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Enhanced Desiccation Tolerance In Mature Cultures Of The Streptophytic Green Alga *Zygnema circumcarinatum* Revealed By Transcriptomics

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Abstract

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 Y(II) ceased. In general, the response to dehydration was much more pronounced in *Z. circumcarinatum*, cultivated in liquid medium for one month, compared to filaments grown on agar plates for seven and twelve months. Cultivation 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 cultivated on agar plates for seven months. Otherwise, both samples showed an induction of stress protection mechanisms such as ROS scavenging (Early light-induced proteins, glutathione metabolism) and DNA repair as well as the expression of chaperones and aquaporins. Additionally, *Z. circumcarinatum*, cultivated 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.

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

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Desiccation Tolerance; Gene Expression; Streptophytic Algae; Transcriptomics; *Zygnema*

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 & Pichrtová 2016). Tolerating water stress is crucial for survival of land plants as well as terrestrial algae and has been addressed extensively (Borstlap 2002, Dinankar & Bartels 2013, Holzinger et al. 2014, Oliver 2007). Most land plants are able to actively regulate their water status (homoiohydry) but 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 & Karsten 2013). Hence, *C. plantagineum* and other resurrection plants were used as models for desiccation stress tolerance (Bartels & Salamini 2001, Dinankar & Bartels 2013). Additionally, other higher plants (Basu et al. 2016, Ma et al. 2015, Oliver et al. 2011) and mosses were studied to unravel the mechanisms of desiccation response and resilience (Gao et al. 2015, Shinde et al. 2012).

Over the past years, substantial information about the impact of desiccation stress on streptophytic green algae, including *Zygnema* spp. and *Klebsormidium* spp., became available (for summary see Holzinger & Karsten 2013, Karsten & Holzinger 2014, Holzinger & 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 (Davey 1991, Hawes 1990). Upon 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 & Pessoney 1971). The maturation process is accompanied by changes in the 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 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 ROS scavenging and increase of the chaperone transcript pool (late embryogenesis abundant (LEA) and heat-shock proteins (Hsps)) (Fernández-Marín et al. 2016, Wang et al. 2004). The above listed defense systems were also induced in the basal streptophyte alga *K. crenulatum* upon harsh desiccation over silica gel as demonstrated by Holzinger et al. (2014).

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In contrast to Klebsormidiophyceae, which are located closer to the basis of the Streptophyta (Becker & Marin 2009), Zygnematophyceae are the sister lineage of the land plants (Wickett et al. 2014). This has been proven through several phylogenetic analyses but is also confirmed by the fact that zygnematophycean algae possess a modified plastid, the '*embryoplast*', which played a key role in the development of the land plants (de Vries et al. 2016, Ruhfeld et al. 2014, Wodniok et al. 2011, Zhong et al. 2013). 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 of Y(II), the kinetics of water loss are different (Herburger & Holzinger 2015, Lajos et al. 2016). Herburger & Holzinger (2015) reported an elevated callose content in the cell walls of *K. crenulatum*, which enables shrinkage of the whole cell, compared to *Z. circumcarinatum* which forms rigid cellulosic secondary walls. In terms of water loss, *Z. circumcarinatum* reduces the protoplast volume more rapidly compared to *Klebsormidium* at a relative air humidity (RH) lower than 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 to *K. crenulatum*, transcriptomic analyses were performed on algal cultures, either grown in liquid medium or on agar plates and, subsequently, subjected to dehydration. Furthermore, differently matured cultures (one, seven and twelve months) were used to investigate the influence of culture age, which is associated with pre-akinete formation in cultures older than seven 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 the Y(II) was monitored. An experimental overview is given in Figure 1. For all samples dehydration stress was applied until Y(II) dropped to zero. Hence, the physiological state of all filaments is comparable. The biomass of the liquid culture (L) and the 12 months old agar plates (P3) maintained photosynthesis the longest until approximately 390 min and 360 min, respectively (Figure 2A/D). Y(II) of the filaments, cultivated for one month on solid medium (P1), drops first at about 90 min (Figure 2B) while Y(II) of the 7 months old culture (P2) reaches zero at approximately 220 min (Figure 2C). Table 1 displays the desiccation time and observed water loss for all samples. When comparing the time required for the cells cultivated on agar plates to reduce Y(II) to zero, increased age is clearly correlated ($r = 0.98$) with a prolonged activity. All three cultures grown on agar (P1, P2, P3) are differing significantly in desiccation time ($p < 0.001$). No significant difference has been observed between P3 and L, however, P2 and P1 differ 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 ($p < 0.01$).

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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.

Figure 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 bp and 24,724 bp, respectively. To assess the completeness of the established reference transcriptome, Benchmarking Universal Single-Copy Orthologs (BUSCO) were used. As displayed in Figure 3B, we found 76% to be complete, 8% to be fragmented while only 16% were missing. Compared to 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 (Figure 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 (Figure 3D, Figure S2 in the supporting information). For all examined e-values ranging from E-3 to E-20, all assembled contigs shared most sequences with *Physcomitrella patens* and *Klebsormidium flaccidum* (Figure S2). The homology comparison of the assembly to all streptophytic and all chlorophytic sequences showed that our assembly shared 21,262 with both groups while 10,672 and 615 sequences were exclusively aligned to streptophytic and chlorophytic proteins, respectively (Figure 3D). In order to evaluate the coverage of metabolic networks, the assigned KO numbers (5.9% of all contigs) were mapped onto the KEGG metabolic pathways map (ko01100, Supplemental Figure S3). In general, we observed a 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 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, that were filtered out during quality control, were used to investigate the presence of putative contaminations in the algal culture (Supplemental Figure 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., was detected. We also observed a large number of putative rRNAs which could not be annotated at all (up to 20% for P3C). Overall, rRNA reads related to *Zygnema* sp. or with no significant similarity to any organism in BLAST analyses constituted about 90% of the rRNA except for the P1C and P1D. For these samples a large contamination with bacterial (up to 40%)

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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 seven months old solid culture (P2C to P2D). The analysis was performed without P1C and P1D as well as P3C and P3D due to high levels of contamination and low RNA extraction yields, respectively. A total of 2,886 (2.1%) transcripts exhibited differential expression (FDR less or equal to 0.001) upon desiccation treatment compared to 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 (Figure 4A, supplementary Table S5). Between 30% and 54% of the upregulated and downregulated contigs in those groups were successfully annotated. The largest part of the 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 150 were repressed (Figure 4B). A total of 2,037 and 392 transcripts exhibited differential expression solely in group L and P2, respectively. To assess the correlation of the replicates of each sample, a principal component analysis was performed and sample correlations were visualized as a heatmap (Supplementary Figure S6). We found that the individual replicates cluster closer according to the sample affiliation than randomly when looking at the first two principal components (Figure S6A). The same is true for the Pearson correlation matrix, where clustering of the associated replicates as well as the controls and treated samples was observed (Figure S6B).

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 analysed 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 the “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, similar parent categories are grouped and, subsequently, tested for enrichment in one sample compared to 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

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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 Figure 5, a network of enriched non-redundant GO terms in up- and downregulated gene sets is displayed with major categories highlighted in light grey. 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 supplemental 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 (Supplemental 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 $\log_2$ hereinafter) are displayed in Table 4.

