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Cloning and stress-responding expression analysis of malonyl CoA-acyl carrier protein transacylase gene of *Nannochloropsis gaditana*

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ABSTRACT

Malonyl CoA-acyl carrier protein transacylase (MCAT, E2.3.1.39) is closely associated with the FASII pathway of fatty acid biosynthesis. However, the information about microalgal MCAT is scarce. In this study, a MCAT gene was isolated from *Nannochloropsis gaditana* with its deduced protein (NgMCAT) characterized with bioinformatic tools. The abundance of the *NgMCAT* transcript under different environmental conditions was determined with real-time quantitative PCR. Results showed that the open reading frame (ORF) of *NgMCAT* was 1059 bp in length, which encoded 352 amino acid residues. The abundance of *NgMCAT* transcript reached the maximum (5.17-fold) at 6 h when sodium nitrate was limited, and reached the maximum (4.25-fold) at 12 h at low temperature (15 °C). The abundance of *NgMCAT* transcript fluctuated at high temperature (35 °C) when the concentration of nitrate and sodium chloride exceeded 150 mg/L and 62 g/L, respectively. In addition, some components of fatty acid that changed with the expression of *NgMCAT* were also observed.

1. Introduction

As a pivotal enzyme in the FASII pathway, malonyl-CoA: acyl carrier protein transacylase (MCAT, E2.3.1.39) is responsible for transferring the malonyl group from malony-CoA to the holo-ACP via formation of free CoASH and malonyl-ACP (Hong et al., 2010). Previous studies show that MCAT catalyzes a two-step reaction in which MCAT binds to malonyl-CoA, forming a stable tetrahedral malonyl-MCAT intermediate, and then the holo-ACP docks on the surface of the intermediate to receive malonyl (Keatinge-Clay et al., 2003; Natarajan et al., 2012).

Recently, many trials have oriented to increasing cellular lipid content of microalgae. It was found that the lipid content of microalgae was associated with diverse environmental factors such as illumination and temperature and the availability of ferric ion, nitrogen, phosphate, sodium chloride, inorganic carbon and among others (Gordillo et al., 1998; Pal et al., 2011). Particularly, under temperature, nitrogen and sodium chloride stress, the lipid content will increase obviously. But high lipid content and biomass productivity are in contradiction with each other (Courchesne et al., 2009). Therefore, reaching high lipid content without losing high biomass productivity is the target of both breeding and culturing. Two-stage culture is a common practice in which cell growth and lipid accumulation are separated into two phases (Schenk et al., 2008). However, stimulating lipid accumulation may result in severely impeded cell growth and photosynthesis. As an alternative, genetic modification of the genes involved in lipid biosynthesis and accumulation promises the obtaining of high lipid contents and biomass synchronically. Biotechnological processes based on transgenic microalgae are still in their infancy and more works should be done for the identification, purification, characterization and stress-responding expression of genes involved in lipid accumulation (Khozin-Goldberg and Cohen, 2011; Radakovits et al., 2010).

As one of the most promising biofuel producers, *Nannochloropsis gaditana* is receiving more and more attention due to its high lipid content and photosynthesis efficiency, avoidance of land and successfulness of cultivation (Gouveia and Oliveira, 2009). Identification of genes associating with lipid metabolism of *N. gaditana* will facilitate its practical application. However, compared with higher plants, only a few genes involved in lipid metabolism have been identified in microalgae. For example, the MCAT gene, a key gene functioning inthe FASII pathway, has been isolated and characterized from many organisms (e.g., *Brassica napus*, *Jatropha curcas*, *Arachis hypogaea*) (Li et al., 2009; Simon and Slabas, 1998; Sudheer Pamidimarri et al., 2010). However, little work addressed this gene in microalgae. In the present study, a full length cDNA of *NgMCAT* was isolated, the abundance of the *NgMCAT* transcript under different environmental conditions were determined and the correlation between the transcript abundance of *NgMCAT* and FAT synthesis level was also discussed. Results presented here might provide valuable information for the future study of lipid accumulation in *N. gaditana* and other microalgae.

