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Effects of Salt Concentrations and Nitrogen and Phosphorus Starvations on Neutral Lipid Contents in the Green Microalga *Dunaliella tertiolecta*

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ABSTRACT: *Dunaliella tertiolecta*, a halotolerant alga, can accumulate large amounts of neutral lipid, which makes it a potential biodiesel feedstock. In this study, neutral lipids of *D. tertiolecta* induced by different salinities or N or P starvation were analyzed by thin-layer chromatography (TLC), flow cytometry (FCM), and confocal laser scanning microscopy (CLSM). High salinities or N or P starvation resulted in a decrease in cell growth and chlorophyll contents of *D. tertiolecta*. Neutral lipid contents increased markedly after 3−7 days of N starvation or at low NaCl concentrations (0.5−2.0 M). N starvation had a more dramatic effect on the neutral lipid contents of *D. tertiolecta* than P starvation. Four putative ME isozymes in different conditions can be detected by using isozyme electrophoresis. Two alternative acetyl-CoA producers, *ACL* and *ACS* genes, were up-regulated under low salinities and N starvation. It was suggested that low salinities and N starvation are considered efficient ways to stimulate lipid accumulation in *D. tertiolecta*.

KEYWORDS: *Dunaliella tertiolecta, nitrogen starvation, phosphorus starvation, neutral lipid, flow cytometry*

INTRODUCTION

Oleaginous microalgae have been considered as a kind of promising alternative source for next-generation renewable fuels. Microalgae are rich in triacylglycerol (TAG, neutral lipid), which can be used for biodiesel production, under stress conditions, especially during nitrogen (N) starvation. The advantages of exploiting microalgae as biofuel feedstock are mainly due to their high lipid contents, high photosynthetic efficiency, short life cycle, less affected by geographical regions, low labor requirement, and ease of scale-up.[1,2] *Dunaliella tertiolecta*, a halotolerant alga, was reported to contain a large amount of lipids (up to 71.0% per dry cell weight), which made it a potential biodiesel feedstock.[3] Numerous papers have indicated that N deprivation improves lipid accumulation in many microalgae, for example, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Chlorella zofingiensis*, *Micractinium pusillum*, and *D. tertiolecta*.[4−7] It was reported that lipid content increased under nitrogen starvation condition with time, and phosphate deprivation had little effect on lipid accumulation in *D. tertiolecta*.[7] However, little is revealed about the molecular mechanisms of lipid production under N or P starvation and other cultivation conditions in *D. tertiolecta*.

In microalgae, the lipid biosynthetic pathway includes fatty acid synthesis and TAG synthesis. To accumulate fatty acids, it is important to have a sufficient supply of NADPH in the cytosol and a continuous provision of acetyl-CoA as an essential precursor for fatty acid synthetase.[2,8] Malic enzyme (ME) plays an important role in regulating the lipid accumulation process in many organisms. ME catalyzes the synthesis of pyruvate from the decarboxylation of malate with the formation of NADPH, which is necessary for the synthesis of fatty acids.

In plants and algae, as for the precursor (i.e., acetyl-CoA) for fatty acid synthesis, it was reported that there are three pathways involved in the formation of acetyl-CoA: acetyl-CoA synthetase (ACS), ATP-citrate lyase (ACL), and pyruvate dehydrogenase (PDH).[9,10] It was recommended that the accumulation of high TAG mainly relies on the enhancement of acetyl-CoA production.[11] Algal ACLs are composed of two different subunits, ACLA and ACLB.[9] It was reported that two alternative acetyl-CoA producers, *ACS* and *ACL*, were up-regulated before TAG accumulation in the oleaginous species *Chlorella desiccate* under nitrogen deprivation.[10]

The traditional gravimetric method has been used for the detection of lipid content in microalgae, but it is time-consuming and needs a relatively large algal sample size.[12] In contrast, the fluorescent staining method provides an inexpensive and efficient way to detect neutral lipid content and requires only a small sample volume. However, the Nile Red staining method would be greatly influenced by many factors including algal species and different measurement conditions.[13] The emitting light of the Nile Red is at 590−640 nm, which may interfere with the detection of chlorophyll fluorescence (detected at 650−700 nm). Compared with Nile Red, a lipophilic bright green fluorescent dye, BODIPY 505/515, has a high oil/water partition coefficient and sharp emission bands.[13,14] BODIPY 505/515 is stimulated by a blue 488 nm laser and detected at 505−515 nm, which is spectrally different from algal chloroplasts.

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In this study, neutral lipids induced by different NaCl concentrations and N or P starvation were analyzed by thin-layer chromatography (TLC), flow cytometry (FCM), and confocal laser scanning microscopy (CLSM). The effects of different conditions on algal biomass and photosynthetic pigment synthesis were also discussed. The isozyme activity of ME in different conditions was investigated by using isozyme electrophoresis. The transcript levels of *ACL* and *ACS* genes from *D. tertiolecta* (*DtACLA*, *DtACLB*, and *DtACS*) in response to N or P deficiency and different salinities were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

Microalgal Strain and Culture Conditions. *D. tertiolecta* FACHB-821 was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. *D. tertiolecta* cells were grown in a *Dunaliella* medium[15] containing 2.0 M NaCl (as normal condition) at 26 °C and 8000 lx provided by cool-white fluorescent lamps under a 16/8 h dark/light cycle for 14−16 days. The optical density (OD) of the algal samples was detected at 630 nm (OD_{630}) by a spectrophotometer (Agilent, USA). After 7 days of normal growth (2.0 M NaCl) in N-sufficient medium (N+) (OD_{630} = 07−0.8), the algal cells were harvested by centrifugation at 2000*g* for 5 min and resuspended twice with a fresh N-deficient medium (N−) without $NaNO_3$ for a second period of N starvation cultivation for 7 days. For phosphorus (P) starvation treatment, algal cells in P-sufficient medium (2.0 M NaCl) (P+) were harvested by centrifugation at 2000*g* for 5 min and resuspended twice with a fresh P-deficient medium (P−) without $NaH_2PO_4{\cdot}2H_2O$ for P starvation cultivation for 7 days. To study lipid accumulation of *D. tertiolecta* in different salt concentrations, algal cells were grown in *Dunaliella* media with 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 M NaCl for 14−16 days, respectively.

