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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

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Abstract Glycerol-3-phosphate dehydrogenase (GPDH) catalyzes the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) and plays a central role in the synthesis of glycerol and triacylglycerides (TAGs). Osmotic stress has been shown to induce the accumulation of glycerol and TAGs in the green microalga *Chlamydomonas reinhardtii*. In a previous study, we identified three *GPDH* homologs (*CrGPDH1*, *CrGPDH2*, and *CrGPDH3*) in this microalga. We found that *CrGPDH2* and *CrGPDH3* were expressed in response to 200 mM NaCl treatment, suggesting that these two genes play roles in glycerol and TAGs synthesis and in osmotic stress tolerance. In this study, we report on the functional characterization of *CrGPDH2* and *CrGPDH3*. A concentration of NaCl as low as 5 mM for 5 min was sufficient to induce the expressions of both genes. We mapped the cDNA ends of *CrGPDH2* and *CrGPDH3* using RLM-RACE and cloned their full-length cDNAs. The expression of these two cDNAs in the *Saccharomyces cerevisiae gpd1Δgpd2Δ* double mutant confirmed that both CrGPDH2 and CrGPDH3 have GPDH activity. The genetic complementation analysis revealed that *CrGPDH2* and *CrGPDH3* were able to restore glycerol production and rescue the salt sensitivity of this mutant. Compared with *CrGPDH3*, *CrGPDH2* conferred higher glycerol production and greater salt tolerance when expressed in the *gpd1Δgpd2Δ* double mutant. Together, these findings show that *CrGPDH2* and *CrGPDH3* encode functional homologs of the *S. cerevisiae GPD1* gene that is involved in glycerol synthesis and osmotic stress tolerance.

Keywords *Chlamydomonas reinhardtii* · Glycerol-3-phosphate dehydrogenase (GPDH) · Glycerol synthesis · Osmotic stress

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Introduction

Chlamydomonas reinhardtii is a freshwater unicellular green alga that is capable of synthesizing glycerol as an osmoregulatory metabolite in response to osmotic stress. This alga can tolerate up to 200 mM NaCl (León and Galván 1994). *C. reinhardtii* is also able to accumulate triacylglycerides (TAGs) in response to NaCl ranging from 20 to 100 mM (Siaut et al. 2011). Glycerol is an important osmoregulatory solute induced by osmotic up-shocks in some halophilic green algae, including several species of *Chlamydomonas* and *Dunaliella*, as well as almost all yeast species studied to date (Ben-Amotz and Avron 1983; Avron 1986; Hohmann 2002). However, the microalgal genes involved in glycerol and lipid biosynthesis have not yet been fully characterized. Glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) catalyzes the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) using NADH as an electron donor. Then, G3P is dephosphorylated to glycerol by the action

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of glycerol phosphatase (GPP) (Gancedo et al. 1968; Ghoshal et al. 2002). G3P plays an important role in adaptation to osmotic stress and is a key metabolite in the synthesis of TAGs (Vigeolas et al. 2007). *GPDH* genes were initially studied in the yeast *Saccharomyces cerevisiae*, wherein *GPD1* and *GPD2*, which encode different GPDH enzymes, are responsible for glycerol synthesis (Ansell et al. 1997). Studies have revealed that the *GPD1* gene product is the most relevant form under osmotic stress conditions, whereas transcription of the *GPD2* gene is induced under anaerobic conditions (Ansell et al. 1997). The overexpression of *S. cerevisiae GPD1* in *Brassica napus* L. resulted in a 40 % increase in the lipid content of mature seeds of this commercial oilseed crop (Vigeolas et al. 2007).

Genes encoding GPDH have been cloned and characterized from the microalgae *Dunaliella salina* and *Dunaliella viridis* (He et al. 2007, 2009; Cai et al. 2013). Their expressions were shown to increase in response to a high salt concentration (He et al. 2007, 2009). The relationship between glycerol biosynthesis and genetic expression of *GPDH* genes has been studied in these microalgae (He et al. 2007, 2009; Chen et al. 2011). Recently, we reported the in silico and expression characterization of three *GPDH* genes from *C. reinhardtii*; *CrGPDH1*, *CrGPDH2*, and *CrGPDH3* (Herrera-Valencia et al. 2012). The expression analysis revealed constitutive expression of *CrGPDH1*, while *CrGPDH2* and *CrGPDH3* were expressed in response to 200 mM NaCl, suggesting that these two genes could be involved in glycerol production and osmotic stress tolerance as well as TAGs synthesis in this microalga (Herrera-Valencia et al. 2012). In the present study, we have extended these previous findings by analyzing the expressions of *CrGPDH2* and *CrGPDH3* in response to lower NaCl concentrations and over time. We report for the first time the cloning and functional characterization of *CrGPDH2* and *CrGPDH3* cDNAs. Both *CrGPDH2* and *CrGPDH3* cDNAs were expressed in the *S. cerevisiae gpd1Δgpd2Δ* double mutant and their products showed GPDH activity, which was correlated with glycerol production. Both of the cDNAs restored the salt tolerance of the salt-sensitive double mutant in YPD medium supplemented with 500 mM NaCl, but only *CrGPDH2* restored its salt tolerance in medium containing 800 mM NaCl.

