MAPK in *Dunaliella terticola* regulates glycerol production in response to osmotic shock

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Running title: *DtMAPK* regulates glycerol production
ABSTRACT

Glycerol production is modulated in some halo-tolerant organisms in response to extracellular osmotic stress. Glycerol-3-phosphate dehydrogenase (GPDH) is a rate-limiting enzyme of glycerol synthesis in both yeast and the green microalgae *Dunaliella*. In yeast, a High-Osmolarity-Glycerol (HOG) pathway, which is a mitogen-activated protein kinase (MAPK) cascade, has been suggested to activate the expression of GPDH and thus the accumulation of glycerol under osmotic stress. In *Dunaliella tertiolecta*, however, the osmo-regulatory mechanisms for its glycerol synthesis are not well understood. In this study, the homologues of MAPK and GPDH in *D. tertiolecta* were cloned using Rapid Amplification of cDNA Ends (RACE) to investigate the molecular basis of the osmo-regulatory mechanisms. The isolated cDNA sequences were named *DtMAPK* (GenBank: KJ930518) and *DtGPDH* (GenBank: KJ930370) respectively. It was found that expression of *DtMAPK* responded to osmotic shocks within 0.5 h, while expression of *DtGPDH* responded within 1 h. In addition, glycerol production and *DtGPDH* expression level paralleled the expression of *DtMAPK* under various osmotic stress conditions. This suggests a close correlation between the expression of these two genes and glycerol production in *D. tertiolecta*. Moreover, suppressed transcription of *DtGPDH* as well as delayed accumulation of intracellular glycerol were observed in *DtMAPK* knock-down cells upon hyper-osmotic shock, which provides further evidence that *DtMAPK* is involved in the regulation of *DtGPDH* expression and thus glycerol synthesis. Our findings demonstrate that a MAPK-mediated signalling pathway that is similar to the yeast HOG pathway may exist in *D. tertiolecta* in response to osmotic stress.
Keywords: *Dunaliella tertiolecta*, osmotic response, osmo-regulation, glycerol production, MAPK, GPDH

Abbreviations

AMT, adenine methyltransferase; ble, bleomycin; GPDH, Glycerol-3-phosphate dehydrogenase; HOG, High-Osmolarity-Glycerol; MAPK, mitogen-activated protein kinase; RACE, Rapid Amplification of cDNA; RNAi, RNA interference; TUB, β-tubulin; WT, wild-type; MR2, *DtMAPK* knock-down MR2 strain.
INTRODUCTION

The eukaryotic and photosynthetic microalga *Dunaliella* adapts to various osmotic stresses by varying its glycerol content (Ben-Amotz & Avron, 1990). In addition to intracellular glycerol accumulation, it has been found that *Dunaliella tertiolecta* also continuously releases large amounts of glycerol into the external medium (Chow et al., 2013). The massive glycerol production in extremely high osmolarity environments confers *Dunaliella* the commercial potential as a glycerol production source (Ben-Amotz & Avron, 1981; Chisti, 2007; Chow et al., 2013). However, the osmo-regulatory mechanisms for the glycerol production of *Dunaliella* under osmotic stress have not been completely understood.

Glycerol 3-phosphate dehydrogenase (GPDH) is an important rate-limiting enzyme for glycerol synthesis, and the intracellular concentration of glycerol functions as the counterbalancing osmolyte in both the unicellular green algae *Dunaliella* and other organisms such as the yeast *Saccharomyces cerevisiae* (Hohmann, 2002; Saito & Posas, 2012). It has been reported that a Mitogen-activated-protein-kinase (MAPK) cascade, also known as the High-Osmolarity-Glycerol (HOG) signalling pathway, is activated to regulate glycerol synthesis during the adaptation to osmotic stress in yeast (Saito & Tatebayashi, 2004; Hohmann, 2009). When yeast cells were stimulated by hyper-osmotic challenges, the accumulated and activated Hog1 triggers a cascade of signalling and transcription events that promote the transcription of GPDH enzyme and synthesis of glycerol (Saito & Posas, 2012). The core molecule Hog1 in the yeast HOG signalling pathway belongs to the MAPK family.

MAPK functions are highly conserved (Galcheva-Gargova et al., 1994; English et al., 2015); MAPK pathways are dynamic signalling modules present in all eukaryotic cells and mainly involved in the regulation of cellular responses to environmental stress inputs.
MAP kinase proteins and genes have also been studied in plants such as *Arabidopsis*, rice, maize, tobacco, as well as the green algae *Dunaliella* sp. A *Dunaliella salina* MAPK gene (*DsMPK*) was reported to be involved in *D. salina* responding to hyper-osmotic shock (Lei *et al.*, 2008). A MAPK-like protein was suggested to be both up-regulated and phosphorylated in *Dunaliella viridis* in response to hypertonic shock (Jimenez *et al.*, 2004). This protein could be detected by antibodies specific to yeast Hog1 and its mammalian homolog p38. Furthermore, an inhibitor of p38 MAP kinase (SB203580) was reported to markedly impair the adaptation of *Dunaliella* to osmotic stress (Jimenez *et al.*, 2004). These investigations suggest that a MAPK signalling pathway may be operating in *Dunaliella* as the mechanism of osmosis regulation. A MAPK cascade similar to yeast HOG pathway may exist to regulate its glycerol production under osmotic stress.

