2017

A multidomain enzyme, with glycerol-3-phosphate dehydrogenase and phosphatase activities, is involved in a chloroplastic pathway for glycerol synthesis in *Chlamydomonas reinhardtii*

Daniela Morales-Sánchez
University of Nebraska-Lincoln, daniela.morales-sanchez@unl.edu

Yeongho Kim
University of Nebraska - Lincoln, ykim24@unl.edu

Ee Leng Terng
University of Nebraska-Lincoln, el.terng@gmail.com

Laura Peterson
University of Kentucky

Heriberto D. Cerutti
University of Nebraska - Lincoln, hcerutti1@unl.edu

The Plant Journal (Accepted manuscript online: 8 March 2017)

Received Date : 03-Oct-2016

Accepted Date : 28-Feb-2017

Article type : Original Article

A multidomain enzyme, with glycerol-3-phosphate dehydrogenase and phosphatase activities, is involved in a chloroplastic pathway for glycerol synthesis in *Chlamydomonas reinhardtii*

Daniela Morales-Sánchez[1], Yeongho Kim[1], Ee Leng Terng[1], Laura Peterson[2] and Heriberto Cerutti[1,*]

[1]School of Biological Sciences and Center for Plant Science Innovation, University of Nebraska–Lincoln, Lincoln, Nebraska 68588, USA, and [2]Department of Physiology, College of Medicine, University of Kentucky, Lexington, Kentucky, USA.

*Corresponding author: Heriberto Cerutti

Tel: +1 (402) 472-0247

E-mail: hcerutti1@unl.edu

Authors email addresses:

DMS: daniela.morales-sanchez@unl.edu

ELT: el.terng@gmail.com

LP: laura.peterson@uky.edu

YK: yeongho.kim@unl.edu

Running head: *Chlamydomonas* GPD2 and plastid glycerol synthesis

Keywords: *Chlamydomonas*, algae, glycerol, triacylglycerol, glycerolipid, nitrogen deprivation, osmotic stress, chloroplast, biofuel

SUMMARY

Understanding the unique features of algal metabolism may be necessary to realize the full potential of algae as feedstock for the production of biofuels and biomaterials. Under nitrogen deprivation, the green alga *C. reinhardtii* showed substantial triacylglycerol (TAG) accumulation and up-regulation of a gene, *GPD2*, encoding a multidomain enzyme with a putative phosphoserine phosphatase (PSP) motif fused to glycerol-3-phosphate dehydrogenase (GPD) domains. Canonical GPD enzymes catalyze the synthesis of glycerol-3-phosphate (G3P) by reduction of dihydroxyacetone phosphate (DHAP). G3P forms the backbone of TAGs and membrane glycerolipids and it can be dephosphorylated to yield glycerol, an osmotic stabilizer and compatible solute under hypertonic stress. Recombinant *Chlamydomonas* GPD2 showed both reductase and phosphatase activities *in vitro* and it can work as a bifunctional enzyme capable of synthesizing glycerol directly from DHAP. In addition, *GPD2* and a gene encoding glycerol kinase were up-regulated in *Chlamydomonas* cells exposed to high salinity. RNA-mediated silencing of *GPD2* revealed that the multidomain enzyme was required for TAG accumulation under nitrogen deprivation and for glycerol synthesis under high salinity. Moreover, a GPD2-mCherry fusion protein was found to localize to the chloroplast, supporting the existence of a GPD2-dependent plastid pathway for the rapid synthesis of glycerol in response to hyperosmotic stress. We hypothesize that the reductase and phosphatase activities of PSP-GPD multidomain enzymes may be modulated by post-translational modifications/mechanisms, allowing them to synthesize primarily G3P or glycerol depending on environmental conditions and/or metabolic demands in algal species of the core Chlorophytes.

INTRODUCTION

Microalgae, photosynthetic eukaryotic microorganisms, can convert CO_2 to a variety of products, including high value chemicals, pharmaceuticals, and biofuel precursors (Borowitzka, 1998; Pulz and Gross, 2004; Spolaore *et al.*, 2006; Giovanardi *et al.*, 2013). Some microalgal species can accumulate high quantities of nonpolar lipids, mainly triacylglycerols, and can potentially be used as feedstock for the production of edible oils and biofuels (Morales-Sanchez *et al.*, 2014; Gimpel *et al.*, 2015). However, microalgae are very diverse and their physiology and metabolism appear to have unique features whose understanding may be required to fulfill the potential of these organisms as sustainable biotechnological resources (Gimpel *et al.*, 2015; Zienkiewicz *et al.*, 2016).

Chlamydomonas reinhardtii is a unicellular alga well established as a model system for examining photosynthesis, physiology, metabolism and the structure and function of flagella (Riekhof *et al.*, 2005; Merchant *et al.*, 2007). *Chlamydomonas* has been shown to accumulate considerable amounts of storage compounds when subject to various stresses, such as nutrient deprivation or high salinity, under photoheterotrophic or photoautotrophic conditions (Siaut *et al.*, 2011; Msanne *et al.*, 2012; Goodenough *et al.*, 2014; Schmollinger *et al.*, 2014; Zienkiewicz *et al.*, 2016). In wild type cells, nitrogen depletion triggered an initial increase in starch content followed by substantial TAG accumulation (Siaut *et al.*, 2011; Msanne *et al.*, 2012). Transcripts encoding certain glycerol-3-phosphate dehydrogenase isoenzymes, particularly GPD2, exhibited markedly enhanced abundance under these conditions (Goodenough *et al.*, 2014; Schmollinger *et al.*, 2014). GPDs catalyze the formation of glycerol-3-phosphate, the backbone of TAGs and membrane glycerolipids, by reduction of dihydroxyacetone phosphate; and they may influence TAG accumulation by affecting G3P levels (Wang *et al.*, 2001; Radakovits *et al.*, 2010).

Indeed, GPDs are commonly involved in glycerolipid metabolism and in the preservation of cellular redox status by consuming NAD(P)H and regenerating $NAD(P)^+$ (Klöck and Kreuzberg, 1989; Wang *et al.*, 2001; Goshal *et al.*, 2002). These enzymes have also been implicated in osmotic stress

acclimation in several algal species of the genera *Dunaliella, Asteromonas* and *Chlamydomonas* (Ben-Amotz *et al.*, 1982; Klöck and Kreuzberg, 1989; Gee *et al.*, 1993; Goshal *et al.*, 2002; Oren, 2016). GPDs play a role in the response to osmotic and salinity stress by affecting the synthesis of glycerol, a known osmoprotectant (Ben-Amotz *et al.*, 1982; Goshal *et al.*, 2002). Gee *et al.* (1993) identified biochemically three GPD isoforms in the halotolerant microalga *Dunaliella tertiolecta*: a chloroplast osmoregulatory form, which is stimulated by NaCl, a chloroplast glyceride form whose activity is increased during cell growth, and a minor cytosolic form. Genes encoding similar enzymes from *Dunaliella salina* (*DsGPDH2* and *G3PDH*) and from *Dunaliella viridis* (*DvGPDH1* and *DvGPDH2*) have also been cloned and characterized in relationship to salinity tolerance (He *et al.*, 2007; He *et al.*, 2009; Chen *et al.*, 2011; Cai *et al.*, 2013). The *C. reinhardtii* genome encodes five $NAD(P)^+$-dependent GPDs (Merchant *et al*., 2007) and a partially purified enzymatic activity was described in earlier work (Klöck and Kreuzberg, 1989). Recently, Herrera-Valencia *et al.* (2012) characterized *in silico* three *Chlamydomonas* GPD isoforms. Additionally, based on transcript abundance analyses, they proposed that *GPD4* (referred to as *GPDH1*) might be constitutively expressed whereas *GPD2* and *GPD3* appeared to be induced in response to salinity stress.

Bioinformatic analyses revealed that the *Chlamydomonas* GPD2, GPD3 and GPD4 isoforms as well as their putative *Dunaliella* orthologs have an unusual structure consisting of canonical glycerol-3-phosphate dehydrogenase domains fused, at their N-terminus, with a putative phosphoserine phosphatase (PSP) motif (He *et al.*, 2007; He *et al.*, 2009; Herrera-Valencia *et al.,* 2012; Cai *et al.*, 2013). The role of the PSP domain in these chimeric proteins is currently unknown, although we and others have hypothesized that it may function in the dephosphorylation of G3P to glycerol (He *et al.*, 2009; Cai *et al.*, 2013). We report here on the enzymatic and physiological characterization of *C. reinhardtii* GPD2, as the archetypical form for these PSP-GPD fusion proteins. Our observations indicate that GPD2-like enzymes can catalyze directly the synthesis of glycerol, by using as substrates DHAP and NAD(P)H, and are localized to the chloroplast, suggesting a unique pathway for rapid glycerol synthesis during acclimation to hyperosmotic stress. In *Chlamydomonas* (and likely in the

core Chlorophytes) GPD2-like enzymes are necessary for both TAG accumulation under nitrogen deprivation and for glycerol production under high salinity.

RESULTS

Bioinformatic analyses show that *Chlamydomonas* GPD2 has a multidomain structure, consisting of fused PSP and GPD motifs

Six genes encoding glycerol-3-phosphate dehydrogenase enzymes were identified by homology searches of the *C. reinhardtii* genome. Five genes code for $NAD(P)^+$-dependent GPDs, namely Cre12.g511150 (*GPD1*), Cre01.g053000 (*GPD2*), Cre01.g053150 (*GPD3*), Cre10.g421700 (*GPD4*), and Cre09.g387763 (*GPD5*). The sixth gene, namely Cre13.g577450 (*mtGPD*), encodes a FAD^+-dependent GPD, which is likely located to the mitochondrion. Of the five $NAD(P)^+$-dependent GPD homologs, only GPD1 and GPD5 exhibit the canonical protein organization, consisting of an N-terminal $NAD(P)^+$-binding domain and a C-terminal substrate-binding domain (Figure 1a). The other three homologs (GPD2, GPD3 and GPD4) have a unique structure, including at their N terminus an additional motif, a haloacid dehalogenase (HAD)-like superfamily domain most closely related to phosphoserine phosphatase (Figure 1a).

To gain insight into the evolution and diversity of these chimeric $NAD(P)^+$-dependent GPD homologs, phylogenetic analysis were performed independently with the GPD and the HAD-like domains, including available algal sequences as well as those from assorted eukaryotes and eubacteria. However, PSP-GPD fusion enzymes appear to be limited to algal species in the Chlorophyceae and Trebouxiophyceae classes (Figure 1b and 1c). The GPD domains of the algal chimeric proteins clustered in phylogenetic trees with canonical glycerol-3-phosphate dehydrogenases of diverse eukaryotes (Figure 1b), suggesting a common ancestry with these enzymes characterized as cytosolic in metazoans and fungi (Wang *et al.*, 2001; Lee *et al.*, 2012). Additionally, homology-

modeling studies previously suggested that the GPD domains of *Chlamydomonas* GPD2, GPD3, and GPD4 had fold topology features similar to human GPD1 (Herrera-Valencia *et al.,* 2012). In contrast, *Chlamydomonas* GPD1 and GPD5 clustered with eubacterial (as well as plant and algal) glycerol-3-phosphate dehydrogenase sequences (Figure 1b), suggesting that they might have been acquired through an endosymbiotic event or possibly through horizontal gene transfer. The PSP (HAD-like) domains of the algal chimeric GPDs grouped together in phylogenetic trees (Figure 1c), but as a sister clade to canonical eukaryotic phosphoserine phosphatases (commonly involved in L-serine biosynthesis). Thus, it is tempting to speculate that the PSP-GPD fusion enzymes may have arisen, in an early green algal lineage, by duplication of a gene encoding an ancestral phosphoserine phosphatase with subsequent (or concomitant) translational fusion of one of the duplicated copies to a gene encoding a eukaryotic-like GPD.