Materials and methods

Chlamydomonas reinhardtii CC-125 (mt+) was obtained from the Chlamydomonas Center (www.chlamy.org). Axenic cultures were maintained on Tris-acetate-phosphate (TAP) (Harris 1989) at 25 °C±2 under a 16 h/8 h (light/dark) cycle at light intensity of 90 μmol photons m^{-2} s^{-1} on a rotary shaker (140 rpm). Exponentially growing cells were inoculated into 250-mL Erlenmeyer flasks containing 50 mL liquid TAP medium at an initial cell concentration of 10,000 cells mL^{-1} and were allowed to grow for 7 days before the appropriate NaCl treatment was applied. To evaluate the effect of osmotic stress on the expression of *CrGPDH2* and *CrGPDH3*, cells were incubated in medium containing 0, 5, 10, 15, 20, 25, 50, 100, 150, and 200 mM NaCl for 2 h. Cells were also incubated in medium containing either 5 or 200 mM NaCl for different times (5, 10, 60, and 120 min) and cells without NaCl treatment were used as control (time 0). The cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80 °C until further analysis. All reagents and solvents were of analytical grade.

Yeast strains, medium, and culture conditions The *S. cerevisiae* strains wild-type *W303-1A* (*MATa leu2-3*/*112*; *ura3-1*; *trp1-1*; *his3-11*/*15*; *ade2-1*; *can1-100*; *GAL*; *SUC2*) and the *gpd1Δgpd2Δ* double mutant (*W303-1A*; *gpd1Δ*::*TRP1*; *gpd2Δ*::*URA3*) were kindly provided by Professor Peter Dahl, Gothenburg University, Germany (Ansell et al. 1997). Strains were cultivated routinely on a rotary shaker (250 rpm) in YPD medium (Sigma-Aldrich, USA) or Synthetic Leu-dropout medium (Clontech Laboratories, Inc., USA) for selection at 30 °C.

Gene expression analysis by RT-PCR To evaluate the expressions of *CrGPDH2* and *CrGPDH3*, *C. reinhardtii* cultures were exposed to various NaCl treatments, and then total RNA was extracted using TRIzol reagent (Invitrogen, USA). The RNA was treated with RNase-free DNase (Promega, USA) following the manufacturer's instructions. The cDNAs were synthesized using 10 μg total RNA and 200 U SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. The PCR analyses were conducted as described by Herrera-Valencia et al. (2012). The thermal cycling conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by final extension at 72 °C for 10 min.

Cloning of *C. reinhardtii* CrGPDH2 and CrGPDH3 cDNAs Mapping of the 5′ and 3′ ends of *CrGPDH2* and *CrGPDH3* was performed by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) using the GeneRacer kit (Invitrogen). Total RNA was extracted using TRIzol reagent (Invitrogen) and then used for cDNA synthesis. First-strand synthesis was carried out with oligo(dT) primer (Invitrogen). The PCR primers (Table S1 in Supplementary material) were designed based on the in silico *CrGPDH2* and *CrGPDH3* sequences obtained from the *C. reinhardtii* genome sequence v5.3.1 at Phytozome (http://www.phytozome.net/chlamy.php). The 5′ RACE was performed using the specific reverse primers shown in Table S1 (Supplementary material) in combination with the GeneRacer 5′ primer (Invitrogen). The resulting amplicons

