Short title: PLD activates MAPKs in drying lichen microalgae

*** Corresponding author:**

Francisco Gasulla

Departamento de Ciencias de la Vida, Facultad de Ciencias Biológicas, Edificio de Ciencias, Campus Universitario, Ctra. Madrid-Barcelona km. 33,600, 28805 Alcalá de Henares (Madrid), Spain

Telephone number: 0034 91 885 64 32

Title: Phospholipase D controls the activation of MAPK signalling cascades in rapid cellular responses to osmotic stress in lichen microalgae[1].

Authors: Francisco Gasulla[2, *], Eva Barreno, Carlos Jiménez, María L. Parages[3], Joaquín Cámara, Peter Doermann, Dorothea Bartels

Departamento de Botánica & ICBIBE, Fac. C. Biológicas, Universitat de València, 46100 Burjassot, Spain (F.G., E.B.); Institute of Molecular Physiology and Biotechnology of Plants (IMBIO), University of Bonn, D-53115 Bonn, Germany (F.G., P.D., D.B.); Departamento de Ecología, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain (C.J., M.L.P., J.C.)

One sentence summary: Protective mechanisms are induced in lichen microalgae during natural drying events, the phospholipase D pathway is involved in the modulation of these rapid cellular responses through the phosphorylation of proteins.

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Present address: [2] Departamento de Ciencias de la Vida, Fac. C. Biológicas, Universidad de Alcalá de Henares, 28805, Alcalá de Henares, Spain.

[3]

Corresponding author email: francisco.gasulla@uv.es

Abstract

Classically, lichen phycobionts are described as poikilohydric organisms able to undergo desiccation due to the constitutive presence of molecular protection mechanisms. However, little is known about the induction of cellular responses in lichen phycobionts during drying. The analysis of lipid composition of the lichen microalgae *Asterochloris erici* submitted to desiccation revealed the unusual vegetative accumulation of highly polar lipids (oligogalactolipids and phosphatidylinositol), which prevent the fusion of membranes during stress, but also the active degradation of cone-shaped lipids (monogalactosyldiacylglycerol and phosphatidylethanolamine) to stabilize membranes in desiccated cells. The level of phosphatidic acid increased seven-fold during desiccation, implicating a possible role of phospholipase D (PLD) in the response to osmotic stress. Inhibition of PLD with 1-butanol markedly impaired the recovery of photosynthesis activity in A. erici upon desiccation and salt stress (2M NaCl). These two hyperosmotic stresses caused the phosphorylation of c-Jun N-terminal kinases (JNK) and p38-like mitogen activated protein kinases (MAPK) and the dephosphorylation of extracellular signal-regulated kinases (ERK). The incubation with 1-butanol reduced the phosphorylation and dephosphorylation of JNK and ERK-like proteins, respectively, which indicates an up-stream control of MAPK cascades by PLD. Accordingly, desiccation caused the phosphorylation of several proteins in A. erici, most of them involved in protein turnover as revealed by the analysis of the phosphoproteome. The results demonstrate that lichen phycobionts possess both constitutive and inducible protective mechanisms to acquire desiccation tolerance. Among others these responses are controlled by the PLD pathway through the activation of MAPK cascades.

Introduction

Lichens are symbiotic associations between a fungus (mycobiont) and a population of green algae (phycobiont) and/or cyanobacteria (cyanobiont) that results in a unique entity of a holobiont (Margulis and Barreno, 2003). Together with other small poikilohydric plants, they form the group of the "fully desiccation-tolerant plants", which are able to survive to rapid drying processes (hours) due to the constitutive presence of protective mechanisms (Oliver and Bewley, 1997). In contrast, the group of "modified desiccation-tolerant plants" or the resurrection tracheophytes only survive if the rate of water loss is slow (days) since they need to accumulate protective factors (Alpert and Oliver, 2002; Oliver et al., 2011). The lichen phycobiont *Asterochloris erici* (Ahmadjian) Skaloud *et* Peksa is able to survive rapid desiccation processes through the constitutive presence of protection mechanisms such as a powerful antioxidant system, LEA proteins, and de-epoxidated xanthophylls among others (Gasulla et al., 2009, Kranner et al., 2008). Proteome analysis has revealed no major changes in *A. erici* during dehydration and rehydration, regardless of the drying rate, which supports the hypothesis that desiccation tolerance relies mainly on constitutive mechanisms (Gasulla et al., 2013a). However, *A. erici* recovers faster and cellular injuries are less severe after slow drying than after a rapid drying regime (Gasulla et al., 2009; Gasulla et al., 2013a) suggesting that a minimal period is required to develop strategies which facilitate transition to the desiccated state. Several studies carried out with lichens support this hypothesis, such as the induction of the antioxidant system (Kranner et al., 2005), the activation of an alternative mechanism of light energy dissipation that protects photosystem II (Veerman et al., 2007; Gasulla et al., 2009; Heber et al., 2011), or the de-epoxidation of violaxanthin to zeaxanthin (Fernández-Marín et al., 2010) during desiccation.

Transcriptomic studies have shown that dehydration triggers changes in the expression of hundreds of genes in poikilohydric plants such as the lichen-alga *Trebouxia gelatinosa* (Candotto et al., 2014), the lichen *Cladonia rangiferina* (Junttila et al., 2013), the desiccation-tolerant moss *Tortula ruralis* (Oliver et al., 2004, 2009), or the aeroterrestrial alga *Klebsormidium crenulatum* (Holzinger et al., 2014). However, there is no direct evidence that the modification of the transcriptome is correlated with changes in the proteome. In *T. ruralis* protein synthesis ceases almost immediately during dehydration (see Bewley 1979 for a review). Likewise, proteomic analyses revealed that desiccation in *A. erici* causes only minor changes of the proteome (Gasulla et al., 2013a). It has been suggested that in poikilohydric plants the increase/decrease in transcript abundance may be the result of mRNA stabilization (Wood & Oliver 1999) and selective degradation (O'Mahony and Oliver, 1999) and not of *de novo* transcription. Accordingly, the induction of protective mechanisms in lichen phycobionts during desiccation could not be attributed to the synthesis of proteins, and therefore a post-translational regulation is conceivable.

The phospholipid metabolism has been suggested to be involved in plant responses to various forms of abiotic stress. The formation of phospholipid-based signal molecules may well be among the primary events (within minutes) in the signal cascade that leads from the perception of water stress to metabolic adaptation (Bartels and Salamini 2001). Phospholipase D (PLD) catalyzes the hydrolysis of structural phospholipids, e.g. phosphatidylcholine, producing phosphatidic acid (PA) and a free head group. PA may affect cellular processes via different modes of action, such as functioning in signaling cascades by recruiting target proteins to particular membranes and/or influencing their activity, e.g. acting as an activator of protein kinases (Testerink and Munnik 2011). Enzymatic addition of phosphate groups by kinases modulates protein function, increases or decreases the activities of enzymes, redirects the sub-cellular localization of proteins, promotes or disrupts protein-protein interactions or tags proteins for degradation (Ma, 1993).

All plants possess Mitogen Activated Protein Kinases (MAPK) homologues, which play a critical role in the cell signalling network (Ligterink 2000, Mishra et al., 2006, Sinha et al., 2011). Despite the fact that signal transduction in algae has only recently become a focus of research, different studies with both macro- and microalgae (Jiménez et al., 2004, 2007, García-Gómez et al., 2012, Parages et al., 2012, 2013, 2014a, b) have demonstrated that p38-, c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK) components are present in algae, and that they have a crucial role in acclimation to stress and in cell division. The inhibition of p38- or JNK-like phosphorylation in both micro- and macroalgae highly impaired acclimation under stress conditions, eventually leading to cell death (Jiménez et al., 2004; Parages 2012). Decrease in adaptability and, eventually, in cell survival in the presence of specific inhibitors of the signalling pathways suggests the existence of signalling mechanisms in algae similar to those found in mammalian cells, and that algae survive under stress conditions by activating several cell programmes, among them p38- and JNK-like MAPKs pathways.

Phospholipase D is a potential candidate to be involved in the rapid cellular responses of lichen phycobionts to dehydration stress. It is reasonable to assume that under natural conditions, where lichen drying occurs within a few hours (even in minutes), algae need to preferentially employ fast cellular responses, like post-translational protein modifications triggered by the PLD pathway, instead of slow responses such as changes in gene expression or protein synthesis. Thus, the aim of this study was to explore the possibility that the lichen microalgae *A. erici* respond to hyperosmotic stress by activating PLD and MAPK signaling pathways, including p38 and JNK kinases, and whether inhibition of these pathways compromises the ability of the cells to adapt to environmental stresses.

Results

Modification of lipid composition in *A. erici* during desiccation

The lipid analysis by quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) revealed that the level of total polar lipids in *A. erici* decreased about 15 %, from 100.1 to 84.9 nmol mg^{-1} DW, during desiccation (Figure 1). Most of this decrease was due to the degradation of the main membrane lipid monogalactosyldiacylglycerol (MGDG), which was reduced from 34.4 to 22.6 nmol mg^{-1} DW. The second most pronounced decrease was associated with phosphatidylethanolamine (PE), which decreased from 4.78 to 2.64 nmol mg^{-1} DW. Minor decreases were observed for phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylserine (PS) and sulfoquinovosyldiacylglycerol (SQDG). The oligogalactolipids, tri- and tetragalactosyldiacylglycerol (TGDG and TeGDG), were constitutively present in *A. erici,* with 1.75 and 0.14 nmol mg^{-1} DW, respectively, and their levels did not change during desiccation. The levels of the two lipids, digalactosyldiacylglycerol (DGDG) and phosphatidic acid (PA), increased in response to dehydration. DGDG became the most abundant polar lipid in dried *A. erici*, it increased from 21.3 to 24.1 nmol mg^{-1} DW. The ratio of MGDG:DGDG declined from 1.62 to 0.94 during desiccation. In relation to the control amount, PA was the lipid most actively synthesized, and reached 0.652 nmol mg^{-1} DW, which represents a 7-fold increase during desiccation. Regarding non-polar lipids, diacylglycerol (DAG) and triacylglycerols (TAG) were 64 % higher and 21% lower, respectively in the dried samples compared to hydrated algae.

The Q-TOF MS/MS analysis allows to measure individual lipid molecular species, and therefore to follow lipid moieties during membrane lipid remodelling. Molecular species containing 34 carbons (34:x) were dominant in MGDG of hydrated algae, with 94.1 % of total MGDG (Fig. 2). The decrease of MGDG during desiccation was nearly fully (99.5 %) attributable to 34:x molecular species. Likely, most of the MGDG was hydrolysed by galactolipases resulting in the accumulation of 34:x DAG (Fig. 4). Another fraction of the 34:x MGDG was presumably converted into DGDG and oligogalactolipids; approximately 45 % of the DGDG increase was due to 34:x molecular species, while 34:5 TGDG and 34:6 TeGDG accumulated. The significant increase in 36: x DGDG and TGDG molecular species indicates that there is also an important *de novo* synthesis during desiccation of *A. erici*. The decline in 16:x/18:x/18:x TAG molecular species indicates that *de novo* synthesis of galactolipids uses the bulk of TAG as precursor.

The contribution of 34:x and 36:x molecular species to the PA increase was similar, 53% and 47%, respectively, whereas the opposite was observed for PC and PE. Thus, the accumulation of PA might be due to the activation of PLD in response to dehydration and the subsequent hydrolysis of phospholipids.