Algal Biomass Analysis. *D. tertiolecta* cells were grown in a defined medium containing 2.0 M NaCl for 14 days. The optical density of the algal samples (using blank medium to dilute into different proportions, 1:1, 1:2, 1:5, 1:10, and 1:15) was detected at 630 nm (OD_{630}) by a spectrophotometer (Agilent, USA), and the blank medium without algal cells was used as the control sample. For the measurement of algal dry cell weight (DCW), culture samples (100 mL) were harvested by centrifugation at 8000 rpm for 5 min, washed twice with distilled water, and dried at 80 °C to a constant weight for 24 h. Then the DCW was obtained by determining the OD_{630}, $y = 993.21x - 16.359$, $R^2 = 0.9988$, where y = DCW (mg/L) and x = OD_{630} value ($0.05 < OD_{630} < 0.9$). The OD_{630} of the algal sample should be determined after proper dilution with fresh medium to give an absorbance range of 0.05−0.9.

The cell counts of algal samples (using blank medium to dilute into different proportions, 1:1, 1:2, 1:5, 1:10, and 1:15) were detected by direct counting by using a light microscope (magnification ×40) with the hemocytometer. Then the relationship curve between OD_{630} and cell number can be obtained, $y = 3418.3x + 226.33$, $R^2 = 0.9908$, where y = cell number ($\times 10^4$) and x = OD_{630} value.

Extraction and Estimation of Photosynthetic Pigments from *D. tertiolecta*. Two milliliters of algal cells was harvested by centrifugation at 8000 rpm for 5 min. Then, the cell pellets were washed twice with fresh medium and mixed with 2 mL of 100% methanol. The mixtures were incubated overnight at 4 °C in the dark and then centrifuged at 3000*g* for 10 min to recover the supernatant with the pigments. The absorbance of the supernatant was read at 665.2 and 652.4 nm by UV−visible spectrophotometer (Agilent, USA) to measure chlorophyll *a* (Chl a) and chlorophyll *b* (Chl b) contents and at 470 nm to measure total carotenoids (Car) content. The Chl a, Chl b, and Car contents could be determined by the equations of Lichtenthaler et al.:[16]

$$\text{Chl a}\ (\mu g/mL) = 16.72A_{665.2} - 9.16A_{652.4}$$

$$\text{Chl b}\ (\mu g/mL) = 34.09A_{652.4} - 15.28A_{665.2}$$

$$\text{Car}\ (\mu g/mL) = (1000A_{470} - 1.63\text{Chl a} - 104.96\text{Chl b})/221$$

Lipid Extraction from *D. tertiolecta*. One hundred and twenty milliliters of algal samples at the log phase (OD_{630} = 0.8−0.9) was harvested by centrifugation at 8000 rpm for 5 min and washed twice with 1% NaCl solution. Then the cell pellet was resuspended with 6 mL of chloroform/methanol solution (2:1, v/v) and sonicated (300 W, 3 min) on ice with a JY92-IID ultrasonic cell disruptor (Ningbo Scientz Biotechnology Ltd. Co., China). Then water and methanol were mixed to create the mixture of chloroform/methanol/water (1:1:0.9, v/v/v) and incubated for 10 min. Next, the mixture was centrifuged at 10000*g* for 10 min at 4 °C for phase separation. The upper layer (methanol−water layer) was removed, and the chloroform layer containing lipid was separated out.

TLC Analysis of Lipid. Lipid extracts (20 μL) from the chloroform extraction mentioned above were separated by TLC (silica gel 60 F254, 10 × 10 cm, 0.25 mm thickness, Merck, Germany) in a mixture of *n*-hexane/diethyl ether (3:1, v/v). Glyceryl trioleate (0.2 mg) was used as a reference substance for lipids. After separation by TLC, lipids can be reversibly stained by iodine vapor at 37 °C for 10 min. The neutral lipid content of *D. tertiolecta* was detected by ImageJ software (ver1.41, NIH) and determined as a percentage of DCW.

FCM Analysis. BODIPY 505/515 (Invitrogen, USA) was used to stain neutral lipids in the organelles, also called lipid bodies.[14] BODIPY 505/515 was prepared in 0.1% dimethyl sulfoxide (DMSO; v/v) to give a final concentration of 1.0 mM. First, 0.2 mL of algal cells was centrifuged at 3000 rpm for 5 min. Then the cell pellets were washed twice using the fresh medium, and 1−2 μL of 1.0 mM BODIPY 505/515 was added for staining. Samples stained with BODIPY 505/515 were detected on a board in the FACSAria Flow Cytometer (Becton Dickinson, USA) equipped with a blue 488 nm laser and an optical filter FL1 (530/30 nm). The data were analyzed using FlowJo software (Tree Star, USA).

CLSM Analysis. Microscopy analysis of algal cells stained with BODIPY 505/515 was performed using a CLSM instrument (Zeiss LSM 710 NLO, Germany). The green BODIPY fluorescence was excited with a blue 488 nm laser and detected at 505−515 nm. The red autofluorescence of the algal chloroplasts was detected at 650−700 nm.