Alignment of the N-terminal sequences of *Chlamydomonas* GPD2, GPD3, and GPD4, as well as of their *Dunaliella* and *Chlorella* homologs, with the phosphoserine phosphatases from *Mycobacterium avium* and *Methanococcus jannaschii* indicated conservation of three short HAD-like typical motifs (Figure 1d). The amino acids of these motifs cluster together in the canonical PSP three dimensional structure to form the active site, and the first aspartate of the conserved DXDX[T/V] motif I (Figure 1d) is used as a nucleophilic residue for catalysis (Collet *et al.*, 1998; Peeraer *et al.*, 2004). Homology modeling of the three-dimensional structure of the GPD2 HAD-like domain indicated a similar overall fold to the *M. avium* PSP template (Figure S1a). In particular, GPD2 showed similar clustering of the residues critical for catalysis and for binding of the Mg^{2+} cofactor (Figure S1b). These observations suggested that the PSP domain of GPD2 might be enzymatically active but, based on the phylogenetic analysis (Figure 1c), it appears to have diverged from canonical phosphoserine phosphatases and might act on a different, although likely related, substrate. Since G3P has a similar stereochemical configuration as L-phosphoserine (the natural substrate of PSPs) we decided to explore the possibility that algal GPD2-like enzymes may have G3P phosphatase activity and catalyze directly the two-step conversion of DHAP to glycerol.

GPD2 is a bifunctional protein, with reductase and phosphatase activities, capable of synthesizing directly glycerol from DHAP

To examine the enzymatic activities of GPD2, recombinant proteins of the wild type isoform, two mutant versions, as well as canonical GPD1 were produced by *in vitro* transcription/translation in a continuous-exchange cell-free wheat germ system. *Chlamydomonas* GPD1 was used as a positive control for the standard activity of a glycerol-3-phosphate dehydrogenase, since it lacks a PSP domain. GPD2 mutants were generated by site-directed mutagenesis of specific residues within the PSP domain (Collet *et al.*, 1998; Peeraer *et al.*, 2004), in order to eliminate any potential phosphatase activity. In GPD2-NV2Mut the first aspartate (presumably the nucleophilic residue for catalysis) in the conserved DVDCT motif I was replaced with asparagine (D93N) whereas in GPD2-NVN3Mut both aspartates in this conserved motif were replaced with asparagines (D93N and D95N).

We first tested for glycerol-3-phosphate dehydrogenase activity of the recombinant proteins by measuring a decrease in NADH as it is oxidized to NAD^+, a reaction stoichiometrically coupled with the reduction of DHAP to G3P (Vigeolas *et al.*, 2007; Casais-Molina *et al.*, 2016). All four proteins caused a similar decrease in NADH concentration, implying equivalent GPD activities under the experimental conditions used (Figure 2a), although this was achieved with a smaller amount of recombinant protein for GPD1 (see Methods S1). As a control, an aliquot of the wheat germ lysate programmed with an empty vector did not affect the NADH concentration. These observations corroborated that *Chlamydomonas* GPD2 has glycerol-3-phosphate dehydrogenase activity, as previously demonstrated by complementation of a *Saccharomyces cerevisiae gpd1Δgpd2Δ* double mutant via expression of a *GPD2* cDNA (Casais-Molina *et al.*, 2016). Moreover, the GPD activity of GPD2 was no affected by mutations in its PSP domain (Figure 2a).

We next examined whether GPD2 may have phosphatase activity in reactions containing glycerol-3-phosphate as the substrate. Production of inorganic orthophosphate (Pi) was measured colorimetrically as phosphomolybdate (Heinonen and Lahti, 1981). In these reactions, only wild type

GPD2 showed substantial release of Pi whereas GPD1 (lacking a PSP domain) and GPD2-NV2Mut or GPD2-NVN3Mut (with mutated residues in the putative PSP catalytic site) exhibited no enzymatic activity (Figure 2b). Similar results were obtained by measuring glycerol production (Figure 3a) instead of Pi release. These findings indicated that the GPD2 PSP domain can use G3P as a substrate, prompting us to explore whether the chimeric enzyme might catalyze directly the two-step conversion of DHAP to glycerol. Indeed, wild type GPD2 yielded inorganic phosphate and glycerol in reactions containing as substrates DHAP and NADPH (Figures 2c and 3b) or DHAP and NADH (Figures 2d and 3c). However, the absence of NAD(P)H in the reactions, required to reduce DHAP to G3P, abolished the GPD2-mediated production of Pi (Figure 2e) and glycerol (Figure 3d). In all these assays, GPD1, GPD2-NV2Mut and GPD2-NVN3Mut demonstrated no activity, indicating that an intact PSP domain is required for the production of Pi and glycerol from DHAP and NAD(P)H. Additionally, none of the recombinant proteins, including wild type GPD2, showed phosphatase activity with L-phosphoserine as the substrate.

These observations strongly suggested that GPD2-like enzymes can catalyze the reduction of DHAP to G3P (via their GPD domains) followed by dephosphorylation of G3P to glycerol (via their PSP domain). Consistent with a two-step conversion from DHAP to glycerol, the initial rate of reaction for the production of inorganic phosphate by GPD2 is slower (~0.024 mmol/min) when using DHAP and NADH as substrates (which would require the synthesis of G3P prior to its dephosphorylation) than when providing directly G3P as the substrate (~0.25 mmol/min). Similar conclusion can be reached when analyzing the initial rate of reaction for the synthesis of glycerol by GPD2 using as substrates either DHAP and NADH or G3P.

Chlamydomonas GPD2 is localized to the chloroplast

To gain insight on where GPD2 may function we examined its subcellular localization. Bioinformatic analyses with ChloroP and PredAlgo tools suggested that GPD2, GPD3 and GPD4 have N-terminal chloroplast transit peptides (CTPs), whereas GPD1 and GPD5 would not be targeted to an organelle

(Table S1). These analyses and the experimental work were carried out with the protein sequences predicted by Phytozome v11.0 gene models, except for GPD3 since the gene model ova_au5.g2031_t1 appears to be more accurate (including a longer N-terminal sequence similar to that in the very closely related GPD2). To assess experimentally the subcellular localization of GPD2 and GPD1, the 5' end of the *GPD2* coding sequence (CDS) (including the predicted CTP and the PSP domain) and the full-length *GPD1* CDS were fused in frame at the 5' end of the mCherry fluorescent protein CDS. These transgenes, under the control of the *PsaD* promoter, were then introduced by electroporation into *C. reinhardtii* CC-124. The intracellular distribution of GPD2-mCherry and GPD1-mCherry was examined in the transgenic strains by laser scanning confocal microscopy. The fluorescent signal from GPD2-mCherry overlapped with that from chlorophyll fluorescence (Figures 4 and S2), indicating that the recombinant protein is located predominantly in the chloroplast. In contrast, GPD1-mCherry appears to be localized throughout the cytosol and, possibly, also in the nucleus (Figures 4 and S2). Non-transgenic, wild type CC-124 was used as a negative control, to verify the absence of any background signal in the mCherry channel (Figures 4 and S2). This subcellular localization suggested that GPD2-like enzymes may synthesize G3P and/or glycerol in the chloroplast of core Chlorophytes.

GPD2 and GPD3 are required for both triacylglycerol accumulation under nitrogen depletion and glycerol production under high salinity

In order to assess the *in vivo* role of GPD2-like enzymes, expression of the closely related *GPD2* and *GPD3* genes was simultaneously suppressed by RNA interference (RNAi) in transgenic strains derived from *Chlamydomonas* CC-125. Several RNAi strains (Ri14, Ri16 and Ri18), with partial *GPD2*/*GPD3* suppression, were used for the experimental analyses. The growth of these RNAi lines in nutrient replete medium under photoautotrophic conditions was very similar to that of the wild type (Figure S3). We also examined the relative abundance of the most prevalent membrane lipids: monogalactosyldiacylglycerol (MGDG), diacylglycerol-*N,N,N*-trimethylhomoserine (DGTS),

phosphatidylethanolamine (PE), phosphatidylglycerol (PG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylinositol (PI). However, when cultured in standard minimal (HS) medium, no statistically significant differences were observed between the wild type and the RNAi strains in relative or total amounts of these membrane lipid classes (Figure S4a). For analyses under environmental stress conditions, CC-125 and the RNAi lines were pre-cultured to middle logarithmic phase in HS medium. Cells were then harvested by centrifugation, resuspended in HS medium containing 100 mM NaCl (HS+Na) or in HS medium lacking nitrogen (HS-N) and cultured under photoautotrophic conditions during 6 hours for the high salinity experiment or during 2-3 days for the nitrogen deprivation analyses.

Under nitrogen starvation *Chlamydomonas* cells halt cell division and channel excess carbon into storage compounds (Siaut *et al.*, 2011; Msanne *et al.*, 2012; Goodenough *et al.*, 2014; Schmollinger *et al.*, 2014). The primary product of CO_2 fixation, 3-phosphoglycerate, can feed directly into starch synthesis or can be used for TAG synthesis, as a precursor of the glycerol backbone and of acetyl-CoA for fatty acid production (Hu *et al.*, 2008; Radakovits *et al.*, 2010). Thus, the synthesis of starch and TAGs may compete with each other, although the relationship between these two metabolic pathways appears to be more complex than mere competition (Li *et al.*, 2010; Work *et al.*, 2010; Siaut *et al.*, 2011; Krishnan *et al.*, 2015). To evaluate neutral lipid accumulation during nitrogen starvation, *Chlamydomonas* cells were examined by fluorescence microscopy after staining with the nonpolar lipid fluorophore Nile Red. Lipid body formation, which normally increases substantially in nitrogen-stressed *C. reinhardtii* (Siaut *et al.*, 2011; Msanne *et al.*, 2012; Goodenough *et al.*, 2014), was reduced in the *GPD2/GPD3* RNAi lines compared with the wild type (Figure S5). Consistent with this observation, TAG content, determined as fatty acid methyl esters analyzed by gas chromatography-flame ionization detection, was significantly lower in Ri16 and Ri18 relative to CC-125 after two days of nitrogen deprivation (Figure 5a). Moreover, under these conditions, the cellular content of nearly all major membrane glycerolipids was also reduced in the *GPD2/GPD3* RNAi strains (Figure S4b), although their relative levels (expressed in mol %) remained almost identical to those in the wild type (Figure S4a).