were subjected to a second round of PCR using specific nested 5′ primers (Table S1, Supplementary material) in combination with the GeneRacer nested 5′ primer (Invitrogen). The 3′ RACE was performed using the specific forward primers shown in Table S1 (Supplementary material) in combination with the GeneRacer 3′ primer (Invitrogen). The PCR products were subjected to a second round of PCR using the specific nested 3′ primers shown in Table S1 (Supplementary material) in combination with the GeneRacer nested 3′ primer (Invitrogen). The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, USA) and cloned into the pGEM-T Easy vector (Promega). Plasmids were sequenced at the Clemson University Genomics Institute. Sequences were assembled, edited, and aligned using the programs SeqMan, EDIT, and MegAlign, respectively, in the Lasergene software package version 7.2 (DNASTAR, USA). The entire open reading frames (ORFs) of *CrGPDH2* and *CrGPDH3* were amplified by RT-PCR using the primers shown in Table S2 (Supplementary material), in combination with the high-fidelity Expand Long Template PCR system (Roche, USA). First-round PCR was carried out using the cDNA template obtained by RLM-RACE, using a forward primer that bound both *CrGPDH2* and *CrGPDH3* and a reverse primer that selectively bound *CrGPDH2* or *CrGPDH3* (Table S2, Supplementary material). Then, appropriate PCR products were used as the template to amplify either *CrGPDH2* or *CrGPDH3* using the combination of nested primers shown in Table S2 (Supplementary material). Nested PCR products were cloned and sequenced, and then sequences were assembled, edited, and aligned as described above. The percentage of amino acid identity between sequences was determined by ClustalW alignment with MegAlign program in the Lasergene software package version 7.2.1 (DNASTAR, USA). GPDH domain alignment was performed with ClustalX V2.0 and illustrated with BoxShade v3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Yeast transformation The *S. cerevisiae gpd1Δgpd2Δ* double mutant was used for genetic transformation. The yeast strong constitutive GPD promoter (from the glyceraldehyde-3-phosphate dehydrogenase gene) and the CYC1 terminator, both from p424GPD plasmid (Mumberg et al. 1995), were cloned into pGADT7 AD (Clontech) using the restriction enzyme sites *SacI* and *KpnI* to generate the plasmid psGC (LEU2, GPD promoter, CYC1 terminator, AmpR) for yeast transformation. The *CrGPDH2* and *CrGPDH3* cDNAs were cloned into *BamHI*/*EcoRI* sites of psGC, yielding psGPDH2 and psGPDH3, respectively. The *S. cerevisiae gpd1Δgpd2Δ* double mutant was transformed using the Yeastmaker™ Yeast Transformation Kit (Clontech) according to the manufacturer's protocol. The transformants were cultivated in Leu-dropout medium (Clontech) for selection at 30 °C. The *gpd1Δgpd2Δ* double mutant cells were transformed either with psGC (empty plasmid), psGPDH2, psGPDH3, or with both plasmids (psGPDH2 and then psGPDH3).

Protein extraction Cells from 30-mL samples of yeast cultures (OD_{610}=0.6) were harvested by centrifugation, washed twice in ice-cold triethanolamine buffer (Sigma-Aldrich) and frozen in liquid nitrogen. The parental strain *W303-1A* was cultured until the OD_{610} reached 0.6 and then supplemented with 200 mM NaCl for 4 h to induce *GPD1* expression. Samples were homogenized for 1 min in 1.5 mL protein extraction buffer [20 mM triethanolamine and 1 mM dithiothreitol (DTT)], freshly supplemented with 2 mM β-mercaptoethanol and 0.5 μL of protease inhibitor mix per mL (Sigma-Aldrich), using an Ultra Turrax T25 Polytron (Janke & Kunkel, Germany). Then, the cells were disrupted at 4 °C with 5-s bursts of ultrasonic sound at 5-s intervals over a total period of 25 s using an XL-2000 Sonicator 3000 (Misonix, USA). Unbroken cells and debris were removed by centrifugation at 4 °C for 30 min at 10,000 rpm, and the supernatant was stored at −80 °C until further analysis. The total protein concentration was determined using the Quick Start Bradford Protein Assay Kit 1 (Bio-Rad) with bovine serum albumin as the standard.

GPDH enzyme assay The activity of GPDH was measured as described by Vigeolas et al. (2007) with some modifications. The assay mixture contained 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffer, 1 mM DHAP, 0.2 mM NADH, and different amounts of protein (20, 40, 60, and 80 μg) in a final volume of 2 mL. The reaction was conducted at 30 °C and was initiated by adding the protein extract. The decrease in NADH was monitored by measuring the change in absorbance at 340 nm over 20 min using a spectrophotometer (model DU 650, Beckman Coulter, Inc., USA). One unit (U) of enzyme activity was defined as the amount of enzyme reducing 1 μmol NADH per min, calculated using the extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1} (Galafassi et al. 2013). Specific activity was calculated as units per milligram of protein (U mg^{-1} protein). Activity was calculated from the linear rate during the initial reaction period.

Glycerol quantification and NaCl tolerance assay The *gpd1Δgpd2Δ* double mutant yeast strain is deficient in glycerol synthesis and sensitive to osmotic stress; therefore, it was used for the genetic complementation study as described previously by Lee et al. (2008) and Lenassi et al. (2011). To measure glycerol content, yeast cells were grown in YPD medium (OD_{610}=0.6), harvested by centrifugation at 1000×*g* for 5 min, and resuspended in medium supplemented with 200 mM NaCl. Cells were collected after 4 or 12 h incubation at 30 °C with shaking (250 rpm), and the glycerol concentration was measured as described by Watanabe et al.

(2004), using free glycerol reagent (Sigma-Aldrich) according to the manufacturer's protocol. Reproducibility was confirmed in three independent experiments. For the NaCl tolerance assays, yeast cells were cultured in YPD medium (Lee et al. 2008) until the OD_{610} reached 0.6, and then 20 μL of ten-fold serial dilutions of each yeast culture was spotted onto YPD medium supplemented with NaCl at indicated concentrations. Plates were incubated for 4 days at 30 °C. Reproducibility was confirmed in two independent experiments.