Photosynthesis and photorespiration—Transcriptomic analysis of photosynthetic processes revealed a strong downregulation of components of both PSs in group L. A repression of transcripts encoding parts of PS I and PS II with fold changes of -2.3 to -3.4 was observed. The strongest downregulated transcript was the PS II 22kDa protein (PsbS) with a fold change of -4.6. In group P2, only PS I subunit IV and PS II 22kDa protein exhibited 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 PS II light harvesting complex protein 2.2 displayed a 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 a weaker downregulation. Upon desiccation, the light harvesting complex PS II, ATPase 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 chloroplastic low molecular mass ELIP was repressed. The solid culture of *Z. circumcarinatum* (P2) showed an upregulation of the chloroplastic ELIP (ELI), ELIP1 and the chloroplastic high molecular mass ELIP. The chloroplastic high molecular mass ELIP exhibits the strongest induction with a fold change of 12.2.

Desiccation also caused a repression of enzymes involved in photorespiration in both comparisons. The transcript pools of the (S)-2-hydroxy-acid oxidase, serine-glyoxylate transaminase and glycine dehydrogenase showed a decline for L and P2 while group L also exhibits a downregulation of glutamate-glyoxylate aminotransferase and glycerate dehydrogenase. The detected fold changes ranged from -2.5 to -4.2.

Carbohydrate metabolism—Starch degradation and sucrose formation were induced during dehydration in group L while group P2 shows only minor effects. A variety of starch consuming enzymes were upregulated such as glycogen phosphorylase, alpha- and beta-amylase, isoamylase, 4-alpha-glucanotransferase as well as the sucrose synthesizing enzymes sucrose-phosphatase and sucrose-synthase. The strongest induction was observed for glycogen phosphorylase with an 8.3-fold upregulation while the others exhibit fold changes between 2.2 and 4.3.

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

Transporter 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 groups L and 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, which were showing 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 and calcium-dependent protein kinases (CPK), such as the leucine-rich repeat receptor-like serine/ threonine-protein kinases BAM2 and FLS2 as well as the calcium-dependent protein kinases 17 and 20, were the most prominent transcripts. Overall, more signaling related contigs were repressed than upregulated.

Stress response—An upregulation 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 the 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, the proteasome assembly chaperone 2 exhibits the largest change of 11.2-fold. Transcripts similar to the Nijmegen breakage syndrome 1 protein and DNA-damage-repair/ toleration protein, both involved DNA repair, also showed an enhanced expression.

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Astonishingly, the 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, consisting only of LEA4 proteins in group L while group P2 showed the upregulation of both LEA4 and LEA5 proteins. The LEA5 gene exhibited an 9.8-fold increase in expression. The BLAST analysis of several Chlorophyta and Streptophyta (Figure 6) reveals LEA group distribution among Viridiplantae species. We observed that all studied streptophyte genomes contained at least LEA proteins from group LEA2, LEA4 and LEA5 while chlorophytes mostly only possessed LEA2 proteins with some exceptions (in our example, *D. salina*).

Overall, photosynthesis appears to be repressed in group L while several ELIPs are upregulated in both groups. Desiccation stress also causes an upregulation of transcripts involved in the lipid metabolism and transport in L and P2. Furthermore, group L exhibits an induction of the carbohydrate metabolism. Both groups increase 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 towards dehydration as photosynthesis was maintained longer compared to younger cells (Herburger et al. 2015). These findings are in agreement with our observations for *Z. circumcarinatum* cultivated on agar (Figure 2B-D). Culture P1 abandoned photosynthetic activity first, 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, 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 to 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 completely removed by blotting. During the desiccation treatment, the excess water had to evaporate before the algal biomass could be desiccated effectively.

The dehydration of *Z. circumcarinatum*, cultivated in liquid medium for one month, led to a strong repression of transcripts encoding components of PS I and II as well as light-harvesting proteins indicating photoinhibition. In contrast, *K. crenulatum* (Holzinger et al. 2014) showed an upregulation of genes related to photosynthesis in response to desiccation. Holzinger et al. (2014) suggest that this mechanism serves as a preparation to rapidly resume photosynthesis upon rehydration. Similar results were obtained for *Trebouxia gelatinosa* (Carniel et al. 2016). However, Carniel et al. (2016) compared a couple of different transcriptomic studies on desiccation of Viriplantae detecting a repression of photosynthetic transcripts in *Syntrichya ruralis* (desiccation tolerant moss), *C. plantagineum* (resurrection

plant), *Haberlea rhodopensis* (resurrection plant) and *Xerophyta humilis* (resurrection plant). Moreover, the resurrection plant *Myrothamnus flabellifolia* also exhibited a downregulation of photosynthesis genes when desiccated (Ma et al. 2015). Surprisingly, mainly genes, encoding parts of PS I and II, the electron transport chain and the ATP synthase, were repressed in *Myrothamnus flabellifolia* (Ma et al. 2015). This is also true for *Z. circumcarinatum*, as demonstrated in the present study. Ma et al. (2015) argue that thereby excitation energy and, thus, ROS production likely 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 *Oryza sativa* (rice) seedlings (Turan & Tripathy 2015). The observed impairment of the chlorophyll biosynthesis is probably occurring to avoid ROS formation (Farrant et al. 2003). As an additional protection, the expression of ELIPs was enhanced in group L and P2. ELIPs are photoprotectants and belong to the chlorophyll *a/b*-binding (CAB) superfamily, respond to abiotic stress, mainly to high light and UVR, and are located in the thylakoid membrane (Hayami et al. 2015, Hutin et al. 2003, Norén et al. 2003). Similar reactions were observed for other green algae, for example, *Chlamydomonas reinhardtii* and *Dunaliella 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). Less studies have been dedicated to the relationship of cold stress and ELIP expression in algae. Król et al. (1997) reported an induction of ELIPs in *Dunaliella salina* when exposed to low temperatures. *Spirogyra varians* (Zygnematales) also exhibits an accumulation of ELIP-like transcripts when cultivated at 4°C (Han & Kim 2013). Nevertheless, the accumulation of ELIPs is also commonly associated with desiccation stress (Dinakar & Bartels 2013, Ma et al. 2015, Zeng et al. 2002). These proteins protect the thylakoid membranes against photooxidative damage by scavenging free chlorophyll molecules and act as sinks for excitation energy (Heddad et al. 2012, Zeng et al. 2002). 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 (Hutin et al. 2003, Marraccini et al. 2012, Zeng et al. 2002).