Electrophoresis Analysis of Malic Enzyme (ME) Isozymes. One hundred and twenty milliliters of algal samples cultivated under different conditions was collected by centrifugation at 2000*g* for 5 min. Then the pellets were washed twice using fresh medium and mixed with 2 mL of extraction buffer (100 mM Tris, 20 mM ascorbic acid, pH 6.9). The mixture was disrupted by ultrasonication (300 W, 3 min) on ice with a JY92-IID ultrasonic cell disruptor (Ningbo Scientz Biotechnology Ltd. Co., China) and then centrifuged at 13000*g* for 30 min to recover the supernatants (crude extracts). Protein concentrations were detected using the Modified Bradford Protein Assay Kit (Sangon Biotech, China).

Native-PAGE analysis of the crude extracts was performed with a 5% stacking gel and a 10% separating gel. Electrophoresis was run at 120 V for 1.5 h. The gel was assayed for ME isozymes activity by staining in 50 mL of solution containing 50 mM Tris-HCl (pH 8.0), 700 mg of L-malate, 15 mg of nicotinamide adenine dinucleotide phosphate (NADP), 1 mg of phenazine methosulfate (PMS), 15 mg of nitroblue tetrazolium (NBT), and 50 mg of $MgCl_2$.

Quantitative Real-Time PCR (qRT-PCR) Analysis. To analyze the transcript levels of *DtACLA*, *DtACLB*, and *DtACS* under different NaCl concentations, the total RNA was extracted from the algal samples cultivated in 0.5−4.0 M for 16 days. To test the transcript levels of *DtACLA*, *DtACLB*, and *DtACS* under P starvation, the total RNA was extracted from the algal samples cultivated in P− medium for 1, 3, 5, and 7 days. The untreated algal samples were cultivated in normal condition for 0, 1, 3, 5, and 7 days for control. The procedure of qRT-PCR analysis was mentioned previously.[9] The primers for qRT-PCR analysis were listed as follows: for *DtACLA* (GeneBank: KU316086), 5′-GCAGGACATGGATGAGCGTA-3′ and 5′-GTAGATCACGCTGGCTCCTC-3′; for *DtACLB* (GeneBank: KU316087), 5′-ACATCGTCTCAAGCCTCGTG-3′ and 5′-TTG-

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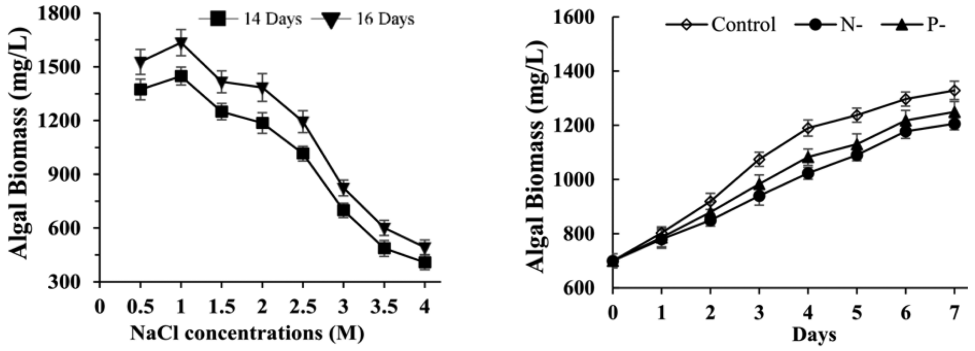

Figure 1. Effects of different conditions on algal biomass of *D. tertiolecta*: (A) effects of different NaCl concentrations on algal biomass; (B) effects of N or P starvation on algal biomass.

AGGCCCTCCACAAAGTC-3′; for *DtACS* (GeneBank: KT692941), 5′-ATTCTGGACGACAAAGGCGT-3′ and 5′-TGGGTAGGGCCC-AAAGTAGT-3′; and for endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*DtGAPDH*), 5′-TGACTTCAAGGGCATGGA-CC-3′ and 5′-GTCGTACCAGGCAACAACCT-3′. Each sample was repeated three times to ensure statistical significance.

Statistical Analysis. Data were expressed as the mean ± SD of three parallel measurements (t test, $p < 0.05$). A p value of <0.05 was considered significant.

RESULTS

Effect of Different Culture Conditions on Cell Growth of *D. tertiolecta*. To evaluate the cell growth of *D. tertiolecta* under different conditions, the algal biomass of different samples was measured. As shown in Figure 1A, under 0.5−2.5 M NaCl, the algal biomass was much higher than under 3.0−4.0 M NaCl. It was indicated that lower salinities are very favorable for cell growth of *D. tertiolecta*. The algal cells were at the late log phase on day 14 and at the stationary phase on day 16. The algal biomass was increased with days. For N or P starvation (Figure 1B), algal biomass was lower than with the untreated samples. Obviously, N or P deprivation resulted in a decrease of cell growth of *D. tertiolecta*. Algal biomass under N starvation was a little lower than that under P starvation.

Analysis of Photosynthetic Pigments. To evaluate the variation of photosynthesis in *D. tertiolecta* under different culture conditions, pigment contents were determined spectrophotometrically. Obviously, Chl a was the main pigment in *D. tertiolecta*. As shown in Figure 2A, at day 16 upon 1.0−2.5 M NaCl concentration, Chl a, Chl b, and Car contents were rather high and then decreased with NaCl concentration from 2.5 to 4.0 M. Chl a and Chl b contents in algal cells of N− medium decreased with time, whereas Car content had no significant changes during 7 days of N starvation (Figure 2B). Chl a, Chl b, and Car contents all decreased with time under P starvation (Figure 2C). It was indicated that high NaCl concentrations, N starvation, and P starvation can lead to a reduction of photosynthetic pigments.