Results

Expression analysis of *CrGPDH2* and *CrGPDH3* in response to different NaCl treatments

The expression analysis of *CrGPDH2* and *CrGPDH3* was performed by RT-PCR (Fig. 1). Very low transcript levels of both *CrGPDH2* and *CrGPDH3* were detected at 0 mM NaCl, but higher transcript levels were detected after all of the 2-h NaCl treatments, with NaCl concentrations as low as 5 mM and up to 200 mM NaCl. The expected amplicons of 191 and 280 bp were detected for *CrGPDH2* and *CrGPDH3*, respectively (Fig. 1a). A time-course expression analysis showed that both *CrGPDH2* and *CrGPDH3* were induced after only 5 min in 5 and 200 mM NaCl (Fig. 1b, c). The expression profile for the *Actin* gene from *C. reinhardtii* (*CrActin*) was similar in all NaCl treatments evaluated, indicating that there were similar amounts of RNA template for cDNA synthesis and validating the absence of genomic DNA contamination, since the *CrActin* amplicon with the 220-bp intron was not detected.

Structure of *CrGPDH2* and *CrGPDH3* cDNAs

The 5′ and 3′ regions of *CrGPDH2* and *CrGPDH3* were mapped using RLM-RACE (Fig. S1 and Fig. S2 in Supplementary material). The full-length cDNAs were 2873 bp long for *CrGPDH2*

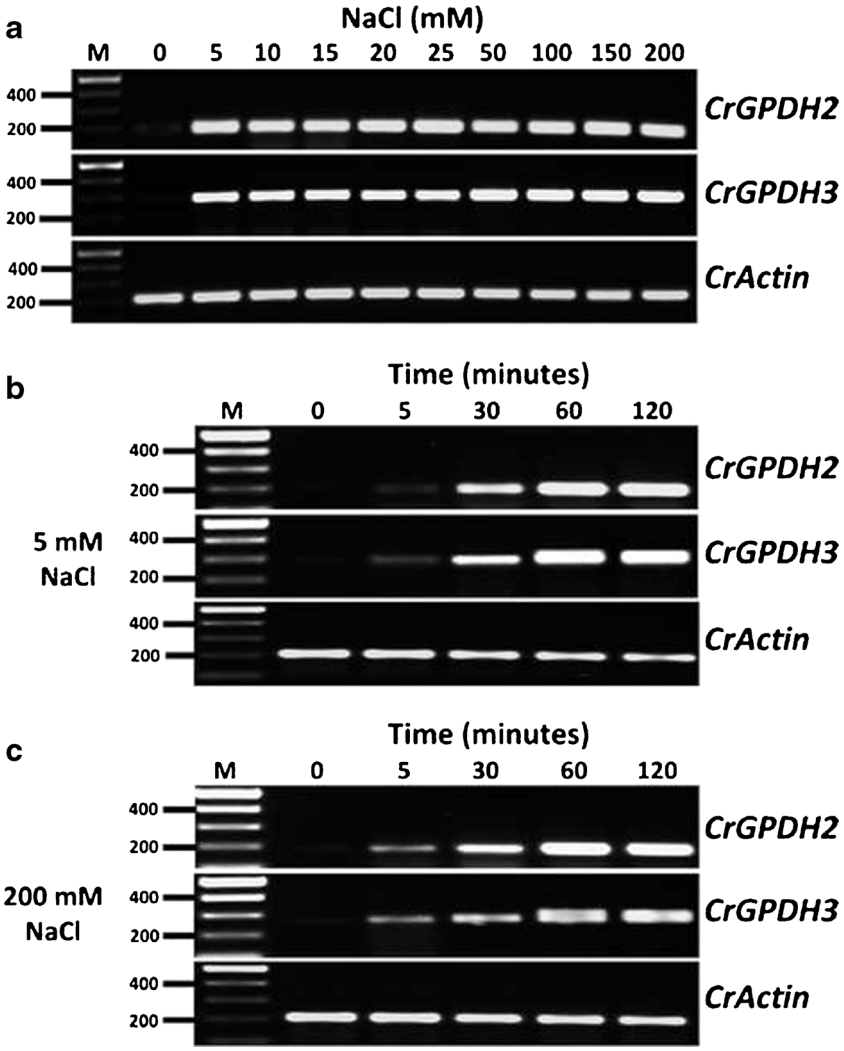

Fig. 1 Expression analysis by RT-PCR of *CrGPDH2* and *CrGPDH3* in *Chlamydomonas reinhardtii* under different NaCl treatments. *Chlamydomonas reinhardtii* cells were collected for analysis after the following treatments: **a** 2 h in 0, 5, 10, 15, 20, 25, 50, 100, 150, and 200 mM NaCl; **b** 5, 30, 60, and 120 min in 5 mM NaCl, cells without NaCl treatment were used as control (time 0); and **c** 5, 30, 60, and 120 min in 200 mM NaCl, cells without NaCl treatment were used as control (time 0). Primers for the *CrActin* gene spanning an intron of 220 bp were used as positive control. Molecular weight marker (*M*) is 2-log DNA ladder (New England Biolabs)