A decrease in photorespiratory transcripts upon desiccation was detected of both groups of *Z. circumcarinatum* suggesting a repression of photorespiration. In contrast, photorespiration 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. 1999). However, protection of the PSs by photorespiration is not essential during desiccation (Wingler et al. 2000). Furthermore, dehydration is reported to reduce photorespiratory activity in plants which may be caused by the 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 sugars, polyols and proteins (Dinankar & Bartels 2013, Fernández-Marín et al. 2016, Hinsha et al. 1996, Holzinger & Pichrtová 2016, Ma et al. 2015). By increasing the amount of osmoprotectants in the cell, a negative osmotic potential

is achieved, membranes are stabilized and protein protection is enhanced (Bisson & Kirst 1995). Nagao et al. (2008) found that *K. flaccidum* accumulates the osmolyte sucrose during cold acclimation which contributes to a higher freezing tolerance. Moreover, the alga *Chlorella vulgaris* exhibits an increase in sucrose and raffinose content in response to cold shock treatment (Salerno & Pontis 1989). However, sucrose is also typically formed upon desiccation stress in plants and algae (Cruz de Carvalho et al. 2014, Dinankar & Bartels 2013, Holzinger & Pichrtová 2016, Ramanjulu & Bartels 2002, 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 an enhancement of the "starch and sucrose metabolism". A similar strategy is pursued by *K. crenulatum* inducing transcripts encoding sucrose synthase and the sucrose phosphate synthase (Holzinger et al. 2014). The authors suggest that raffinose family oligosaccharides also function as osmoprotectants because several enzymes, belonging to the galactinol/raffinose metabolism, exhibited a higher expression in desiccated cells (Holzinger et al. 2014). *Z. circumcarinatum* did not show this expression pattern in response to water stress. However, the sucrose phosphate synthase of *Z. cirumcarinatum* contains a conserved phosphorylation site (results not shown) which is typically found in angiosperms and known to become modified upon osmotic stress (Winter & Huber 2000). Hence, the sucrose metabolism of *Z. circumcarinatum* is most likely not only regulated by transcription but also posttranslational modifications.

Callose is an important polysaccharide found to be involved in response 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* (Herburger & Holzinger 2015). Complementary, the desiccation transcriptome of *Klebsormidium* revealed an upregulation of the callose synthase complex confirming the significance of this carbohydrate during dehydration events (Holzinger et al. 2014). Albeit the occurrence of this enzyme in the *Zygnema* transcriptome no differential expression was detected. These results are in agreement with Herburger & Holzinger (2015) who reported a stable callose content throughout desiccation for 2.5 h. As the callose synthase is located in the plasma membrane and the protoplast is retracting from the cell wall upon dehydration callose incorporation is prevented (Herburger & 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 (Dinankar & Bartels 2013, Holzinger et al. 2014, Perlikowski et al. 2016, Valledor et al. 2013). 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 alpha-galactosidase is upregulated in group L. However, parts of the glycero- 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). *Z. 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 (Anderberg et al. 2011 & 2012, Barkla et al. 1999). AQPs in embryophytes 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 (PIPs) and TIPs (Danielson et al. 2008). Anderberg et al. (2011) studied different chlorophytes and found AQPs of these groups only in Trebouxiophyceae (PIP, GIP). *Z. 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. Both groups, L and P2, showed a strong induction of TIPs during water stress which was also observed for other AQPs in the plants *Arabidopsis thaliana* and *C. plantagineum* (Dinankar & Bartels 2012, Ramanjulu & Bartels 2002) as well as the alga *T. gelatinosa* (Carniel et al. 2016). Carniel et al. (2016) argue that AQPs are protection 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 (Jarzyniak & Jarsiński 2014).Thus, desiccation tolerance is dependent on sugar transportation within the cell. Liu et al. (2016) reported an 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 analyzing *Caragana korshinskii* (leguminous shrub; Li et al. 2016) and *Saccharum* spp. (sugar cane; Zhang et al. 2016).

The signaling in plants during desiccation generally involves several hormones such as abscisic acid, cytokinin and ethylene (Campo et al. 2014, Holzinger & Becker 2015, Van de Poel et al. 2016, Zhou et al. 2014) as well as calcium-dependent and serine/threonine-protein kinases (Campo et al. 2014, Ramanjulu & 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 land plant evolution (de Vries et al. 2016). In contrast to *K. crenulatum* (Holzinger & Becker 2015), the up-regulation 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 obtained 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. Dong et al. 2016, Cruz de Carvalho 2008, Cruz de Carvalho et al. 2012, Han et al. 2011, Heinrich et al. 2016, Kranner et al. 2005). For example, high light stress triggers the expression of ROS scavenging enzymes in the green alga *C. reinhardtii* and the marine diatom *Thalasiosira pseudonana* (Dong et al. 2016, Ericksson et al. 2015). Similarly, the dinoflagellate *Symbiodinium* involves 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 suggests ROS generation upon desiccation as *Z. circumcarinatum* expresses a multitude of enzymes related to ROS protection: the glutathione-S-transferase conjugates glutathione (GSH) to hydrophobic molecules (Rezaei et al. 2013), the peroxisomal catalase acts directly on ROS (Gorrini et al. 2013), peroxiredoxin catalyzes the reduction of peroxides (Dayer et al. 2008), the peptide methionine sulfoxide reductase reduces methionine sulfoxide back to methionine (Weissbach et al. 2002) and (chloroquine-resistance transporter)-like transporter 3 transports GSH (Noctor 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 a liquid cultivation. An 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 increases the transcript pool of the (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 (Cruz de Carvalho 2008, Heinrich et al. 2015), was addressed by a 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 damages associated with desiccation (Akutsu et al. 2007, Cruz de Carvalho 2008).

Abiotic stress generally leads to aggregation and conformational changes in protein which is lethal to the cell (Wang et al. 2004). In response, plants and algae express chaperones and Hsps which are assisting the refolding of proteins and protect them from aggregation (Al-Whaibi 2011, Mitra et al. 2013, Schulz-Raffelt et al. 2007, Van de Poel 2016, Wang et al. 2004). 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 an 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. Similarily, 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 an increased expression of certain chaperones and Hsps in *Selaginella lepidophylla* (lycophyte, Carniel et al. 2016), *Physcomitrella patens* (moss, Wang et al. 2009), *Pyropia orbicularis* (red alga, López-Cristoffanini et al. 2015) and *Asterochloris erici* (green alga, Gasulla et al. 2013). *Z. circumcarinatum* showed an 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 an 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 raised 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 the co-chaperones DnaJ (Wang et al. 2014). Wang et al. (2014) reported an increase in drought tolerance in transgenic tobacco resulting from an overexpression of the chloroplast-targeted chaperone DnaJ. Furthermore, an 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. latissimi* and *T. pseudonana* (Dong et al. 2016, Heinrich et al. 2012a). Furthermore, our data shows an 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 the protein quality control are proteasomes which selectively eliminate dysfunctional proteins (Hanssum et al. 2014). In response to changing conditions and environmental stress, the demand of 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 which 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 as water stress specific chaperones (Goyal et al. 2005, Hatanaka et al. 2014, Shinde et al. 2012) which can be grouped depending on their sequence motifs/patterns (Hundertmark & Hincha 2008). The drought induced expression of LEA proteins has been already 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 & Hincha (2008), the *Z. circumcarinatum* transcriptome covered sequences belonging to LEA4 and LEA5 (Figure 6). LEA4 is likely the group, which emerged first, as it shows the biggest diversity of all and is present in all investigated algae and plants except *D. salina*. LEA5 was probably also emerging early because it was found in all streptophyta and *C. reinhardtii*. All analyzed streptophyta genomes featured LEA2 proteins but this group is 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.