TLC Analysis of Lipid Accumulation. To detect lipid accumulation under different culture conditions in *D. tertiolecta*, the intracellular lipid levels were analyzed by TLC. As shown in Figures 3A and 4A, it was obvious that larger amounts of lipids can be accumulated under 0.5−1.5 M NaCl at day 16. At day 14, the lipid content was highest (23.4 ± 0.89%) upon 1.0 M NaCl, whereas at day 16, the highest lipid content was 38.8 ± 1.47% at 0.5 M NaCl concentration. Lipid contents were

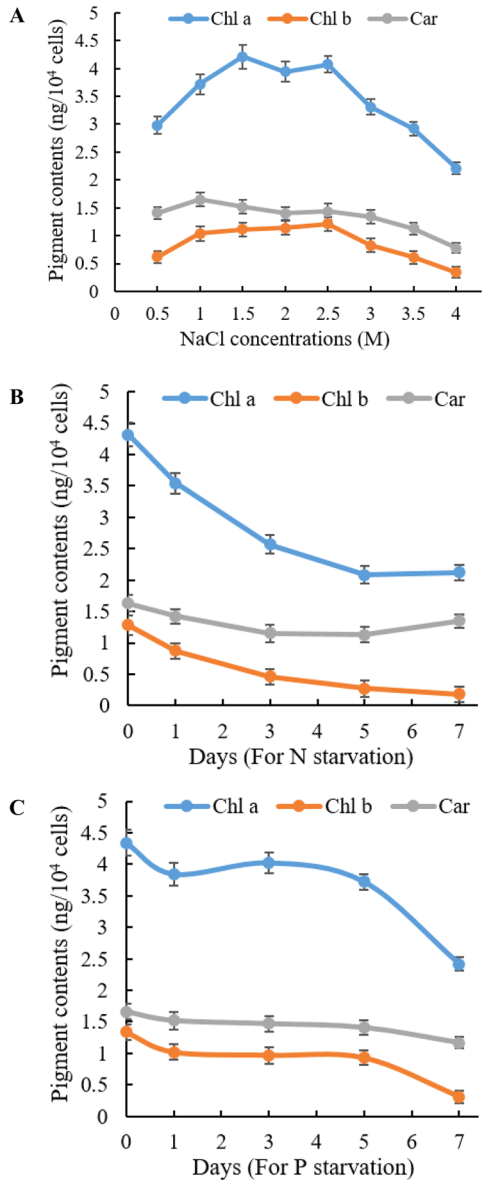

Figure 2. Effects of different conditions on photosynthetic pigment synthesis in *D. tertiolecta* cells: (A) effect of different NaCl concentrations on pigments synthesis; (B) effect of N starvation on pigment synthesis; (C) effect of P starvation on pigment synthesis.

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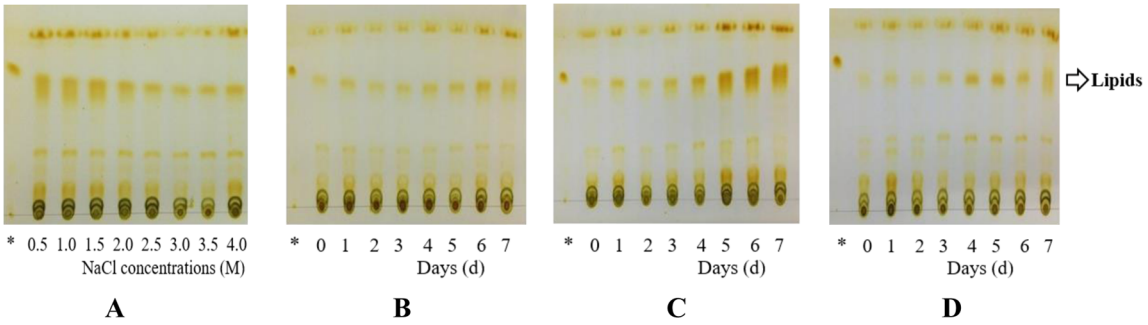

Figure 3. Neutral lipids of *D. tertiolecta* cells cultivated in different conditions separated by TLC method: (A) TLC analysis of lipid accumulation in cells cultivated in different salt concentrations; (B) TLC analysis of lipid accumulation in cells under normal condition (2.0 M NaCl, used as the control of N or P condition); (C) TLC analysis of lipid accumulation in cells under N starvation during 7 days; (D) TLC analysis of lipid accumulation in cells under P starvation during 7 days. The asterisk indicates glyceryl trioleate.

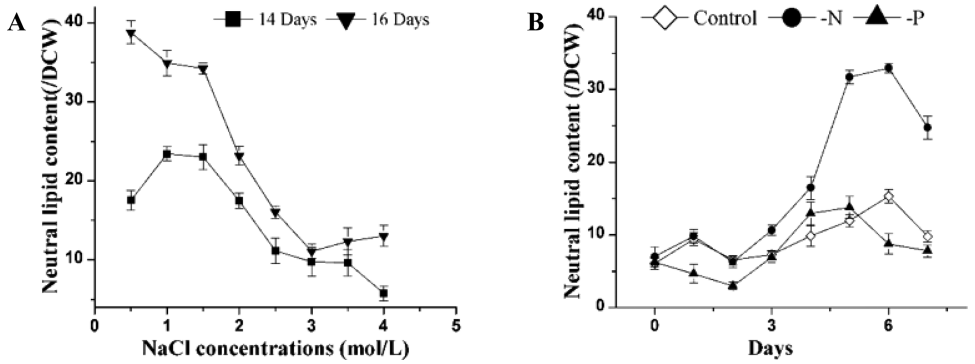

Figure 4. Effects of different culture conditions on neutral lipid contents of *D. tertiolecta*: (A) neutral lipid contents of *D. tertiolecta* cells under different NaCl concentrations at days 14 and 16; (B) neutral lipid contents of *D. tertiolecta* cells under N or P starvation.

decreased with NaCl concentration from 2.0 to 4.0 M. As for N starvation condition shown in Figures 3C and 4B, during the first 2 days of treatment, there were nearly no changes in lipid accumulation of cells cultivated in N+ or N− media. A significant lipid accumulation was observed from day 3 to day 7 of N starvation treatment compared with the untreated sample (Figure 3B,C). At day 6, lipid contents reached the maximum of 32.93 ± 0.62% by DCW (Figure 4B). As for P starvation condition shown in Figures 3D and 4B, only at days 4 and 5 were lipid contents a little higher than those in normal cultivation (control). It seemed that P deprivation had little effect on lipid accumulation. It was indicated that N starvation cultivation can contribute to much more lipid accumulation in *D. tertiolecta* than P starvation condition.