The expression of GPDH enzyme as well as synthesis of glycerol is regulated by the MAPK protein Hog1 in yeast. To demonstrate that a MAPK cascade, which is similar to yeast HOG pathway, may exist in *Dunaliella* and regulate its glycerol synthesis in response to osmotic stress, the yeast Hog1 homologue MAPK encoding gene *DtMAPK*, as well as the GPDH encoding gene *DtGPDH* were isolated and characterized in *D. tertiolecta*. The sequence of *DtMAPK* is highly conserved. It was found that *DtMAPK* was involved in the regulation of *DtGPDH* expression and glycerol synthesis in *D. tertiolecta*. Based on the investigations, a MAPK mediated signalling pathway is suggested to control glycerol synthesis, which sheds light on the molecular basis of the osmo-regulatory mechanism in *D. tertiolecta*. 
MATERIALS AND METHODS

Strains and culture conditions

*Dunaliella tertiolecta* LB-999 was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX). *D. tertiolecta* cells were grown in batch cultures, in sterile modified ATCC-1174 DA liquid medium (American Type Culture Collection at Manassas, Virginia) with various NaCl concentrations (0.5 M, 1 M, 2 M, 3 M and 4 M). The cultures were agitated by a rotary shaker at 100 rpm and incubated at 25 °C, under a 14 h Light / 10 h Dark photoperiod of approximately 50 µmol photons m⁻² s⁻¹ light intensity provided by cool white fluorescent lights. Irradiance was measured using a Model LI-189 Quantum Meter (LI-COR, USA).

Determination of cell density and glycerol concentration

Cell density was monitored by microscopy counting using a hemacytometer (Neubauer Improved Grid) loaded with 10 µl of cell culture. Before counting, cells were fixed with 2% (v / v) formaldehyde solution.

Glycerol content was measured using the Free Glycerol Determination kit (FG0100, Sigma-Aldrich, St Louis, MO). Cells were collected by centrifugation at 5000 × g at 4 °C for 10 minutes and then washed once with isotonic culture medium. The cell pellet was resuspended into distilled water and boiled for 10 min to release intracellular glycerol. The culture supernatant and intracellular glycerol contents were assayed. Both extracellular and intracellular glycerol production were calculated on per cell basis.
RNA extraction and total cDNA synthesis

Total RNA was isolated from *D. tertiolecta* cells using the RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA samples were prepared from a minimum of $10^7$ cells for each sample. The quality and concentration of RNA were measured using NanoDrop (NanoDrop 2000 UV-Vis Spectrophotometer, Thermo Scientific) immediately after extraction. The cDNA was prepared using 1µg RNA per sample by reverse transcription using the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA) according to the manufacturer’s instructions.

Cloning of *DtMAPK* and *DtGPDH* in *D. tertiolecta* by Rapid Amplification of cDNA Ends (RACE)

RACE primers for *D. tertiolecta* MAPK gene (*DtMAPK*) were designed from the conserved regions of MAPK from *D. salina* (NCBI GenBank: ABN03944.1), *Chlamydomonas reinhardtii* (NCBI GenBank: XP_001700291.1), *Volvox carteri* (NCBI GenBank: XP_002955338.1), *Arabidopsis thaliana* (NCBI GenBank: ABR46165.1) and the budding yeast *Saccharomyces cerevisiae* (NCBI GenBank: NP_013214.1). All primer sequences were minimized based on the *D. tertiolecta* codon bias from the website (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3047&aa=1&style=GCG) or the sequence of MAPK from *D. salina*. The forward primers DtMAPK-dgF1 (5'-AGGAGCATACGGTGTGGTTTG-3') and DtMAPK-dgF2 (5'-AAAGTGGCCATCAAGAAAAAT-3') were designed from the conserved peptide sequences GAYGVVC and KVAIKK. The reverse primers DtMAPK-dgR1 (5'-TAGCTCAGGGGCTCTGTACCA-3') and DtMAPK-dgR2 (5'-
TAGTCCTTGCCGGGAACA-3') were designed from the conserved peptide sequence WYRAPEL and FPGKDY. *DtMAPK* related cDNA fragments were amplified by PCR and then sequenced.