and 3252 bp long for *CrGPDH3* (Fig. S1 and Fig. S2 in Supplementary material). The cDNA sequences of *CrGPDH2* and *CrGPDH3* have been deposited in GenBank under the accession numbers KM923898 and KM923899, respectively. We found four *CrGPDH2* cDNAs with 3′ UTRs of different lengths. Therefore, four different polyadenylation sites were detected (Fig. S1 in Supplementary material). The 3′ UTR of *CrGPDH3* corresponded to the sequence predicted in the in silico analysis (Fig. S2 in Supplementary material). A possible polyadenylation signal sequence, TGTAA, was identified in the 3′ UTR of *CrGPDH2*, while two possible polyadenylation signal sequences, TGTAG and TGTAA, were identified in the 3′ UTR of *CrGPDH3*. Since a TGTAA sequence was found in the 3′ UTRs of both *CrGPDH2* and *CrGPDH3*, this sequence might be the polyadenylation signal for these genes. The *CrGPDH2* cDNA contained an ORF encoding a polypeptide of 723 amino acids (Fig. S1 in Supplementary material) with a predicted molecular mass of 77.8 kDa. The *CrGPDH3* cDNA contained an ORF encoding a polypeptide of 725 amino acids (Fig. S2 in Supplementary material) with a predicted molecular mass of 78.1 kDa. The deduced amino acid sequences of CrGPDH2 and CrGPDH3 showed 94.2 % identity. The positions of introns in *CrGPDH2* and *CrGPDH3* were verified by comparing the cDNA sequences and the genomic and coding DNA sequences obtained from the Phytozome database. We found that the *CrGPDH3* cDNA contained an intron at the 811th nucleotide position; in the database coding sequence, this is incorrectly predicted as a 24-bp exon (Fig. S2 in Supplementary material). In *CrGPDH3*, there was a translational start codon (ATG) in the 5′ UTR at 153 bp upstream of the in silico sequence predicted for this gene. Interestingly, the nucleotide sequence around the start codon, ACCATGA (position 130–136 in Fig. S2 in Supplementary material), was almost identical to the Kozak consensus sequence (ACCATGG) required for optimal translation initiation in eukaryotic cells (Kozak 1986). The amino acid sequence alignment of the GPDH domains of CrGPDH2 and CrGPDH3 revealed that they varied in nine amino acids, five of which corresponded to changes between amino acids that belonged to the same classification according to their R group (similar amino acids) and four of which belonged to a different classification (Fig. 2). The alignment included GPDH sequences from *D. viridis* and *S. cerevisiae*. Interestingly, the Asn at position 272 in CrGPDH2 was conserved among *D. viridis* DvGPDH1 and DvGPDH2 and *S. cerevisiae* ScGPD1, but not in CrGPDH3 (Fig. 2).

CrGPDH2 and CrGPDH3 are functional GPDH enzymes

The protein extracts used in the GPDH activity assay were obtained from cultures of the yeast *gpd1Δgpd2Δ* double mutant transformed with empty plasmid (psGC) or with plasmids containing *CrGPDH2* cDNA (psGPDH2), *CrGPDH3* cDNA (psGPDH3), or both cDNAs (psGPDH2-3). As shown in Fig. 3, the highest specific GPDH activity was in the parental strain *W303-1A* (approx. 48 mU mg^{-1} protein), and the lowest was in