Conclusion

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 seven months old agar culture of *Zygnema* was analyzed. Our results found a 3-fold stronger transcriptional response of filaments grown in liquid medium compared to older filaments cultivated on agar. These findings are a clear indication of a pre-acclimation to low water availability of the algal culture grown on agar for seven 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 the 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 shall 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 cultivated for one month in liquid Waris-H medium (McFadden & Melkonian 1986) or on 1.5% agar plates, containing BBM medium, as previously described by Herburger et al. (2015). The algal strain clusters in phylogenetic analysis close to *Zygnema 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 one month old liquid culture (L) and one month (P1), seven months (P2) and twelve months old cultures (P3) grown on agar plates (solid medium; Figure 1). The liquid culture of *Z. circumcarinatum* was harvested by centrifugation (300 xg) 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. The triplicates of all samples were placed into separate desiccation chambers, which were previously described (Karsten et al. 2014), and desiccated over a saturated KCl solution at a RH of approximately 86%

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according to Pichrtová et al. (2014). Y(II) was monitored during the desiccation event, using a portable 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 of the water content was determined according to the following formula:

Reduction [%] = 100 – desiccated biomass/fresh biomass x 100

Both for Y(II) and water loss, differences between each group were assessed in R by a one-way ANOVA ($p < 0.01$) followed by Tukey's post-hoc test (HSD, $p < 0.01$). Correlation coefficients were calculated according to Pearson (1895).

RNA isolation and sequencing

Extraction of RNA was performed as previously described by Holzinger et al. (2014) with some modifications. Six filters from each cultivation (L, P1, P2, P3), containing either control samples (C, n = 3) or samples, subjected to desiccation (D, n = 3), were treated with 450 µl of Life Guard Soil Preservation solution (MO BIO Laboratories, Carlsbad, CA USA) and frozen in liquid nitrogen. Subsequently, 2% CTAB buffer (in 100 mM Tris, 50 mM EDTA, pH=8) was added and the samples were ground in a mortar as previously described by Heinrich et al. (2012b). Furthermore, 20 µl DTT were added to the mixture followed by a centrifugation step. The supernatant was mixed with ethanol and, finally, RNA was extracted using the peqGold Plant RNA Kit (peqlab/VWR International, Erlangen, Germany) according to the manufacturer's instructions. RNA samples were further purified as described by Rippin et al. (2016). After DNase I treatment (Thermo Fisher Scientific, Waltham, MA, USA), the RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany) was utilized for concentration and clean-up. After assessing RNA quantity using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) the RNA content of one replicate of P2D and all replicates of P3C and P3D, was insufficient for sequencing. Thus, the P2D replicate was excluded and the triplicates of each of the samples P3C and P3D were pooled in equal amounts. Finally, a reference was pooled from all samples in equal amounts.

After RNA quality control with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), two expression libraries for the reference and one for each of the sample replicates and the two triplicate pools were prepared by Eurofins Genomics (Ebersberg, Germany). Furthermore, mRNA was enriched using oligo-(dT) beads followed by fragmentation, random-primed cDNA synthesis and Illumina compatible adaptor ligation. The two normalized reference libraries were sequenced on one lane of an Illumina MiSeq, 300 bp paired end mode, and all sample libraries together on three different lanes of an Illumina HiSeq 2500, 125 bp single mode, using the MiSeq Control Software 2.5.0.5 or HiSeq Control Software 2.2.38, RTA 1.18.54 or RT 1.18.61 and bcl2fastq-1.8.4 (Illumina, San Diego, CA, USA).

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 & Edwards 2011). Subsequently,

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rRNA sequence reads were separated by SortMeRNA 2.1 (Kopylova et al. 2012) employing the SILVA SSU NR Ref 119 and LSU Ref 119. Before assembly, COPEread (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 annotating all contigs with Diamond 0.8.24 (Buchfink et al. 2015) against a custom-made database which contained the protein sequences of *Physcomitrella patens* (Phytozome database version 12), *Klebsormidium flaccidum* (Hori et al. 2014) and *Naegleria gruberi* (Fritz-Laylin et al. 2010). Contigs scoring an e-value higher than E-20 against *N. gruberi* were removed. Control and treatment reads were subjected to trimming with Trimmomatic 0.35, rRNA filtering with SortMeRNA 2.1 and were subsequently mapped to the reference assembly using Bowtie2 2.2.9 (Langmead & Salzberg 2012) estimating the abundance with RSEM (Li & Dewey 2011). For differential gene expression analysis, the R package edgeR (Robinson et al. 2010) was employed, analyzing LC, LD, P2C and P2D and proceeding only with differentially expressed genes possessing an FDR (Benjamini & Hochberg 1995) smaller than 0.001 and a fold change of at least 4. Contig annotation was performed with the Trinotate pipeline 3.0.0 (http://trinotate.github.io/), including TransDecoder 2.1 (http://transdecoder.github.io/), NCBI BLAST+ 2.3.0 (Altschul et al. 1990), HMMER 3.1b (Finn et al. 2011), SignalP 4.1 (Petersen et al. 2011), TMHMM 2.0c (Krogh et al. 2001), RNAmmer 1.2 (Lagesen et al. 2007) as well as the databases Swiss-Prot and PFAM 3.1b2. The e-value cutoff was set to E-10. Two gene set enrichment analyses were carried out in R, GO term enrichment with GoSeq 1.26.0 (Young et al. 2010) and KEGG pathway enrichment with clusterProfiler 3.2.11 (Yu et al. 2012), setting the FDR in both cases to 0.001. Diamond 0.8.24 was used for annotations against the Phytozome database version 12 and the *Chlorella* sp. NC64A genome (Blanc et al. 2010), the *Klebsormidium crenulatum* transcriptome (Holzinger et al. 2014) and the *Klebsormidium flaccidum* genome. Filtered rRNA reads were clustered into OTUs utilizing USEARCH 5.2.2 (Edgar 2013) and annotated against the Silva SSU database 123.1 by feeding them into the QIIME 1.9.1 (Caporaso et al. 2010) script assign_taxonomy.py (e-value cutoff E-6). All raw reads and assembled contigs were submitted to the SRA database and will become available upon publication of the study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA Analysis of variance

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BBM	Bold's basal medium
BLAST	Basic local alignment search tool
CTAB	Cetyl trimethylammonium bromide
DTT	Dithiothreitol
ERD	Early-response-to-dehydration protein
FDR	False discovery rate
GO	Gene ontology
HSD	Honestly significant difference
KEGG	Kyoto encyclopedia of genes and genomes
KO	KEGG orthology
OTU	Operational taxonomic unit
PAM	Pulse-amplitude modulated fluorometer
ROS	Reactive oxygen species
RuBisCo	Ribulose-1,5-bisphosphat-carboxylase/-oxygenase
UVR	Ultraviolet radiation
Y(II)	Effective quantum yield of photosystem II

