Molecular Characterization of a Tolerant Saline-Alkali *Chlorella* Phosphatidate Phosphatase That Confers NaCl and Sorbitol Tolerance

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The gene encoding a putative phosphatidate phosphatase (PAP) from tolerant saline-alkali (TSA) *Chlorella*, *ChPAP*, was identified from a yeast cDNA library constructed from TSA *Chlorella* after a NaCl treatment. *ChPAP* expressed in yeast enhanced its tolerance to NaCl and sorbitol. The *ChPAP* protein from a GFP-tagged construct localized to the plasma membrane and the lumen of vacuoles. The relative transcript levels of *ChPAP* in *Chlorella* cells were strongly induced by NaCl and sorbitol as assessed by northern blot analyses. Thus, *ChPAP* may play important roles in promoting Na-ion movement into the cell and maintaining the cytoplasmic ion balance. In addition, *ChPAP* may catalyze diacylglycerol pyrophosphate to phosphatidate in vacuoles.

Keywords: tolerant saline-alkali *Chlorella*, NaCl, sorbitol, subcellular localization, gene expression

INTRODUCTION

Phosphatidate phosphatase (PAP) has the effects of catalyzing the dephosphorylation of phosphatidate (PA), and generating diacylglycerol and inorganic phosphate (Smith et al., 1957). It is also an essential enzyme in lipid metabolism, plays important roles in lipid synthesis, and is involved in the generation or degradation of lipid signaling molecules (Brindley and Pilquil, 1984; Carman, 1997; Nanjundan and Possmayer, 2001). The PAP enzymes are divided into Mg<sup>2+</sup>-dependent PAP1 or Mg<sup>2+</sup>-independent PAP2 [also be called lipid phosphate phosphatase (LPP) or diacylglycerol pyrophosphate (DGPP) phosphatase] based on the cofactor requirement for catalytic activity (Jamal et al., 1991; Brindley et al., 2002). The PAP1 enzymes play roles in cell homeostasis and lipid synthesis (Han et al., 2006; Sherr et al., 2017; Hassaninasab et al., 2019), and PAP1 enzyme, PAH1, performs catalytic function to regulate phospholipid synthesis on the nuclear and endoplasmic reticulum (Eastmond et al., 2010; Hassaninasab et al., 2019). The absence of *Pahp1* (encoded PAH1) leads to the upregulated of *V-ATPase* (Sherr et al., 2017). The expression of *PAH1* is induced in the absence of Zn (Soto-Cardalda et al., 2012). The PAH1 protein of the fungal pathogen *Candida albicans* restricts viral replication by affecting phospholipid synthesis and plays significant roles in hyphal growth and environmental stress regulation (Mu et al., 2019). In this study, the PAP2 enzymes are highlighted. PAP2, encoded by DPP1 and LPP1, is a vacuole membrane-associated enzyme that catalyzes DGPP to form PA and then catalyzes PA to form diacylglycerol by removing the phosphate (Wu et al., 1996; Han et al., 2001). PAP2 enzymes contain a three-domain lipid phosphatase catalytic motif containing the conserved sequences.
KxxxxxxRP (domain 1), PSGH (domain 2), and SRxxxxxHxxxD (domain 3). The conserved arginine residue in domain 1 and the conserved histidine residues in domains 2 and 3 are essential for the catalytic activities of PAP2 enzymes (Hemrika et al., 1997; Neuwald, 1997; Stukey and Carman, 1997; Toke et al., 1998, 1999ab; Zhang et al., 2000; Han et al., 2004). The PAP2 enzymes are localized on the hydrophilic surfaces of the membrane (Stukey and Carman, 1997; Toke et al., 1998, 1999ab; Zhang et al., 2000; Han et al., 2004), the vacuole (Han et al., 2001, 2004), and Golgi (Huh et al., 2003) and have broad substrate specificity levels and may function under stress conditions (Oshiro et al., 2003). In the early studies, PAP2 enzymes are found that are responsible for lipid signaling in yeast and mammals (Carman, 1997; Nanjundan and Possmayer, 2001; Brindley, 2004; Pyne et al., 2005). In plants, Arabidopsis thaliana AtLPP1 appears to be more highly expressed in the leaves and roots compared with other tissues, and the expression level of AtLPP1 increased in Arabidopsis after ionization and UV-B irradiation (Brindley, 2004; Pierrugues et al., 2011). In addition, the AtLPP2 appears to be expressed at similar levels in all the plant's tissues, and AtLPP2 is involved with abscisic acid signaling and regulation of stomatal movements (Paradis et al., 2011).