FCM Analysis of Lipid Accumulation under Different Culture Conditions of *D. tertiolecta*. In FCM, forward scatter (FSC) is nearly proportional to cell volume or cell size and side scatter (SSC) is influenced by cell morphology, especially by intracellular structures.[17] The value of FL1 (530/30 nm) reflects the green fluorescence intensity. Because BODIPY 505/515 is a lipophilic green fluorescent dye for determination of neutral lipids, the stronger green fluorescence intensity means a higher lipid accumulation. As shown in Figure 5A, under different NaCl concentrations, it seemed that the size of single algal cell increased with the NaCl concentration. Green fluorescence intensities were much higher upon 0.5−2.0 M NaCl than those upon 2.5−4.0 M NaCl, which indicated that 0.5−2.0 M NaCl concentrations were suitable for lipid accumulation in *D. tertiolecta*. As for nutrient-limited condition shown in Figure 5D, no matter under normal cultivation, N starvation, or P starvation, green fluorescence intensities increased with time; the green fluorescence intensities were much higher under N starvation condition. Compared with the untreated samples (0 days), green fluorescence intensities of algal samples at day 7 of normal cultivation, N starvation, and P starvation were enhanced by 1.76-, 3.37-, and 2.24-fold, respectively. It was suggested that N starvation condition is much more favorable for lipid accumulation.

CLSM Observation of Cell Morphology under Different Culture Conditions of *D. tertiolecta*. To visualize the variation of lipid body formation, *D. tertiolecta* cells stained with BODIPY 505/515 were analyzed by CLSM instrument. In Figure 6A, strong green fluorescence signals were observed under 0.5−2.0 M, whereas weaker green fluorescence signals were detected under 2.5−4.0 M. Besides, the red Chl autofluorescence intensities in cells under 0.5−2.0 M NaCl were stronger than those under 2.5−4.0 M NaCl, which was in agreement with the results of pigment detection (Figure 2A). As for the N starvation condition as shown in Figure 6B, compared with the control, stronger green fluorescence signals could be detected at days 3−7, whereas green fluorescence intensities had no significant difference between the normal condition (control) and P starvation condition. All of these microscopy results were quite in accordance with the TLC results (Figures 3 and 4) and FCM results (Figure 5). Weaker Chl autofluorescence signals were observed under N or P starvation at days 3−7, which indicated that long-term N or P deprivation resulted in a dramatic decrease of Chl synthesis.

Electrophoresis Analysis of ME Isozymes under Different Culture Conditions of *D. tertiolecta*. From Figure 7, four putative ME isozymes (blue bands) can be separated. We inferred that the white bands in Figure 7 may be

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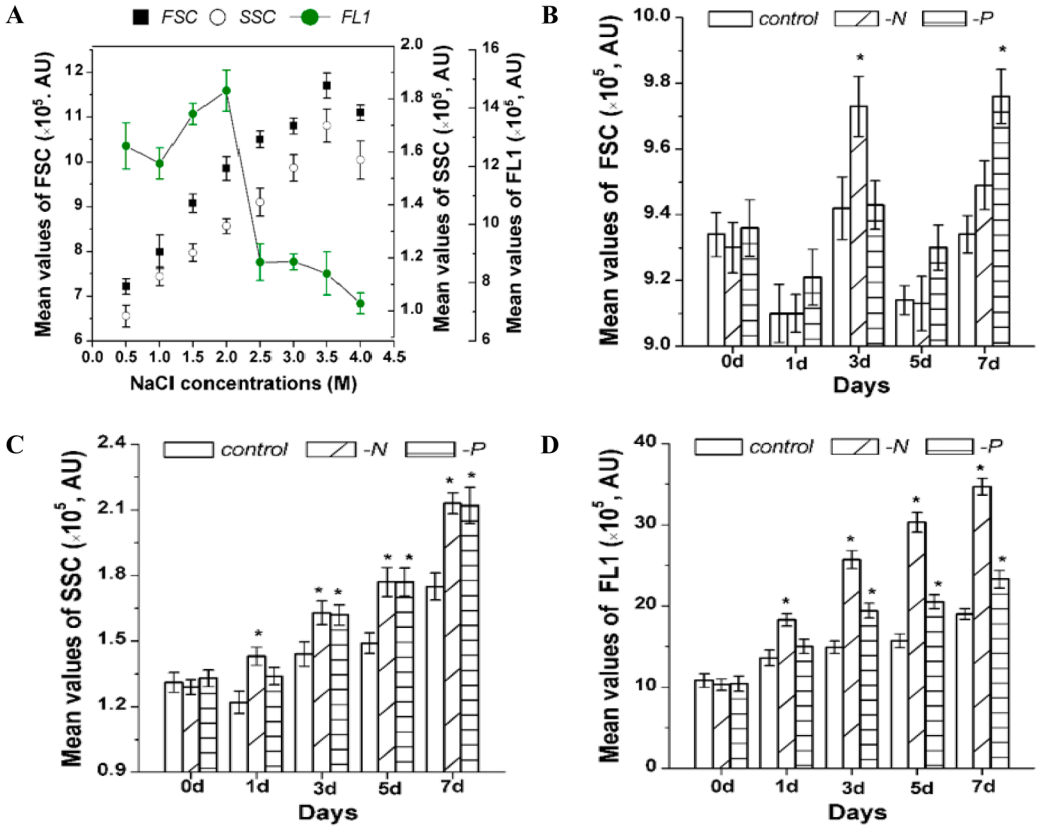