Abbreviations: MCAT, malonyl CoA-acyl carrier protein transacylase; RT-PCR, reverse transcription PCR; nr, non-redundant protein database; BLAST, Basic Local Alignment Search Tool; MW, molecular weight; pI, isoelectronic point; CDD, Conserved Domains Database; ORF, open reading frame.

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2. Materials and methods

2.1. Algal strain and culture condition

N. gaditana HH-1 was maintained in the Research Center for Marine Ecology, The First Institute of Oceanography, State Oceanic Administration of China. A f/2 medium (Guillard and Ryther., 1962) was prepared with seawater and autoclaved at 121 °C for 20 min. The seawater used in this experiment was collected from a nearby shore of Qingdao and filtered through a 0.44 μm membrance. *N. gaditana* was cultured in the f/2 medium at 25 °C and 100 μmol m^{-2} s^{-1} (under cool-white fluorescent lamps) with a rhythm of 14 h light/10 h dark. The culture was shaken three times a day. Algal cells were harvested at 6×10^7 cells mL^{-1} by centrifuging at 4500 rpm for 5 min and resuspended in a fresh f/2 medium.

To determine the effect of temperature on *NgMCAT* expression, the alga was inoculated into a 300 mL f/2 medium to 5×10^6 cells mL^{-1} and cultured at 15 °C, 25 °C and 35 °C, respectively. To confirm the effect of nitrogen content on the expression of *NgMCAT*, the alga was inoculated into a 300 mL f/2 medium without sodium nitrate (nitrogen limited) or with 75 mg L^{-1} sodium nitrate (nitrogen optimal) or 150 mg L^{-1} sodium nitrate (nitrogen excess) to 5×10^6 cells mL^{-1}. To analyze the response of *NgMCAT* to sodium chloride, the alga was inoculated into a 300 mL f/2 medium with 32 g L^{-1} sodium chloride (normal salinity) or 64 g L^{-1} sodium chloride (high salinity) to 5×10^6 cells mL^{-1}. Algal cells cultured under different conditions were harvested at 7 different time intervals (0 h, 6 h, 12 h, 24 h, 36 h, 48 and 72 h; 0 h as control) by centrifugation at 4500 rpm and 4 °C for 5 min, washed three times with deionized water, frozen in liquid nitrogen immediately and used for total RNA extraction and compositions analysis of fatty acid, three parallel samples each.

2.2. Total RNA preparation

According to the manufacturer's introduction, the total RNA was extracted with a TransZol Plant RNA Extraction Kit (Transgen, Beijing, China). The harvested cells were ground into powder in liquid nitrogen, mixed with 1 mL of a Trizol reagent and homogenized at room temperature for 30 s. Total RNA was precipitated with isopropanol, washed with ethanol (75%) and dissolved in 30 μL of RNA-free water, treated with Dnase I (Promega, USA) and stored at −80°C. The integrity and purity of total RNA were assessed with a spectrophotometer (Amersham, USA) and agarose gel electrophoresis.

2.3. Rapid amplification of cDNA ends

The first-strand cDNA was synthesized with RT-PCR (Transgen, Beijing, China) following the kit manufacturer's instruction. A partial *NgMCAT* cDNA sequence was obtained from the transcriptome library that we have deposited at DDBJ/EMBL/GenBank with accession number GAGR01000000 (Zheng et al., 2013). Two pairs of nested PCR primers, 5GSP1 (outward), 5GSP2 (inward) and 3GSP1 (outward), 3GSP2 (inward) were designed for 5′ RACE and 3′ RACE, respectively (Additional file 1). The PCR product with expected length was excised and purified with an Agarose Gel DNA Fragment Recovery Kit (Transgen, Beijing, China), ligated with PMD19-T (Transgen, Beijing, China), transferred into *Escherichia coli* and sequenced commercially (Sango Co., Ltd, Shanghai, China).