The PAP2 gene has been also identified in microalgae (eukaryotic microbes), including Chlorella variabilis (Blanc et al., 2010), Chlorella protothecoides (Gao et al., 2014), Chlamydomonas reinhardtii (Deng et al., 2013), and Coccomyxa subellipsoida (Blanc et al., 2010). However, there are limited reports on the cloning and functional analyses of PAP2 genes of microalgae. Some studies indicated that the expression levels of citrate synthase and phosphoenolpyruvate carboxylase 1 in Chlamydomonas reinhardtii are decreased, but the PAP2 has higher expression in the RNAi transgenic Chlamydomonas strains (Deng et al., 2013, 2014). In our previous study, we determined that tolerant saline-alkali (TSA) Chlorella can survive in an environment containing 600-mm NaCl, and the TSA Chlorella PAP gene was isolated from a TSA Chlorella full-length cDNA yeast library constructed under 1-M NaCl stress conditions (Qiao et al., 2015). Here, we determined the growth rates of transgenic yeast on a solid medium under high salinity and drought conditions. The subcellular localization of the ChPAP protein in yeast cells was detected using confocal microscopy, and the effects of high salinity and drought conditions on ChPAP expression were investigated.

MATERIALS AND METHODS
Chlorella Source, Culture, and Gene
The TSA Chlorella was previously isolated from extreme saline-alkali soil on the Songnen Plain, Heilongjiang Province, China (Wang et al., 2011), which is rich in different salt types, including NaCl and NaHCO3, and grown in liquid Bold's basal medium (BBM, Bold and Wynne, 1984). The culture conditions were 23°C under a 16-h light/8-h dark photoperiod. The illumination intensity was 40-mmol photons m-2 s-1. The TSA Chlorella cells were maintained in solid BBM, and the sub-culturing and rapid propagation of TSA Chlorella was cultured in liquid BBM. A full-length cDNA yeast library of TSA Chlorella was constructed (Qiao et al., 2018). A sequence screened from the 1-M NaCl-treated TSA Chlorella library had close similarity levels to sequences of other species' PAP genes. Accordingly, it was named ChPAP.

Sequence Analysis
The full-length ChPAP sequence was analyzed using BlastX and ORFfinder on the NCBI Web site. GeneDoc 3.0 software was used to align the sequences of the ChPAP protein and other species. The maximum-likelihood-based phylogenetic tree was constructed using MEGA 5.1 software. The transmembrane domains in the ChPAP sequence were predicted using the TMHMM server v. 2.0.

Plasmid Construction, Yeast Transformation, and Stress-Tolerance Assays
The ChPAP cDNA fragment harboring the open reading frame was amplified from TSA Chlorella using PCR with the ChPAP-forward (5′-GGATTCATGGTGCACCGATGTTG-3′; BamHI restriction site) and -reverse (5′-GTCGACTCAACACGACCATGCTGC-3′; KpnI restriction site) primers and Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd., Nanjing, China). For yeast transformation, ChPAP was ligated into the pYES2 vector (Invitrogen, United States) digested with BamHI and NotI restriction enzymes to construct the plasmid pYES2-ChPAP, which was transferred into InVSC1 yeast cells using the PEG/LiAC method (Gietz et al., 1992). First, to test the tolerance of transgenic yeast under different stress conditions, yeast cells containing, independently, the pYES2-ChPAP and empty pYES2 vectors were cultivated in liquid SD-Uracil (pH 5.8) medium at 30°C for 2 days. The concentration of the transgenic yeast cells was adjusted to an OD600 value of 0.5 and then diluted to 10−1, 10−2, 10−3, and 10−4 with sterile H2O. A total of 4.5 μl of each dilution series was placed into a solid yeast (1% yeast extract, 2% peptone, and 2% galactose) medium supplemented independently with 0.8-M NaCl, 1.0-M NaCl, and 1.6-M sorbitol. The empty pYES2 vector in yeast cells was used as a control, and the growth of yeast cells at 30°C was observed and photographed for 5–10 days.

Subcellular Localization of the ChPAP Protein in Yeast
To determine the subcellular localization of ChPAP, the expression plasmid pYES2-ChPAP-EGFP was constructed. The ChPAP full-length sequence with restriction sites was cloned using PCR with EGFP-specific forward GFP-F (5′-GGATCCATGGTGCACCGATGTTG-3′) and reverse GFP-R (5′-GGATCCCAACACGACCATGCTGC-3′) primers. The PCR product was ligated into the pEGFP fusion

http://www.ncbi.nlm.nih.gov
http://www.cbs.dtu.dk/services/TMHMM/
fragment was digested at the BamHI and NotI sites in the 
peGFP plasmid to construct the empty pYES2 vector.