In addition, the RNAi strains showed somewhat enhanced accumulation of starch, compared with CC-125, when subject to nitrogen depletion (Figure S6). Under nutrient stress conditions, the GPD2/GPD3 enzymes likely increase carbon flux toward membrane glycerolipid and TAG production (and reroute it from starch accumulation) by influencing the synthesis of the glycerol backbone. We also attempted to determine changes in glycerol content in the *Chlamydomonas* strains but this was difficult to assess because glycerol amounts were fairly low in cells grown photoautotrophically in minimal medium and decreased slightly in nitrogen-deprived cells, without any obvious difference among the wild type and the *GPD2/GPD3* RNAi lines (Figure 5c).

Salt stress has also been shown to induce TAG accumulation in *C. reinhardtii* under photoheterotrophic conditions (Siaut *et al.*, 2011). Likewise, we observed a slight increase in TAG content in cells cultured in HS+Na under photoautotrophic conditions and, the same as under nitrogen deprivation, this increase was of somewhat lower magnitude in the *GPD2/GPD3* RNAi strains (Figure 5b). In response to hyperosmotic stress, *Chlamydomonas* also synthesizes large amounts of glycerol as an osmoregulatory metabolite (Husic and Tolbert, 1986). However, the accumulation of glycerol triggered by exposure of cells to 100 mM NaCl was substantially diminished in the Ri16 and Ri18 RNAi strains relative to the wild type (Figure 5d). Thus, our overall results indicate that *Chlamydomonas* GPD2/GPD3 are mainly required for glycerol and/or membrane glycerolipid/TAG production under certain environmental stresses, but they appear to be dispensable in cells grown in nutrient replete medium.

Few genes encoding enzymes of glycerol metabolism show altered expression in *C. reinhardtii* subject to nitrogen deprivation or salinity stress

Production of glycerol-3-phosphate and glycerol under nitrogen starvation or hyperosmotic stress likely involves interconversions catalyzed by several enzymes (Wang *et al.*, 2001; Oren, 2016) (Figures 6 and 7). Besides GPD2-like chimeric proteins, DHAP conversion to glycerol can be carried

out by two canonical enzymes, a standard GPD (like GPD1 or GPD5) and a glycerol-3-phosphate phosphatase (GPP) (Figure 7). Conversely, glycerol can be converted to G3P by a glycerol kinase (GK) and to DHAP by a glycerol 2-dehydrogenase and a dihydroxyacetone kinase (DAK) (Figure 7). In *Dunaliella*, several of these enzymes were proposed to constitute a glycerol cycle, involving specific pathways for glycerol synthesis and for glycerol removal during the osmoregulatory process (Ben-Amotz *et al.*, 1982; Chen *et al.*, 2012; Oren, 2016). Putative orthologs of these enzymes encoded in the *C. reinhardtii* genome were identified by homology searches, and their roles are indicated in Figure 7 (yellow box). However, the true *Chlamydomonas* ortholog of glycerol 2-dehydrogenase (i.e., dihydroxyacetone reductase) is difficult to determine based solely on sequence alignments and we chose to examine AKR3 (aldo/keto reductase 3) as the best match to a putative *D. salina* dihydroxyacetone reductase (ACL13982). Additionally, the intracellular compartmentalization of enzymes is expected to affect carbon flux and product generation. The subcellular location of several of these enzymes has been experimentally determined in *C. reinhardtii* (Terashima *et al.*, 2011; Johnson and Alric, 2013; Polle *et al.*, 2014; this work), whereas the location of others, as represented in Figure 7, has been inferred based on bioinformatic analyses (e.g., AKR3, DAK1, GK, GPP and GPD5).

To assess whether genes encoding enzymes of glycerol metabolism may be transcriptionally activated in cells exposed to environmental stress, their transcript abundance, in wild type CC-125 and in the Ri16 *GPD2/GPD3* RNAi strain, was examined by RT-PCR (Figure 6). As previously reported (Goodenough *et al.*, 2014; Schmollinger *et al.*, 2014; Casais-Molina *et al.*, 2016), *GPD2* mRNA levels were substantially enhanced in CC-125 under nitrogen deprivation or hyperosmotic stress. However, this up-regulation was largely suppressed in the Ri16 RNAi strain (Figure 6). All other examined genes showed comparable transcript abundance upon nitrogen depletion as in nutrient replete medium, in both CC-125 and Ri16. In contrast, in cells exposed to high salinity, both *GPD2* and *GK* appeared to be up-regulated in wild type CC-125. Of note, enhanced transcript abundance for

these genes was not detected in Ri16, even though only *GPD2/GPD3* mRNAs are targeted for RNA interference by the introduced inverted repeat transgene (see Discussion).

DISCUSSION

The *C. reinhardtii* GPD2-like isoforms are chimeric proteins consisting of a phosphoserine phosphatase-like motif fused to canonical glycerol-3-phosphate dehydrogenase domains. Phylogenetic analyses indicated that putative orthologs of these multidomain polypeptides are unique to algae within the Chlorophyceae and Trebouxiophyceae classes of Chlorophytes. Canonical GPDs catalyze the reversible redox conversion of dihydroxyacetone phosphate to glycerol-3-phosphate and our observations substantiated that recombinant GPD2 has G3P dehydrogenase activity *in vitro*, as previously established by complementation of a *S. cerevisiae gpd1Δgpd2Δ* double mutant (Casais-Molina *et al.*, 2016). This reductase activity of GPD2 was not affected by mutations in its PSP domain. Moreover, two *D. viridis* GPD2 homologs with deleted PSP domains were still capable of reverting the high salt sensitivity of the *S. cerevisiae gpd1Δ* mutant (He *et al.*, 2009), implying their competence to synthesize G3P. These collective results suggest that GPD2-like enzymes can catalyze the reduction of DHAP to G3P via their GPD domains and that this activity appears to be independent from the presence of a functional PSP motif.

Homology modeling of the N-terminal region of GPD2 indicated a similar fold, and similar clustering of residues essential for catalysis, to the *M. avium* phosphoserine phosphatase. However, given the phylogenetic divergence of the GPD2 PSP domain from canonical phosphoserine phosphatases, we explored the possibility that it might dephosphorylate glycerol-3-phosphate. Indeed, in *in vitro* reactions, recombinant GPD2 yielded inorganic orthophosphate and glycerol when using G3P as the substrate. However, this activity was eliminated by mutations in the GPD2 DVDCT motif. The reaction mechanism of phosphoserine phosphatases, members of the HAD-like superfamily, involves a conserved DXDX(T/V) motif where the first aspartate functions as a nucleophilic residue for

catalysis (Collet *et al.*, 1998; Peeraer *et al.*, 2004; Larrouy-Maumus *et al.*, 2014). Substitution of asparagines for the first aspartate (D93N) or both aspartates (D93N and D95N) in the DVDCT motif of GPD2 abolished its phosphatase activity towards G3P. Equivalent mutations in human L-3-phosphoserine phosphatase resulted in complete inactivation of the enzyme (Collet *et al.*, 1998), supporting a similar catalytic mechanism for the PSP domain of GPD2.

Land plants such as *Arabidopsis thaliana* encode two low-molecular-weight, typical G3P phosphatases (Caparrós-Martin *et al.*, 2007). Subcellular fractionation revealed that AtGPP1 is located in plastids, consistent with it having a predicted chloroplast transit peptide, whereas AtGPP2 is found in the cytosol (Caparrós-Martin *et al.*, 2007). In contrast, green algae of the Chlorophyceae and Trebouxiophyceae classes appear to encode a single GPP in their genomes, more closely related to the cytosolic form from *A. thaliana*. Thus, it is tempting to speculate that these algae may have lost during evolution the chloroplast targeted canonical GPP and that this activity may have been replaced by the divergent PSP domain of the GPD2-like enzymes.

The *Chlamydomonas* GPD2 protein can catalyze *in vitro* the two step conversion of DHAP to glycerol [in the presence of NAD(P)H]. These results and the GPD2 plastid localization suggested that PSP-GPD multidomain enzymes may synthesize G3P and/or glycerol within the chloroplast in core Chlorophytes (Figure 7). Consistent with our observations, partial protein purification and biochemical studies in *D. tertiolecta* had also implicated a chloroplastic GPD isoform, which seemed to form a tight complex with a G3P phosphatase activity, in the osmoregulatory synthesis of glycerol (Goyal, 2007; Chen and Jiang, 2009). In *Chlamydomonas*, suppression of *GPD2*/*GPD3* expression by RNAi showed that the chimeric enzymes are required for membrane glycerolipid and TAG production under nitrogen deprivation as well as for glycerol accumulation under high salinity. GPD2-like enzymes may play a key role in algal responses to these environmental stresses by enhancing carbon flux toward membrane lipids/TAG and/or glycerol synthesis (and diverting it from starch production)

(Figure 7). As already mentioned, G3P is the precursor for the backbone of glycerolipids, with TAGs accumulating as carbon and energy reserves under nutrient deprivation (Hu *et al.*, 2008; Radakovits *et al.*, 2010; Msanne *et al*., 2012; Johnson and Alric, 2013; Morales-Sánchez *et al*., 2013; Zienkiewicz *et al.*, 2016). On the other hand, glycerol is involved in algal acclimation to high salinity as osmotic stabilizer and compatible solute that allows maintenance of enzyme activities under conditions of low water activity (Ben-Amotz and Avron, 1973; Ben-Amotz *et al.*, 1982; Goshal *et al.*, 2002; Goyal, 2007; Chen and Jiang, 2009; Chen *et al.,* 2011; Oren, 2016).

An important, possibly a controlling role of GPD2-like enzymes in lipid and glycerol metabolism in *C. reinhardtii* under environmental stress is also suggested by the significant up-regulation of the corresponding genes under both nitrogen deprivation and hyperosmotic stress (Goodenough *et al.*, 2014; Schmollinger *et al.*, 2014; Casais-Molina *et al.*, 2016; this work). However, there are also obvious differences between these two conditions when considering the regulation of other genes encoding enzymes of glycerol metabolism. Under high salinity, we observed increased transcript abundance for GPD2, conceivably involved in direct glycerol synthesis from DHAP, but also for GK, conceivably involved in glycerol removal (via glycerolipid synthesis and/or the glycolytic/gluconeogenic pathway). As proposed before, algal cells may modulate the internal glycerol concentration for osmoregulatory purposes by the coordinated action of antagonistic enzymes involved in either glycerol synthesis or glycerol catabolism (Ben-Amotz *et al.*, 1982; Chen *et al.*, 2012). Intriguingly, *GK* gene expression might be regulated by sensing metabolite (possibly glycerol) levels since its up-regulation was minimized in the *GPD2/GPD3* RNAi strains with reduced accumulation of glycerol under high salinity.