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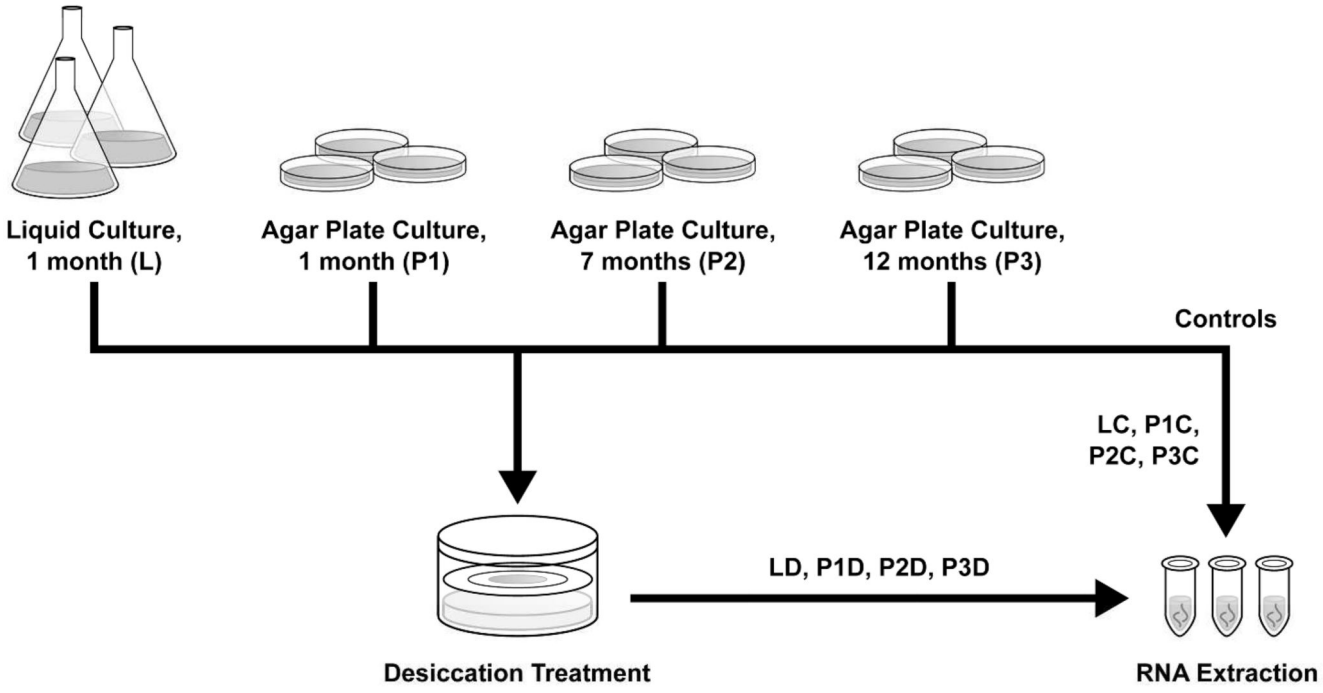

Figure 1.

Experimental Setup. Four different culture conditions were chosen: one month old culture grown in liquid medium (L), one month old culture grown on solid medium (P1), seven months old culture grown on solid medium (P2), 12 months old culture grown on solid medium (P3). Samples were taken as controls and for desiccation treatment and RNA was extracted subsequently.

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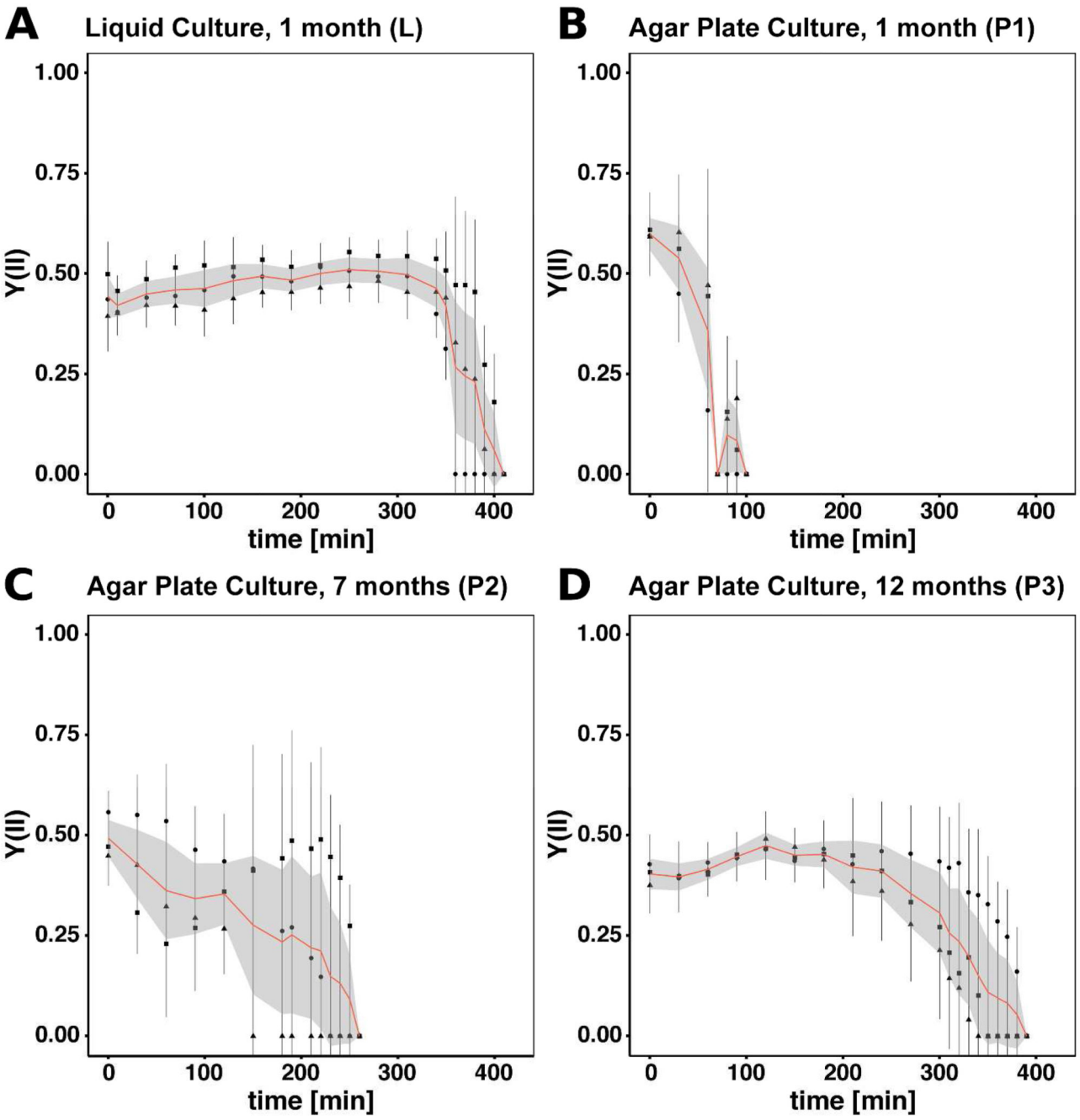

Figure 2.

Y(II) measured over the course of desiccation for all samples. Each triplicate was measured three times at different positions of the filter. The bar indicates standard deviation per replicate while square, triangle and circle indicate the mean value. The mean of all replicates is displayed in red giving the lower and upper Gaussian confidence limit in light grey. A) One month old culture grown in liquid medium (L). B) One month old culture grown on solid medium (P1). C) Seven months old culture grown on solid medium (P2). D) 12 months old culture grown on solid medium (P3).