Figure 5. Analysis of mean fluorescence intensity of FSC, SSC, and neutral lipid stained with BODIPY 505/515 under different culture conditions (based on a single algal cell) (t test, $p < 0.05$): (A) fluorescence intensity of neutral lipid stained with BODIPY 505/515 under different NaCl concentrations; (B–D) fluorescence intensity of FSC, SSC, and neutral lipid stained with BODIPY 505/515 under N or P starvation, respectively.

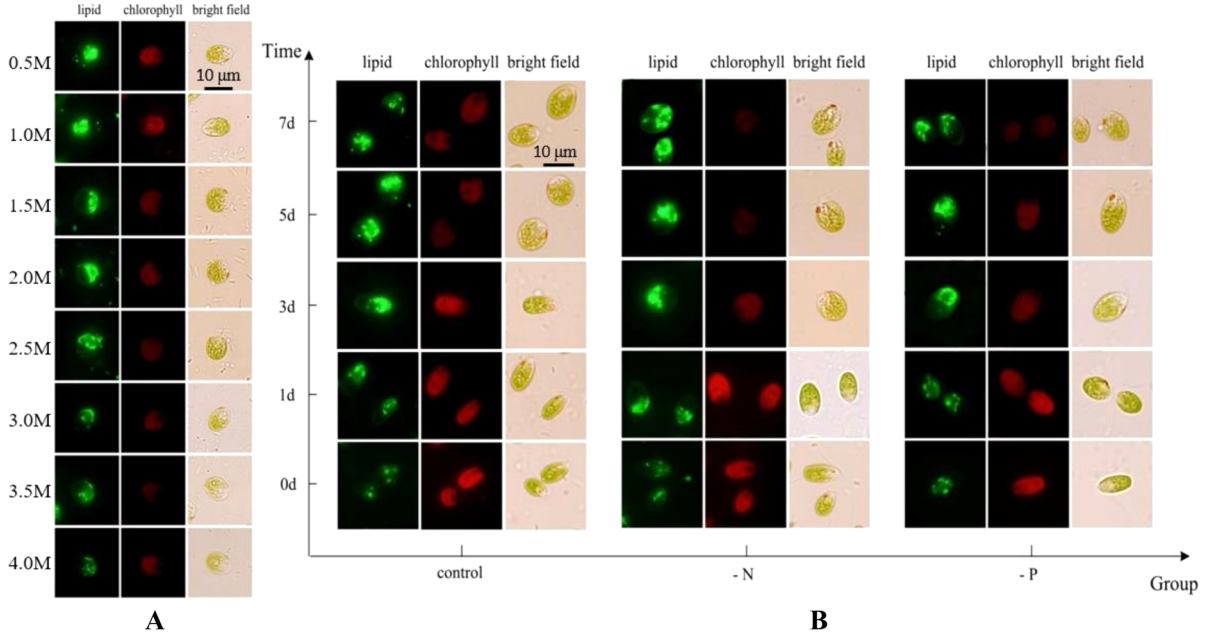

Figure 6. Cell morphology of *D. tertiolecta* cells stained with BODIPY 505/515 cultivated under different conditions: (A) cell morphology of *D. tertiolecta* cultivated under different NaCl concentrations; (B) cell morphology of *D. tertiolecta* cultivated under normal condition (control) and N or P starvation condition.

superoxide dismutase (SOD) isozymes, as SODs can lead to the reduction of nitroblue tetrazolium (NBT),[18] one of the components of the staining solution. SOD isozymes have three types: Fe-SOD, Mn-SOD, and CuZn-SOD.[19] Under different salinities and N or P starvation, the activities of ME isozymes had great effects. It seemed that ME2 and ME3 from *D. tertiolecta* (DtME2 and DtME3) can exist in different culture conditions and have high activities. As shown in Figure

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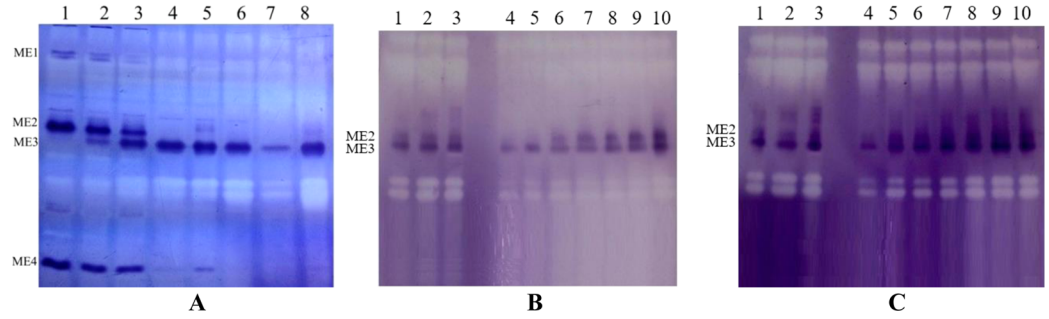

Figure 7. Variations of ME isozyme pattern induced by different conditions in *D. tertiolecta*: (A) activities of ME isozymes under different NaCl concentrations (lanes 1−8, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 M NaCl, respectively); (B) activities of ME isozymes under N starvation (lanes 1−3, untreated samples at days 1, 3, and 5, respectively; lanes 4−10, N starvation at days 1−7, respectively); (C) activities of ME isozymes under P starvation (lanes 1−3, untreated samples at days 1, 3, and 5, respectively; lanes 4−10, P starvation at days 1−7, respectively).