The 5’- and 3’- ends of the *D. tertiolecta MAPK* cDNA (*DtMAPK*) were cloned respectively using the SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA). *DtMAPK* 5’-RACE product was generated using 10×Universal Primer A Mix (UPM) or Nested Universal Primer A (NUP) provided with the kit as a forward primer and *MAPK* gene-specific primers *DtMAPK*-gsR1 (5’-ATCGCGGTGCAGGATGGCAG-3’) or *DtMAPK*-gsR2 (5’-CCACACCGTATGCTCCTTTGCCA-3’) as a reverse primer. *DtMAPK* 3’-RACE product was generated using a gene-specific forward primer *DtMAPK*-gsF1 (5’-TGCCAAACGCACACTGCGTG-3’) or *DtMAPK*-gsF2 (5’-CGCCTGATGTGGTGCGAGGC-3’) and UPM or NUP as a reverse primer.

RACE primers for amplifying the cDNA of *D. tertiolecta GPDH* (*DtGPDH*) were designed from the conserved regions of the GPDH from *D. salina* (NCBI GenBank: AY845323). The 5’- and 3’- ends of *DtGPDH* were cloned similar to *DtMAPK*. *DtGPDH* 5’-RACE product was generated using the UPM or NUP from the kit as a forward primer and *GPDH* gene-specific primers *DtGPDH*-gsR1 (5’-GTTGATGGTGAACAAG-3’) or *DtGPDH*-gsR2 (5’-TGTACTCGATCAGGTTGC-3’) or *DtGPDH*-gsR3 (5’-GAAGGCATCTGCTCCATC-3’) as a reverse primer. *DtGPDH* 3’-RACE product was generated using a gene-specific forward primer *DtGPDH*-gsF1 (5’-AGTTCATCTCCCCCTCAGTGCGAGA-3’) or *DtGPDH*-gsF2 (5’-AGGCCCTGGGCCCAGAAGAGGATCG-3’) (He *et al.*, 2009) and UPM or NUP as a reverse primer.
Sequence analysis

The RACE-PCR products were cloned into pGEM®-T Easy Vector System (Promega, Madison, WI) for sequencing. RACE 5’- and 3’-overlapping sequences were assembled using the EMBOSS merger program to obtain the full-length sequences. The assembled sequences were named \textit{DtMAPK} (NCBI GenBank: KJ930517) and \textit{DtGPDH} (NCBI GenBank: KJ930370) respectively, and analyzed for open reading frames using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced amino acid sequences were compared with previously characterized protein sequences using the Blast algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of \textit{DtMAPK}-RNAi plasmid

The pGreen-0229 binary vector HY104 (Yu \textit{et al.}, 2004) was modified and used as the \textit{D. tertiolecta} transformation vector. To generate the \textit{DtMAPK} knock-down construct \textit{DtMAPK}-RNAi (Error! Reference source not found.), an 851-bp adenine methyltransferase (\textit{AMT}) gene (NCBI GenBank: X06618.1) promoter from \textit{Chlorella virus} was amplified using the PCR primer pair AMT-F-KpnI (5’-aaaGGTACCAGTAATGTGTTAATTGC3’), which introduces a cutting site for the restriction enzyme \textit{KpnI}, and AMT-R-XhoI (5’-ccgCTCGAGTTAATAATATAGTGTATTTTAG-3’), which introduces the \textit{XhoI} site, and cloned into pGreen-0229 upstream of the gene fragments to drive its expression in \textit{D. tertiolecta} (Mitra \textit{et al.}, 1994). A 526-bp bleomycin resistance gene (\textit{ble}) derived from \textit{Streptoalloteichus hindustanus} was amplified using the primers ble-F-XhoI ((5’-AAACTCGAGATGGCCAAGCTGACCAGC-3’) introducing an \textit{XhoI} site and ble-R2-ClaI
(5’-CACATCGATTAGTCTGCTCCTCGGC-3’) introducing a Clal site and inserted to the pGreen-0229 backbone as a dominant selectable marker to confer zeocin-resistance.

A 3349bp coding fragment of DtMAPK (bp 75-408) was used for the construction of DtMAPK-RNAi, which expressed self-complementary or hairpin RNA-containing sequences homologous to the target DtMAPK driven by the AMT promoter (Error! Reference source not found.). Sense and anti-sense sequences of this fragment were amplified by PCR from the total cDNA of D. tertiolecta. Primer pair for the sense fragment amplification were the forward primer DtMAPK-SF-PstI (5’-AACTGCAGAGTCCATCAACTATGAGGCCG-3’), which introduces a PstI site, and the reverse primer DtMAPK-SR-BamHI (5’-CGGGATCTCGTACACCACATACAGGTCC-3’) introducing a BamHI site. Primers for the amplification of the anti-sense fragment were DtMAPK-AsF-ClaI (5’-CCATCGATTCGTACACCACATACAGGTCC-3’), which introduces a Clal site, and DtMAPK-AsR-EcoRI (5’-CGGAATTCTAGTCCATCAACTATGAGGCCG-3’), which introduces an EcoRI site. The amplified gene-specific anti-sense and sense fragments were then inserted into the modified pGreen-0229 vector before and after the β-glucuronidase encoding gene (GUS) fragment respectively.