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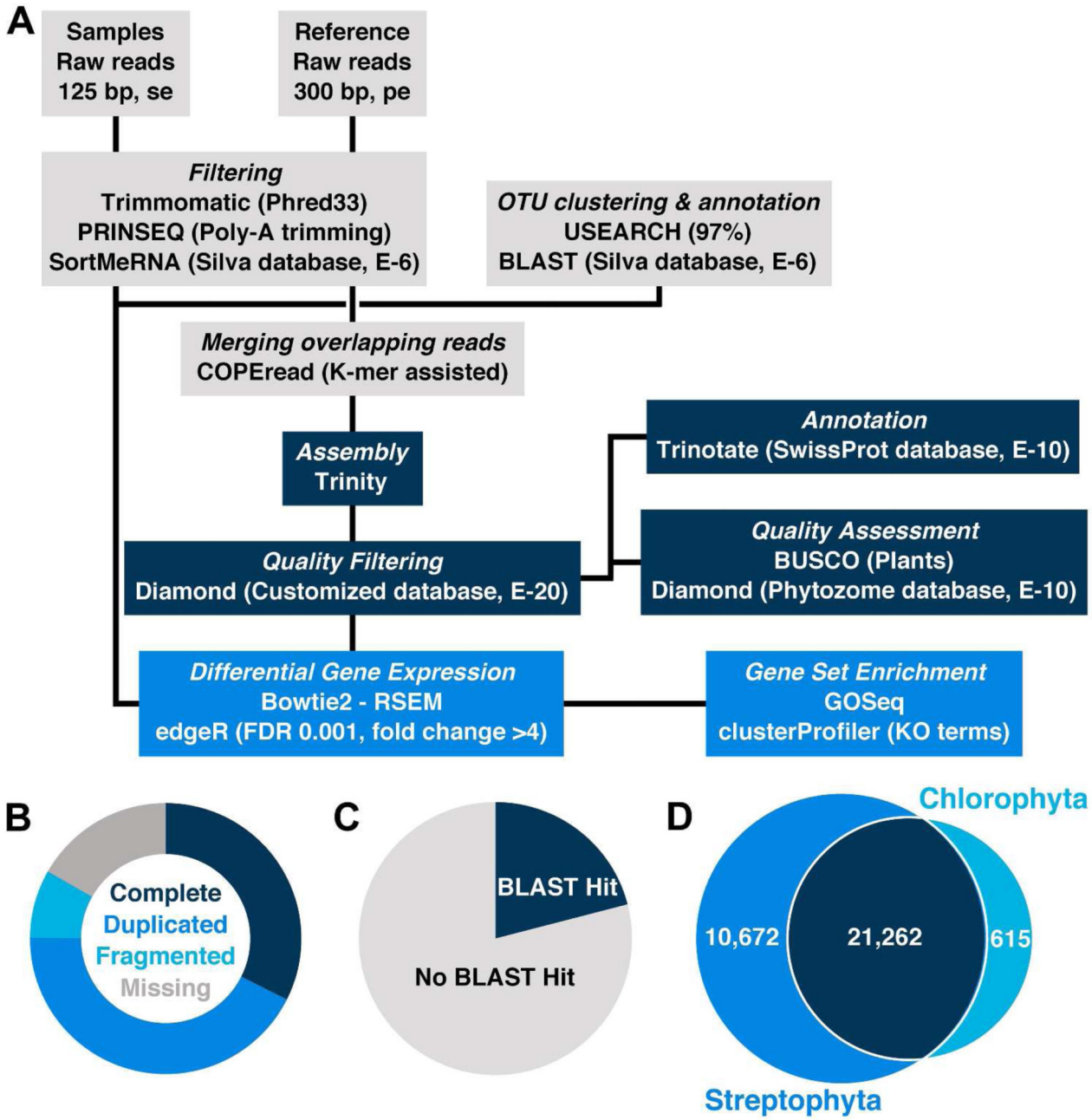

Figure 3.

A) Bioinformatic Pipeline. Raw Reads of all samples (controls and treated) as well as the reference (pool of all samples) were filtered using Trimmomatic, PRINSEQ and SortMeRNA. The remaining paired-end reads of the reference were merged, if possible, using COPEread and subsequently assembled with Trinity. The filtered rRNA reads of the samples were clustered and annotated using USEARCH and BLAST. The assembly was further quality filtered against a customized database containg sequences originating from *Physcomitrella*, *Klebsormidium* and *Naegleria*. After quality assessment with BUSCO and

Diamond the contigs were annotated with Trinotate. The sample reads were mapped onto the contigs using Bowtie2, read counts were calculated with RSEM and differential gene expression was performed with edgeR. Finally, GOSeq and clusterProfiler were used for gene set enrichment analyses. More details on the procedure can be found in the material and methods section. B) The donut chart displays the results of the BUSCO analysis which was carried out to assess the completeness of the assembly. The categories complete, duplicated, fragmented and missing are represented by 310, 408, 78 identified and 160 not identified orthologs, respectively. C) In total, 28,427 contigs of the assembly could be annotated at E-10 while 107,145 could not. D) Venn-diagram depicting the number of contigs mapping to sequences of selected Streptophyta (blue), Chlorophyta (turquoise) or both (dark blue) at E-10.

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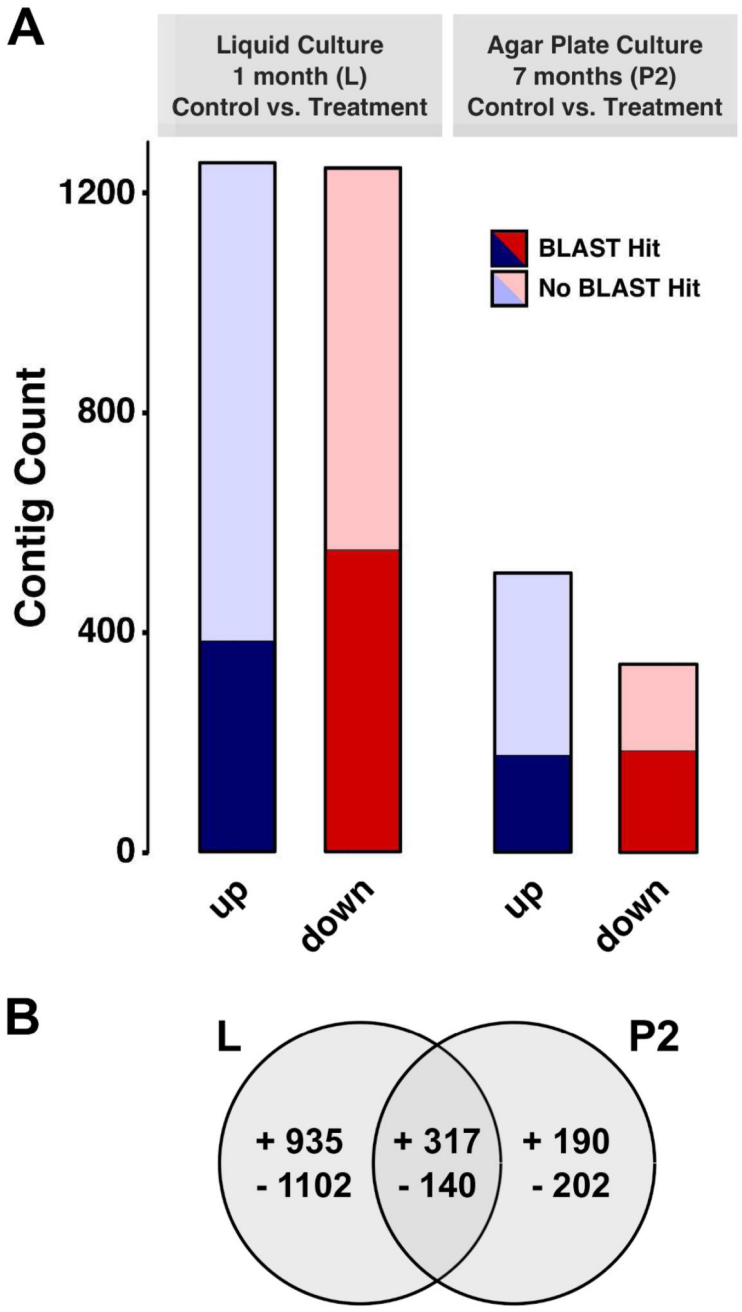