In contrast, under nitrogen deprivation, most genes involved in glycerol metabolism did not change expression and a significant increase in transcript abundance appeared to be limited to *GPD2*. Moreover, GPD2-like enzymes may synthesize predominantly G3P under these conditions (as well as

in nutrient replete medium) since the generation of intraplastidic G3P (barring its import from the cytosol) would be required for the synthesis of plastid glycerolipids. *Chlamydomonas* GPD1 and GPD5, based on the protein sequences predicted by Phytozome v11.0 gene models, do not localize to organelles and the PSP-GPD fusion polypeptides may be the only enzymes capable of catalyzing G3P formation inside the chloroplast (possibly constitutively expressed GPD4 in nutrient replete medium and primarily up-regulated GPD2/GPD3 under nitrogen depletion, explaining the reduction in plastid glycerolipid abundance in the nitrogen deprived Ri16 RNAi strain). However, a potential role of GPD1 in this metabolic pathway cannot be ruled out (Figure 7) since its transcript may be translated from an alternative start codon resulting in a longer protein capable of localizing to organelles (Table S1, GPD1_pasa).

There is extensive evidence suggesting post-translational modulation of enzymes involved in glycerol metabolism in diverse organisms. In *C. reinhardtii* under salt stress, substantial changes in metabolite levels preceded adjustments in enzyme abundance (Mastrobuoni *et al.*, 2012). In *S. cerevisiae* the two homologous glycerol-3-phosphate dehydrogenases, Gpd1 and Gpd2, are negatively regulated post-translationally through phosphorylation (Lee *et al.*, 2012). Conversely, in the case of the *Phycomyces blakesleeanus* G3P phosphatase, it has been proposed that phosphorylation causes its activation (Van Schaftingen and Van Laere, 1985). Protein phosphorylation has also been implicated in the response of *D. viridis* to hypertonic shock (Jiménez *et al.*, 2004). Additionally, in *D. salina, de novo* protein synthesis was not required for the osmotic response and the considerable increase in glycerol content triggered by high salinity (Sadka *et al.*, 1989). Thus, it seems possible that key enzymes of glycerol metabolism are largely controlled by post-translational modifications/mechanisms and that transcriptional up-regulation under stress conditions simply reinforces enzymatic capacity.

In *Chlamydomonas* under nitrogen starvation GPD2-like proteins may function predominantly as reductases, synthesizing G3P, which can be used in the chloroplast for glycerolipid synthesis and/or exported to the cytosol for membrane lipid/TAG assembly in the endoplasmic reticulum. For

glycerolipid synthesis, this reaction would be energetically favored over the generation of glycerol as the end product. Moreover, the capacity of isolated *Chlamydomonas* chloroplasts to synthesize and export G3P, as a product of starch degradation, has been previously demonstrated (Klöck and Kreuzberg, 1987). In contrast, under hyperosmotic stress, activation (or de-repression) of the PSP domain of GPD2-like proteins may lead to the rapid synthesis of intraplastidic glycerol, which may then translocate to the cytosol for osmoregulatory purposes. Supporting this hypothesis, an experiment mentioned by Wegmann (1986) indicated that isolated *Dunaliella* chloroplasts can perform glycerol synthesis.

In summary, GPD2-like proteins are chimeric PSP-GPD enzymes with both G3P dehydrogenase and G3P phosphatase activities. Their chloroplast localization implies the existence of a plastid pathway for rapid glycerol synthesis, which appears to be triggered in response to hyperosmotic stress in algae of the core Chlorophytes. We propose that the reductase and phosphatase activities of these multidomain proteins may be regulated independently by post-translational modifications/mechanisms, allowing them to synthesize primarily G3P or glycerol depending on environmental conditions and/or metabolic demands. However, further work will be necessary to assess the validity of this hypothesis.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

C. reinhardtii CC-124 and CC-125 (Harris, 1989) as well as derived transgenic strains (described below) were used in all reported experiments. Unless noted otherwise, cultures were incubated under continuous illumination on an orbital shaker at 25 ºC and ambient level of CO_2 (Msanne *et al.*, 2012). For subcellular localization experiments, CC-124 and derived transgenic strains were grown in Tris-Acetate-Phosphate (TAP) medium (Harris, 1989) to the middle of the logarithmic phase. For other

experiments, CC-125 and derived RNAi strains were pre-cultured to middle logarithmic phase in minimal (HS) medium (Sueoka, 1960). Cells were collected by centrifugation, washed twice, and resuspended in HS medium, HS medium lacking nitrogen (HS-N) or HS medium containing 100 mM NaCl (HS+Na) at a density of ~2 x 10^6 cells mL^{-1}. Strains were then cultured under strictly photoautotrophic conditions during 6 hours for the high salinity experiment or during 2-3 days for the nitrogen deprivation analyses. At the end of the experimental period, cells were harvested by centrifugation at 2000x*g* for 5 minutes and the pellets frozen in liquid nitrogen and stored at -70 ºC for further analyses.

Phylogenetic and bioinformatic analyses

GPD and PSP related sequences were identified by BLASTP searches against GenBank, Phytozome v11.0 (https://phytozome.jgi.doe.gov/pz/portal.html) and Uniprot, using as queries the *Chlamydomonas* GPD2 (Cre01.g053000) PSP or GPD domains, or the whole sequences of GPD1 (Cre12.g511150) or PSP1 (Cre04.g217955). Protein sequences were aligned by Clustal-omega (Pedro *et al.*, 2016), and maximum-likelihood phylogenetic trees were built using PhyML (Guindon and Gascuel, 2003). Most phylogenetic analyses were done with prebuilt scripts in the ETE3 toolkit (Huerta-Cepas *et al.*, 2016). Independent phylogenetic trees were constructed with the PSP or the GPD domain sequences and accession numbers are indicated in the corresponding figures. Homology modeling of the three dimensional structure of GPD2 was performed by using the Phyre2 web portal (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

Generation of full-length GPD1, GPD2, GPD2-NV2Mut and GPD2-NVN3Mut coding sequences for recombinant protein production

Total RNA isolated from nitrogen deprived CC-124 was used as template for cDNA synthesis by reverse transcription, as previously described (Carninci *et al.*, 1998). For *GPD1*, the full-length *GPD1* CDS was amplified by PCR with primers GPD1-F/GPD1-R (Methods S1). This PCR product was cloned into the vector pIVEX 1.3 WG (5 Prime). For *GPD2*, the coding sequence was obtained by the

independent amplification of three partially overlapping PCR products, which were then assembled into a full-length fragment to generate the pIVEX-GPD2 construct (Methods S1). Modified versions of the *GPD2* CDS, namely GPD2-NV2Mut and GPD2-NVN3Mut, were obtained by site-directed mutagenesis. Plasmid pIVEX-GPD2 was used as the template for the generation of the mutants with the QuikChange II-E site-directed mutagenesis kit (Agilent Technologies) as described in Methods S1.

Synthesis of recombinant proteins and in vitro enzymatic assays

GPD1, GPD2, GPD2-NV2Mut and GPD2-NVN3Mut proteins were produced from the pIVEX 1.3 WG vector constructs by *in vitro* transcription/translation in a continuous-exchange cell-free wheat germ system (Biotechrabbit), in accordance with the manufacturer's instructions. Phosphatase activity was analyzed following Gancedo *et al.* (1968), with some minor modifications (Methods S1). Glycerol production was monitored by using the free glycerol reagent (Sigma-Aldrich), following the manufacturer's directions. Glycerol-3-phosphate dehydrogenase activity was examined according to the protocols of Vigeolas *et al.* (2007) and Casais-Molina *et al.* (2016), as described in Methods S1. The amount and size of the recombinant proteins produced in the cell-free wheat germ system were checked by immunoblotting (Figure S7 and Method S1), as previously reported (van Dijk *et al.*, 2005).

Construction of GPD2-mCherry and GPD1-mCherry fusions, generation of transgenic strains, and confocal fluorescence microscopy

The *GPD2* and *GPD1* coding sequences were amplified by RT-PCR using as template total RNA isolated from nitrogen deprived CC-124. The *GPD2* 5' end CDS, comprising a putative chloroplast transit peptide and the PSP domain, was obtained using primers GPD2-mCherry-F/GPD2-mCherry-R, whereas the full-length *GPD1* CDS was amplified with primers GPD1-mCherry-F/GPD1-mCherry-R

(Methods S1). The PCR fragments were inserted into the pQU vector, to generate a translational fusion with the mCherry CDS expressed from the *PsaD* promoter. This plasmid also contains the *Ble*^{r} gene for zeocin resistance (Plucinak *et al.*, 2015). The transgenes were transformed into CC-124 by electroporation, as previously described (Plucinak *et al.*, 2015), and colonies selected on TAP-agar plates containing 16 μg mL^{-1} zeocin. To examine the subcellular localization of the fusion proteins, images of *Chlamydomonas* cells were captured using a Nikon A1 confocal imaging system mounted on a Nikon Eclipse 90i microscope with a 100x objective. mCherry and chlorophyll fluorescence signals were acquired sequentially with 561.5 nm excitation and 570-620 emission and 641 nm excitation and 662-737 emission, respectively, and pseudo-colored green and red for visualization.

Generation of *GPD2/GPD3* RNAi transgenic strains

Chlamydomonas strains containing an inverted repeat (IR) transgene homologous to *GPD2* and *GPD3* were obtained as described under Methods S1, following established protocols (Rohr *et al.*, 2004; Kim and Cerutti, 2009). The *MAA7/GPD2* tandem IR transgene can induce co-silencing of *GPD2* and *GPD3* as well as of *MAA7* (encoding tryptophan synthase β subunit), conferring resistance to 5-fluoroindole to transformed cells (Rohr *et al.*, 2004; Kim and Cerutti, 2009). This construct was stably introduced into CC-125 as previously described (Rohr *et al.*, 2004).

Lipid analyses, starch determination and glycerol quantification

Total lipids from *C. reinhardtii* were extracted by the method of Bligh and Dyer (1959), with some modifications (Methods S1), and fatty acid methyl esters (FAMEs) from TAGs or membrane lipids identified and quantified as previously described (Msanne et al., 2012; Tsai *et al.*, 2015). Starch measurements were performed according to the protocol already described by Ball *et al.* (1990), using an ethanol-washed chlorophyll-free cell pellet (Methods S1). Glycerol content was determined with the free glycerol reagent (Sigma-Aldrich).

Reverse transcriptase (RT)-PCR assays

Reverse transcription reactions from total cell RNA were performed as previously described (Carninci *et al.*, 1998). The synthesized cDNA was then used as the template for standard or real time PCR reactions (Sambrook and Russell, 2001). Primers and PCR conditions are described in Methods S1.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the National Science Foundation (to H.C.). We also acknowledge the support of the Nebraska Experimental Program to Stimulate Competitive Research (EPSCoR). D.M.-S. holds a scholarship (No. 46378) from CONACyT-Mexico. The authors declare no conflict of interest.

SHORT SUPPORTING INFORMATION LEGENDS

Additional supporting information may be found in the online version of this article.

Figure S1. Homology modeling of the three-dimensional structure of the GPD2 HAD-like domain and its catalytic site.

Figure S2. Subcellular localization of *Chlamydomonas* GPD1 and GPD2.