Transformation of D. tertiolecta

Constructed plasmids were transformed into D. tertiolecta cells using the Agrobacterium mediated Dunaliella transformation method as described previously (Kumar et al., 2004; Fang et al., 2012) with slight modifications. The DtMAPK-RNAi construct was transformed into Agrobacterium tumefaciens GV3101 via electroporation (2.5 kV). The transformed agrobacteria were subsequently co-cultured with logarithmic phase (5-day old) D. tertiolecta.
liquid culture in 0.05 M NaCl 1174 DA medium with 100 µM acetosyringone for 2 days. The transformed *D. tertiolecta* were plated onto 0.5 M NaCl 1174 DA medium agar plates containing 20 µg ml⁻¹ zeocin (Zeocin™, Invitrogen) as the selection marker. Colonies that appeared within 2 weeks were subcultured in 96-well plates containing liquid selective medium (0.5 M NaCl ATCC-1174 DA medium containing 20 µg ml⁻¹ zeocin). Individual *D. tertiolecta* colonies (transformants) were processed with genotyping PCR using primers ble-gen-F (5′-GGAGCGGGTGAGTTCTGG-3′) and ble-gen-R (5′-CTCGCCGATCTCGGCAT-3′) to confirm the presence of gene integration.

Osmotic stress treatments

*D. tertiolecta* wild-type (WT) cells or *DtMAPK*-RNAi transformants were subjected to osmotic stress treatments following the process described below. Cells were first grown in sterile 2 M NaCl ATCC-1174 DA liquid medium under photoperiod illumination for 5 days (logarithmic phase), and then harvested by centrifugation at 5000 × g for 10 min at 4 °C. The microalgae pellets were respectively suspended into iso-volumetric ATCC-1174 DA medium containing 0.5 M (hypo-osmotic shock), 1 M (hypo-osmotic shock), 2 M (iso-osmotic control), 3 M (hyper-osmotic shock) or 4 M (hyper-osmotic shock) NaCl accordingly.

Treated cells were harvested at 0.5 h, 1 h, 2 h, 4 h, 6 h and 8 h (short-term osmotic shock) or 5 days (long-term osmotic shock) accordingly by centrifugation at 5000 × g for 10 min at 4 °C. The cell pellets were either frozen in liquid nitrogen followed by storage at -80 °C (for RNA extraction), or analyzed immediately in subsequent steps. Each osmotic shock experiment was repeated at least three times.
Gene expression analysis by quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) was performed with the 2 × Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, Waltham, MA, USA) according to the manufacturer’s instructions, using an ABI PRISM® 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Specific primers for \( DtMAPK \) (forward: 5’-AGCCCCTGAGCTACTGCTCTCAT-3’; reverse: 5’-GTTCAGCTGGTGCACGTAGTCCT-3’), \( DtGPDH \) (forward: 5’-ATTAACCTGCTTTGCGGATGT-3’; reverse: 5’-CATAGCTGCTGGCAATCAAA-3’), and \( D. tertiolecta \) \( \beta \)-tubulin gene (\( DtTUB \)) (forward: 5’-CAGATGTGGGATGCCAAGAACAT-3’; reverse: 5’-GTTCAGCATCTGCTCATCCACCT-3’) were designed to analyze gene expression. \( DtTUB \) was used as the endogenous control. Relative expression of \( DtMAPK \) or \( DtGPDH \) was calculated using the equation \( 2^{-\Delta\Delta Ct} \) (Livak & Schmittgen, 2001). All reactions were carried out in parallel triplicates and the experiments were repeated 3 times.

Statistical analysis

All values are expressed as the means ± standard deviation of at least triplicate assays to measure technical variation and typically multiple experiments were performed with independent batches of cells to assess biological reproducibility. Statistical analyses were performed using SPSS Version 15 (SPSS Inc., Chicago, IL). Student’s t-test was used for comparisons and Linear Regression was used for correlation analysis. A p-value \( \leq 0.05 \) was considered as statistically significant.
RESULTS

Identification and characterization of DtMAPK and DtGPDH in D. tertiolecta

Rapid amplification of cDNA ends (RACE) was performed in D. tertiolecta. A mitogen-activated protein kinase (MAPK) homolog was identified and named DtMAPK. The entire coding region of the isolated DtMAPK is 14719bp in length, and encodes a putative protein containing 472 amino acids (GenBank: AIN35080.1). A BLASTX search against the National Center for Biotechnology Information (NCBI) protein database (http://www.ncbi.nlm.nih.gov/protein) revealed that the amino acid sequence is 98% and 81% similar to two MAPK proteins reported in D. salina (DsMPKs, NCBI GenBank: ABN03944.1 and AFJ54625.1) respectively, and 75% similar to MAPK from Chlamydomonas reinhardtii (CrMAPK, NCBI GenBank: XP_001700291.1). A conserved MAPK family protein catalytic domain (including an active site, an ATP-binding site, a substrate-binding site, an activation loop and a KIM docking site) of the Serine/Threonine Kinases, TEY Mitogen-Activated Protein Kinases from plants was predicted to be located from position 43 to position 241. These features strongly suggest that the isolated DtMAPK cDNA encodes a putative mitogen-activated protein kinase of the MAPK family in D. tertiolecta.