Inhibition of cellular signaling pathways and physiological consequences

We employed the technique of the modulate chlorophyll fluorescence to assess the physiological effect of blocking the PLD and MAPK pathways in *A. erici* under hyperosmotic stress. During dehydration the maximum photosynthesis yields (F_v/F_m) remained constant and close to control values (0.671) until the RWC reached 25 %, then the photosynthesis yield decreased sharply and photosynthesis ceased at 5 % RWC (Fig. 3). It was not possible to conclude whether PLD and MAPK inhibitors have any effect on the response of *A. erici* to severe osmotic stress due to high variation of the F_v/F_m measurements. Lichen algae are able to maintain the maximum photosynthesis rates until the RWC reaches about 25 %, but beyond this point F_v/F_m varies widely (Gasulla et al., 2009).The difficulty in maintaining all samples under the same RWC made it impossible to compare the F_v/F_m values of the differently treated samples. We also examined the effect of the inhibitors on recovery of *A. erici* after desiccation. F_v/F_m values of desiccated *A. erici* cells recovered to control values within one hour upon rehydration, with the exception of those treated with 1-butanol (PLD inhibitor) and SP600125 (JNK pathway inhibitor). In these samples F_v/F_m were about 5 % and 6 % lower, respectively, than in non-inhibited samples (Figure 5). However, while SP600125 treated samples recovered control values after 2 h, 1-butanol treated samples did not recover within the first 24 h of rewatering. The small effect of the inhibitors might be due to the loss of their activity over time because desiccation took about five hours and then samples remained dry overnight. In view of the above results, we decided to mimic dehydration stress using a hyperosmotic saline stress. In this way it is possible to apply constant osmotic stress. After inhibitor treatments, *A. erici* samples were incubated in a 2 M NaCl solution (water potential Ψ = -10 MPa). This represents moderate water stress in poikilohydric lichens but it is a critical physiological threshold (Barták *et al,* 2005; Chakir and Jensen, 1999; Kawamitsu et al., 2000; Váczi and Barták 2006). The submission of *A. erici* to salt stress caused a rapid decrease of photosynthesis activity; F_v/F_m values were about 18 % lower (0.545-0.560) after one hour in 2 M NaCl than in control samples (0.671) (Fig. 5). The inhibition of the PLD enzyme had a strong negative effect on the resistance of *A. erici* to salt stress; F_v/F_m dropped to 0.250 when algal cells were incubated in 1-butanol, about 62.6 % lower than in control. No effect of the p38 and JNK pathway inhibitors was observed during the first hour of saline treatment. During the following four hours there was a slight recovery of photosynthetic activity in non-inhibited (3NBBM medium and DMSO) and 1-butanol treated samples but photosynthesis did not recover in samples treated with SB203580 (p38 pathway inhibitor) and SP600125. After 24 h of hyperosmotic stress, F_v/F_m values decreased to 0.490-0.510, about 25 % lower than in control samples. The decay of photosynthesis activity was higher in the JNK-pathway inhibited samples, F_v/F_m was reduced to 0.450. On the contrary, photosynthesis activity continued recovering in 1-butanol-treated samples and F_v/F_m raised to 0.356 after 24 h of salt stress, although this value was still far from control samples.

These results demonstrate that PLD, but also JNK and p38-kinase pathways have an important role in the response of the lichen phycobiont *A. erici* to osmotic stress. The higher damage observed in

PLD- than in JNK- and p38-inhibited samples indicates that PLD is upstream of the signaling cascade triggering cellular responses like the activation of kinase pathways.

Phosphorylation pattern of MAPKs during hyperosmotic stress

Phosphorylation of the three MAPKs (e.g. JNK, p38 and ERK) has been studied by immunoblotting using specific antibodies against the phosphorylated forms. Immunobloting with phospho-JNK, phospho-p38 and phospho-ERK antibodies displayed a main 48, 38 and 44 KDa band, respectively (Fig. 7 and 8). The use of blocking peptides clearly reduced the intensity of the bands detected by the three phospho-antibodies, which demostrates the presence of phospho-p38-, -JNK- and -ERK-like proteins (data not shown).

Water loss led to a modification in the degree of phosphorylation of JNK in *A. erici*, which showed different behavior in control than in 1-butanol-treated cells. As shown in Figure 7A, there was a double-peak of phosphorylation in control cells 1 and 3 h after the onset of desiccation, however, no activation was found in the presence of 1-butanol. The maximum peak of phosphorylation of the JNK in control cells at 3 h coincided with a water loss in the cells in the range of >60 %; from this point onwards (water loss of 75-95 %), JNK phosphorylation decreased to initial levels. These data may indicate that acclimation to desiccation occurred in a narrow humidity range; cells of *A. erici* were not stressed when the water level was still in the range of 50-60 %. Only when the water level decreased to 50 % of the initial hydration, new JNK phosphorylation was observed; F_v/F_m showed higher values at low humidity when JNK was not inhibited during acclimation (control cultures in the absence of 1-butanol; Fig. 5A). However, extreme desiccation (water loss > 80 %) nearly completely inactivated photosynthesis and basic metabolism, including a return of the phosphorylation of JNK to its basal level found before desiccation. A very different behavior was found in the response of p38 to desiccation. As shown in Figure 7B, p38 MAPK was rapidly phosphorylated both in control and in 1-butanol-treated cells. In all cases, p38 phosphorylation peaked one hour after the initiation of desiccation (water loss of > 20 %), and remained high for the whole duration of the experiments (water loss up to 95 %). As expected, ERK phosphorylation also varied during desiccation of *A. erici* both in control and in 1-butanol-treated cells (Fig. 7C). ERK activity dropped during the first two hours. However, while it recovered in control cells after 3 h to values even higher than initial ones, it remained low in the presence of 1-butanol. This peak of ERK phosphorylation in control cells coincided with the maximum phosphorylation state of the JNK.

Hypersalinity represents an osmotic stress that induced modifications of the phosphorylation state of JNK, p38 and ERK. JNK-like MAPK showed variations during the submission to salt stress, and between treatments (with and without 1-butanol). JNK was rapidly and transiently phosphorylated in the absence of 1-butanol (Fig. 8A). Maximal phosphorylation occurred 2 h after the immersion in

2M NaCl. After the maximal phosphorylation detected at 2 h, phosphorylation decreased to values similar to initial ones after 4 h, remaining stable for 24 h. However, a certain increase of JNK phosphorylation also occurred in the presence of 1-butanol during the first 2 h after saline treatment (Fig. 8A); a partial decrease at 3 h was followed by another rise to the end of the experiments. Rapid phosphorylation of the p38-like MAPK occurred right after 2 M NaCl exposure (Fig. 8B), reaching the maximum peak after 1 h in control cultures and after 4 h in the presence of 1-butanol. The high level of phosphorylation lasted for the whole experiment in both cases. Then, salt stress induces the activation of the p38 cascade, but 1-butanol seems not to inhibit it. Evolution of ERK phosphorylation was also studied during acclimation to 2 M NaCl. As seen in Figure 8C, a partial and transient deactivation of ERK occurred during the first hours, however a phosphorylation level similar to the initial one was detected after 24 h. In contrast, in the presence of 1-butanol ERK was nearly completely dephosphorylated after hyperosmotic shock, not even recovering similar levels of phosphorylation as in control cells after 24 h. It is known that ERK is not directly involved in acclimation to stress, but that its phosphorylation is required for cell division. A temporary deactivation of cell division occurred after osmotic stress, which was more pronounced in the presence of 1-butanol. When cells were acclimated to the new conditions, ERK recovered its phosphorylation and presumably *A. erici* underwent normal cell division. Thus, the phosphorylation pattern of ERK in 1-butanol as compared to control cultures showed three main differences after exposure to high salinity: (i) the drop in the phosphorylation state was more pronounced in 1-butanol-treated cells; (ii) The low levels of phosphorylation lasted for longer period; (iii) Recovery in the presence of 1-butanol was not completed after 24 h. From these data it can be concluded that both JNK and p38 behaved differentially in relation to hypersalinity. JNK showed a transient activation in control cells, while p38 remained activated during the whole length of the hyperosmotic treatment. 1-butanol partially inhibited the response of JNK, however its effect on p38 was nearly negligible.

These data, together with those of F_v/F_m (Fig.6) measurements in the presence of specific phosphorylation inhibitors of either JNK or p38 may indicate that initial JNK phosphorylation seems to be crucial for keeping photosynthetic activity as well as cell division after hyperosmotic shock in *A. erici*.

Phosphoproteome analysis

To investigate the targets of the kinase pathways total proteins and phosphoproteins were analyzed from hydrated and dried *A. erici* samples. The two dimensional separation (2D PAGE) of total proteins did not display any visual change in abundance or in phosphorylation patterns (Figure S1). However, the 2D PAGE analysis of the phosphoproteome showed that dehydration induced the phosphorylation of some proteins in *A. erici* (Fig. 9). Two dimensional gels were repeated three times

with phosphoproteins isolated from independent experiments. Strong signals for eight phosphoproteins were detected after desiccation; all of them were acidic proteins with isoelectric point (pI) ranging between 3 and 7, while no phosphorylation was observed for basic proteins (pI 7-10, Figure S2).

To identify the corresponding phosphoproteins, spots were excised from the gels and subjected to in-gel tryptic digestions. The identification of the resulting peptides by MALDI-TOF/TOF revealed that proteins involved in cellular processes were phosphorylated during desiccation (Table 1). These proteins include the translation elongation factor EFTs/EF1B, alpha-tubulin, ubiquitin-specific peptidase 41, malate dehydrogenase NAD-dependent, GrpE nucleotide exchange factor, 60S ribosomal protein 10L, 20S proteasome beta subunit. Only the protein of spot 8 was not identified.

A principle component analysis (PCA) was performed with the amounts of the same three phosphoprotein replicas obtained from the 2D PAGE analysis. Protein amounts were calculated from the peptide peak areas from the MS/MS chromatograms. The PCA analysis did not show a clear grouping (Figure 10). Four proteins changed significantly in abundance, two were up-regulated and two others were down-regulated in response to dehydration (Table 2). The synthesis Thi4 protein and an unknown protein increased about two fold during desiccation, while the heat shock protein DnaJ and the WD40 protein decreased eight and four fold, respectively. All of them were among the less abundant phosphoproteins (data not shown) and for this reason their change in abundance during desiccation was not observed in 2D gels. None of the eight proteins excised from gels and subsequently identified (Table 1) were among the differentially expressed proteins. Therefore, the stronger signal in the desiccation Pro-Q Diamond stained gels can be attributed to a higher degree of phosphorylation and not to changes in abundance.

Discussion

Lipid analysis demonstrates that desiccation-tolerance is based on constitutive and induced mechanisms

Membranes belong to the first targets of degradation during dehydration. Protection of membrane integrity is essential to maintain metabolic homeostasis (Sahsah et al., 1998). In this way, the analysis of the lipid membrane composition revealed that lipids play an important role in the acquisition of desiccation tolerance in lichen phycobionts as they participate in both constitutive and induced protection mechanisms.