Figure S3. Growth of wild type CC-125 and *GPD2/GPD3* RNAi strains (Ri16 and Ri18) under photoautotrophic conditions in nutrient replete minimal medium (HS).

Figure S4. Analysis of major membrane lipids in the wild type CC-125 and the *GPD2/GPD3* RNAi strain Ri16 grown under photoautotrophic conditions in nutrient replete minimal medium (+N) or in HS medium lacking nitrogen (-N).

Figure S5. Nonpolar lipid accumulation in wild type *C. reinhardtii* CC-125 and *GPD2/GPD3* RNAi strains (Ri16 and Ri18) subject to nitrogen deprivation.

Figure S6. Starch accumulation in wild type *C. reinhardtii* CC-125 and *GPD2/GPD3* RNAi strains (Ri14 and Ri16) subject to nitrogen deprivation.

Figure S7. Immunoblot analysis of recombinant proteins produced by *in vitro* transcription/translation in a continuous-exchange cell-free wheat germ system.

Table S1. Prediction results of protein subcellular localization according to ChloroP and PredAlgo.

Methods S1. Supporting experimental procedures.

REFERENCES

Ball, S. G., Dirick, L., Decq, A., Martiat, J.-C. and Matagne, R. F. (1990) Physiology of starch storage in the monocellular alga *Chlamydomonas reinhardtii. Plant Sci*. **66**, 1-9

Ben-Amotz, A. and Avron, M. (1973) The role of glycerol in osmotic regulation of the halophilic alga *Dunaliella parva. Plant Physiol.* **51**, 875-878.

Ben-Amotz, A., Sussman, I. and Avron, M. (1982) Glycerol production by *Dunaliella*. *Experientia* **38**, 49-52

Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*, **37**, 911-917

Borowitzka, M. A. (1998) Vitamins and fine chemicals from microalgae. In *Micro-algal biotechnology* (Borowitzka, M. A., Borowitzka, L. J., eds). Oxford University Press, Oxford, pp 153-196

Cai, M., He, L. H. and Yu, T. Y. (2013) Molecular clone and expression of a NAD+-dependent glycerol-3-phosphate dehydrogenase isozyme gene from the halotolerant alga Dunaliella salina. *PLoS*

One **8,** e62287

Caparrós-Martin, J. A., Reiland, S., Kochert, K., Cutanda, M. C. and Culiáñez-Macià, F. A. (2007) Arabidopsis thaliana AtGpp1 and AtGpp2: two novel low molecular weight phosphatases involved in plant glycerol metabolism. *Plant Mol. Biol.* **63**, 505-517

Carninci, P., Nishiyama, Y., Westover, A., Itoh, M., Nagaoka, S., Sasaki, N., Okazaki, Y., Muramatsu, M. and Hayashizaki, Y. (1998) Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. *Proc. Natl. Acad. Sci. USA* **95**, 520-524

Casais-Molina, M. L., Peraza-Echeverria, S., Echevarria-Machado, I. and Herrera-Valencia, V. A. (2016) Expression of *Chlamydomonas reinhardtii* CrGPDH2 and CrGPDH3 cDNAs in yeast reveals that they encode functional glycerol-3-phosphate dehydrogenases involved in glycerol production and osmotic stress tolerance. *J. Appl. Phycol.* **28**(1), 219-226

Chen, H. and Jiang J. G. (2009) Osmotic responses of *Dunaliella* to the changes of salinity. *J. Cell. Physiol.* **219**, 251-258

Chen, H., Lao, Y. M. and Jiang, J. G. (2011) Effects of salinities on the gene expression of a (NAD+)-dependent glycerol-3-phosphate dehydrogenase in *Dunaliella salina*. *Sci. Total Environ*. **409**, 1291-1297

Chen, H., Lu, Y. and Jiang, J.-G. (2012) Comparative analysis on the key enzymes of the glycerol cycle metabolic pathway in *Dunaliella salina* under osmotic stresses. *PLoS One* **7**, e37578

Collet, J., Stroobant, V., Pirard, M., Delpierre, G. and Schaftingen, E. V. (1998) A new class of phosphotransferases phosphorylated on an aspartate residue in an amino-terminal DXDX(T/V) motif. *J. Biol. Chem.* **273**, 14107-14112

Gancedo, C., Gancedo, J. M. and Sols, A. (1968) Glycerol metabolism in yeast: pathways of utilization and production. *Eur. J. Biochem.* **5**, 165-172

Gee, R., Goyal, A., Byerrum, R. U. and Tolbert, N. E. (1993) Two isoforms of dihydroxyacetone phosphate reductase from the chloroplast of *Dunaliella tertiolecta. Plant Physiol.* **103**, 243-249

Gimpel, J. A., Henriquez, V. and Mayfield S. P. (2015) In metabolic engineering of eukaryotic microalgae: potential and challenges come with great diversity. *Front. Microbiol.* **6**, 1376

Giovanardi, M., Ferroni, L., Baldisserotto, C., Tedeschi, P., Maietti, A., Pantaleoni, L. and Pancaldi, S. (2013) Morphophysiological analyses of *Neochloris oleoabundans* (Chlorophyta) grown mixotrophically in a carbon-rich waste product. *Protoplasma* **250**, 161-174

Goodenough, U., Blaby, I., Gallaher, S. D., Goodson, C., Johnson, S., Lee, J. H., Merchant, S. S., Pellegrini, M., Roth, R., Rusch, J., Singh, M., Umen, J. G., Weiss, T. L. and Wulan, T. (2014) The path to triacylglyceride obesity in the sta6 strain of Chlamydomonas reinhardtii. *Eukaryot. Cell* **13**, 591-613

Goshal, D., Mach, D., Agarwal, M. and Goyal, A. (2002) Osmoregulatory isoform of dihydroxyacetone phosphate reductase from *Dunaliella tertiolecta:* purification and characterization. *Protein. Expr. Purif.* **24**, 404-411

Goyal, A. (2007) Osmoregulation in *Dunaliella*, Part I: Effects of osmotic stress on photosynthesis, dark respiration and glycerol metabolism in *Dunaliella tertiolecta* and its salt-sensitive mutant (HL 25/8). *Plant Physiol. Biochem.* **45**, 696-704

Guindon, S. and Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**, 696-704

Harris, E. H. (1989) The *Chlamydomonas* sourcebook: a comprehensive guide to biology and laboratory use, 1st edition, Academic Press, San Diego.

He, Q. H., Qiao, D.R., Bai, L.H., Zhang, Q.L., Yang, W.G., Li, Q. and Cao, Y. (2007) Cloning and characterization of a plastidic glycerol 3-phosphate dehydrogenase cDNA from *Dunaliella salina*. *J. Plant Physiol.* **164**, 214-220

He, Y., Meng, X., Fan, Q., Sun, X., Xu, Z. and Song, R. (2009) Cloning and characterization of two novel chloroplastic glycerol 3-phosphate dehydrogenases from *Dunaliella viridis*. *Plant Mol. Biol.* **71**, 193-205

Heinonen, J. K. and Lahti, R. J. (1981) A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Anal Biochem.* **113**, 313-317

Herrera-Valencia, V. A., Macario-Gonzales, L. A., Casais-Molina, M. L., Beltran-Aguilar, A. G. and Peraza-Echeverria, S. (2012) *In silico* cloning and characterization of the glycerol 3-phosphate dehydrogenase (GPDH) gene family in the green microalga *Chlamydomonas reinhardtii. Curr. Microbiol.* **64**, 477-485

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. and Darzins, A. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.* **54**, 621-639

Huerta-Cepas, J., Serra, F. and Bork, P. (2016) ETE 3: reconstruction, analysis and visualization of phylogenomic data. *Mol. Biol. Evol.* **33**, 1635-1638

Husic, H. D. and Tolbert, N. E. (1986) Effect of Osmotic Stress on Carbon Metabolism in Chlamydomonas reinhardtii: Accumulation of Glycerol as an Osmoregulatory Solute. *Plant Physiol.* **82,** 594-596

Jiménez, C., Berl, T., Rivard. C.J., Edelstein, C.L. and Capasso, J.M. (2004) Phosphorylation of MAP kinase-like proteins mediate the response of the halotolerant alga *Dunaliella viridis* to hypertonic shock. *Biochim. Biophys. Acta* **1644,** 61-69

Johnson, X. and Alric, J. (2013) Central carbon metabolism and electron transport in *Chlamydomonas reinhardtii*: metabolic constraints for carbon partitioning between oil and starch. *Eukaryot. Cell* **12**, 776-792

Kim, E. J. and Cerutti, H. (2009) Targeted gene silencing by RNA interference in *Chlamydomonas*. *Methods Cell Biol.* **93**, 99-110

Klöck, G. and Kreuzberg, K. (1987) Sn-Glycerol-3-phosphate is a product of starch degradation in isolated chloroplasts from *Chlamydomonas reinhardtii*. *Z. Naturforsch.* **42c**, 567-569

Klöck, G. and Kreuzberg, K. (1989) Kinetic properties of a *sn*-glycerol 3-phosphate dehydrogenase purified from the unicellular alga *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta* **991**, 347-352

Krishnan, A., Kumaraswamy, G. K., Vinyard, D. J., Gu, H., Ananyev, G., Posewitz, M. C. and Dismukes, G. C. (2015) Metabolic and photosynthetic consequences of blocking starch biosynthesis in the green alga Chlamydomonas reinhardtii sta6 mutant. *Plant J.* **81,** 947-960

Larrouy-Maumus, G., Kelly, G. and de Carvalho, L. P. (2014) Chemical mechanism of glycerol 3-phosphate phosphatase: pH-dependent changes in the rate-limiting step. *Biochemistry* **53**, 143-151

Lee, Y. J., Jeschke, G. R., Roelants, F. M., Thorner, J. and Turk, B. E. (2012) Reciprocal phosphorylation of yeast glycerol-3-phosphate dehydrogenase in adaptation to distinct types of stress. *Mol. Cell Biol.* **32**, 4705-4717

Li, Y., Han, D., Hu, G., Sommerfeld, M. and Hu, Q. (2010) Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii. Biotechnol. Bioeng.* **107**, 258-268

Mastrobuoni, G., Irgang, S., Pietzke, M., Assmus, H. E., Wenzel, M., Schulze, W. X. and Kempa, S. (2012) Proteome dynamics and early salt stress response of the photosynthetic organism *Chlamydomonas reinhardtii*. *BMC Genomics* **13**, 215

Merchant, S. S., Prochnik, S. E., Vallon, O., Harris, E. H., Karpowicz, S. J., Witman, G. B., Terry, A., Salamov, A., Fritz-Laylin, L. K., Maréchal-Drouard, L., Marshall, W. F., Qu, L. H., Nelson, D. R., Sanderfoot, A. A., Spalding, M. H., Kapitonov, V. V., Ren, Q., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S. M., Grimwood, J., Schmutz, J., Cardol, P,. Cerutti, H., Chanfreau, G., Chen, C. L., Cognat, V., Croft, M.T., Dent, R., Dutcher, S., Fernández,