Figure 4.

A) The total of up- (dark, light blue) and downregulated (dark, light red) contigs of *Z. circumcarinatum* under desiccation in group L and P2. Only contigs with a FDR of less than or equal to 0.001 were considered. All contigs were annotated against the Swiss-Prot database using BLASTx with an e-value of less than or equal to E-10. B) Overview of total up- and downregulated contigs of *Z. circumcarinatum* upon desiccation in group L and P2. Only contigs with a FDR of less than or equal to 0.001 were considered. The Venn diagram displays the number of regulated genes shared between both groups.

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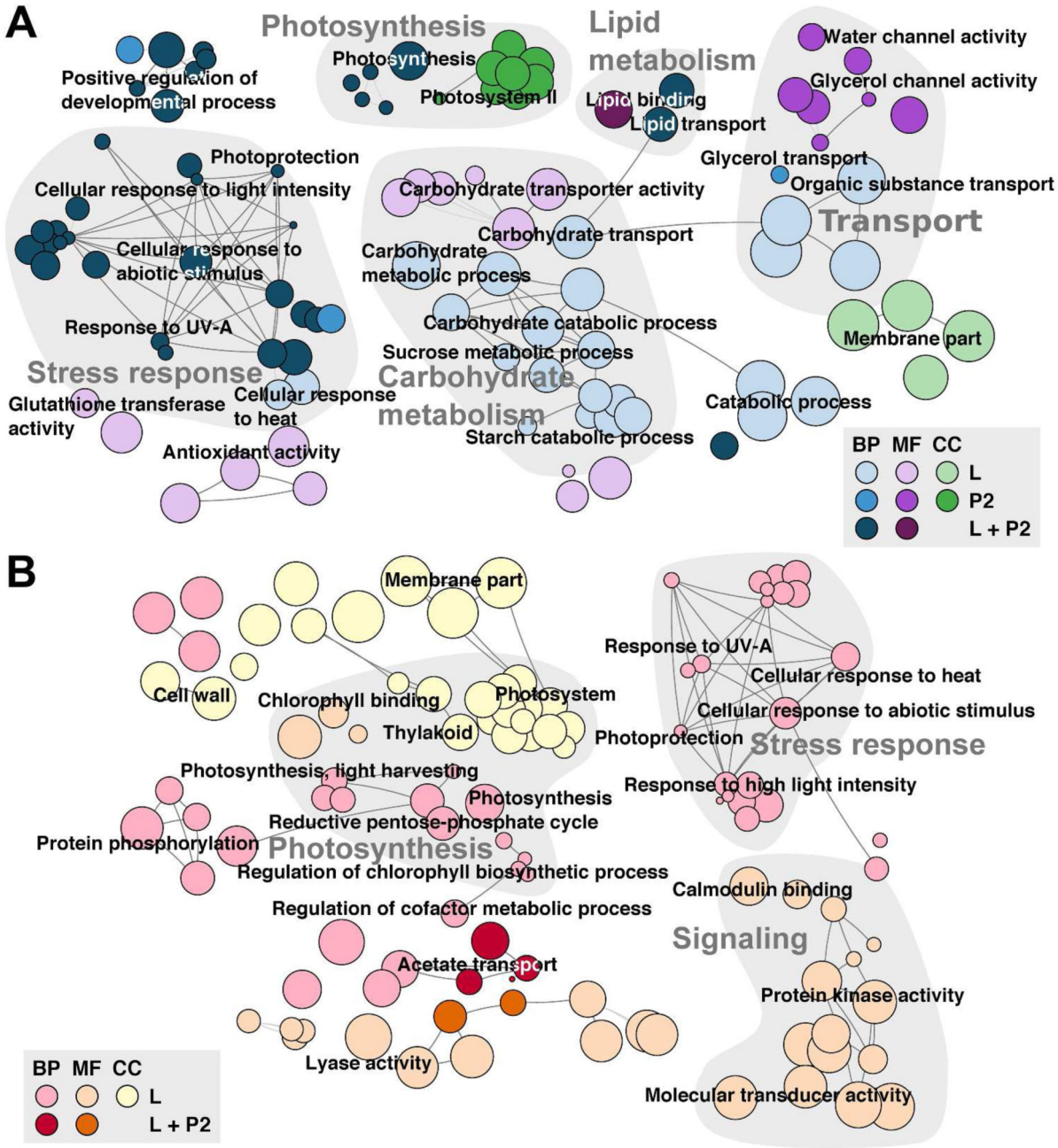

Figure 5.

GO Network displaying all enriched categories in both groups L and P2 as well as A) up- 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 grey are selected groups such as photosynthesis, lipid metabolism, transport, carbohydrate metabolism, stress response and signaling.