In untreated *A. erici* cells the basal levels of the chloroplast lipids TGDG and TeGDG were surprisingly high, they represented the 1.75 % and 0.14 % of total polar lipids, respectively (Figure 1). Usually, oligogalactolipids are barely detectable in plants growing under optimal conditions and less

than 0.1-0.2 % can only be found in plants exposed to freezing or drought stress (Gasulla et al., 2013b; Moellering et al., 2010). It has been proposed that the accumulation of oligogalactolipids protects chloroplast membranes during stress, because their large polar head-groups lead to an increase in the distance and in repulsive hydration forces between adjacent membranes avoiding membrane fusion (Moellering et al., 2010). Likewise, the constitutive level of the extraplastidial phospholipid PI was also unusually high in *A. erici*, about 14 % of polar glycerolipids, while generally its proportion in vascular plants is lower than 5 % (Gasulla et al., 2013b). The accumulation of PI may be correlated with the acquisition of desiccation-tolerance in resurrection plants (Gasulla et al., 2013b). PI possesses a sugar alcohol head group and therefore, in analogy with the chloroplast galactolipids, its accumulation might enhance the repulsive hydration force between adjacent bilayers to avoid the fusion of extraplastidial membranes during dehydration. The hydroxyl groups of membrane lipids (galactolipids, PI) could establish hydrogen bonds with macromolecules acting as "water replacement" to avoid their precipitation in the tightly packed cytoplasm of a desiccated cell (Cuming, 1999; Hoekstra et al., 2001). In conclusion, the high constitutive levels of glycolipids and of PI seems to be related with the permanent protection of membranes against desiccation-induced injuries.

Besides these constitutive lipid-based protective mechanisms, dehydration triggered modifications in the lipid membrane composition to improve the stabilization of membranes in dried cells. One of these changes was the decrease in the MGDG:DGDG ratio, from 1.62 to 0.94, mainly caused by the degradation of MGDG and to a minor extent by the synthesis of DGDG (Figure 1). This is the first time that such a response is reported in a poikilohydric plant, while the decrease of the MGDG:DGDG ratio in osmotic stressed vascular plants has been widely documented (Monteiro de Paula et al., 1993; Gigon et al., 2004; Moellering et al., 2010; Gasulla et al., 2013b). MGDG molecules are cone-shaped due to the small size of the head group and tend to form inverted hexagonal II (H_{II}) structures that facilitate membrane fusions or fissions, whereas DGDG and oligogalactolipids are cylindrical-shaped and form lamellar bilayers (Cullis et al., 1985; Sprague, 1987; Webb and Green, 1991). Thus, the conversion of conic MGDG into cylindrical DGDG and oligogalactolipids is a common plant strategy to stabilize thylakoid membranes during osmotic stress. In parallel, the PI to PE ratio was increased from 2.94 in fresh to 4.60 in dried *A. erici* cells, mainly due to the degradation of PE. The increase in the ratio PI:PE in extraplastidial membranes has also been related with the adaptation to drought stress in vascular plants (Gasulla et al., 2013b). PE is the only cone-shaped phospholipid in extraplastidial membranes (Cullis et al., 1985). In this regard, the degradation of PE is analogous to the decrease of MGDG in the chloroplast since the removal of a non-billayer-forming lipid contributes to the stabilization of extraplastidial membranes during desiccation.

These results demonstrate that in desiccation-tolerant poikilohydric plants, besides constitutive protection mechanisms, an active physiological response exists to facilitate the transition to the anhydrobiosis state, but this raises the question which cellular pathways control these responses? The

modification of the membrane lipid composition observed in *A. erici* cannot be attributed to the control of transcription and/or translation since lipid enzymes do not accumulate during desiccation (Gasulla et al., 2013), and therefore the regulation should be located on post-translational level. Consistent with this hypothesis is that the *Arabidopsis* SFR2 enzyme, which converts MGDG into DGDG and oligogalactolipids, is constitutively present but remains inactive during normal growth until it is activated by osmotic stress (Thorbly et al., 2004).

PLD is involved in the cellular response and adaptation to hyperosmotic stress

One mechanism potentially involved in adaptation to water deficit is the accumulation of PA in *A. erici* during the response to desiccation. PA can be generated via the PLC/DGK and the PLD pathways (Munnik, 2001). Phospholipase C (PLC) hydrolyses phospholipids to yield phosphorylated head groups and diacylglycerol that is rapidly phosphorylated by the diacylglycerol kinase (DGK) to PA. PLD cleaves structural phospholipids releasing PA and a free head group. It is possible to distinguish between the two pathways by the ability of PLD to transphosphatidylate primary alcohols such as ethanol or 1-butanol to form phosphatidylethanol or phosphatidylbutanol (Munnik, 2001; Munnik et al., 1998). Employing this strategy, it was shown that activation of phospholipase D represents the major biosynthesis biosynthetic route for PA during osmotic stress is due to the activation of PLD (Frank et al., 2000; Munnik et al., 2000; Katagiri et al., 2001). Another important trait of the PLD pathway is that it can be activated within minutes upon the application of salt or water stress (Munnik and Meijer, 2001). Studies carried out with genetic transformed plants have linked PLD-derived PA accumulation to the adaptation of plants to high salinity and hyperosmotic stress (see a review in Hong et al., 2009). These observations led us to hypothesize that the PLD pathway is involved in the regulation of rapid cellular responses of lichen phycobionts to dehydration stress. As mentioned above, 1-butanol competes with water during the PLD reaction and therefore, the presence of non-limiting concentrations of 1-butanol favors the synthesis of phosphatidyl-butanol instead of PA. Thus, *A. erici* samples were incubated with 0.5 % (v/v) 1-butanol before desiccation and saline treatments. Blocking the PA production had a slight but significant negative effect on the capacity of recovery of *A. erici* after desiccation. In non-butanol treated samples, normal photosynthesis activity was recovered after two hours of rehydration, while in 1-butanol treated samples the maximum photosynthetic yield (F_v/F_m) remained about 10 % lower. As expected, PLD had a minor role in the survival of *A. erici* during desiccation since desiccation tolerance ultimately relies on constitutive mechanisms, but activation of the PLD pathway improves the fitness of phycobionts and allows to recover the metabolic activity quickly upon rehydration. Considering that under natural conditions phycobionts have to feed the myocobiont -which represents 90-95 % of the thallus biomass- and that the periods of photosynthesis activity are very short -from minutes to a few hours-, a reduction of 10

% in photosynthesis can be critical for the lichen survival. The submission of *A. erici* to a more severe hyperosmotic stress confirmed the important role of PLD in the cellular response. PLD inhibition markedly impaired the adaptation of *A. erici* to salt stress. In control samples the incubation in a 2 M NaCl solution caused an immediate decrease of 18 % in F_v/F_m, while the preincubation with 1-butanol resulted in a drop of 62 %. Despite of the damage, there was a partial recovery of photosynthesis activity in both control and butanol-treated samples during the first 4 hours. Surprisingly, after 24 hours of salt stress F_v/F_m declined in control samples, whereas butanol-treated samples continued to recover, which might be attributed either to the activation of PLD-independent mechanisms or to the PA-PLD synthesis due to the dissipation of the 1-butanol in the salt solution. In conclusion, the synthesis of PA by PLD is involved in adaptation to osmotic stress in lichen phycobionts like in vascular plants .

MAPK cascades are activated in response to hyperosmotic stress

Several roles have been proposed for PLD in the modulation of plant functions that allow their adaptation to biotic or abiotic stress, including vesicular transport, membrane degradation and intracellular signaling (Bargmann et al., 2009). Among these functions, PA has the capacity to activate MAPK cascades (Liscovithc et al., 2000; Zhang et al., 2003). The activation of MAPKs requires the double phosphorylation of their threonine and tyrosine residues, which is achieved through a sequential phosphorylation cascade by MAPKK kinase (MAPKKK) and MAPK kinase (MAPKK). Several studies have shown that PA binds to proteins kinases like Raf-1, protein kinase C or PDK1, which trigger MAPK phosphorylation cascades (Ghosh et al., 1996; Zhang et al., 2003; Anthony et al., 2004; Rentel et al., 2004).

We explored the possibility that PLD may be involved in the posttranslational control of osmotic-stress induced phycobiont responses through the activation of MAPK cascades. Desiccation of *A. erici* triggered the phosphorylation of both JNK-like and p38-like MAPKs, while JNK phosphorylation was transitory, p38 remained highly phosphorylated during the whole desiccation process (24 h) in control cells. The phosphorylation of JNK and p38-like proteins in response to dehydration has also been recently documented by Parages et al., (2014a) in intertidal macroalgae corresponding with emersion. The submission of *A. erici* to hypersaline stress also caused the transient phosphorylation of JNK- and p38-like proteins. The activation of JNK-like proteins in response to both hyperosmotic stress was less pronounced in the presence of 1-butanol, which indicates a direct relation between PLD and MAPKs. The participation of both JNK and p38 signaling pathways (and in extension of PLD) influenced the response of ERK-like proteins. Some authors have shown a parallel response of both JNK and ERK in mammalian cells, i.e., both MAPKs follow the same pattern of phosphorylation under stress or in apoptosis (i.e. Xu et al., 2006); however, other authors have

confirmed that these two MAPKs may follow reversed patterns, and ERK is dephosphorylated when JNK is phosphorylated (i.e. Chuang et al., 2000). In microalgae we have shown that ERK is dephosphorylated under stress, and only after JNK phosphorylation triggers the acclimation to the new environmental conditions, a recovery of ERK phosphorylation occurs (Jiménez et al., 2007; García-Gómez et al., 2012; Jiménez, C., personal observations). The reason for this behaviour lies in the involvement of ERK in cell division control; Jiménez et al., (2007) demonstrated that when ERK dephosphorylation was induced with the specific inhibitor PD98059 in the microalga *Dunaliella viridis*, the cell cycle was arrested. As expected, incubation in 2 M NaCl or under desiccation triggered the de-phosphorylation of ERK proteins. ERK was less phosphorylated in the presence of 1-butanol, and did not recover to initial levels even after 24 h, probably due to the lack of activation of JNK during the first hours after of stress. It is also interesting to mention that ERK was never fully de-phosphorylated, even after 95 % water loss. Then, some cell division may still be expected under those conditions. Previous work has demonstrated that even though photobionts divide more rapidly in wet periods, some cell division still occurs in dry lichens (Greenhalgh and Anglesea, 1979; Palmqvist, 2000; Schofield et al., 2003)

Another tool that helps to validate the participation of MAPK-dependent phosphorylation in the cellular adaptation of *A. erici* to stress is the use of specific inhibitors of MAPKs. The inhibition of p38 and JNK activities using SB203580 and SP600125, respectively, markedly impaired the adaptation of *Dunaliella viridis* to hyperosmotic stress (Jiménez et al., 2004). Likewise, Parages (2012) demonstrated that blocking the JNK-like pathway in stressed intertidal macrophytes leads to algal death. Here the inhibition of the JNK pathway had a slight negative effect on the recovery of photosynthesis activity upon desiccation. The result of JNK and p38 inhibition was more evident when *A. erici* was submitted to a salinity stress. The incubation with SB203580 and SP600125 impaired the recovery of photosynthesis after 4 h of 2 M NaCl exposure, with the damage being higher in SP600125 treated samples after 24 h. In both inhibition experiments, cellular injuries caused by hyperosmotic stresses were lower than in butanol-treated samples, which indicates that PLD might be upstream of MAPKs in the signaling. PLD has several modes of action and MAPK cascades (especially p38) can also be activated by other PLD-independent effectors in response to stress (Zhu 2002; Testerink and Munnik 2005), which might explain the recovery of 1-butanol treated samples as well as the higher damage suffered by PLD-inhibited than by MAPK-inhibited *A. erici* samples.

These results indicate that lichen phycobionts possess MAPK-like signaling components that sense and respond to osmotic stress, permitting cell acclimation and survival under changing environmental conditions. MAPK cascades seem to be controlled upstream of PLD through the synthesis of PA, although other molecular pathways might additionally be involved in the cellular response.