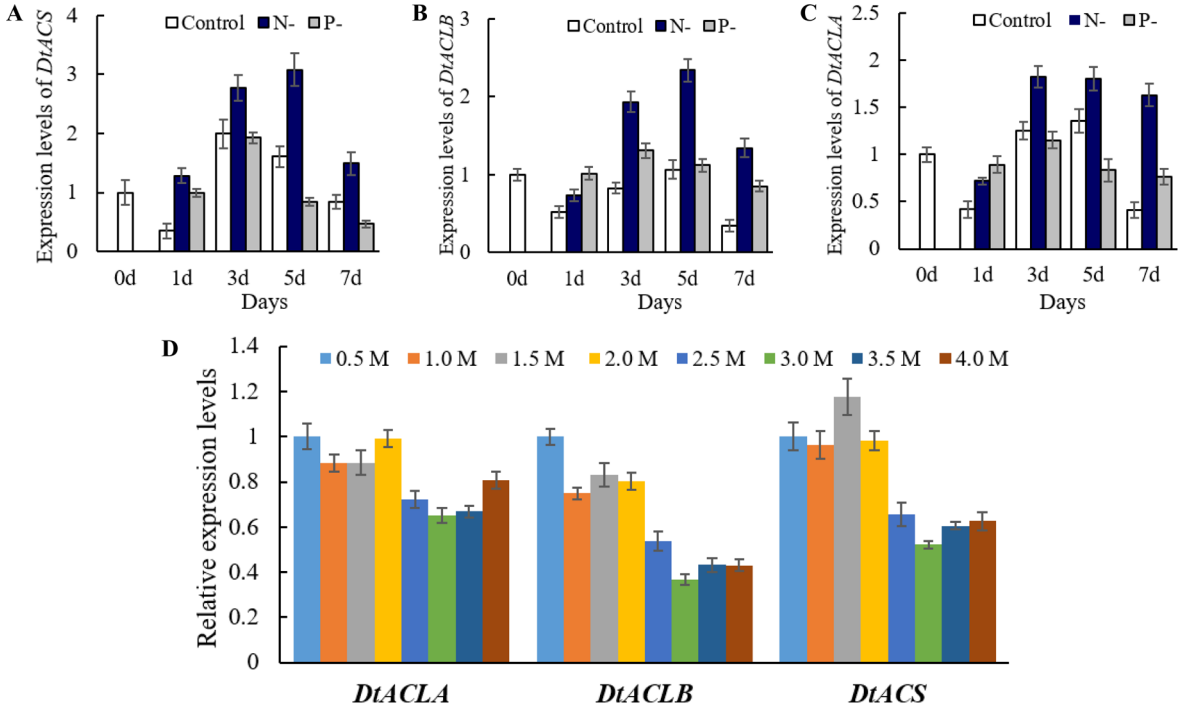

Figure 8. Effects of N starvation, P starvation, and salinity on gene expressions of *DtACLA*, *DtACLB*, and *DtACS*: (A) effects of N or P starvation on gene expressions of *DtACLA*; (B) effects of N or P starvation on gene expressions of *DtACLB*; (C) effects of N or P starvation on gene expressions of *DtACS*; (D) effects of different NaCl concentrations on gene expressions of *DtACLA*, *DtACLB*, and *DtACS*.

7A, DtME1, DtME2, and DtME4 could be expressed only under lower NaCl concentrations, whereas DtME3 still had high activity under high salinities. As for N or P starvation (2.0 M NaCl), only DtME2 and DtME3 can be detected (Figure 7B,C).

MEs are widely found in bacteria, fungi, algae, plants, and animals. MEs take part in various metabolic pathways, for example, photosynthesis, lipid synthesis, and energy metabolism.[8] On the basis of the substrate specificity and coenzyme preference, MEs are classified into three types:[20] (1) NAD^+-ME (EC 1.1.1.38) is able to decarboxylate oxaloacetic acid (OAA) in the presence of NAD^+; (2) $NAD(P)^+$-ME (EC 1.1.1.39) prefers to use NAD^+ rather than $NADP^+$, but it is unable to decarboxylate OAA; (3) $NADP^+$-ME (EC 1.1.1.40) catalyzes the decarboxylation of OAA in the presence of $NADP^+$. The released CO_2 from malate by ME enzyme can be refixed through the Calvin cycle for photosynthesis.[8] In the mitochondrial matrix, malate can be catalyzed to form pyruvate (by ME enzyme) or OAA, both of which can enter the tricarboxylic (TCA) cycle for energy metabolism.[8] ME catalyzes the decarboxylation from malate to pyruvate with the formation of NADPH, which is necessary for the synthesis of fatty acids. In eukaryotes, MEs are located in the mitochondria or cytosol. In plants and green algae, MEs can also exist in the chloroplasts.[8] In combination with the results of chlorophyll analysis (Figures 2 and 6), DtME1 and DtME4 may be located in chloroplasts and involved in photosynthesis. They existed only in lower salinities (Figure 7A), and chlorophyll synthesis had no effects on these conditions. DtME2 seemed more related to lipid accumulation and may be located in cytosol and participate in lipid synthesis; although DtME3 is quite common in various conditions, DtME3 may be located in mitochondria and involved in energy metabolism.

qRT-PCR Results. We have previously reported that the transcript levels of *DtACLA*, *DtACLB*, and *DtACS* were enhanced under N-deficient cultivation, compared with the

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untreated *D. tertiolecta* cells.[9,21] These related data are also shown in Figure 8A−C. As for P starvation cultivation, it seemed that the expression levels of *DtACLA*, *DtACLB*, and *DtACS* are not much different from the untreated algal cells (control). On the basis of the results of TLC and FCM analysis (Figures 3, 4, and 5), P starvation had little effect on lipid accumulation. It was quite consistent that the expression of *ACL* and *ACS* genes involved in lipid synthesis also had not much influence under P starvation. As for effects of NaCl concentrations on gene expressions of *DtACLA*, *DtACLB*, and *DtACS*, it was obvious that under 0.5−2.0 M NaCl, the transcription levels of these genes were a little higher than under 2.5−4.0 M NaCl (Figure 8D). All of these results indicated that the expression levels of *DtACLA*, *DtACLB*, and *DtACS* were related to lipid accumulation.