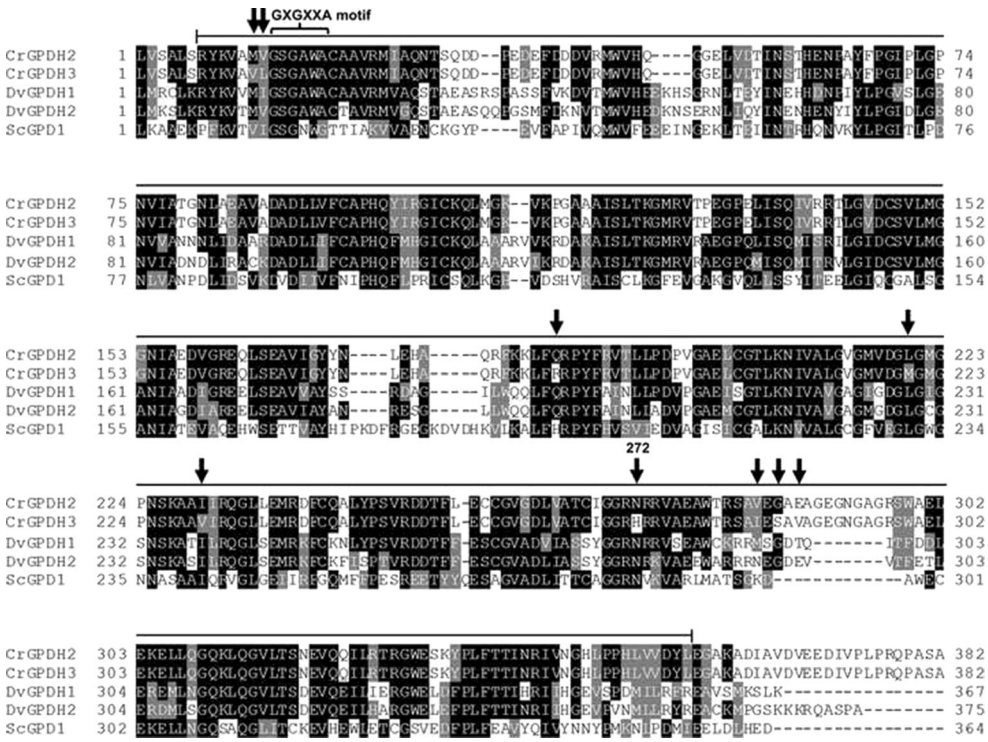

Fig. 2 Comparison of the deduced amino acid sequences of the GPDH domain of CrGPDH2 (KM923898), CrGPDH3 (KM923899), *Dunaliella viridis* DvGPDH1 (EU624406), and DvGPDH2 (EU624407) and GPD1 from *Saccharomyces cerevisiae* (ScGPD1: X76859.1). *Line* above alignment indicates GPDH domain. Putative NADH-binding motif is shown in parentheses (GXGXXA). Similar amino acids are shown in *gray*; identical amino acids are shown in *black*. *Black arrows* indicate amino acids that vary between CrGPDH2 and CrGPDH3; *number above the arrow* indicates position of amino acid identical in CrGPDH2 and ScGPD1 but different in CrGPDH3

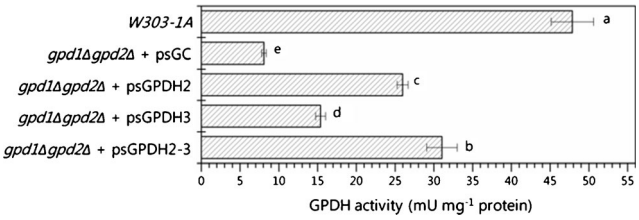

Fig. 3 Enhanced GPDH activity in *Saccharomyces cerevisiae* *gpd1Δgpd2Δ* double mutant transformed with *CrGPDH2* and *CrGPDH3* cDNAs. GPDH activity was determined in yeast *gpd1Δgpd2Δ* double mutant transformed with plasmid containing *CrGPDH2* (psGPDH2), *CrGPDH3* (psGPDH3), or with both plasmids (psGPDH2-3). Parental strain *W303-1A* and double mutant transformed with empty plasmid (psGC) were included as controls. Data are means (±SD) of three experiments, each carried out in duplicate. Different *lowercase letters* indicate significant difference between means (one-way ANOVA, Fisher's LSD test, $P<0.05$)

the strain transformed with the empty plasmid psGC (8 mU mg^{-1} protein). The specific GPDH activities in the *gpd1Δgpd2Δ* double mutant transformed with psGPDH2, psGPDH3, and psGPDH2-3 were approximately 26, 15, and 31 mU mg^{-1} protein, respectively. These activities were significantly higher than that in the same mutant transformed with psGC only, indicating that the GPDH activities resulted from the heterologous expression of *CrGPDH2* and/or *CrGPDH3* cDNAs in the mutant and confirming that the *C. reinhardtii* cDNAs encode functional GPDH enzymes.

CrGPDH2 and *CrGPDH3* cDNAs are involved in glycerol production and osmotic stress tolerance

Next, we analyzed the physiological and functional roles of *CrGPDH2* and *CrGPDH3* cDNAs in glycerol production and osmotic stress tolerance by introducing *CrGPDH2*, *CrGPDH3*, or both cDNAs into the *gpd1Δgpd2Δ* double mutant. As shown in Fig. 4, mutant cells transformed with the empty plasmid psGC produced very little glycerol, whereas glycerol production of transformants containing *CrGPDH2* was restored and was similar to that of wild type after 12 h under NaCl treatment (Fig. 4b). Glycerol accumulation in transformants harboring both *CrGPDH2* and *CrGPDH3* was similar to that in transformants harboring *CrGPDH2* (Fig. 4b). Although transformants containing *CrGPDH3* showed significantly higher glycerol production than that in the mutant transformed with the empty plasmid psGC, the amount of glycerol was strikingly less than that produced in cells transformed with psGPDH2 or psGPDH2-3 (Fig. 4b). Both *CrGPDH2* and *CrGPDH3* were able to restore NaCl tolerance to the salt-sensitive *gpd1Δgpd2Δ* double mutant, although *CrGPDH2* conferred higher tolerance than *CrGPDH3* to salt stress (Fig. 5). Transformants containing *CrGPDH2* were able to grow on plates containing 500 mM NaCl and showed some growth on 800 mM NaCl when the culture was undiluted. In contrast, transformants harboring *CrGPDH3* showed less growth on 500 mM NaCl and were unable to grow in medium

