPs of these groups only in Trebouxiophyceae (PIP, GIP). *Zygnema circumcarinatum*, a charophyte alga, is more closely related to land plants and expresses TIPs, NIPs and SIPs, while only TIPs appeared to play an important role in desiccation. Groups L and P2 both showed strong induction of TIPs during water stress, which was also observed for other AQPs in the plants *A. thaliana* and *C. plantagineum* (Ramanjulu and Bartels 2002, Dinakar and Bartels 2013) as well as the alga *T. gelatinosa* (Carniel et al. 2016). Carniel et al. (2016) argue that AQPs protect against damage during rehydration by increasing the permeability of biomembranes to water.

As mentioned above, the formation of sugars and other osmolytes is increased during water stress; however, these molecules also need to be distributed within the cell (Jarzynka and Jarsin´ski 2014). Thus, desiccation tolerance is dependent on sugar transportation within the cell. Liu et al. (2016) reported increased drought tolerance in *A. thaliana* associated with the expression of the hexose facilitator AtSWEET4. Similar results, indicating desiccation-induced expression of sugar transporters, were obtained for *Caragana korshinskii* (leguminous shrub; Li et al. 2016) and *Saccharum* spp. (sugar cane; Zhang et al. 2016).

Signaling in plants during desiccation generally involves several hormones, such as abscisic acid, cytokinin and ethylene (Campos et al. 2014, Zhou et al. 2014, Holzinger and Becker 2015, Van de Poel et al. 2016) as well as calcium-dependent and serine/threonine-protein kinases (Campos et al. 2014,
Ramanjulu and Bartels 2002). Especially abscisic acid plays a major role in the desiccation stress response, linking host and plastid signaling, which is considered a key step in the evolution of land plants (de Vries et al. 2016). In contrast to K. crenulatum (Holzinger and Becker 2015), the upregulation of hormone-specific signaling transcripts was not evident in Zygnema. However, we found an intricate expression pattern of threonine/serine- and calcium-dependent protein kinases in response to dehydration in Z. circumcarinatum. Other studies found desiccation-induced signaling networks with similar complexity, e.g. for K. crenulatum and V. vinifera (Holzinger et al. 2014, Salman et al. 2016).

Stress protection
The formation of ROS, occurring during different abiotic stress conditions, is extremely harmful, making ROS scavenging crucial for cell survival (e.g. Kraner et al. 2005, Cruz de Carvalho 2008, Han et al. 2011, Cruz de Carvalho et al. 2012, Dong et al. 2016, Heinrich et al. 2016). For example, high light stress triggers the expression of ROS-scavenging enzymes in the green alga C. reinhardtii and the marine diatom Thalasiosira pseudonana (Erickson et al. 2015, Dong et al. 2016). Similarly, the dinoflagellate Symbiodinium uses a number of ROS defense proteins and antioxidants when exposed to heat stress (Gierz et al. 2017). The same holds true for cold stress, which induces the accumulation of antioxidants in S. varians to counteract ROS formation (Han et al. 2011). Our data suggest ROS generation upon desiccation as Z. circumcarinatum expresses a multitude of enzymes related to ROS protection: glutathione-S-transferases conjugates GSH to hydrophobic molecules (Rezaei et al. 2013), peroxisomal catalase acts directly on ROS (Gorni et al. 2013), peroxiredoxin catalyzes the reduction of peroxides (Dayer et al. 2008), peptide methionine sulfoxide reductase reduces methionine sulfoxide back to methionine (Weissbach et al. 2002) and (chloroquine-resistance transporter)-like transporter 3 transports GSH (Nooter et al. 2011). Interestingly, the induction of the glutathione-S-transferase, peroxisomal catalase, peroxiredoxin and peptide methionine sulfoxide reductase was higher in group P2 than L, suggesting a more pronounced ROS stress response of filaments grown on agar plates than filaments from liquid culture. Upregulation of peroxiredoxin and the peptide methionine sulfoxide reductase was also detected in T. pseudonana exposed to high light stress (Dong et al. 2016). Hence, both enzymes are certainly involved in a general ROS coping mechanism. Moreover, group L strongly increased the transcript pool of (chloroquine-resistance transporter)-like transporter 3. These transporter proteins play a major role in GSH homeostasis in A. thaliana as they connect the plastid and the cytosolic thiol pool (Maughan et al. 2010). Thus, (chloroquine-resistance transporter)-like transporters are essential to counteract ROS stress (Maughan et al. 2010). Furthermore, DNA damage, which is also linked to ROS formation (Cruze de Carvalho 2008, Heinrich et al. 2015), was addressed by strong upregulation of repair enzymes in desiccation-stressed Z. circumcarinatum. For example, the Nijmegen breakage syndrome 1 protein was highly induced, with fold changes of 9.5 and 9.7 in group L and P2, respectively, suggesting a higher risk of DNA damage associated with desiccation (Akutsu et al. 2007, Cruz de Carvalho 2008).