The isolated DtGPDH is 27349bp long with an ORF of 2106-bp. The predicted polypeptide codes for 701 amino acids with a molecular weight of 76.9 kDa and pI of 6.09. The putative amino acid sequence of DtGPDH shared a high homology with other Dunaliella GPDHs with the highest homology of 99% with the osmo-regulatory chloroplastic isoform DsGPDH2 in D. salina (NCBI GenBank: AY845323). The first 30 amino acids at the N terminal of the translated DtGPDH were predicted to be a chloroplast targeting peptide.
Similar to other GPDHs cloned from *Dunaliella*, the DtGPDH enzyme contains phosphoserine phosphatase (position 106 to position 330) and GPDH (position 333 to position 682) catalytic domains.

Glycerol production and expression of *DtMAPK* and *DtGPDH* in *D. tertiolecta* are dependent on external osmolarity.

In this investigation, glycerol content of *D. tertiolecta* as well as the expression levels of both *DtMAPK* and *DtGPDH* was determined at different external salinities (Error! Reference source not found., Fig. 2). Error! Reference source not found., Fig. 2 A& B show the intracellular and extracellular glycerol accumulated per cell over a 5 day period after the cells were transferred from 2 M NaCl to different salinities ranging from 0.5 M to 4 M NaCl. As can be seen, both intracellular and extracellular glycerol production by *D. tertiolecta* were proportional to the external salt concentrations (linear regression, p < 0.05). Intracellular glycerol content increases with increasing salinity (Error! Reference source not found., Figure 2 A). This was expected as the intracellular glycerol functions as an osmolyte to maintain osmolality balance across the cell membrane. Extracellular glycerol is also positively correlated with external salinity (Error! Reference source not found., Figure 2 B) and *D. tertiolecta* has higher total glycerol production at high salt conditions. The results confirm that glycerol production of *D. tertiolecta* is regulated accordingly in the adaptation to various osmotic pressure conditions.

Moreover, Error! Reference source not found., Figure 2 C and D show that the mRNA levels of both *DtGPDH* and *DtMAPK* genes were positively correlated to the external salinity (linear regression, p < 0.05). Gene expression levels were higher in *D. tertiolecta* cells with
higher salt stress condition. This observation is similar to the trend of glycerol production in *D. tertiolecta* at various salinity (Error! Reference source not found., Figure 2 A and B). Hence, glycerol production as well as the expression of *DtMAPK* and *DtGPDH* is dependent on external osmolarity, suggesting that correlations may be exist between the gene expressions and glycerol synthesis in *D. tertiolecta* in response to osmotic stress.

Expression kinetics of *DtMAPK* in response to osmotic shocks

The expression kinetics of *DtMAPK* was further investigated on the response to changes in extracellular osmolarity. Error! Reference source not found., Figure 3 shows the relative fold change of *DtMAPK* mRNA level in response to a 2 M to 4 M hyper-osmotic shock as well as a 2 M to 0.5 M hypo-osmotic shock. As can be seen, transcriptional level of *DtMAPK* was regulated in response to osmotic shocks within the first 0.5 h after osmotic shock treatment. *DtMAPK* expression was up-regulated by approximately 5 folds within 2 h upon hyper-osmotic shock treatment (Error! Reference source not found., Figure 3 A) while suppressed after hypo-osmotic shock treatment (Error! Reference source not found., Figure 3 B). Hence the expression of *DtMAPK* rapidly responds to osmotic shocks in *D. tertiolecta*.

Expression kinetics of *DtGPDH* in response to hyper-osmotic shock

To explore the correlation between *DtMAPK* and *DtGPDH* in *D. tertiolecta*, kinetic expression of *DtGPDH* was determined under hyper-osmotic condition where glycerol production of *D. tertiolecta* was increased. Error! Reference source not found., Figure 4 shows the fold change of *DtGPDH* in *D. tertiolecta* in response to a hyper-osmotic shock.
treatment from 2 M NaCl medium to 4 M NaCl medium. It can be seen that \textit{DtGPDH} was up-regulated by hyper-osmotic stress within 1 h after the osmotic treatment. Transcriptional level of \textit{DtGPDH} was increased by 10-15 times during the following 4-8 hours after hyper-osmotic shock. The prior up-regulation of \textit{DtMAPK} expression within 0.5 h after hyper-osmotic treatment suggests that \textit{DtMAPK} may be involved in the activation of \textit{DtGPDH} transcription, which is required for the increase in glycerol synthesis of \textit{D. tertiolecta} under hyper-osmotic stress.