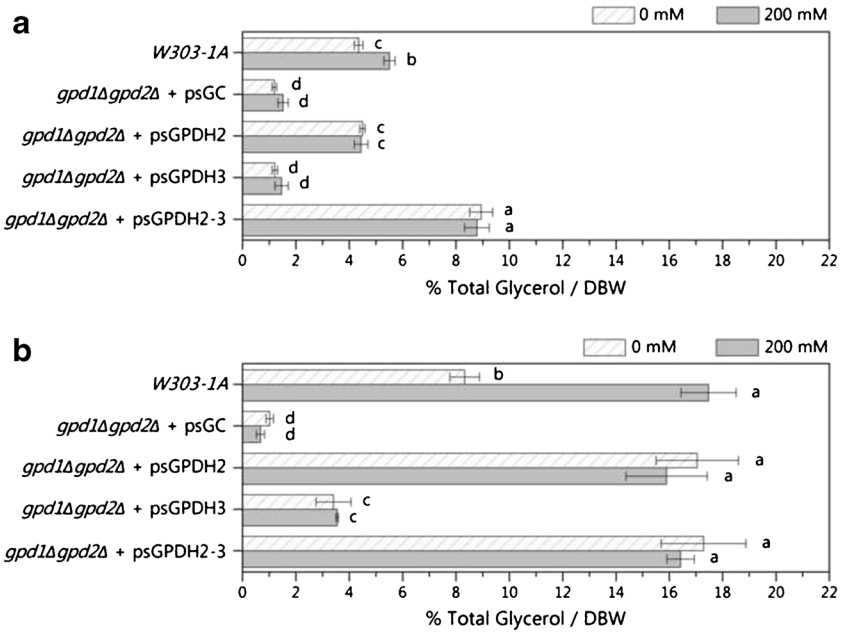

Fig. 4 Functional complementation of glycerol-deficient *Saccharomyces cerevisiae* *gpd1Δgpd2Δ* double mutant by *CrGPDH2* and *CrGPDH3* cDNAs. Total glycerol content was measured in *gpd1Δgpd2Δ* double mutant transformed with plasmid containing *CrGPDH2* (psGPDH2), *CrGPDH3* (psGPDH3), or with both plasmids (psGPDH2-3). Parental strain *W303-1A* and double mutant transformed with empty plasmid (psGC) were included as controls. Glycerol was quantified in cultures incubated in YPD medium without NaCl or after a treatment with 200 mM NaCl for **a** 4 h and **b** 12 h. *DWB* dry weight biomass. Data are means (±SD) of three independent experiments. Different *lowercase letters* indicate significant difference between means (one-way ANOVA, Fisher's LSD test, $P<0.05$)

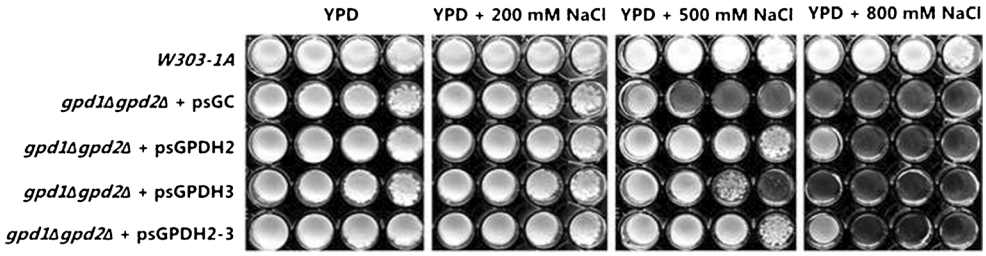

Fig. 5 Enhanced osmotic tolerance of *S. cerevisiae gpd1Δgpd2Δ* double mutant transformed with *CrGPDH2* and *CrGPDH3* cDNAs. *gpd1Δgpd2Δ* double mutant was transformed with plasmid containing *CrGPDH2* (psGPDH2), *CrGPDH3* (psGPDH3), or with both plasmids (psGPDH2-3). Parental strain *W303-1A* and double mutant transformed with empty plasmid (psGC) were included as controls. Experimental and control cells were serially diluted ten-fold, then spotted onto YPD plates without NaCl or supplemented with indicated concentrations of NaCl and grown at 30 °C for 4 days

containing 800 mM NaCl. Transformants harboring both *CrGPDH2* and *CrGPDH3* showed similar growth to that of transformants harboring *CrGPDH2*. These findings showed that *CrGPDH2* and *CrGPDH3* cDNAs are involved in glycerol production and osmotic stress tolerance.