Several proteins are phosphorylated during desiccation

Finally, we tried to identify the targets of the MAPKs through the analysis of the phosphoproteome. The phosphoproteome pattern did not change, only the intensity of eight spots increased in response to desiccation. A similar phosphoproteome analysis in the resurrection plant *Craterostigma plantagineum* showed that 34 phosphoprotein spots changed during a de-/rehydration cycle (Röhrig et al., 2008). The difference in the number of phosphoproteins that change during stress correlates with the two strategies that the plants employ to cope with desiccation. While in vascular plants the slow drying rate (days) allows to induce stress mechanisms, poikilohydric plants need constitutive mechanisms to cope with rapid drying rates (hours). Despite of the small number of phosphorylated proteins, for the first time it is demonstrated that post-translational modifications are also part of the response network induced in poikilohydric plants when exposed to osmotic stress.

Two of them were enzymes involved in protein synthesis, the elongation factor EF1B, whose phosphorylation could have important biological consequences for the regulation of translation (Janssen et al, 1998), and RPL10, a structural protein of the 60S ribosome that organizes the architecture of the aminoacyl-tRNA binding and contains two PKC phosphorylation sites (Oh et al., 2002). The phosphorylation of these two proteins may be related with the early stop of the protein translation in poikylohydric plants during desiccation (Bewley et al., 1979). Two of the phosphorylated proteins were enzymes with a role in the degradation of proteins, the proteasome 20S beta-subunit protein that forms part of the 26S proteasome (Ciechanover, 1998 Coffino, 2001), and the ubiquitin specific peptidase 41 like protein (USP41) (Jentsch et al., 1991). Ubiquitination of targeted proteins and recruitment into proteosomes is a strategy of resurrection plants to remove damaged proteins from the cell in order to maintain cellular function (O'Mahony and Oliver 1999; Chen and Wood 2003).

Proteins with an important role in cellular protection were also phosphorylated in *A. erici* in response to desiccation. Among these proteins was the NAD-dependent malate dehydrogenase, which might be related with the accumulation of organic acids and the improvement of drought tolerance (El-Tohamy et al., 2013; Levi et al., 2011; Sun and Hong, 2011). The α-tubulin was another protein phosphorylated in *A. erici* during desiccation. α-tubulin phosphorylation also occurs in *A. thaliana* and rice in response to hyperosmotic stress causing the disassembly of microtubules (Ban et al., 2013), in which PA PLD-mediated has been directly related (Komis et al., 2006; Zhang et al., 2012). Thus, the drying-induced phosphorylation of α-tubulin, together with the accumulation of β-tubulin (Gasulla et al., 2013a), indicates that cytoskeleton remodeling plays a key role in desiccation-tolerance in lichen phycobionts, remodeling that might be regulated by PLD. Finally, the GrpE nucleotide exchange factor was also phosphorylated. GrpE is a cochaperone that accelerates the release of ADP from DnaK

(a chaperon protein) and the new incorporation of ATP (Zylicz et al. 1987; Mally and Witt, 2001). The phosphorylation of the GrpE protein in *A. erici* suggests that besides the accumulation of chaperones during desiccation (Gasulla et al., 2013a) the regulation of their activity is crucial for cell protection. These results show that poikilohydric organism can activate protection mechanisms through rapid post-transcription modifications without the necessity of protein synthesis.

Conclusions

Classically, lichen phycobionts are described as poikilohydric organisms able to undergo desiccation owing to the constitutive presence of molecular protection mechanisms. Here, we demonstrate that there is also a cellular response to hyperosmotic stress that involves the activation of signaling cascades and the induction of protection mechanisms similar to those employed by homeohydric plants. One clear example of this duality is found in the membrane lipids of *A. erici*. Besides a high constitutive content of polar glycolipids that prevents the fusion of adjacent membranes, drying triggers the degradation of cone-shaped lipids to stabilize membranes. Several lines of evidence indicate that the PLD pathway is involved in the control of rapid cellular responses to hyperosmotic stress in *A. erici*: PA accumulates during desiccation; a PLD-inhibition impairs recovery upon hyperosmotic stress; PLD activity is related with the phosphorylation of JNK and ERK MAPK-like proteins. Thus, we suggest that PA PLD-derived acts as a second messenger in the cellular signaling cascades activating MAPKs pathways. Accordingly, several proteins are phosphorylated in *A. erici* during desiccation, mostly enzymes involved in the synthesis and degradation of proteins, but also in gluconeogenesis, cytoskeleton and protein stability. These novel results offer new hypotheses concerning the mechanisms and processes that poikilohydric plants employ to adapt to aeroterrestrial environments where water availability fluctuates within short time intervals.

Material and methods

Biological material

An axenic strain of the lichen photobiont *Asterochloris erici* (Ahmadjian) Skaloud *et* Peksa (SAG 32.85 = UTEX 911; collection of algae, University of Texas at Austin, TX, USA) was used for our study. Stock cultures of the alga were maintained in 10 ml tubes with 3xN Bold's Basal Medium (3NBBM) supplemented with 10 g casein and 20 g glucose per liter (Trebouxia Medium, TM; Ahmadjian, 1973) at 20 °C under a 12 h photoperiod with 30 μmol m^{-2} s^{-1} white-light illumination. Two week-old cultures were stirred, then 1 ml of the cell suspension was taken and the cell number was quantified using a haemocytometer. The final cell density was adjusted with sterilized medium to

10^6 cells/ml. Inoculation proceeded by applying 50 µl of this suspension on sterile cellulose-acetate discs then placed on agar TM in Petri plates (Goldsmith et al., 1997). All experiments were carried out 21 days after inoculation.

Desiccation treatments

For desiccation experiments the discs supporting the algae culture were picked from the agar plates, transferred to an open empty Petri plate and placed into the growth chamber with a relative humidity of 50 %. Under this regime, samples dried and reached a stable weight within 4-5 h.

Extraction and quantification of lipids

Lipid extraction for mass spectrometry was done according to Roughan et al., (1978). One algal disc (approx. 100 mg) was transferred into a glass tube and incubated in boiling water for 20 min. The water was removed, then a first extraction was done with 1 ml $CHCl_3$/ MeOH (1:2) and the organic phase collected. The lipid extraction was repeated with 1 ml $CHCl_3$/ MeOH (2:1) and the organic phases were combined. One volume of $CHCl_3$ and 0.75 volumes of aqueous 300 mM ammonium acetate were added to the combined chloroform extracts. Samples were vortexed and centrifuged (2000 g, 5 min). The organic phase was collected and lipids were stored at -20 °C. The remaining biomass was dried at 80 °C and weighed to determine the dry weight (DW). The solvent of the lipid extract was evaporated under a stream of N_2. Total lipids were dissolved in 1 ml of $CHCl_3$:MeOH (2:1), divided into two 500 µl aliquots and concentrated again by evaporation.

For phospholipid and galactolipid quantification by Q-TOF MS/MS measurements see Gasulla et al., (2013b) and Welti et al., (2002). Triacylglycerol and diacylglycerol were quantified by Q-TOF MS/MS according to Lippold et al., (2012) and vom Dorp et al., (2013). Oligonucleotides were measured by Q-TOF MS/MS as described in Gasulla et al., (2013b) and Moreau et al., (2008).

Inhibitor treatments

Algal discs were immersed in TM plus 0.5 % 1-butanol, 100 µM SB203580 or 100 µM SP600125 (Calbiochem, La Jolla, CA) dissolved in DMSO, at these concentrations these molecules are very selective inhibitors of the PLD (Munnik et al., 1995), p38 (Capasso et al., 2001) and JNK (Bennet et al., 2001) pathways, respectively. After two hours, algal discs were recovered and left for a few seconds on a filter paper to remove the excess of solution. Then, samples were either subjected to a desiccation/rehydration cycle or to a hypersaline stress (immersion in a 2 M NaCl solution).

Chlorophyll-fluorescence measurements

Chlorophyll *a* fluorescence was measured *in vivo* with a modulated light fluorometer (Dual-PAM, Walz, Effeltrich, Germany). The discs were placed on a microscope slide wrapped with moist filter paper to maintain a hydrated state. All the samples were kept in the dark for 15 min just before fluorescence measurements were taken. The minimum (dark) fluorescence yield (F_o) was obtained after excitation of the algae with a weak measuring beam from a light emitting diode. The maximum fluorescence yield (F_m) was determined with an 800 ms saturating pulse of white light (SP, 8,000 µmol m^{-2} s^{-1}). Maximal variable fluorescence (F_v) was calculated as F_m - F_o. The parameter F_v/F_m represents the maximum quantum efficiency of photosystem II chemistry when all the reaction centres are open after a period of dark adaptation (Baker and Oxborough, 2004).

MAPKs extraction and immunodetection

Fifty mg of *A. erici* cultures grown on cellulose-acetate discs on TM agar were transferred to 2 ml conical tubes and were deeply frozen in liquid N_2. 100 µl of 10 % SDS were added on frozen biomass grounded using a Retsch MM400 mixer mill (Retsch, Haan, Germany). Samples were placed in a pre-cooled sonicating water bath (Branson 2510, Branson Ultrasonic Corporation, Danbury, CT, USA) for 5 min, and subsequently samples were gently mixed with 400 µl of MAPK lysis buffer (50 mM β-glycerophosphate pH 7.2, 0.1 mM sodium vanadate, 2 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 2 µg ml^{-1} leupeptin, 4 µg ml^{-1} aprotinin). A second sonicating water bath step was applied for 5 min, and finally cell debris was removed by centrifugation (4 ºC, 30 min, 15000 x g). The supernatant was employed for protein quantification (BCA- bicinchoninic acid method), PAGE and western blotting.

Gel electrophoresis, immunoblotting and detection of phosphorylated MAPKs were performed according to methods described by Parages et al., (2012). Equal amounts of protein extracts (50 µg) were loaded in each lane. Western blots were analysed using Amersham ECL Advance (GE Healthcare, Buckinghamshire, UK) as chemoluminiscence agent and visualised on a Kodak Gel Logic 1500 Imaging System (Eastman Kodak Company, Rochester, NY, USA). Antibodies against the phosphorylated forms of p38, JNK and ERK MAPKs, as well as their specific blocking peptides, were purchased from Cell Signaling Technology (Beverly, MA, USA) and used according to Parages et al., (2012) and to manufacturer's recommendations. Band intensity analyses were carried out using ImageJ 1.440 software (National Institute of Health, USA).

All experiments were repeated two to three times with similar results.

Phosphoproteome analysis

Total proteins were isolated using a phenol-based procedure based on the technique described by Wang et al., (2003). Phosphoproteins were isolated following the modified metal oxide affinity chromatography procedure (Röhrig et al., 2008), separated by two-dimensional polyacrylamide mini-gel electrophoresis and detected by phosphoprotein-specific staining Pro-Q Diamond (Invitrogen), which detects phosphate groups attached to Ser, Thr and Tyr residues. Proteins stained with fluorescent dyes were visualized using a Typhoon 9200 scanner (GE Healthcare). To identify putative phosphoproteins, spots were picked from the 2-D gels, the proteins digested in gel with trypsin and analyzed by MALDI-TOF/TOF (Method S1). Collected mass spectral data were identified with the aid of databases of cDNA clones from *Asterochloris* sp. (isolated from *Cladonia grayi*, JGI Genome Portal).

In parallel, enriched phosphoprotein samples were digested with sequencing grade trypsin (Promega) and analyzed by liquid chromatography and tandem mass spectrometry (LC–MS/MS). The Paragon algorithm of ProteinPilot was used to search (user private; 102203 sequences) database. The PeakView v 1.1 (ABsciex) software was used to generate the peptides areas from Protein Pilot result files (Method S2). The areas of every assigned peptide were analyzed by Marker View 1.1. (ABsciex). The data were normalized by 'median peak ratios' with C1 as reference. Then, a PCA analysis was done with the following parameters: weighting, none; scaling, pareto.