Generation of the \textit{DtMAPK} knock-down \textit{D. tertiolecta} strain by RNA interference

To further elucidate the biological function of \textit{DtMAPK} in the osmo-regulation of \textit{D. tertiolecta}, a \textit{DtMAPK} knock-down \textit{D. tertiolecta} strain was obtained by RNA interference (RNAi) technology. The DtMAPK-RNAi plasmid was constructed to express a hairpin RNA containing the endogenous \textit{DtMAPK} coding fragment (Error! Reference source not found. Figure 1), and then transformed into \textit{D. tertiolecta} by the \textit{Agrobacterium}-mediated gene transfer system. A 397-bp PCR product corresponding to the \textit{ble} selection marker and anti-sense fragment targeted on \textit{DtMAPK} was amplified by genotyping PCR, demonstrating the successfully gene integration in \textit{D. tertiolecta} MR2 strain (Error! Reference source not found. Figure 5).

The transcriptional level in \textit{D. tertiolecta} MR2 strain was measured during a hyper-osmotic shock (Error! Reference source not found. Figure 6). The knock-down effect of \textit{DtMAPK} gene was confirmed in the MR2 strain by three hyper-osmotic shock treatment experiments in parallel. As compared to \textit{DtMAPK} expression in the wild-type which was up-regulated by 4 folds within 2 h upon hyper-osmotic shock, Error! Reference source not
Figure 6 shows that DtMAPK expression in the MR2 strain was not significantly increased during the sudden increase in salinity. DtMAPK expression of the MR2 strain was similar to the iso-osmotic control 2 h or 4 h after hyper-osmotic stress exposure. Moreover, when the MR2 and wild-type cells were subjected to the same treatment, DtMAPK expression in MR2 strain was always lower than that in *D. tertiolecta* wild-type, as shown in Error! Reference source not found. Figure 6. These findings indicate that DtMAPK has been knocked down successfully in the MR2 strain even under hyper-osmotic stress.

Expression of *DtGPDH* was suppressed in the *DtMAPK* knock-down MR2 strain

To evaluate whether *DtGPDH* was regulated by the expression of *DtMAPK*, transcriptional level of *DtGPDH* was determined in the above generated *DtMAPK* knock-down *D. tertiolecta* MR2 strain. Error! Reference source not found. Figure 7 shows the relative expression of *DtGPDH* in the MR2 strain 2 h and 4 h after a hyper-osmotic shock from 2 M NaCl to 4 M NaCl. As can be seen, expression of *DtGPDH* was suppressed in the *DtMAPK* knock-down MR2 strain. Lower *DtGPDH* expression levels were observed in MR2 strain compared to *D. tertiolecta* wild-type, in both the iso-osmotic control group and the 2 M to 4 M hyper-osmotic shock treatment group. The inhibition of *DtGPDH* expression in the MR2 strain suggests that *DtGPDH* transcription in *D. tertiolecta* may be regulated by *DtMAPK*, which is a possible explanation for the osmo-regulatory mechanism for glycerol production in *D. tertiolecta* in response to an osmotic shock.
Osmo-regulation of glycerol production was delayed in the _DtMAPK_ knock-down MR2 strain.

Glycerol content of _D. tertiolecta_ upon hyper-osmotic shock was evaluated in the _DtMAPK_ knock-down MR2 strain to demonstrate the effect of _DtMAPK_ on the osmo-regulation of glycerol production. As the extracellular glycerol concentration in the media was negligible compared to the intracellular glycerol concentration after _D. tertiolecta_ cells were transferred to fresh media for experimental treatments, only the intracellular glycerol concentration of the cells upon osmotic shock treatment was shown (Error! Reference source not found. Figure 8). It was observed that the intracellular glycerol content of _D. tertiolecta_ wild-type was generally increased 2 h and 4 h after a 2 M to 4 M hyper-osmotic shock treatment. However, the _DtMAPK_ knock-down MR2 strain had significantly lower glycerol content than the wild-type 2 h after hyper-osmotic shock treatment (student t test, p < 0.05), with glycerol concentrations similar to the level of iso-osmotic shock treated cells (Error! Reference source not found. Figure 8 A). Therefore, unlike the wild-type strain, MR2 strain did not respond to a hyper-osmotic shock within the first 2 h upon treatment. The osmo-regulation of glycerol production was delayed in the _DtMAPK_ knock-down cells.

On the other hand, the intracellular glycerol content determined 4 h after treatment showed no significant difference between the wild-type and MR2 strain, under both hyper-osmotic shock and iso-osmotic conditions (Error! Reference source not found. Figure 8 B). This correlates with an up-regulation of _DtGPDH_ in MR2 strain 4 h after hyper-osmotic shock treatment, which was absent under iso-osmotic conditions (Error! Reference source not found. Figure 7 B). The delayed increase in _DtGPDH_ expression in MR2 compared to wild type under hyper-osmotic stress led to a slower osmotic response, as shown by the increase in
DISCUSSION

The isolated *DtMAPK* is highly conserved.