Discussion

Previously, it was demonstrated that *C. reinhardtii* synthesizes glycerol in response to osmotic stress (Husic and Tolbert 1986) and that a significant amount of glycerol is produced after incubation with 200 mM NaCl for more than 24 h (León and Galván 1994). Those authors also showed that almost no glycerol accumulated under mild osmotic stress (e.g., 50 mM NaCl). In our previous study, we showed that a 200-mM NaCl treatment induced the expressions of *CrGPDH2* and *CrGPDH3*, and in the absence of this treatment, the expression of both genes was barely detectable by end-point PCR (Herrera-Valencia et al. 2012). We extended the expression analyses of these genes and found that both *CrGPDH2* and *CrGPDH3* were induced under all the NaCl treatments tested. Interestingly, even a low NaCl concentration of 5 mM was sufficient to trigger the expression of these genes after only 5 min, which might have implications for the adaptation of *C. reinhardtii* to changes in its environment.

Based on in silico characterization, a phylogenetic analysis and an expression analysis, we had previously suggested that *CrGPDH2* and *CrGPDH3* encode GPDH enzymes that could be involved in glycerol production in response to NaCl stress in *C. reinhardtii* (Herrera-Valencia et al. 2012). In the present study, we have demonstrated that both gene products have GPDH activity. Both were able to recover glycerol production in, and the salt tolerance of, the *S. cerevisiae gpd1Δgpd2Δ* double mutant, indicating that *CrGPDH2* and *CrGPDH3* are functional homologs of *S. cerevisiae GPD1*. Homologs of *GPD1* have been characterized by *S. cerevisiae* genetic complementation mainly in other yeast species, for example in the salt-tolerant yeast *Zygosaccharomyces rouxii* (Watanabe et al. 2004), the osmotolerant yeasts *Candida magnoliae* (Lee et al. 2008) and *Candida glycerinogenes* (Chen et al. 2008), and the halotolerant yeast *Pichia farinosa* (Peng et al. 2010). Genes encoding GPDHs have also been cloned and characterized from the halotolerant green microalga *D. salina* (He et al. 2007; Cai et al. 2013). Two genes from *D. viridis*, *DvGPDH1* and *DvGPDH2*, were characterized by yeast genetic complementation and enzymatic assays (He et al. 2009). He et al. (2009) showed that both DvGPDH1 and DvGPDH2 were able to function as GPDH enzymes. However, the authors did not offer an explanation as to why DvGPDH2 activity was markedly lower than that of DvGPDH1.

In the present study, we found that the GPDH activity was significantly higher in the *gpd1Δgpd2Δ* double mutant transformed with *CrGPDH2* than in that transformed with *CrGPDH3*, despite the similar expression patterns of these genes and the strong similarity of their protein products (94.2 %). Thus, it is interesting that their enzymatic activity was different, as well as the extent to which they restored glycerol production and NaCl tolerance in the mutant; the transformants harboring *CrGPDH2* showed significantly higher glycerol production and NaCl tolerance. A similar case was reported by Lenassi et al. (2011) for the extremely halotolerant *Hortaea werneckii*, a black yeast. In that case, the *HwGPD1B* gene, but not the *HwGPD1A* gene, was capable of complementing GPDH function in the *S. cerevisiae gpd1Δ* mutant by improving glycerol production and NaCl tolerance. This was in spite of the fact that *HwGPD1B* and *HwGPD1A* had similar gene expression patterns and 98 % amino acid sequence identity. Further analyses showed that there was a nine amino acid difference between HwGPD1A and HwGPD1B. Two of these amino acids, Asn and Asp, were identical between *S. cerevisiae* GPD1 (ScGPD1) and HwGpd1B but different in HwGpd1A, suggesting that they could be critical for the function of these proteins. In the present study, we found that only nine amino acids varied between CrGPDH2 and CrGPDH3 in the GPDH domain. One of these changes, the Asn at position 272, could be responsible for the differences in enzymatic activity between CrGPDH2 and

CrGPDH3, since this amino acid is identical between CrGPDH2 and ScGPD1, but is replaced by a His residue in CrGPDH3. This Asn corresponds to the Asn identified in HwGpd1B by Lenassi et al. (2011). Since DvGPDH1 and DvGPDH2 share this conserved Asn, another amino acid variation might be responsible for the different activities of these enzymes (He et al. 2009). Further research using site-directed mutagenesis should define the role of the conserved Asn 272 in CrGPDH2 and shed light on the roles of other amino acids in the GPDHs of microalgae and yeasts.

The findings of this study indicate that *CrGPDH2* and *CrGPDH3* encode functional GPDH enzymes involved in glycerol production and osmotolerance. Since GPDH is responsible for the synthesis of G3P, which is essential for TAGs synthesis, and considering that CrGPDH2 activity was stronger than CrGPDH3 activity, it would be very interesting to determine whether the overexpression of *CrGPDH2* in *C. reinhardtii* could increase TAGs synthesis. If so, *CrGPDH2* could be used to generate new strains of *C. reinhardtii* or other microalgae with enhanced accumulation of glycerol and TAGs as a renewable feedstock for numerous industrial products and biofuels.

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