Supplemental Material

The following supplemental materials are available.

Figure S1. Changes in the proteome of *A. erici* in response to desiccation.

Figure S2. Changes in the phosphoproteome of *A. erici* in response to desiccation (pH 3-10).

Method S1. Protein identification

Method S2. ProteinPilot v4.5. search engine (ABSciex).

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List of author contributions

F.G. conceived and designed the research, performed most of the research, analyzed the data and wrote the article; E.B. designed the research and wrote the article; C.J. designed the research (MAPKs experiments), analyzed the data and wrote the article; M.L.P. designed and performed immunoblotting assays; J.C. performed immunoblotting assays; P.D. performed lipid analysis, analyzed the data and wrote the article; D.B. designed the research, analyzed the data and wrote the article.

References

Ahmadjian V (1973) Methods of isolation and culturing lichen symbionts and thalli. *In* Ahmadjian V, Hale ME, eds, The lichens. Academic Press, New York, pp 653–660

Alpert P, Oliver MJ (2002) Drying without dying. *In* Black M, Pritchard HW, eds, Desiccation and survival in plants: drying without dying. CABI Publishing, Wallingford, Oxon, pp 3−43

Anthony RG, Henriques R, Helfer A, Meszaros T, Rios G, Testerink C, Munnik T, Deak M, Koncz C, Bogre L (2004) A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in *Arabidopsis*. EMBO Journal **23:** 572–581

Baker NR, Oxborough K (2004) Chlorophyll fluorescence as a probe of photosynthetic productivity. *In* Papageorgiou C, Govindjee, eds, Chlorophyll a fluorescence: a signature of photosynthesis. Adv photosynthesis and respiration, vol 19. Springer, The Netherlands, pp 65–82

Ban Y, Kobayashi Y, Hara T, Hamada T, Hashimoto T, Takeda S, Hattori T (2013) α-tubulin is rapidly phosphorylated in response to hyperosmotic stress in rice and Arabidopsis. Plant Cell Physiol **54:**848–858

Bargmann BO, Laxalt AM, ter Riet B, van Schooten B, Merquiol E, Testerink C, Haring MA, Bartels D, Munnik T (2009) Multiple PLDs required for high salinity and water deficit tolerance in plants. Plant Cell Physiol **50**: 78–89

Barták M, Gloser J, Hájek J (2005) Visualized photosynthetic characteristics of the lichen Xanthoria elegans related to daily courses of light, temperature and hydration: a field study from Galindez Island, maritime Antarctica. Lichenologist **37:** 433−443

Bartels D, Salamini F. (2001) Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at molecular level. Plant Physiol **127:** 13461−353

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA **98:** 13681–13686

Bewley JD (1979) Physiological aspects of dessication-tolerance. Annu Rev Plant Physiol **30:** 195–238

Candotto F (2014) Meccanismi di risposta di simbionti lichenici allo stress foto-ossidativo. PhD Thesis. Università Degli Studi Di Trieste, Trieste.

Capasso JM, Rivard CJ, Berl T (2001) The expression of the γ subunit of Na-K-ATPase is regulated by osmolality via C-terminal Jun kinase and phosphatidylinositol 3-kinasedependent mechanisms. Proc Natl Acad Sci USA **98:** 13414−13419

Chakir S, Jensen M (1999) How does *Lobaria pulmonaria* regulate photosystem 2 during progressive desiccation and osmotic water stress? A chlorophyll fluorescence study at room temperature and at 77 K. Physiol Plant **105:** 257–265

Chen X, Wood A (2003) The 26S proteasome of the resurrection plant *Tortula ruralis*: cloning and characterization of the TrRPT2 subunit. Biol Plantarum **46**: 363–368

Chuang SM, Wang IC, Yang JL (2000) Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium. Carcinogenesis **21:** 1423–1432

Ciechanover A (1998) The ubiquitin–proteasome pathway: on protein death and cell life. EMBO J **17:** 7151–7160

Coffino, P (2001) Regulation of cellular polyamines by antizyme. Nature Reviews Mol. Cell Biol **2:** 188–194

Cullis PR, Hope MJ, de Kruijff B, Verkleij AJ, Tilcock CPS (1985) Structural properties and functional roles of phospholipids in biological membranes. *In* Kuo JF, ed, Phospholipids and cellular regulation. CRC Press, Boca Raton, pp 1–59

Cuming AC (1999) LEA proteins. *In* Shewry PR, Casey R, eds, Seed Proteins. Kluwer Academic Publishers, Dordrecht, pp 753–780

El-Tohamy WA, El-Abagy HM, Badr MA, Gruda N (2013) Drought tolerance and water status of bean plants (*Phaseolus vulgaris* L.) as affected by citric acid application. J Appl Bot Food Qual **86**: 212 – 216

Fernández-Marín B, Becerril JM, García-Plazaola JI (2010) Unravelling the roles of desiccation-induced xanthophyll cycle activity in darkness: a case study in *Lobaria pulmonaria*. Planta **231**:1335-42

Frank W, Munnik T, Kerkmann K, Salamini F, Bartels D (2000) Water deficit triggers phospholipase D activity in the resurrection plant Craterostigma plantagineum. Plant Cell **12**: 111–123

García-Gómez C, Parages ML, Jiménez C, Palma A, Mata MT, Segovia M (2012) Cell survival after UV radiation stress in the unicellular chlorophyte *Dunaliella tertiolecta* is mediated by DNA repair and MAPK phosphorylation. J Exp Bot **63:** 5259–5274

Gasulla F, Gómez-de Nova P, Esteban-Carrasco A, Zapata J.M, Barreno E, Guéra A (2009) Dehydration rate and time of desiccation affect recovery of the lichen algae *Trebouxia erici*: alternative and classical protective mechanisms. Planta **231:** 195–208

Gasulla F, Jain R, Barreno E, Guéra A, Balbuena TS, Thelen JJ, Oliver MJ (2013a) The response of *Asterochloris erici* (Ahmadjian) Skaloud et Peksa to desiccation: a proteomic approach. Plant Cell Environ **36:** 1363–1378

Gasulla F, Vom Dorp K, Dombrink I, Zähringer U, Gisch N, Dörmann P, Bartels D. (2013b) The role of lipid metabolism in the acquisition of desiccation tolerance in *Craterostigma plantagineum*: a comparative approach. Plant J **75:** 726–741

Ghosh S, Strum JC, Sciorra VA, Daniel L, Bell RM (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-0-tetradecanoylphorbol-13-acetate-stimulated madin-darby canine kidney cells. J Biol Chem **271:** 8472–8480

Gigon A, Matos AR, Laffray D, Zuily-Fodil Y, Pham-Thi AT (2004) Effect of drought stress on lipid metabolism in the leaves of Arabidopsis thaliana (ecotype Columbia). Ann Bot **94**: 345–351

Goldsmith SJ, Thomas MA, Gries C (1997) A new technique for photobiont culturing and manipulation. Lichenologist **29:** 559–569

Greenhalgh GN, Anglesea D (1979). The distribution of algal cells in lichen thalli. Lichenologist **11**: 283 – 292.

Heber U, Soni V, Strasser RJ (2011) Photoprotection of reaction centers: thermal dissipation of absorbed light energy vs charge separation in lichens. Phys Plantarum **142:** 65–78

Hoekstra FA, Golovina EA, Buitink J (2001) Mechanisms of plant desiccation tolerance. Trends Plant Sci **6:** 431–438

Holzinger A, Kaplan F , Blaas K, Zechmann B , Komsic-Buchmann K , Becker B (2014) Transcriptomics of Desiccation Tolerance in the Streptophyte Green Alga *Klebsormidium* Reveal a Land Plant-Like Defense Reaction. PLoS ONE **9:** e110630

Hong Y, Devaiah SP, Bahn SC, Thamasandra BN, Li M, Welti R, Wang X (2009) Phospholipase Dε and phosphatidic acid enhance *Arabidopsis* nitrogen signaling and growth. Plant J **58:** 376–387

Jentsch S, Seufert W, Hauser HP (1991) Genetic analysis of the ubiquitin system Biochim Biophys Acta **1089:** 127–139

Jiménez C, Berl T, Rivard CJ, Edelstein C, Capasso JM (2004) Phosphorylation of MAP kinase-like proteins mediate the response of the halotolerant alga *Dunaliella viridis* to hypertonic shocks. Biochim Biophys Acta **1644:** 61–69

Jiménez C, Cossío BR, Rivard CJ, Berl T, Capasso JM (2007) Cell division in the unicellular microalga *Dunaliella viridis* depends on phosphorylation of extracellular signal-regulated kinases (ERKs). J Exp Bot **58:** 1001–1011

Junttila S, Laiho A, Gyenesei A, Rudd S (2013). Whole transcriptome characterization of the effects of dehydration and rehydration on *Cladonia rangiferina*, the grey reindeer lichen. *BMC Genomics* **14:** 870

Katagiri T, Takahashi S, Shinozaki K (2001) Involvement of a novel Arabidopsis phospholipase D, AtPLDδ, in dehydration-inducible accumulation of phosphatidic acid in stress signalling. Plant J **26:** 595–605

Kawamitsu Y, Driscoll T, Boyer JS (2000) Photosynthesis during desiccation in an intertidal alga and a land plant. Plant Cell Physiol **41:** 344–353

Komis G, Quader H, Galatis B, Apostolakos P (2006) Macrotubule-dependent protoplast volume regulation in plasmolysed root-tip cells of *Triticum turgidum*: involvement of phospholipase D. New Phytol **171:** 737–750

Kranner I, Cram WJ, Zorn M, Wornik S, Yoshimura I, Stabentheiner E, Pfeifhofer HW (2005) Antioxidants and photoprotection in a lichen as compared with its isolated symbiotic partners. Proc Natl Acad Sci USA **102**: 3141-3146.