A cDNA coding for MAPK has been previously reported in *D. salina* (*DsMPK*) (Lei et al., 2008). Alignment of *DtMAPK* with *DsMPK* displayed a 98% similarity on their amino acid sequences. This suggests that *DtMAPK* is highly conserved and has a close relationship with *DsMPK*. The isolated *DtMAPK* was found to be up-regulated in *D. tertiolecta* upon hyper-osmotic shock and suppressed upon hypo-osmotic shock in this study (Error! Reference source not found. Figure 3). The transiently up-regulation of *DtMAPK* in response to hyper-osmotic stress could be due to a feedback from *DtGPDH* expression and glycerol synthesis 4-6 h after treatment, which is similar to the yeast HOG pathway (Jimenez et al., 2004).

Moreover, a MAPK-like protein in *D. viridis* that could be detected by the yeast Hog1 antibody was reported to be up-regulated in response to hypertonic shock (Jimenez et al., 2004). The yeast MAPK Hog1 is activated and up-regulated by hyper-osmotic pressure as well (Saito & Tatebayashi, 2004). The conservation of MAPK function suggests that *DtMAPK* in *Dunaliella* has a similar function to yeast Hog1 in response to external osmotic stress.
Expression of *DtGPDH* is regulated by *DtMAPK*

Gene expression study on *DtMAPK* and *DtGPDH* showed that both genes were positively correlated to the external salinities (Error! Reference source not found., Figure 2 C and D). *DtGPDH* encodes the enzyme DtGPDH that catalyzes the rate-limiting step in the glycerol synthetic pathway in *D. tertiolecta*. Glycerol production was also positively correlated to the external salinity (Error! Reference source not found., Figure 2 A and B). Thus, the increase in *DtGPDH* expression is expected with increased external salinity. The parallel increase in expression of *DtMAPK* with salinity can be explained by a potential relationship between *DtMAPK* and *DtGPDH*. GPDH expression is initiated by activation of the MAPK Hog1 in yeast (Hohmann, 2009). Therefore, it was proposed in this study that *DtGPDH* was mediated by *DtMAPK* and this has also been evidenced.

Kinetic studies on the expression of *DtMAPK* (Error! Reference source not found., Figure 3 A) and *DtGPDH* (Error! Reference source not found., Figure 4) in response to short-term hyper-osmotic shocks demonstrated that both genes were up-regulated upon hyper-osmotic shock treatment. However, the up-regulation of *DtMAPK* occurred at 0.5 h and peaked at 2 h after osmotic treatment (Error! Reference source not found., Figure 3 A), while the expression of *DtGPDH* started to increase at 1 h after the transfer of cells to a higher salinity. The highest induction of *DtGPDH* occurred at 4-6 h after the hyper-osmotic shock treatment (Error! Reference source not found., Figure 4). Therefore, it is suggested that *DtGPDH* is downstream of the expression of *DtMAPK* upon osmotic shock, resulting in a delay in its up-regulation.
DtMAPK is responsible for the immediate osmo-regulation of glycerol synthesis in D. tertiolecta

In this study, osmo-regulation in the DtMAPK knock-down MR2 strain was impaired as compared to D. tertiolecta wild-type upon hyper-osmotic shock (Error! Reference source not found, Figure 7 and Error! Reference source not found, Figure 8). A delayed trigger of glycerol synthesis was observed in the DtMAPK knock-down MR2 cells treated with hyper-osmotic stress, while no difference in glycerol contents was observed in the cells subjected to an iso-osmotic treatment (Error! Reference source not found, Figure 8). These findings suggest that DtMAPK is essential for the fast initial regulation of glycerol synthesis by activating the expression of DtGPDH in response to osmotic stress.

Similarly, it has been known that the transcription of GPDH in the budding yeast is activated in response to hyper-osmotic challenge by the HOG pathway, which is mediated by the MAPK protein Hog1 (Saito & Tatebayashi, 2004; Hohmann, 2009). Hence it can be suggested that a yeast HOG-like MAPK signalling pathway is involved in the osmo-regulation of D. tertiolecta under osmotic stress by activating the transcription of DtGPDH and thus, glycerol synthesis.

Other mechanisms might be involved in the osmo-regulation of D. tertiolecta

Glycerol production was delayed in DtMAPK knock-down MR2 cells in response to hyper-osmotic shock, as its intracellular glycerol content did not increase in response to a hyper-osmotic shock until 4 h after treatment (Error! Reference source not found, Figure 8). In addition, DtGPDH expression in MR2 cells was slightly up-regulated 4 h after the hyper-
osmotic shock treatment, though it was still inhibited as compared to wild-type (Error! Reference source not found., Figure 7B). It has been found that more than one MAPK-like protein was transiently phosphorylated under stress conditions in *D. viridis* (Jimenez *et al.*, 2004). These different modules might function collaboratively or take part in the osmo-regulation of *Dunaliella* species. Therefore, other possible mechanisms and modules may be involved in the osmo-regulation in *DtMAPK* knock-down cells.