Abiotic stress generally leads to aggregation and conformational changes in proteins, which is lethal to the cell (Wang et al. 2004). In response, plants and algae express chaperones and Hsps, which assist in the refolding of proteins and protect them from aggregation (Wang et al. 2004, Schulz-Raffelt et al. 2007, Al-Wahaibi 2011, Mitra et al. 2013, Van de Poel 2016). For example, C. reinhardtii raises the transcription level of Hsp90A when heat-shocked at 40°C (Schulz-Raffelt et al. 2007). Kobayashi et al. (2014) found increased expression of small Hsps in the red alga Cyanidioschyzon merolae and C. reinhardtii in response to heat stress. Heinrich et al. (2012a) and Dong et al. (2016) reported that the brown macroalga Saccharina latissima and the diatom T. pseudonana, respectively, induce various chaperones and Hsps when treated with high light intensities. Similarly, cold stress triggers the expression of two chaperones in the Antarctic diatom Chaetoceros neogracile (Park et al. 2008). The same holds true for desiccation, which causes increased expression of certain chaperones and Hsps in Selaginella lepidophylla (lycophyte; Carniel et al. 2016). P. patens (moss; Wang et al. 2009), Pyropia orbicularis (red alga; López-Cristóffanini et al. 2015) and A. erici (green alga, Gasulla et al. 2013). Zygnema circumcarinatum showed induction of chaperones and Hsps as well, which is probably a preparation for protein refolding upon rehydration (Carniel et al. 2016). Clp proteins are chaperones capable of refolding protein complexes and are induced in response to various stress factors (Lee et al. 2006). In O. sativa, a number of Clp proteins showed increased transcription during drought stress (Hu et al. 2009), while the cytosolic chaperone ClpB1 of A. thaliana is involved in chloroplast development and acclimation to increased temperatures (Lee et al. 2006). However, Z. circumcarinatum probably raises the transcription level of the chaperone ClpB1 in response to desiccation stress to enable remodeling of aggregated and misfolded proteins. Another important group of stress-induced proteins are co-chaperones, such as DnaJ (Wang et al. 2014). Wang et al. (2014) reported an increase in drought tolerance in transgenic tobacco resulting from overexpression of the chloroplast-targeted chaperone DnaJ. Furthermore, induction of DnaJ in Saccharum in response to desiccation was demonstrated by de Andrade et al. (2015). The applied desiccation treatment caused Z. circumcarinatum to induce the co-chaperone as part of its stress response. Interestingly, DnaJ expression is also triggered by high light exposure in S. latissim and T. pseudonana (Heinrich et al. 2012a, Dong et al. 2016). Furthermore, our data show upregulation of the transcription factor Hsp31 upon water stress, which is in agreement with the findings in O. sativa by Wang et al. (2011). Another important component of protein quality control is the proteasomes, which selectively eliminate dysfunctional proteins (Hanssum et al. 2014). In response to changing conditions and environmental stress, the demand for proteasomes increases immensely, forcing the cell to increase the proteasome pool (Hanssum et al. 2014). The assembly process is promoted by proteasome assembly chaperones such as proteasome assembly chaperone 2 (Le Tallec et al. 2007). The desiccated filaments of...
Z. circumcarinatum will most likely accumulate a number of misfolded proteins that need to be degraded. To be able to assemble proteasomes, the alga requires adequate chaperones. Additionally, a number of putative LEA proteins are upregulated. LEA proteins are proposed to be water-stress-specific chaperones (Goyal et al. 2005, Shinde et al. 2012, Hatanaka et al. 2014), which can be grouped depending on their sequence motifs/patterns (Hundertmark and Hincha 2008). Drought-induced expression of LEA proteins has already been reported for several species, e.g. M. flabellifolia (Ma et al. 2015), P. patens (Shinde et al. 2012) and K. crenulatum (Holzinger et al. 2014), confirming our findings. An interesting aspect is the distribution of these chaperones across Viridiplantae, which reveals evolutionary relationships of the different subfamilies. Based on the classification by Hundertmark and Hincha (2008), the Z. circumcarinatum transcriptome covered sequences belonging to LEA4 and LEAS (Fig. 6). LEA4 is likely the group that emerged first, as it shows the greatest diversity and is present in all investigated algae and plants except D. salina. LEAS probably also emerged early because it was found in all Streptophyta and C. reinhardii. All analyzed Streptophyta genomes featured LEA2 proteins, but this group was missing in Z. circumcarinatum. The absence of LEA2 is likely linked to the missing induction in response to any condition tested. Finally, the LEA protein group SMP probably evolved during the development of mosses from streptophytic green algae as all embryophyta possess these proteins.