2.4. Sequence analysis

The sequencing reads were assembled into a full length cDNA (*NgMCAT*) with MEGA 5.0 (Kumar et al., 2008). The ORF was predicted with an ORF finder (http://www.ncbiikol.nlm.nih.gov/gorf/gorf.html), from which NgMCAT was deduced. The deduced NgMCAT was used as the query in searching against the non-redundant (nr) protein database of the NCBI (http://www.ncbi.nlm.nih.gov) with BLAST (Basic Local Alignment Search Tool). The MW (molecular weight) and pI (isoelectronic point) were calculated with ProtParam (http://expasy.org/tools/protparam.html). Conserved domains of deduced NgMCAT were identified with the CDD (Conserved Domains Database) search server (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Transmembrane regions were predicted with a TmPred algorithm (http://www.ch.embnet.org/software/TMPRED_form.html). Deduced NgMCAT was also aligned with the retrieved homologs with Clustal X 1.81 with similarity calculated with a closed GeneDoc program. The phylogenetic tree was constructed with Mega 5.0 using a neighbor-joining algorithm with 1000 permutations (Tamura et al., 2011).

2.5. Real-time quantitative PCR

The expression of *NgMCAT* was analyzed using RT-qPCR with the ABI PRISM® 7,500 Real Time PCR system (Applied Biosystems) and TransStart™ Top Green qPCR Kit (Transgen, China). According to the manufacturer's instruction, a pair of primers (MCAT-A, MCAT-S) (Additional file 1) were designed and used to quantify the abundance *NgMCAT* transcript. The abundance of β-action and 18S ribosomal RNA gene was used as internal reference.

The first-strand cDNA was used as the template. RT-qPCR reaction was performed in a volume of 25 μL containing 20 μmol/L primer (each direction), 12.5 μL of 2× TransStart™ Top Green qPCR SuperMix, 0.5 μL of a passive reference dye and 40 ng of cDNA. The mixture was pre denatured at 95 °C for 10 min and followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 15 s and extending at 72°C for 34 s. A melting curve program was followed to verify the specificity of amplification. The $2^{-\Delta\Delta Ct}$ method was used to analyze the transcript abundance of *NgMCAT* (Livak and Schmittgen, 2001), where Ct is the cycle number at which the fluorescent signal rises statistically above the background.

2.6. Composition analysis of fatty acids

The composition analysis of fatty acids was performed as described by Cha and Balasubramanian (Balasubramanian et al., 2013; Cha et al., 2011). Briefly, the samples were dried with a vacuum freeze drier and grinded sufficiently in liquid nitrogen. Then KOH-CH_3OH and HCl-CH_3OH were added successively to form fatty acid methyl esters (FAMEs) (AOCS, 1997). Nonadecanoic acid was also added as an internal standard. Lastly, the FAMEs were analyzed using GC/MS (7890A GC-5975C MSD; Aligent, USA) with an auto-sampler (7683B, Aligent, USA) and a chromatographic column of HP-5MS (30 m × 0.25 mm × 0.25 μm). Helium (99.999%) was used as a carrier gas at a constant flow rate of 1 mL min^{-1}, and the ratio of split injections was 10:1. The temperature programs were as follows: 30 °C–200 °C at 30 °C min^{-1}, 200 °C–215°C at 5 °C min^{-1}, 215 °C–220 °C at 1 °C min^{-1}, and 10 °C min^{-1} to 280 °C for 4 min. Three parallel experiments were replicated for each sample.

2.7. Statistical analyses

Data were analyzed with SPSS software (Version: 16.0). Observations were expressed as mean ± SD (n = 3). Significance was determined at a 95% or 99% confidence.

3. Results

3.1. Isolation and characterization of NgMCAT cDNA

The full length of *NgMCAT* cDNA contained an ORF of 1059 bp, a 5′-UTR of 158 bp and a 3′-UTR of 452 bp. The cDNA encoded a protein of 352 amino acid residues with a deduced pI of 6.06 and MW