The pYES2-ChPAP-EGFP and pYES2-EGFP plasmids were 
transferred independently into yeast cells. The transgenic 
yeast cells were pre-cultured in liquid medium containing 1% yeast 
extract, 2% peptone, and 2% glucose at 30°C for 2 days. 
Afterward, they were washed three times to remove the remaining 
glucose. The EGFp and ChPAP-EGFP plasmids in yeast cells 
were induced to express in liquid yeast (1% yeast extract, 2% 
peptone, and 2% galactose) medium at 30°C for 6 h, and then, 
ChPAP-EGFP yeast cells were incubated at 30°C with 20-μm 
FM4-64 dye for 3 h. The remaining dye was removed by washing 
three times with sterile H2O before samples were observed. 
The fluorescence was detected using laser-scanning confocal 
imaging system (Olympus Fluoview, FV500). The EGFp and 
FM4-64 signals were excited at 488 nm and 543 nm, respectively.

Expression Analysis of ChPAP
To investigate the ChPAP transcript levels under high salinity 
drought stresses, the TSA Chlorella samples were grown 
on medium supplemented independently with 200-mm NaCl 
and 300-mm sorbitol. The samples were collected at 0, 3, 6, 
12, 24, and 48 h and then ground with a mortar and pestle 
in liquid nitrogen for RNA isolation.

The total RNA of TSA Chlorella was extracted using RNAiso 
Plus reagent (TaKaRa, Japan). The ChPAP-specific forward 
(5’-ATGGGCTCAAGGAAGAC-3’) and reverse 
(5’-TCAGGCTACTTCGCTTCAG-3’) primers were used to 
amplify the cDNA probes using a PCR Digoxigenin Probe 
Synthesis kit (Roche, Switzerland). The northern blot analysis 
was performed in accordance with a previously published 
protocol (Qiao et al., 2018).

RESULTS AND DISCUSSION
Characterization of ChPAP Gene
The ChPAP nucleic acid sequence contained an open reading 
frame of 1,002 nucleotides that translated into 333 amino acids. 
The ChPAP amino acid sequence shared close similarities with 
the previously reported PAP sequences of other species, such 
as 70% similarity with C. variabilis and 53% similarity with 
Microactinium conductrix. A comparison of the ChPAP domains 
with those in PAPs of other species revealed the presence of 
conserved domains 1, 2, and 3 (Figure 1), which contained 
two arginine, one histidine, and two histidine residues, 
respectively. These results were consistent with previously 
reported PAP2 protein structures (Hemrika et al., 1997; Neuwald, 
1997; Stukey and Carman, 1997). The phylogenetic analysis 
showed that ChPAP was closely related to the PAP of C. 
variabilis (XP_005848979.1). The TSA Chlorella firstly clustered 
with unicellular microalgae, and then clustered with microbes,

![FIGURE 1](image-url)

**FIGURE 1** | Sequence alignment of the phosphatidate phosphatase (PAP) domains from tolerant saline-alkali (TSA) Chlorella ChPAP2 with those of other species. The conserved residues, including the KxxxxxxRP (domain 1), PSGH (domain 2), and SRxxxxxHxxxD (domain 3) motif, are indicated by boldface in boxes. The ChPAP2 sequence data have been deposited in the GenBank database and assigned accession no. KT750011. The other PAP protein sequences were downloaded from GenBank.
animals, and plants (Figure 2). Thus, ChPAP2 is the PAP gene of TSA Chlorella.

**Overexpression of ChPAP2 in Yeast Enhanced Tolerance to NaCl and Sorbitol**

The five serial dilutions of transgenic yeast cells were spotted into a solid yeast medium. The growth of yeast cells harboring the empty pYES2 vector was similar to that of the ChPAP2 transgenic yeast in medium containing 1% yeast extract, 2% peptone, and 2% glucose without any stress. The ChPAP2 transgenic yeast grew better than the controls in the presence of 0.8-M NaCl, 1-M NaCl, or 1.6-M sorbitol (Figure 3). Thus, the expression of ChPAP2 in yeast cells improved the tolerance to NaCl and sorbitol, which indicated that ChPAP2 functions as a lipid signaling molecule during abiotic stress (Munnik et al., 1996). PAH1 encoded PAP1 was regulated to express under Zn deficiency and enhanced the activity of PAP enzyme (Soto-Cardalda et al., 2012). In addition, Arabidopsis PAP2 was found that was involved with ABA signaling and regulating the stomatal movements (Paradis et al., 2011). However, there was no relevant report on the investigation of PAP2 under abiotic stress.