Kranner I, Beckett R, Hochman A, Nash III TH (2008) Desiccation-tolerance in lichens: a review. Bryologist **111:** 576–593

Levi A, Paterson AH, Cakmak I, Saranga Y (2011) Metabolite and mineral analyses of cotton near-isogenic lines introgressed with QTLs for productivity and drought-related traits. Physiol Plantarum **141:** 265–275

Ligterink W (2000) MAP kinases in plant signal transduction: how many, and what for? Results Probl Cell Differ **27:** 11–27

Lippold F, vom Dorp K, Abraham M, Hölzl G, Wewer V, Lindberg Yilmaz J, Lager I, Montandon C, Besagni C, Kessler F, Stymne S, Dörmann P (2012). Fatty acid phytyl ester synthesis in chloroplasts of *Arabidopsis thaliana*. Plant Cell **24:** 2001–2014

Liscovitch M, Czarny M, Fiucci G, Tang X (2000) Phospholipase D: Molecular and cell biology of a novel gene family. Biochem J **345:** 401–415

Ma H (1993) Protein phosphorylation in plants: enzymes, substrates and regulators. Trends Genet **9:** 228–230

Mally A, Witt SN (2001) GrpE accelerates peptide binding and release from the high affinity state of DnaK. Nat Struct Biol **8:** 254–257

Margulis L, Barreno E (2003) Looking at lichens. Bioscience **53:** 776–778

Mishra NS, Tuteja R, Tuteja N (2006) Signaling through MAP kinase networks in plant. Arch Biochem Biophys **452:** 55–68

Moellering ER, Muthan B, Benning C (2010) Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. Science **330:** 226–228

Monteiro de Paula F, Pham Thi AT, Zuily Fodil Y, Ferrari-Iliou R, Vieira da Silva J, Mazliak P (1993) Effect of water stress on the biosynthesis and degradation of polyunsaturated lipid molecular species in leaves of *Vigna unguiculata*. Plant Physiol Biochem **31:** 707–715

Moreau RA, Doehlert DC, Welti R, Isaac G, Roth M, Tamura P, Nuñez A (2008) The identification of mono-, di-, tri-, and tetragalactosyl-diacylglycerols and their natural estolides in oat kernels. Lipids **43**: 533–548

Munnik T, Arisz SA, de Vrije T, Musgrave A (1995) G protein activation stimulates phospholipase D signaling in plants. Plant Cell **7:** 2197–2210

Munnik T, van Himbergen JAJ, ter Riet B, Braun FJ, Irvine RF, van den Ende H, Musgrave A (1998) Detailed analysis of the turnover of polyphosphoinositides and phosphatidic acid upon activation of phospholipase C and D in *Chlamydomonas* cells treated with non-permeabilizing concentrations of mastoparan. Planta **207:** 133–145

Munnik T, Meijer HJ, Ter Riet B, Hirt H, Frank W, Bartels D, Musgrave A (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. Plant J **22:** 147–154

Munnik T (2001) Phosphatidic acid: an emerging plant lipid second messenger. Trends Plant Sci **6:** 227–233

Munnik T, Meijer HJ (2001) Osmotic stress activates distinct lipid and MAPK signalling pathways in plants. FEBS Letters **498:** 172–178

O'Mahony P, Oliver MJ (1999) The involvement of ubiquitin in vegetative desiccation tolerance. Plant Mol Biol **41:** 657–667

Oh H, Mammucari C, Nenci A, Cabodi, S, Cohen SN, Dotto GP (2002) Negative regulation of cell growth and differentiation by TSG101 through association with p21(Cip1/WAF1). Proc Natl Acad Sci USA **99:** 5430–5435

Oliver MJ, Bewley JD (1997) Desiccation tolerance of plant tissues: a mechanistic overview. Horticult Rev **18:** 171–214

Oliver MJ, Dowd SE, Zaragoza J, Mauget SA, Payton PR (2004) The rehydration transcriptome of the desiccation-tolerant bryophyte *Tortula ruralis*: transcript classification and analysis. BMC Genomics **5:** 1–19

Oliver MJ, Hudgeons J, Dowd SE, Payton PR (2009) A combined subtractive suppression hybridization and expression profiling strategy to identify novel transcripts from *Tortula ruralis* gametophytes. Physiol Plantarum **136:** 437–460

Oliver MJ, Guo L, Alexander D, Ryals JA, Wone BWM, Cushman JC (2011) A sister group contrast delineates the biochemical regulation underlying desiccation tolerance in *Sporobolus stapfianus.* Plant Cell **23:** 1231–1248

Palmqvist K (2000) Carbon economy in lichens. New Phytol **148:** 11 – 36

Parages ML (2012) Role of Mitogen Activated Protein Kinases (MAPKs) cascades in environmental stress signal transduction in intertidal and Arctic macrophytes. PhD Thesis. University of Málaga.

Parages ML, Heinrich S, Wiencke C, Jiménez C (2013) Rapid phosphorylation of MAP kinase-like proteins in two species of Arctic kelps in response to temperature and UV radiation stress. Env Exp Bot **91:** 30–37

Parages ML, Capasso JM, Niell FX, Jiménez C (2014a). Environmental stress response by cyclic phosphorylation of MAP kinase-like proteins in intertidal macrophytes. J Plant Physiol **171:** 276–284

Parages ML, Figueroa FL, Conde-Alvarez RM, Jiménez C (2014b) Phosphorylation of MAPK-like proteins in three intertidal macroalgae under stress conditions. Aquat Biol **22:** 213–226

Rentel MC, Lecourieux D, Ouaked F, Usher SL, Petersen L, Okamoto H, Knight H, Peck SC, Grierson CS, Hirt H, Knight MR (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. Nature **427:** 858–861

Röhrig H, Colby T, Schmidt J, Harzen A, Facchinelli F, Bartels D (2008) Analysis of desiccation-induced candidate phosphoproteins from *Craterostigma plantagineum* isolated with a modified metal oxide affinity chromatography procedure. Proteomics **8:** 3548–3560

Roughan PG, Slack CR, Holland R (1978) Generation of phospholipid artefacts during extraction of developing soybean seeds with methanolic solvents. Lipids **13:** 497–503

Schofield SC, Campbell DA, Funk C, MacKenzie TDB (2003) Changes in macromolecular allocation in nondividing algal symbionts allow for photosynthetic acclimation in the lichen *Lobaria pulmonaria*. New Phytol **159:** 709–718

Sinha AK, Jaggi M, Raghuram B, Tuteja N (2011) Mitogen-activated protein kinase signaling in plants under abiotic stress. Plant Signal Behav **6:** 196-203

Sprague SG (1987) Structural and functional consequences of galactolipids on thylakoid membrane organization. J Bioenerg Biomemb **19:** 691–703

Sun YL, Hong SK (2011) Effects of citric acid as an important component of the responses to saline and alkaline stress in the halophyte *Leymus chinensis* (Trin.). Plant Growth Reg **64:** 129–139

Testerink C, Munnik T (2005) Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trends Plant Sci **10:** 368–375

Testerink C, Munnik T (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. J Exp Bot **62:** 2349–2361

Thorlby G, Fourrier N, Warren G (2004) The *SENSITIVE TO FREEZING2* gene required for freezing tolerance in *Arabidopsis thaliana* encodes a β-glucosidase. Plant Cell **16:** 2192–2203

Váczi P, Barták M (2006) Photosynthesis of lichen symbiotic alga *Trebouxia erici* as affected by irradiance and osmotic stress. Biol Plantarum **50:** 257–26

Veerman J, Vasil'ev S, Paton GD, Ramanauskas J, Bruce D (2007) Photoprotection in the lichen *Parmelia sulcata*: The origins of desiccation-induced fluorescence quenching. Plant Physiol **145:** 997–1005

vom Dorp K, Dombrink I, Dörmann P (2013) Quantification of diacylglycerol by mass spectrometry. *In* Plant Lipid Signaling Protocols, Methods in Molecular Biology, T Munnik and I Heilmann, eds, Humana Press, New York, pp 43–54

Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S, Cresti M (2003) Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electrophoresis **24:** 2369-2375

Webb MS, Green BR (1991) Biochemical and biophysical properties of thylakoid acyl lipids. Biochim Biophys Acta **1060:** 133–158

Welti R, Li WQ, Li MY, Sang YM, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, Wang XM (2002) Profiling membrane lipids in plant stress responses: role of phospholipase Da in freezing-induced lipid changes in *Arabidopsis*. J Biol Chem **277:** 31994–32002

Wood AJ, Oliver MJ (1999) Translational control in plant stress: the formation of messenger ribonucleoprotein particles (mRNPs) in response to dessication of *Tortula ruralis* gametophytes. Plant J **18:** 359–370

Xu C, Yuan X, Pan Z, Shen G, Kim JH, Yu S, Khor TO, Li W, Ma J, Kong ANT (2006) Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. Mol Cancer Ther **5:** 1918–1926

Zhang Q, Lin F, Mao T, Nie J, Yan M, Yuan M, Zhang W (2012) Phosphatidic acid regulates microtubule organization by interacting with MAP65-1 in response to salt stress in *Arabidopsis*. Plant Cell **24:** 4555–4576

Zhang W, Wang C, Qin C, Wood T, Olafsdottir G, Welti R, Wang X (2003) The oleate-stimulated phospholipase D, PLDd, and phosphatidic acid decrease H_2O_2-induced cell death in *Arabidopsis*. The Plant Cell **15:** 2285–2295

Zhu JK (2002) Salt and Drought Stress Signal Transduction in Plants. Annu Rev Plant Biol **53:** 247–273

Zylicz M, Ang D, Georgopolous C (1987) The grpE protein of *Escherichia coli*. J Biol Chem **262:** 17437–17442

Figure 1. Minor and major polar lipids composition. Polar lipids were measured in control (C, white bars) and desiccated (D, black bars) *A. erici* colonies by Q-TOF MS/MS using authentic internal standards, except TGDG and TeGDG which were measured relative to a DGDG standard. Values represent averages and SD of five biological replicates. *$P < 0.05$ or **$P < 0.01$ versus C, *t*-Student test. DGDG, digalactosyldiacylglicerol; MGDG, monogalactosyldiacylglycerol; PA, phospatidic acid; PC, phospatidylcoline; PE, phospatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol; TeGDG, tetragalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol.

Figure 2. **Molecular species composition of polar lipids.** Molecular species of polar lipids were measured in control (C, white bars) and desiccated (D, black bars) *A. erici* colonies by Q-TOF MS/MS using authentic standards, expect TGDG and TeGDG which were measured relative to a DGDG standard. *$P < 0.01$ versus C, *t*-Student test.

Figure 3. Non-polar lipid composition. Diacylglycerol (DAG, relative intensities mg^{-1} DW) and triacylglycerol (TAG, nmol mg^{-1} DW) were measured in control (C, white bars) and desiccated (D, black bars) *A. erici* colonies by Q-TOF MS/MS using internal standards. Values represent averages and SD of 5 biological replicates. *$P < 0.05$ or **$P < 0.01$ versus C, *t*-Student test.

Figure 4. Molecular species composition of non-polar lipids. Molecular species of diacylglycerol (DAG, relative intensities mg^{-1} DW) and triacylglycerol (TAG, nmol mg^{-1} DW) were measured in control (C, white bars) and desiccated (D, black bars) *A. erici* colonies. *$P < 0.05$ or **$P < 0.01$ versus C, *t*-Student test.

Figure 5. Photosynthesis response to desiccation and rehydration in the presence of inhibitors. *A. erici* algal discs were incubated in 3NBBM medium with 0.1 % DMSO, 100 μM SB203580 (p38 pathway inhibitor), 100 μM SP600125 (JNK pathway inhibitor) or 0.5 % 1-butanol (PLD inhibitor) for 2 h and then desiccated. A) The maximum photosynthesis yield (F_v/F_m) was measured and discs weighted to assess the relative water content (RWC) every hour during desiccation. B) After 24 h samples were rehydrated with water and F_v/F_m measured upon 1, 2, 3, 4 and 24 h of rehydration. Values represent averages and SE of 20 biological replicates. *$P < 0.05$ versus C, *t*-Student test. Dashed line, F_v/F_m value of non-treated *A. erici* (control).

Figure 6. Photosynthesis response to hyperosmotic stress in the presence of inhibitors. *A. erici* algal discs were incubated in 3NBBM with 0.1 % DMSO, 100 µM SB203580 (p38 pathway inhibitor), 100 µM SP600125 (JNK pathway inhibitor) or 0.5 % 1-butanol (PLD inhibitor) and then immersed in a 2M NaCl solution. The effect on the maximum photosynthesis yield (F_v/F_m) was measured. Values represent averages and SE of 20 biological replicates. **P* < 0.05 or ***P* < 0.01 versus C, *t*-Student test. Dashed line, F_v/F_m value of non-treated *A. erici* (control).

Figure 7. Immunodetection of MAPKs phosphorylated forms during desiccation. Western blot analysis of phosphorylated forms of the MAPKs as detected by specific phospho-JNK (A), phospho-p38 (B) and phosphor-ERK (C) antibodies in *A. erici* during desiccation in the (control) and presence (+) of butanol. Each lane contains 50 µg protein extract. The graphs show band intensities after scanning versus time (h). Solid lines/closed circles, control; dashed lines/open circles, with butanol.