Conclusions

In this study, the molecular response of the conjugating green alga Z. circumcarinatum to desiccation stress was investigated using differential gene expression analysis. To assess the effect of hardening, the impact of dehydration on a young liquid culture and a 7-month-old agar culture of Zygnema was analyzed. We found a 3-fold stronger transcriptional response of filaments grown in liquid medium compared with older filaments cultured on agar. These findings are a clear indication of pre-acclimation to low water availability of the algal culture grown on agar for 7 months. In agreement with earlier observations, photosynthesis-related genes are highly repressed in group L, while the response of group P2 is much less pronounced. Furthermore, water withdrawal causes membrane modifications and the expression of several transporters, such as aquaporins and carbohydrate transporter proteins. Desiccation also induces the accumulation of sucrose, a common osmolyte, to counteract the rapid loss of water. Finally, a number of stress-related molecules are produced, e.g. ELIPs, chaperones such as LEA proteins, proteins involved in ROS scavenging, and DNA repair proteins. Overall, we conclude that culture age and conditions highly influence the physiological state of the algal filaments and acclimation to water stress. However, it is difficult to mimic natural conditions in a laboratory environment as natural habitats are influenced by stochastic parameters such as weather and soil quality. Thus, future experiments should include the transcriptomic analysis of field samples collected in different seasons with different water availability.

Material and Methods

Algal strain and cultivation

Zygnema sp. (SAG 2419), previously isolated from a sandy shore near the river Saalach in Salzburg, Austria, was used for the experiments. The alga was either cultured for 1 month in liquid Waris-H medium (McFadden and Melkonian 1986) or on 1.5% agar plates containing Bold’s basal medium, as previously described by Herburger et al. (2015). In phylogenetic analysis this algal strain clusters close to Z. circumcarinatum (Herburger and Holzinger 2015); thus, we use this species name in the present study. However, an unambiguous morphological determination of the species was not possible as zygospores were not detected.