**CONCLUSION**

*DtMAPK* and *DtGPDH* coding for MAPK and GPDH respectively were isolated and characterized in *D. tertiolecta* for the first time in this study. Investigation on these genes suggests that a MAPK signalling pathways, similar to yeast HOG pathway, may be involved in the osmo-regulation of *D. tertiolecta*. A potential osmo-regulatory mechanism is proposed (Error! Reference source not found., Figure 9), where the transiently up-regulated *DtMAPK* induces the expression of *DtGPDH*, thus activating glycerol synthesis in *D. tertiolecta*. These findings shed light on the molecular basis of osmo-regulatory mechanisms for glycerol synthesis in *D. tertiolecta*.

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**AUTHOR CONTRIBUTIONS**

RZ: planned and performed the experiments, analyzed the data, wrote the paper; DHPN: performed the experiments, analyzed the data, wrote the paper; LF: contributed materials; YYSC: edited the paper; KYL: planned the experiments, analyzed the data, wrote the paper
REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** Schematic diagram of *DtMAPK*-RNAi construct. Anti-sense and Sense: anti-sense sequence and sense sequence fragments of *DtMAPK* gene coding sequence.

**Fig. 2.** Glycerol production as well as expression of *DtMAPK* and *DtGPDH* in *D. tertiolecta* under various osmotic stresses. Straight lines were generated by linear regression. $R^2$ and p-value are shown for each regression, where $p < 0.05$ suggests a significant linear relationship. The values shown in the data were the average of triplicate experiments in parallel.

**Fig. 3.** Expression of *DtMAPK* in response to osmotic shocks. *DtMAPK* expression level at time 0 (before osmotic shock) was normalized as 1 in each set of experiment. Fold change was calculated and shown respectively based on the iso-osmotic control group (2 M NaCl to 2 M NaCl) taken at the same time points. *DtTUB* was used as the endogenous reference. A:
hyper-osmotic shock (2 M NaCl to 4 M NaCl); B: hypo-osmotic shock (2 M NaCl to 0.5 M NaCl).

**Fig. 4.** Expression of *DtGPDH* in response to a hyper-osmotic shock from 2 M NaCl to 4 M NaCl. *DtGPDH* expression level at time 0 (before osmotic shock) was normalized as 1 for each set of experiment. Fold change was calculated and shown respectively based on the iso-osmotic control group (2 M NaCl to 2 M NaCl) at the same time points taken. *DtTUB* was used as the endogenous reference.

**Fig. 5.** Genotyping PCR of *DtMAPK-RNAi-MR2* strain. MR2: *DtMAPK* knock-down *D. tertiolecta* MR2 strain; WT: *D. tertiolecta* wild-type; NTC: non-template control; P: positive control.

**Fig. 6.** Expression of *DtMAPK* in the *DtMAPK* knock-down MR2 strain could not respond to hyper-osmotic shock. WT (blank): *D. tertiolecta* wild-type; MR2 (slash): *DtMAPK* knock-down MR2 strain. 2 M to 2 M, iso-osmotic control from 2 M NaCl to 2 M NaCl; 2 M to 4 M, hyper-osmotic shock treatment from 2 M NaCl medium to 4 M NaCl medium.

**Fig. 7.** Expression of *DtGPDH* in response to hyper-osmotic shock in the *DtMAPK* knock-down strain MR2. WT (blank): *D. tertiolecta* wild-type; MR2 (slash): *DtMAPK* knock-down MR2 strain. 2 M to 2 M, iso-osmotic control from 2 M NaCl to 2 M NaCl; 2 M to 4 M, hyper-osmotic shock treatment from 2 M NaCl to 4 M NaCl.

**Fig. 8.** Intracellular glycerol content of *D. tertiolecta* wild-type and MR2 strain in response to osmotic shock. WT (blank): *D. tertiolecta* wild-type; MR2 (slash): *DtMAPK* knock-down MR2 strain. 2 M to 2 M: iso-osmotic control from 2 M NaCl to 2 M NaCl; 2 M to 4 M: hyper-osmotic shock treatment from 2 M NaCl to 4 M NaCl.
Fig. 9. Proposed information flow showing the response of *D. tertiolecta* to hyper-osmotic stress. We have shown that *DtGPDH* is up-regulated by the transiently up-regulation of *DtMAPK* and causes activation of glycerol synthesis as an immediate response to high external osmolarity. Unknown mechanisms might be involved leading to up-regulation of *DtGPDH* mRNA levels 4 h after osmotic.