Figure 8. Immunodetection of MAPKs phosphorylated forms during salt stress. Western blot analysis of phosphorylated forms of the MAPKs as detected by specific phospho-JNK (A), phospho-p38 (B) and phosphor-ERK (C) antibodies in *A. erici* submitted to 2 M NaCl osmotic stress in the absence (control) and presence (+) of butanol. Each lane contains 50 µg protein extract. The graphs show band intensities after scanning versus time (h). Solid lines/closed circles, control; dashed lines/open circles, with butanol.

Figure 9. Changes in the phosphoproteome of *A. erici* in response to desiccation. Representative 2D gels of phosphoproteins isolated and enriched from fresh (A, B) and desiccated (C, D) *A. erici* samples. A and C, coomassie staining; B and D, Pro-Q Diamond staining. Arrows indicate proteins with a different phosphorylation degree subsequently identified by MALDI-TOF MS. Gels were repeated three times with proteins of independent experiments. 80 µg of proteins were loaded per gel.

Figure 10. PCA analysis derived from the MALDI-TOF MS/MS peak areas. A PCA analysis was performed with the relative peak area data obtained in the phosphoproteome MALDI-TOF MS analysis. Phosphoproteins were isolated from three independent fresh (C) and desiccated (D) *A. erici* samples.

Table 1. Protein identification of *A. erici* differentially phosphorylated proteins in response to desiccation.

Spot nº	Protein name	Accession number[a]	Score[b]	Matches peptides	% cov	MSMS peptides	Function
1	Translation elongation factor EFTs/EF1B, dimerisation	38791	327	6	4.63	4	Translation elongation activity
2	Alpha tubulin	18787	8965	251	69	31	Microtubule-based movement
3	Ubiquitin-specific peptidase 41	40788	250	7	20.6	3	Ubiquitin-dependent protein catabolism
4	Malate dehydrogenase, NAD-dependent	13168	285	6	22.7	5	Carbohydrate metabolism
5	GrpE nucleotide exchange factor	14016	454	47	76.5	16	Protein folding
6	60S ribosomal protein 10L	13893	896	24	24.3	8	Ribosome biogenesis and assembly translational
7	20S proteasome beta subunit	35556	68	1	12.3	1	Ubiquitin-dependent protein catabolism
8	n.d.						

Spot nº, number of spot in figure 8

[a] Accesion number against the JGI Asterochloris sp. Cgr/DA1pho v2.0 database

b Score = -log (1-Percent Confidence/100)

n.d., non-determined

Table 2. Protein identities differentially expressed in desiccated vs. fresh *A. erici* samples. p-value represents the significance level of t-test performed by the relative peak area obtained in the MALDI-TOF MS analysis. n = 3.

Protein name	Accesion number[a]	Fold change	p-value	Function
WD40 protein	29132	-3,82	0,007	Function unknown
Heat shock protein DnaJ	28577	-8,07	0,039	Heat shock protein binding
Thi4 protein	30369	2,02	0,040	Thiamin biosynthetic process
Predicted protein	40200	1,70	0,042	Function unknown

[a] Accesion number against the JGI Asterochloris sp. Cgr/DA1pho v2.0 database

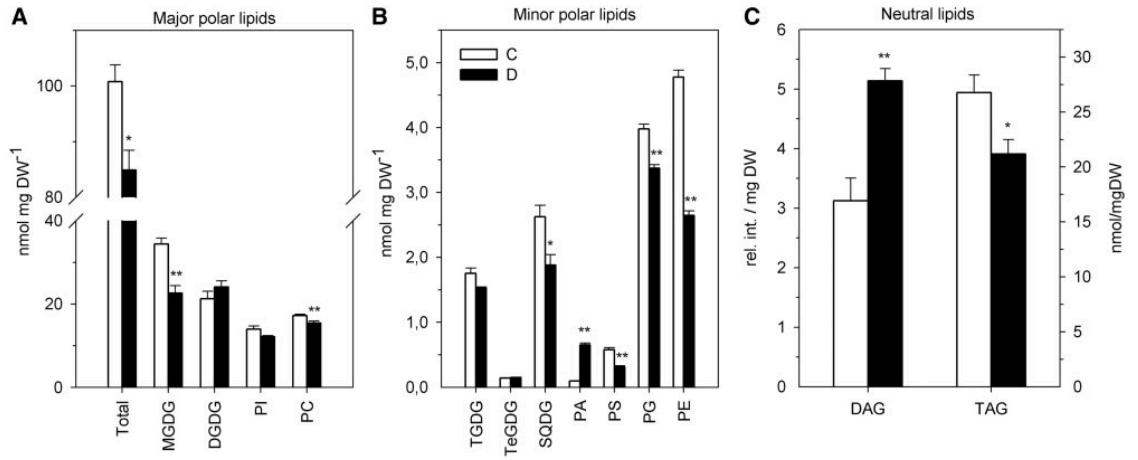

Figure. 1 Changes in lipid composition during desiccation. Polar (A, B) and neutral (C) lipids were measured in control (white bars) and desiccated (black bars) A. erici colonies by Q-TOF MS/MS. Values represent averages and the SE of five biological replicates. *P < 0.05 or **P < 0.01 vs. control, Student t-test. DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phospatidylcholine; PE, phospatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; TeGDG, tetragalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol.

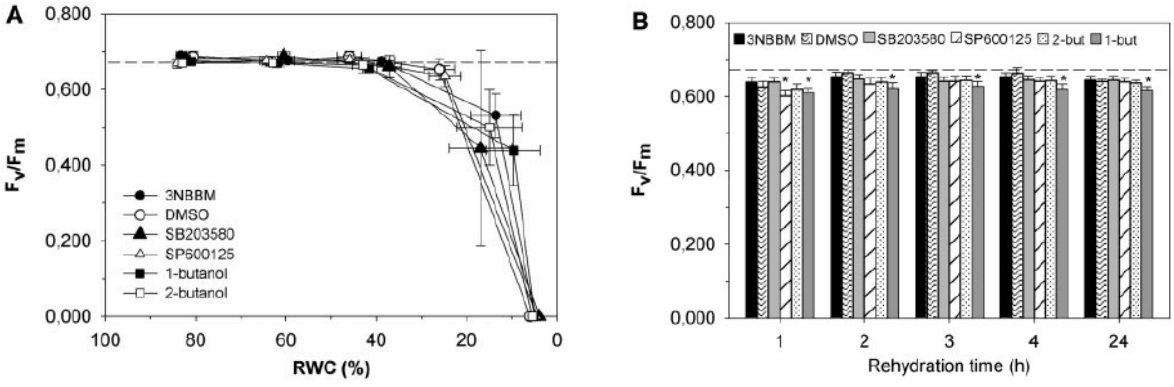

Figure. 2 Photosynthesis response to desiccation and rehydration in preinhibited samples. Asterochloris erici algal discs were incubated in 3NBBM medium with 0.1% DMSO, 100 mM SB203580 (p38 pathway inhibitor), 100 mM SP600125 (JNK pathway inhibitor), 0.5% 1-butanol (PLD inhibitor) or 0.5% 2-butanol (non-active isomer) for 2 h and then desiccated. (A) The maximum photosynthesis yield (F_v/F_m) was measured and discs weighted to assess the relative water content (RWC) every hour during desiccation. (B) After 24 h, samples were rehydrated with water and F_v/F_m was measured upon 1, 2, 3, 4 and 24 h of rehydration. Values represent averages and the SE of 20–30 biological replicates. *P < 0.05 vs.s 3NBBM, Student t-test. Dashed line, F_v/F_m value of non-treated *A. erici*.

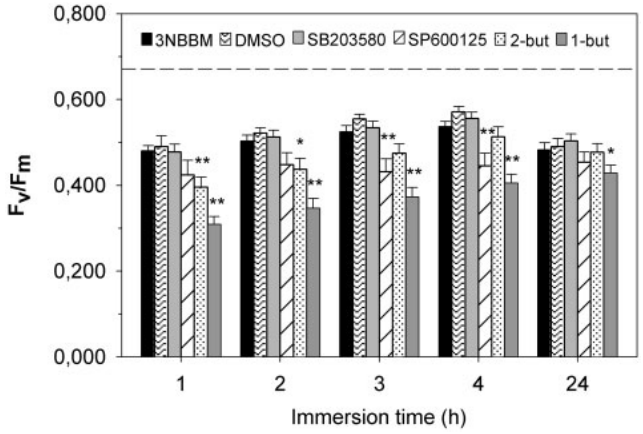

Figure. 3 Photosynthesis response to hyperosmotic stress in pre-inhibited samples. Asterochloris erici algal discs were incubated in 3NBBM with 0.1% DMSO, 100 mM SB203580 (p38 pathway inhibitor), 100 mM SP600125 (JNK pathway inhibitor), 0.5% 1-butanol (PLD inhibitor) or 0.5% 2-butanol (non-active isomer) and then immersed in a 2M NaCl solution. The effect on the maximum photosynthesis yield (F_v/F_m) was measured. Values represent averages and the SE of 20–30 biological replicates. *P < 0.05 or **P < 0.01 vs. 3NBBM, Student t-test.
Dashed line, F_v/F_m value of non-treated *A. erici.*

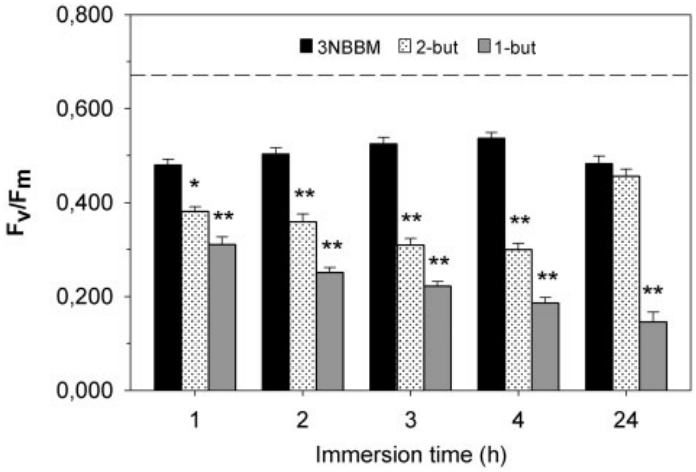

Figure. 4 Photosynthesis response to hyperosmotic stress in the presence of butanol. Asterochloris erici algal discs were incubated in 3NBBM with 0.5% 1-butanol (PLD inhibitor) or 0.5% 2-butanol (non-active isomer) and then immersed in a 2M NaCl solution with 0.5% 2-butanol or 0.5% 1-butanol, respectively. The effect on the máximum photosynthesis yield (F_v/F_m) was measured. Values represent averages and the SE of 15 biological replicates. *P < 0.05 or **P < 0.01 vs. 3NBBM, Student t-test. Dashed line, F_v/F_m value of non-treated *A. erici.*