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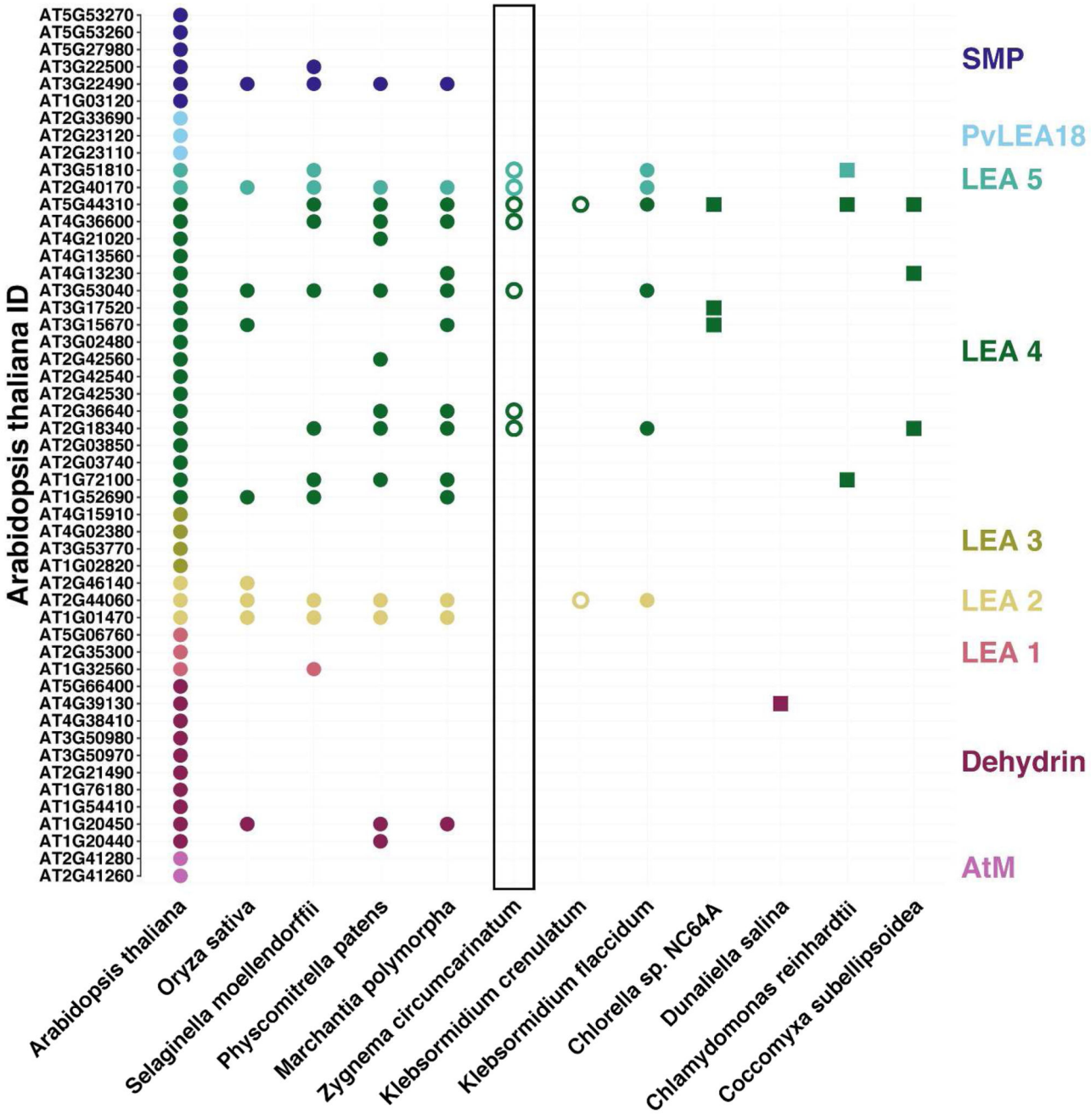

Figure 6.

LEA proteins found in *A. thaliana* and described by Hundermark & Hincha (2008). Annotations were retrieved from Phytozome v11.0 for *A. thaliana*, *C. reinhardtii*, *C. subellipsoidea*, *D. salina*, *M. polymorpha*, *P. patens* and *S. moellendorffii*. 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 *Klebsormidium 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.

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Table 1

Physiological results of desiccation stress experiment. Desiccation time means the time that elapsed from the start of the desiccation treatment until Y(II) dropped to zero.

Sample ID	Cultivation	Desiccation Time [min]	Water loss [%]
L	liquid medium, 1 mo	390 ± 26.5	93.7 ± 2
P1	solid medium, 1 mo	90 ± 17.3	81 ± 3.4*
P2	solid medium, 7 mo	223 ± 40.4	92.8 ± 3.2
P3	solid medium, 12 mo	360 ± 26.5	89 ± 0.01

n=3 if not indicated otherwise, *n=2

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Table 2

Enriched KEGG pathways.

Group	Regulation	KEGG ID	Pathway
L	up	ko00500	Starch and sucrose metabolism
	down	ko00195	Photosynthesis
		ko00630	Glyoxylate and dicarboxylate metabolism

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Table 3

Outcome of GO enrichment analysis displaying solely root category distribution (CC = “Cellular component”, MF = “Molecular function”, BP = “Biological process”). Detailed information is included in supplemental Table S9.

Group	Regulation	CC	MF	BP
L	up	4	16	60
	down	26	31	50
P2	up	8	8	39
	down	0	2	4

Table 4

Selection of contigs showing differential expression in response to desiccation stress (The complete list can be found in Table S5). Contigs are divided into the following groups: Photosynthesis and photorespiration, carbohydrate metabolism, lipid metabolism, transporter proteins, signaling, stress protection. Selected contigs are displayed with ID, annotation, e-value and fold change (log_2 transformed) for group L and P2.

Contig ID	Annotation	E-value	L	P2
Photosynthesis and photorespiration				
TR14384|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|c0_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 22kDa protein (PsbS)	1.08E-83	-4.6	-4.1
TR24382|c0_g1_i1	Photosystem II protein (PsbY)	4.85E-13	-3.4	-
TR71565|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_g1_i2	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_i2	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|c11_g29_i1	Early light-induced protein, chloroplastic (ELI)	1.83E-15	-	12.2
TR58021|c0_g5_i1	Early light-induced protein 1, chloroplastic (ELIP1)	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
TR68913|c1_g1_i2	Glycine dehydrogenase	0	-2.6	-4.2
TR54933|c0_g2_i1	Glutamate-glyoxylate aminotransferase	0	-3.0	-
TR48225|c1_g1_i1	Glycerate dehydrogenase	0	-3.5	-
Carbohydrate metabolism				

Contig ID	Annotation	E-value	L	P2
TR23256|c0_g1_i1	Glycogen phosphorylase	0	8.3	-
TR75230|c1_g1_i1	alpha-Amylase	1.95E-175	4.3	-
TR70181|c1_g1_i2	beta-Amylase	0	2.7	-
TR24697|c0_g3_i4	Isoamylase	6.66E-40	2.6	-
TR25586|c0_g1_i1	4-alpha-Glucanotransferase	1.47E-45	2.2	-
TR45454|c0_g2_i1	Sucrose-phosphatase	6.91E-108	3.6	-
TR61067|c0_g1_i2	Sucrose synthase	0	3.0	-
Lipid metabolism				
TR39622|c0_g1_i1	Lysophospholipid acyltransferase	4.12E-110	2.0	-
TR53082|c0_g1_i1	Diacylglycerol kinase	2.68E-160	2.0	-
TR16611|c0_g1_i2	alpha-Galactosidase	1.91E-154	2.3	3.5
TR42973|c1_g1_i1	Sulfoquinovosyltransferase	4.73E-12	3.7	-
TR31318|c0_g1_i1	Phospholipase D1/2	0	3.7	-
TR28615|c2_g10_i1	Phosphoethanolamine N-methyltransferase	5.8E-102	2.9	3.2
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	-
Stress protection				
TR10757|c0_g3_i1	Chaperone protein ClpB1	7.43E-16	3.4	-
TR35960|c0_g2_i2	Proteasome assembly chaperone 2	4.89E-28	11.2	-
TR41947|c0_g1_i3	Chaperone protein DnaJ	1.06E-10	3.9	-
TR75210|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	Peroxisomal catalase	0	3.7	6.7
TR58823|c0_g2_i1	Peroxiredoxin	6.92E-54	2.3	3.4
TR57779|c0_g1_i1	Peptide methionine sulfoxide reductase	4.35E-79	2.5	3.0

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Contig ID	Annotation	E-value	L	P2
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	-
TR69744|c2_g23_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