E., Fukuzawa, H., González-Ballester, D., González-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K., Kalanon, M., Kuras, R., Lefebvre, P. A., Lemaire, S. D., Lobanov, A. V., Lohr, M., Manuell, A., Meier, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J. V., Moseley, J., Napoli, C., Nedelcu, A. M., Niyogi, K., Novoselov, S. V., Paulsen, I.T., Pazour, G., Purton, S., Ral, J. P., Riaño-Pachón, D. M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R., Wilson, N., Zimmer, S. L., Allmer, J., Balk, J., Bisova, K., Chen, C. J., Elias, M., Gendler, K., Hauser, C., Lamb, M. R., Ledford, H., Long, J. C., Minagawa, J., Page, M. D., Pan, J., Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A. M., Yang, P., Ball, S., Bowler, C., Dieckmann, C.L., Gladyshev, V. N., Green, P., Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R. T., Brokstein, P., Dubchak, I., Goodstein, D., Hornick, L., Huang, Y. W., Jhaveri, J., Luo, Y., Martínez, D., Ngau, W. C., Otillar, B., Poliakov, A., Porter, A., Szajkowski, L., Werner, G., Zhou, K., Grigoriev, I. V., Rokhsar, D. S. and Grossman, A. R. (2007) The Chlamydomonas genome reveals the evolution of key animal and plant functions. *Science* **318**, 245-250

Morales-Sánchez, D., Tinoco-Valencia, R., Kyndt, J. and Martinez, A. (2013) Heterotrophic growth of *Neochloris oleoabundans* using glucose as carbon source. *Biotech Biofuels* **6**, 100

Morales-Sánchez, D., Tinoco-Valencia, R., Caro-Bermudez, M. A. and Martinez, A. (2014) Culturing *Neochloris oleoabundans* microalga in a nitrogen-limited, heterotrophic fed-batch system to enhance lipid and carbohydrate accumulation. *Algal Res.* **5**, 61-69

Msanne, J., Xu, D., Konda, A. R., Casas-Mollano, J. A., Awada, T., Cahoon, E. B. and Cerutti, H. (2012) Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. *Phytochemistry* **75**, 50-59

Oren, A. (2016) Glycerol metabolism in hypersaline environments. *Environ. Microbiol*. Aug 11. doi: 10.1111/1462-2920.13493. [Epub ahead of print]

Pedro, H., Maheswari, U., Urban, M., Irvine, A., G., Cuzick, A., McDowall, M. D., Staines, D.

M., Kulesha, E., Hammond-Kosack, K. E. and Kersey, P. J. (2016) PhytoPath: an integrative resource for plant pathogen genomics. *Nucleic Acids Res.* **44**, 688-693

Peeraer, Y., Rabijns, A., Collet, J. F. and Van Schaftingen, E. (2004) How calcium inhibits the magnesium-dependent enzyme human phosphoserine phosphatase. *Eur. J. Biochem.* **271**, 3421-3427

Plucinak, T. M., Horken, K.M., Jiang, W., Fostvedt, J., Nguyen, S. T. and Weeks, D. P. (2015) Improved and versatile viral 2A platforms for dependable and inducible high-level expression of dicistronic nuclear genes in *Chlamydomonas reinhardtii*. *Plant J*. **82**, 717-729

Polle, J. E., Neofotis, P., Huang, A., Chang, W., Sury, K. and Wiech, E. M. (2014) Carbon partitioning in green algae (Chlorophyta) and the enolase enzyme. *Metabolites* **4**(3), 612-628

Pulz, O. and Gross, W. (2004) Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.*, 65, 635-648.

Radakovits, R., Jinkerson, R. E., Darzins, A. and Posewitz, M. C. (2010) Genetic engineering of algae for enhanced biofuel production. *Eukaryot. Cell* **9**, 486-501

Riekhof, W. R., Sears, B. B. and Benning, C. (2005) Annotation of genes involved in glycerolipid biosynthesis in Chlamydomonas reinhardtii: discovery of the betaine lipid synthase BTA1Cr. *Eukaryotic Cell.* **4**, 242-252

Rohr, J., Sarkar, N., Balenger, S., Jeong, B. R. and Cerutti, H. (2004) Tandem inverted repeat system for selection of effective transgenic RNAi strains in *Chlamydomonas. Plant J.* **40**, 611-621

Sadka, A., Lers, A., Zamir, A. and Avron, M. (1989) A critical examination of the role of the novo protein synthesis in the osmotic adaptation of the halotolerant alga *Dunaliella*. *FEBS Lett.* **244**, 93-98

Sambrook, J. and Russell, D. W. (2001) Molecular cloning – A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Schmollinger, S., Muhlhaus, T., Boyle, N. R., Blaby, I. K., Casero, D., Mettler, T., Moseley J. L., Kropat, J., Sommer, F., Strenkert, D., Hemme, D., Pellegrini, M., Grossman, A. R., stitt, M.,

Schroda, M. and Merchant, S. S. (2014) Nitrogen-sparing mechanisms in *Chlamydomonas* affect the transcriptome, the proteome, and photosynthetic metabolism. *Plant Cell* **26**, 1410-1435

Siaut, M., Cuine, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Beyly, A., Beisson, F., Triantaphylides, C., Li-Beisson, Y. and Peltier, G. (2011) Oil accumulation in the model green alga *Chlamydomonas reinhardtii:* characterization, variability between common laboratory strains and relationship with starch reserves. *BMC Biotechnol.* **11**, 7

Spolaore, P., Joannis-Cassan, C., Duran, E. and Isambert, A. (2006) Commercial applications of microalgae. *J. Biosci. Bioeng.* **101**, 87-96

Sueoka, N. (1960) Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA* **46**, 83-91

Terashima, M., Specht, M. and Hippler, M. (2011) The chloroplast proteome: a survey from the *Chlamydomonas reinhardtii* perspective with a focus on distinctive features. *Curr. Genet.* **57**, 151-168

Tsai, C. H., Zienkiewicz, K., Amstutz, C. L., Brink, B. G., Warakanont, J., Roston, R. and Benning, C. (2015) Dynamics of protein and polar lipid recruitment during lipid droplet assembly in Chlamydomonas reinhardtii. *Plant J.* **83,** 650-660

van Dijk, K., Marley, K. E., Jeong, B. R., Xu, J., Hesson, J., Cerny, R. L., Waterborg, J. H. and Cerutti, H. (2005) Monomethyl histone H3 lysine 4 as an epigenetic mark for silenced euchromatin in *Chlamydomonas*. *Plant Cell* **17**, 2439-2453

Van Schaftingen, E. and Van Laere, A. J. (1985) Glycerol formation after the breaking of dormancy of *Phycomyces blakesleeanus* spores. Role of an interconvertible glycerol-3-phosphatase. *Eur. J. Biochem.* **148**, 399-404

Vigeolas, H., Waldeck, P., Zank, T. and Geigenberger, P. (2007) Increasing seed oil content in oil-seed rape (*Brassica napus L.*) by over-expression of a yeast glycerol-3-phosphate dehydrogenase under the control of a seed-specific promoter. *Plant Biotechnol. J.* **5**, 431-441

Wang, Z. X., Zhuge, J., Fang, H. and Prior, B. A. (2001) Glycerol production by microbial fermentation: a review. *Biotechnol. Adv.* **19**, 201-223

Wegmann, K. (1986) Osmoregulation in eukaryotic algae. *FEMS Microbiol. Rev.* **39**, 37-43

Work, V. H., Radakovits, R., Jinkerson, R. E., Meuser, J. E., Elliott, L. G., Vinyard, D. J., Laurens, L. M., Dismukes, G. C. and Posewitz, M. C. (2010) Increased lipid accumulation in the Chlamydomonas reinhardtii sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. *Eukaryot. Cell* **9**, 1251-1261

Zienkiewicz, K., Du, Z. Y., Ma, W., Vollheyde, K. and Benning, C. (2016) Stress-induced neutral lipid biosynthesis in microalgae – Molecular, cellular and physiological insights. *Biochim. Biophys. Acta* **1861**(9 Pt B), 1269-1281

FIGURE LEGENDS

Figure 1. Glycerol-3-phosphate dehydrogenases (GPDs) encoded in the *C. reinhardtii* genome. (a) Schematic representation of the *Chlamydomonas* GPD homologs. Color bars indicate conserved protein domains. The length of each protein, in amino acids, is indicated by the numbers at the end. For GPD3, gene model ova_au5.g2013_t1 was used in all analyses. For other proteins, Phytozome v11.0 gene models were used in the analyses. (b) Maximum-likelihood phylogenetic tree based on the alignment of the canonical GPD domains of protein sequences from *C. reinhardtii* (bold), green algae (green branches) and several eukaryotes and prokaryotes. Numbers on branches indicate the percentage of 1,000 bootstrap replications supporting a particular node. Abbreviations: Ath, *Arabidopsis thaliana*; Bpr, *Bathycoccus prasinos*; Cel, *Caenorhabditis elegans*; Cme, *Cyanidioschyzon merolae*; Cre, *Chlamydomonas reinhardtii*; Cso, *Chlorella sorokiniana*; Csu, *Coccomyxa subellipsoidea*; Cva, *Chlorella variabilis*; Dfr, *Desulfovibrio frigidus*; Dhy, *Desulfovibrio hydrothermalis*; Dme, *Drosophila melanogaster*; Dre, *Danio rerio*; Dsa, *Dunaliella salina*; Dvi, *Dunaliella viridis*; Dzo, *Desulfovibrio zosterae*; Eco, *Escherichia coli*; Ehu, *Emiliania huxleyi*; Gel,

Geopsychrobacter electrodiphilus; Hoc, *Haliangium ochraceum*; Hsa, *Homo sapiens*; Mko, *Methylomonas koyamae*; Mma, *Maricaulis marinus*; Mtu, *Methylobacter tundripaludum*; Mva, *Methylomarinum vadi*; Ncr, *Neurospora crassa*; Olu, *Ostreococcus lucimarinus*; Ota, *Ostreococcus tauri*; Pae, *Pseudomonas aeruginosa*; Pca, *Pelobacter carbinolicus*; Ppa, *Physcomitrella patents*; Ppr, *Pelobacter propionicus*; Pso, *Phytophthora sojae*; Ptr, *Phaeodactylum tricornutum*; Pul, *Pythium ultimum*; Sce, *Saccharomyces cerevisiae*; Smi, *Scytonema millei;* Tin, *Thermodesulfatator indicus*; Tps, *Thalassiosira pseudonana*; Vca, *Volvox carteri*. (c) Maximum-likelihood phylogenetic tree based on the alignment of the HAD-like domains of GPD2-like and PSP protein sequences from *C. reinhardtii* (bold), green algae (green branches) and several eukaryotes and prokaryotes. (d) Multiple sequence alignment of HAD-like domains. The first column indicates the protein and species names. GPD2, GPD3 and GPD4, *C. reinhardtii* GPDs; GPD_Dun, *Dunaliella salina* GPD; GPD_Chlo, *Chlorella variabilis* GPD; PSP_Myc, *Mycobacterium avium* phosphoserine phosphatase; PSP_Meth: *Methanococcus jannaschii* phosphoserine phosphatase. Numbers indicate the amino acid residues to the N-terminal end of each protein and numbers in parentheses indicate the amino acid residues in gaps between the aligned segments. The highlighted amino acids are conserved in the HAD superfamily (Peeraer *et al.*, 2004).