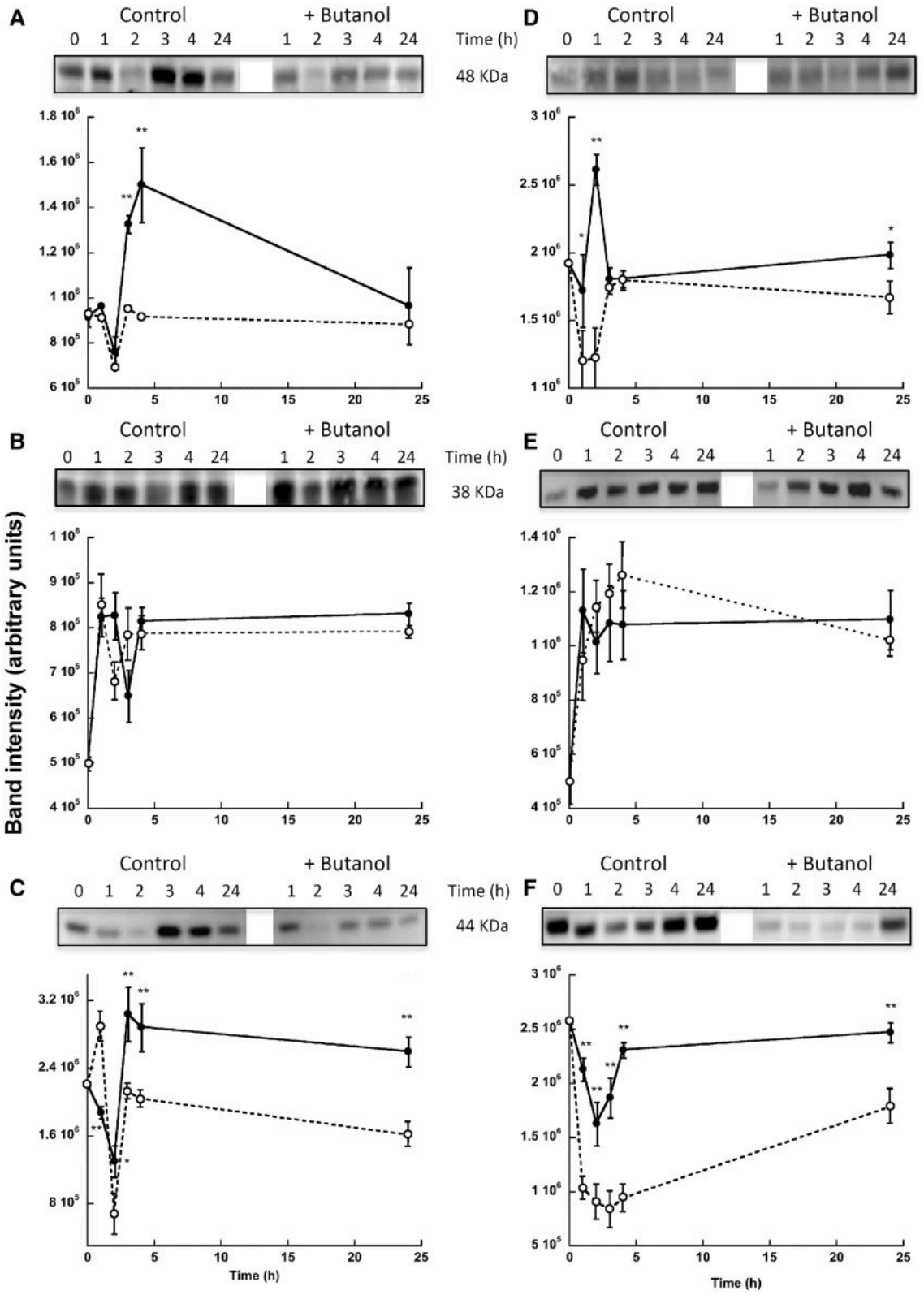

Figure. 5 Immunodetection of MAPK phosphorylated forms during desiccation. Western blot analysis of phosphorylated forms of the MAPKs as detected by specific phospho-JNK (A, D), phospho-p38 (B, E) and phosphor-ERK (C, F) antibodies in A. erici during desiccation (A–C) or 2M NaCl treatment (D–F) in the absence (control) and presence (+) of 1-butanol. Each lane contains 50 mg of protein extract. The graphs show band intensities after scanning vs. time (hours). Solid lines/filled circles, control; dashed lines/open circles, with 1-butanol. Values represent averages and the SE of three biological replicates. *P < 0.05 or **P < 0.01, 1-butanol vs control, Student t-test.

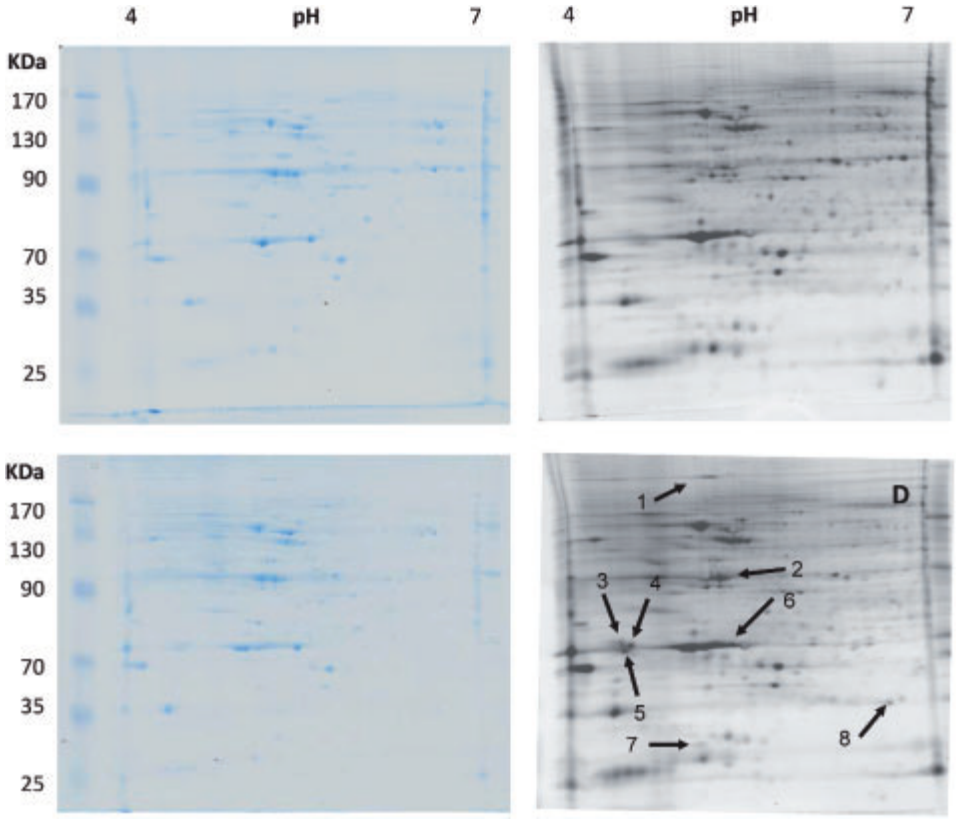

Figure. 6 Changes in the phosphoproteome of A. erici in response to desiccation. Representative 2D gels of phosphoproteins isolated and enriched from fresh (A, B) and desiccated (C, D) A. erici samples. (A and C) Coomassie staining; (B and D) Pro-Q Diamond staining. Arrows indicate proteins with a different phosphorylation degree subsequently identified by MALDI-TOF MS. Gels were repeated three times with proteins of independent experiments. An 80 ug aliquot of proteins was loaded per gel

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7	20S proteasome beta-4 subunit	35556	68	1	12.3	1	Ubiquitin-dependent protein catabolism
8	ND						

Spot no., number of spot in Fig. 6D.
[a] Accession number against the JGI Asterochloris sp. Cgr/DA1pho v2.0 database.
[b] Score = −log (1 − percentage confidence/100).
ND, not-determined.

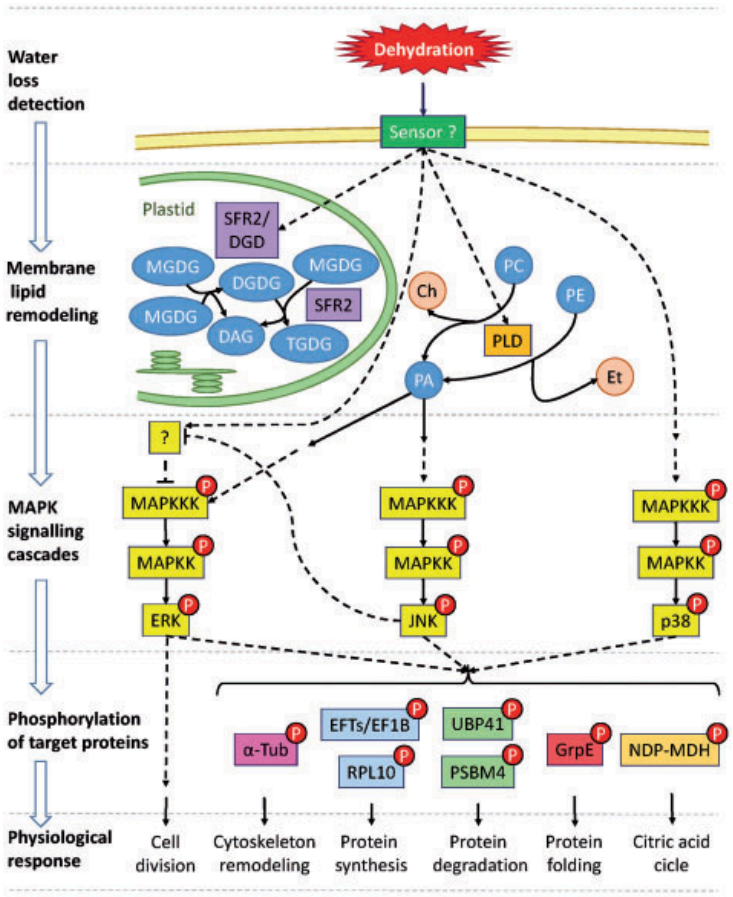

Figure. 7 Schematic drawing of the proposed signaling pathways involved in cellular responses to desiccation in A. erici. Dehydration causes an osmotic stress that triggers lipid membrane remodeling. In chloroplasts, a fraction of MGDG is converted into DGDG by the DGDG synthase DGD and another fraction into DGDG and TGDG by SFR2, which contributes to the stabilization of thylakoid membranes. In non-chloroplast membranes, the phospholipids PC and PE are hydrolyzed by PLD, releasing the signal molecule PA and a polar head group. Subsequently, the threemain MAPK cascades, ERK, JNK and p38, are induced, with PA being involved in the regulation of ERK and JNK pathways, whereas p38 is not PLD dependent. Activation of kinase enzymes leads to the phosphorylation of target proteins involved in physiological responses that facilitate the transition to the anhydrobiosis state and the recovery upon rehydration. For more detailed information, see the text. Enzymes and biological molecules are highlighted in colored boxes and circles, respectively. Solid arrows depict known pathways, while dashed arrows indicate proposed or unknown pathways. a-Tub, a-tubulin; Ch, choline; DAG, diacylglycerol; DGD, digalactosyldiacylglycerol synthase; DGDG, digalactosyldiacylglycerol; DMSO, dimethylsulfoxide; EFTs/EF1B, translation elongation factor EF1B; ERK, extracellular signal-regulated kinases; Et, ethanolamine; GrpE, GrpE nucleotide exchange factor; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; MAPKK, mitogen- activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MGDG, monogalactosyldiacylglycerol; NDP-MDH, malate dehydrogenase NAD-dependent; P, phosphorylation; PA, phospatidic acid; PC, phospatidylcholine; PE, phospatidylethanolamine; PLD, phospholipase D; PSBM4, 20S proteasome b-4 subunit; RPL10, 60S ribosomal protein 10L; SFR2, stress freeze response 2; TGDG, trigalactosyldiacylglycerol; UBP41, ubiquitin-specific peptidase 41.