Desiccation experiment

Four different cultures of Z. circumcarinatum were prepared: a 1-month-old liquid culture (L) and 1-month- (P1), 7-month- (P2) and 12-month-old cultures (P3) grown on agar plates (solid medium; Fig. 1). The liquid culture of Z. circumcarinatum was harvested by centrifugation (300 × g) and blotted onto cellulose membrane filters (pore size 0.45 mm; Sigma Aldrich, St. Louis, MO, USA), while the biomass, grown on agar plates, was transferred directly to the filters without any blotting. Triplicates of all samples were placed into separate desiccation chambers, which were described previously (Karsten et al. 2014), and desiccated over a saturated KCl solution at an RH of approximately 86% according to Pichrová et al. (2014). Y(II) was monitored during the desiccation event, using a portable pulse-amplitude modulated fluorometer (PAM) (Model 2500, Heinz Walz, Effeltrich, Germany), and samples were taken when Y(II) reached zero. The water loss of the biomass was determined prior to and after incubation in the desiccation chamber. The reduction in water content was determined according to the following formula:

\[
\text{Reduction} (\%) = 100 - \left( \frac{\text{desiccated biomass}}{\text{fresh biomass}} \right) \times 100.
\]

For both Y(II) and water loss, differences between each group were assessed in R by one-way ANOVA (P < 0.01) followed by Tukey’s post hoc test (Honest significant difference, P < 0.01). Correlation coefficients were calculated according to Pearson (1895).

RNA isolation and sequencing

Extraction of RNA was performed as described by Holzinger et al. (2014) with some modifications. Six filters from each culture were prepared: a 1-month-old agar culture (C), 1-month-old liquid culture (C), 7-month- (P1), 12-month-old cultures (P2) and 12-month-old cultures (P3) grown on agar plates (solid medium; Fig. 1). The liquid culture of Z. circumcarinatum was harvested by centrifugation (300 × g) and blotted onto cellulose membrane filters (pore size 0.45 mm; Sigma Aldrich, St. Louis, MO, USA), while the biomass, grown on agar plates, was transferred directly to the filters without any blotting. Triplicates of all samples were placed into separate desiccation chambers, which were described previously (Karsten et al. 2014), and desiccated over a saturated KCl solution at an RH of approximately 86% according to Pichrová et al. (2014). Y(II) was monitored during the desiccation event, using a portable pulse-amplitude modulated fluorometer (PAM) (Model 2500, Heinz Walz, Effeltrich, Germany), and samples were taken when Y(II) reached zero. The water loss of the biomass was determined prior to and after incubation in the desiccation chamber. The reduction in water content was determined according to the following formula:

\[
\text{Reduction} (\%) = 100 - \left( \frac{\text{desiccated biomass}}{\text{fresh biomass}} \right) \times 100.
\]
Bioinformatic analyses

Raw reads of the reference were first quality-trimmed and filtered using Trimmomatic 0.35 (Bolger et al. 2014) and PrinSeq Lite 0.20.4 (Schmieder and Edwards 2011). Subsequently, RNA sequence reads were separated by SortMeRNA 2.1 (Kopylova et al. 2012) employing SILVA SSU NR Ref 119 and LSU Ref 119. Before assembly, COTPEread (Liu et al. 2012) was utilized to stitch overlapping reads together. The assembly of the reference was done with Trinity 2.0.6 (Grabherr et al. 2011) and the quality was assessed with scripts from the Trinity package and BUSCO plants 1.1b (Simão et al. 2015). An additional quality-filtering step was carried out by annotating all contigs with Diamond 0.8.24 (Buchfink et al. 2015) against a custom-made database that contained the protein sequences of *P. patens* (Phytozome database version 12), *K. flaccidum* (Hori et al. 2014) and *Naegleria gruberi* (Fritz-Laylin et al. 2014) and the K. *flaccidum* genome. Filtered rRNA reads were submitted to the SRA database (SRP117803) and will become available upon publication of the study.

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Disclosure

The authors declare that they do not have a conflict of interest.

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