DISCUSSION

N starvation is one of the efficient ways of stimulating lipid accumulation in microalgae. In this study, the TLC result showed that neutral lipid contents increased markedly after 3−7 days of N starvation in the green alga *D. tertiolecta* (Figures 3C and 4B), which was consistent with the further experiments by FCM (Figure 5) and CLSM (Figure 6B). At day 6 of N starvation, lipid contents were highest (Figure 4B). As for P starvation, it seemed that P deprivation had no significant influence on accumulation of neutral lipids in *D. tertiolecta* (Figures 3D and 4B). N starvation possessed a more dramatic effect on the accumulation of neutral lipids in *D. tertiolecta* than P starvation. Similarly, it has been reported that lipid accumulation in *D. tertiolecta* under nitrogen starvation was time-dependent, and P starvation had little effect on accumulation of neutral lipids.[7] It was indicated that the green alga *Micractinium pusillum* accumulated significant amounts of TAGs after 6 days of N starvation.[6] A significant lipid enhancement in *Chlorella sorokiniana* C3 cells could be detected after 8 days of N starvation.[22] The lipid content in *Chlorella pyrenoidosa* cultivated in P-deficient condition experienced only a modest increase.[23] In *Chlamydomonas reinhardtii*, N starvation had a more significant influence on the physiological changes and protein and neutral lipids contents than P starvation.[24] As for salinity, low NaCl concentrations were much better for cell growth (Figure 1A) and lipid accumulation (Figures 3A, 4A, and 6A) in *D. tertiolecta*.

Obviously, high salinities and N or P starvation resulted in a decrease in cell growth (Figure 1) and chlorophyll contents (Figure 2) of *D. tertiolecta*. N is one of the most abundant and important elements and primary constituents of intracellular components. N starvation can cause an obvious reduction in protein and chlorophyll contents, including the decrease of ribosomal polypeptides and some important enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) for CO_2 assimilation.[23] P is a constituent ingredient of ATP and necessary for photophosphorylation, which is of significance for the metabolism and cell growth of microalgae. In P-deficient cultures, the reactions involved in the Calvin cycle were reduced, as photophosphorylation ability and ATP synthesis were decreased. Accordingly, P depletion severely influenced the metabolism of microalgae and caused an obvious reduction in algal biomass in contrast with the control.[23,25] It was also indicated that in *Dunliella salina*, growth-retarding environmental conditions such as nutrient limitation and high NaCl are considered to cause the decrease of chlorophyll.[26]

Salt concentrations and N or P starvation would influence the expression of genes in relation to lipid synthesis in *D. tertiolecta* according to the results of ME isozyme electrophoresis (Figure 7) and qRT-PCR (Figure 8). Low salinities and N starvation can induce the expression of *DtACLA*, *DtACLB*, and *DtACS* (Figure 8). These three genes are two alternative acetyl-CoA producers. A continuous provision of acetyl-CoA is necessary for fatty acids and lipid synthesis. In the oleaginous species *Chlorella desiccate*, *ACS* and *ACL* genes were up-regulated under N deprivation.[10] It was indicated that enzymes in the TAG biosynthesis of *Chlorella pyrenoidosa* such as ME, ACC, and diacylglycerol acyltransferase (DGAT) are highly related to lipid accumulation.[23] The engineered *Chlorella pyrenoidosa* overexpressing *PtME* gene from *Phaeodactylum tricornutum* significantly increased the neutral lipid content by 3.2-fold compared with wild type.[27] Overexpression of the *ME* and *AccD* (one subunit of acetyl-CoA carboxylase) genes in *Dunaliella salina* led to a 12% increase in total lipid content.[28] In *Micractinium pusillum*, it was shown that genes related to isoprenoid biosynthesis, pyruvate and acetyl-CoA synthesis, and TAG degradation were induced under N deficiency, and genes involved in cell growth, photosynthesis, cell cycle regulation, and amino acid synthesis were down-regulated dramatically.[6] The transcriptional profile of the model diatom *Phaeodactylum tricornutum* showed that genes related to glycerolipid metabolism, TCA cycle, and carbon fixation were up-regulated under N starvation, but the expression levels of most light-harvesting complex genes were decreased, and photosynthetic efficiency was declined.[29]

In conclusion, high salinities and N or P starvation resulted in a decrease in cell growth and chlorophyll contents of *D. tertiolecta*. N starvation or lower NaCl concentrations (0.5−2.0 M) led to a significant increase of neutral lipid contents, whereas P deprivation had little effect on lipid accumulation in *D. tertiolecta*. N deprivation led to a more dramatic enhancement of the neutral lipids in *D. tertiolecta* than P deprivation. The DtMEs isozymes displayed different activities in different cultivation conditions. Two alternative acetyl-CoA producers from *D. tertiolecta*, *DtACL* and *DtACS* genes, were up-regulated under low salinities and N starvation. It was suggested that low salinities and N starvation are thought to be efficient ways to stimulate lipid accumulation in *D. tertiolecta*.

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Notes

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