Figure 2. Enzymatic activities of *Chlamydomonas* GPD1 and GPD2. Recombinant proteins of the wild type GPD2 isoform, two mutant versions with substitutions in the PSP domain (GPD2-NV2Mut and GPD2-NVN3Mut) and canonical GPD1 were produced by *in vitro* transcription/translation in a continuous-exchange cell-free wheat germ system. An aliquot of the wheat germ lysate (WGL), supplied with an empty vector, was used as a negative control. Values shown are the mean ± SD of three independent experiments. (a) Glycerol-3-phosphate dehydrogenase activity of the recombinant proteins determined as oxidation of NADH. (b) Phosphatase activity of the recombinant proteins when using G3P as the substrate. (c) Phosphatase activity of the recombinant proteins when using DHAP and NADPH as the substrates. (d) Phosphatase activity of the recombinant proteins when using DHAP and NADH as the substrates. (e) Effect of the absence of NADH, in reactions containing DHAP, on the phosphatase activity of the recombinant proteins.

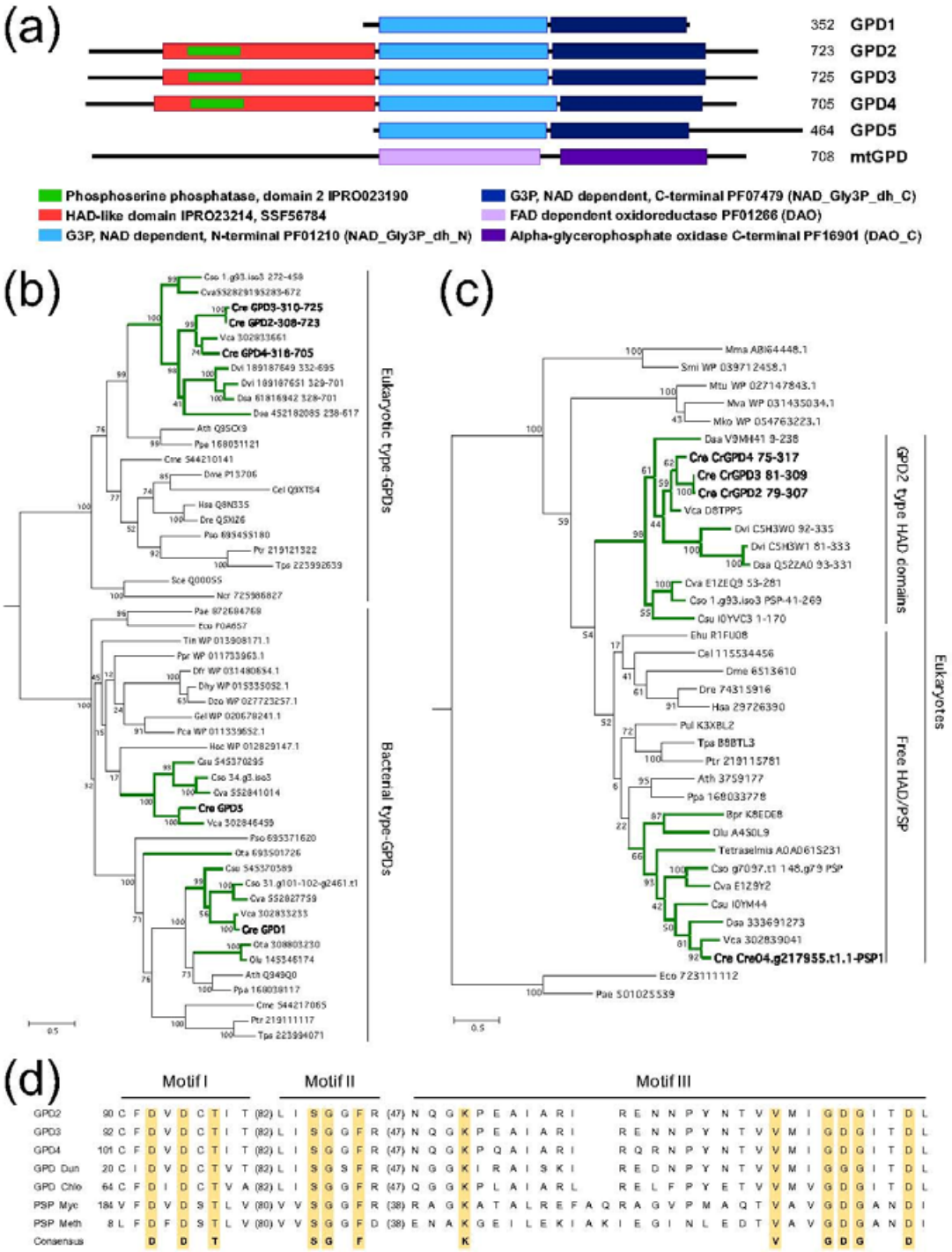
Figure 3. Glycerol production by recombinant GPD2 *in vitro*. Recombinant proteins were produced as indicated in the legend to Figure 2. An aliquot of the wheat germ lysate (WGL), programmed with an empty vector, was used as a negative control. Values shown are the mean ± SD of three independent experiments. (a) Glycerol production by the recombinant proteins when using G3P as the substrate. (b) Glycerol production by the recombinant proteins when using DHAP and NADPH as the substrates. (c) Glycerol production by the recombinant proteins when using DHAP and NADH as the substrates. (d) Effect of the absence of NADH, in reactions containing DHAP, on the production of glycerol by the recombinant proteins.

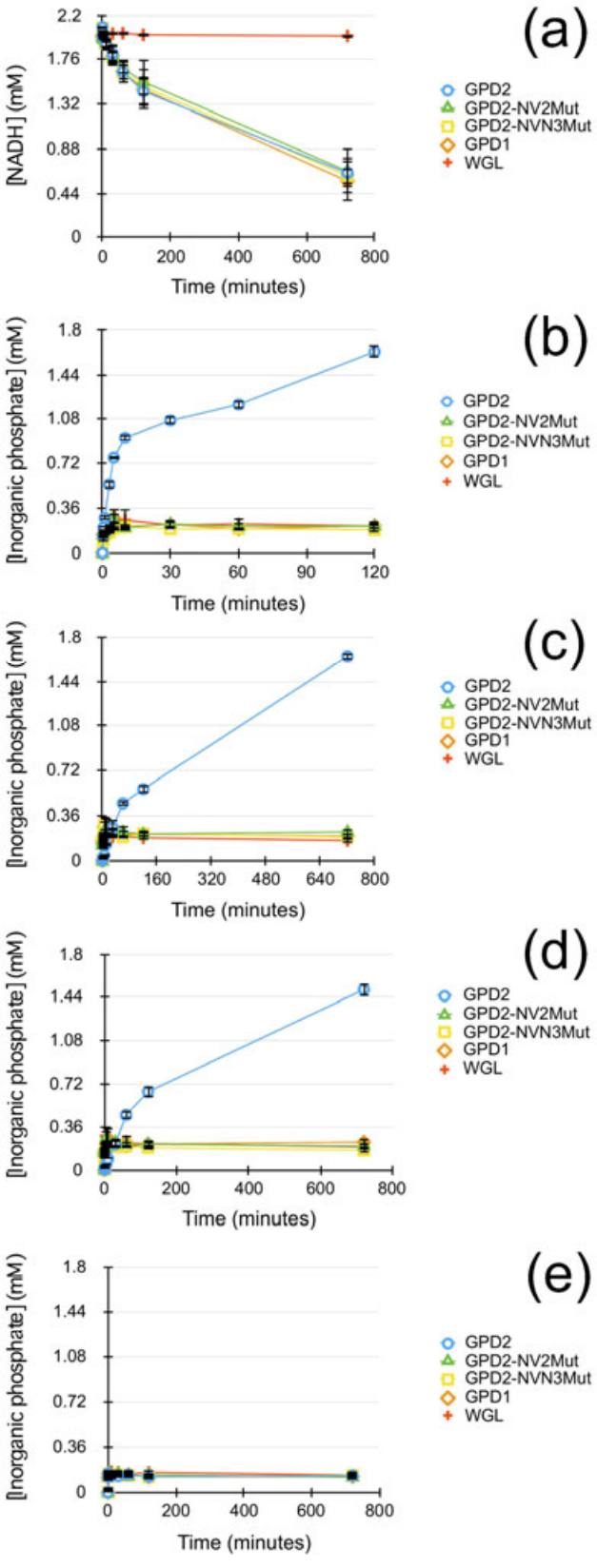
Figure 4. Subcellular localization of *Chlamydomonas* GPD1 and GPD2. Recombinant GPD2 or GPD1 polypeptides, with the fluorescent protein mCherry fused at their carboxyl ends, were expressed under the control of the *PsaD* promoter in transgenic strains derived from CC-124. Representative images of transgenic and wild type cells, acquired by laser scanning confocal microscopy, are shown. Chlorophyll autofluorescence is shown in red and mCherry fluorescence is shown in green. Wild type CC-124 was used to verify the absence of any background signal in the mCherry channel.