**The Expression of ChPAP2 Was Inducible Under NaCl and Sorbitol Stresses**

A northern blot analysis was used to detect ChPAP2 expression patterns. Total RNA was used to analyze the effects of high salinity and drought stresses on ChPAP2 expression. The TSA Chlorella cells were exposed independently to NaCl and sorbitol for 0, 3, 6, 12, 24, and 48 h. The ChPAP2 mRNA expression dramatically increased with the 200-mm NaCl treatment from 6 to 48 h compared with the control (0 h), indicating that ChPAP2 was upregulated (Figure 4A). Thus, in yeast, the increased ChPAP2 expression level may increase NaCl resistance. The PA and DGPP levels also increase under hyperosmotic stress conditions (Munnik et al., 2000). The presence of NaCl in liquid media not only leads to high salinity stress, but also to hyperosmotic stress in plants. We hypothesized that the ChPAP2 expression level may be upregulated, allowing it to catalyze the excess DGPP into PA.

In this study, a sorbitol solution was used as the drought agent (Bocchini et al., 2018; Lu et al., 2019). The ChPAP2 expression levels did not differ significantly compared with those of the control after 3–6 h of exposure to 300-mm sorbitol.

**FIGURE 2** | The maximum-likelihood (ML) phylogenetic relationships of PAP proteins between TSA Chlorella sp. and those of other species. Bootstrap values were calculated 1,000 times, and values below 50% were not included. The TSA Chlorella sp. is indicated by boldface in a box.
The ChPAP2 mRNA level began to rise at 12 h, and the level was the most significantly different from that of the control at 48 h after treatment (Figure 4B). In Saccharomyces cerevisiae, PAP accumulates during exposure to hyperosmotic and dehydration stresses (Munnik et al., 2000). Thus, the upregulated ChPAP2 expression may enhance drought tolerance.

ChPAP2 Localized at the Plasma Membrane and in the Lumen of Vacuoles

The deduced ChPAP2 amino acid sequence was predicted to contain six transmembrane domains (Figure 5A). To determine its subcellular localization, GFP was fused to the C-terminus of ChPAP2 (ChPAP2-GFP). The green fluorescence of the GFP protein alone was almost evenly distributed throughout the yeast cells (Figures 5B1,2). FM4-64 stained the vacuole membrane, and its localization signal was consistent with the vacuolar membrane signal (red fluorescence area; Figure 5B4). Thus, the ChPAP2 protein appeared to localize on the plasma membrane and in the lumen of vacuoles in yeast cells (Figures 5B3,5). The results suggested that ChPAP2 might play important roles in transporting lipids through the plasma membrane and in catalyzing DGPP into PA in the vacuoles.

ChPAP2, as a catalytic enzyme, participates in lipid metabolism. ChPAP2 localized on the plasma membrane may function in maintaining cell membrane stability and the ion balance of the cytoplasm in response to abiotic stresses. The ChPAP2 protein also is localized in the lumen of vacuoles, where it may be involved with lipid translocation and DGPP catalysis to form PA. Therefore, we hypothesized that ChPAP2 participates in catalyzing DGPP in the lumen of vacuoles.

The PAP protein may function as a signaling molecule in planta under stress conditions, and its levels accumulate during hyperosmotic and dehydration stresses (Munnik et al., 1996). Therefore, the PAP enzyme may play a role in regulating specific cellular DGPP and PA pools under stress conditions (Oshiro et al., 2003).

In summary, the ChPAP in TSA Chlorella was upregulated expression in treated with high salinity and drought, and the ChPAP in yeasts could tolerate high salinity and drought stresses. Its protein was localized at the plasma membrane and in the lumen of vacuoles. The ChPAP might translocate excess NaCl and sorbitol from plasma membrane and then segregate them into vacuole to regulate ion balance in the cytoplasm. As a consequence, the PAP as a novel transporter can enhance high salinity tolerance and accumulate excess high salinity. These characteristics make ChPAP for the bioremediation of saline-alkali soil.
DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

JW designed the research. QS, YR, and DS performed the experiments. HZ, JZ, and SG analyzed the data. KQ and AZ wrote the manuscript. All authors revised and approved the manuscript.

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