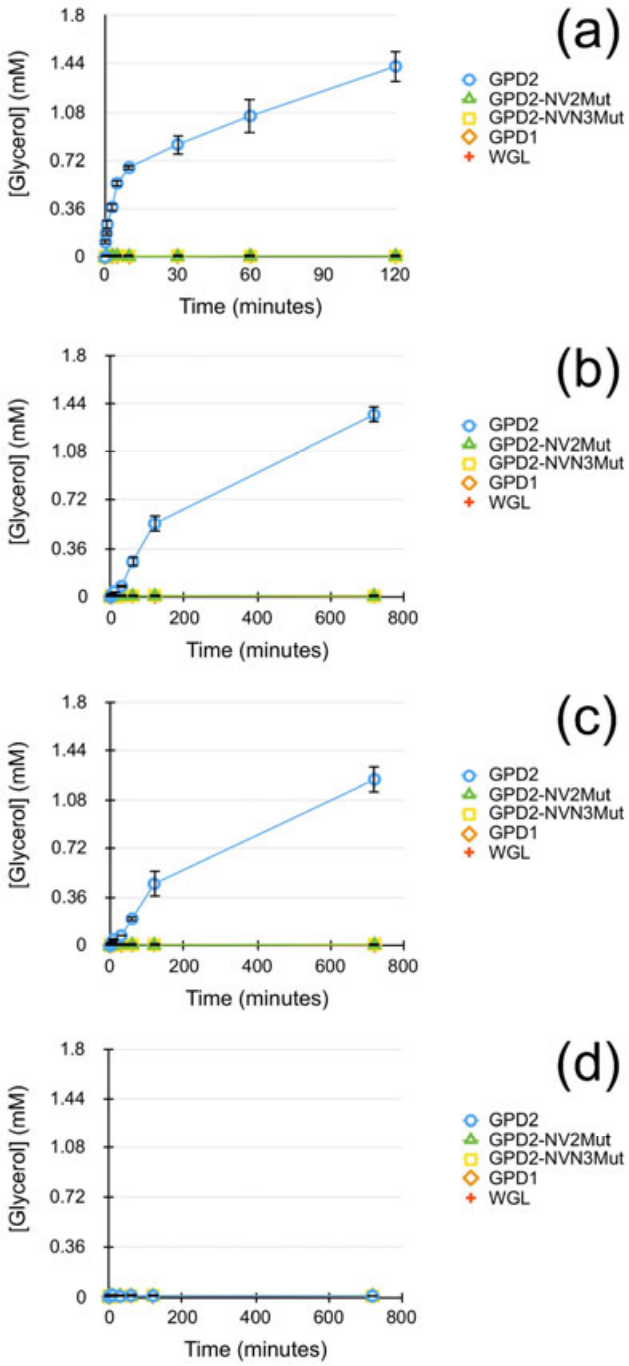
Figure 5. Triacylglycerol (TAG) and glycerol content in wild type CC-125 and *GPD2/GPD3* RNAi strains (Ri16 and Ri18) subject to nitrogen deprivation or high salinity. Expression of the *GPD2* and *GPD3* genes was simultaneously suppressed by RNAi in transgenic strains derived from *Chlamydomonas* CC-125. For nutrient deprivation experiments, cells were incubated for two days in HS medium, either nutrient replete (+N) or nitrogen depleted (-N) [(a) and (c)]. For high salinity experiments, cells were incubated for 6 hours in standard HS medium (0 mM NaCl) or in medium supplemented with NaCl (100 mM NaCl) [(b) and (d)]. Values indicate the mean ± SD of three independent experiments. Different lowercase letters indicate a significant difference among means (one way ANOVA with post-hoc Tukey HSD test, $P<0.01$). (a) - (b) Total fatty acids (FAMEs) in triacylglycerols. (c) - (d) Total glycerol content.

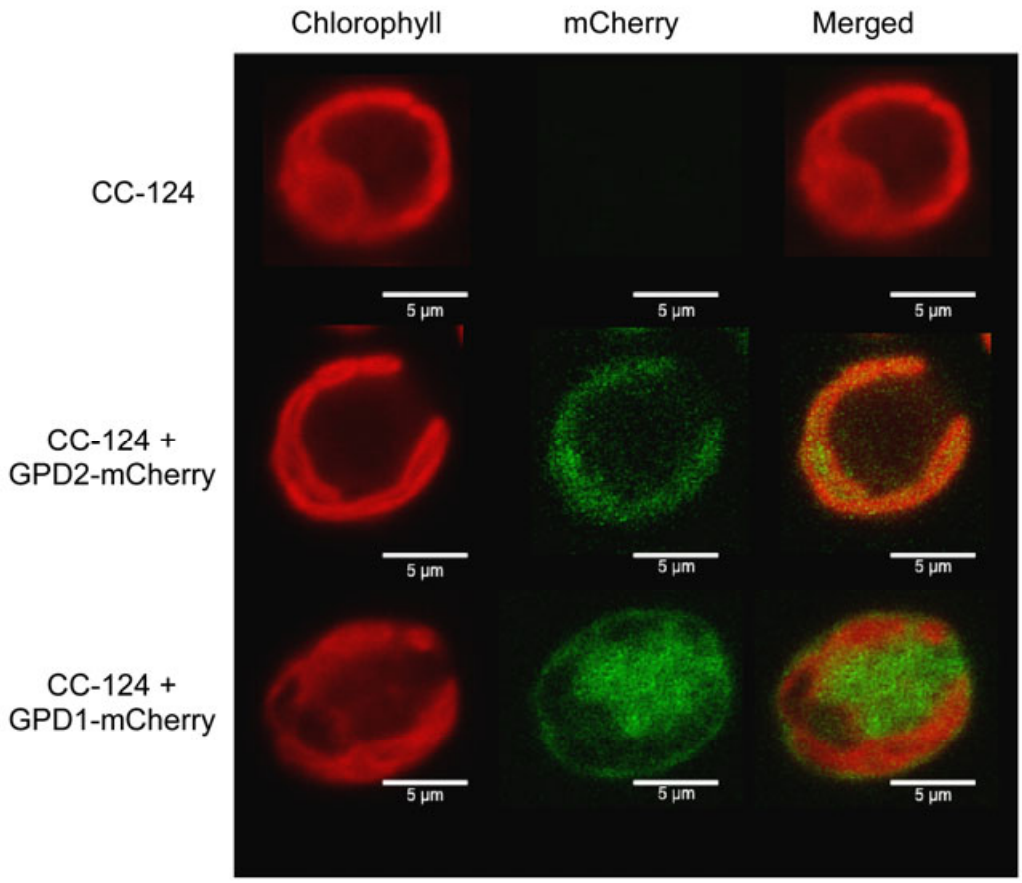
Figure 6. Expression of genes encoding enzymes of glycerol metabolism in wild type CC-125 and *GPD2/GPD3* RNAi strain Ri16 subject to hyperosmotic stress (+Na) or nitrogen deprivation (-N). Cells were cultured as indicated in the legend to Figure 5. In addition to *GPD2* and *GPD4*, examined genes included *GLYCEROL KINASE* (*GK*), *ALDO/KETO REDUCTASE 3* (*AKR3*, encoding a putative glycerol 2-dehydrogenase), *DIHYDROXYACETONE KINASE 1* (*DAK1*) and *GLYCEROL-3-PHOSPHATE PHOSPHATASE* (*GPP*). Amplification of transcripts from *ACT1* (encoding actin) was used as a control for normalization purposes. (a) Relative transcript abundance analyzed by quantitative RT-PCR. Values shown, normalized to those in CC-125 grown in standard HS medium, are the mean ± SD of three independent experiments. Samples indicated with an asterisk are significantly different from the control ($P<0.01$) in a two tailed Student's t test. (b) Transcript abundance was examined by semi-quantitative RT-PCR. The panels show representative reverse images of agarose resolved RT-PCR products stained with ethidium bromide.

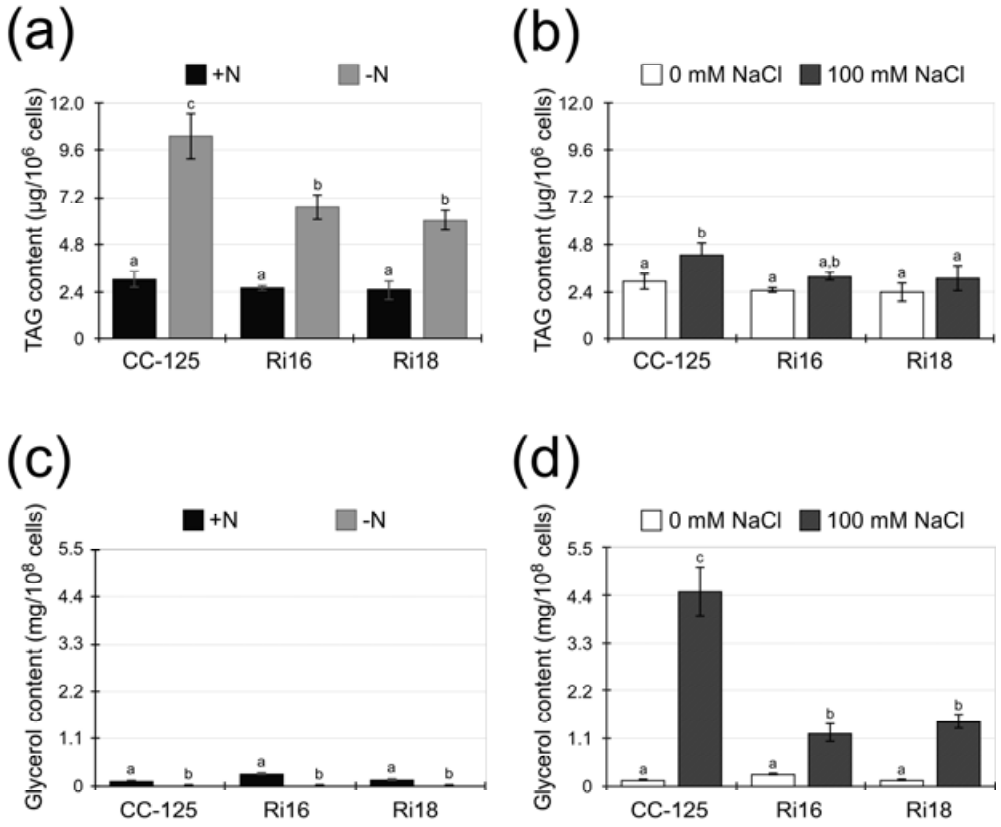
Figure 7. Glycerol metabolism (yellow box) in *Chlamydomonas reinhardtii*, reconstructed on the basis of the annotated genome, and connections to starch and triacylglycerol synthesis. The lower part of glycolysis appears to occur exclusively in the cytosol in *C. reinhardtii* (Terashima *et al.*, 2011; Johnson and Alric, 2013; Polle *et al.*, 2014). Metabolites: 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; FFA, free fatty acid; G1P, glucose-1-phosphate; G3P, glycerol-3-phosphate; GAP, glyceraldehyde-3-phosphate; Gly, glycerol; PEP, phosphoenolpyruvate; Pyr, pyruvate. Enzymes: AGPase, ADP glucose pyrophosphorylase; AKR3, aldo/keto reductase 3 (putative glycerol 2-dehydrogenase); DAK1, dihydroxyacetone kinase 1; ENO, enolase; FAT, fatty acyl-ACP thioesterase; GK, glycerol kinase; GPD1/2/3/4/5, $NAD(P)^+$-dependent glycerol-3-phosphate dehydrogenases; GPP, glycerol-3-phosphate phosphatase; LACS, long-chain acyl-CoA synthetase; PDC, pyruvate dehydrogenase complex; PYK, pyruvate kinase. The abbreviations for enzymes are written in red. Additional abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; ER, endoplasmic reticulum.



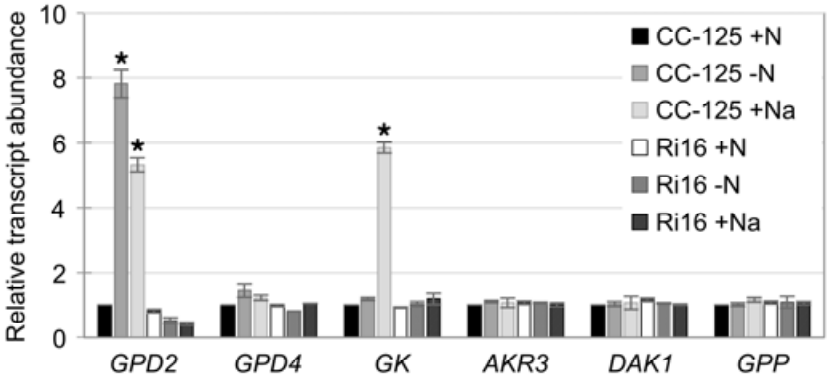








(a)



(b)