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Dimethylsulfoniopropionate biosynthesis in a diatom *Thalassiosira pseudonana*: Identification of a gene encoding MTHB-methyltransferase

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ABSTRACT

Dimethylsulfoniopropionate (DMSP) is one of the most abundant molecules on earth and plays a pivotal role in the marine sulfur cycle. DMSP is believed to be synthesized from methionine by a four-step reaction pathway in marine algae. The genes responsible for biosynthesis of DMSP remain unidentified. A diatom *Thalassiosira pseudonana* CCMP1335 is an important component of marine ecosystems and contributes greatly to the world's primary production. In this study, through genome search, *in vivo* activity and functional studies of cDNA products, a gene encoding *Thalassiosira* methyltransferase (TpMMT) which catalyzes the key step of DMSP synthesis formation of 4-methylthio-2-hydroxybutyrate (DMSHB) from 4-methylthio-2-oxobutyrate (MTHB), was identified. The amino acid sequence of TpMMT was homologous to the methyltransferase from *Phaeodactylum tricornutum* CCAP 1055/1, but not the recently identified bacterium gene. High salinity and nitrogen limitation stresses caused the increase of DMSP content and TpMMT protein in *Thalassiosira*. In addition to TpMMT, the enzyme activities for the first three steps could be detected and enhanced under high salinity, suggesting the importance of four-step DMSP synthetic pathway in *Thalassiosira*.

1. Introduction

Dimethylsulfoniopropionate (DMSP) is one of the most abundant small sulfur compounds, a key component for marine microorganisms and a biogenic precursor for gaseous dimethylsulfide (DMS) [1,2]. DMS is believed to be involved in the regulation of global climate change by forming cloud condensation nuclei in the atmosphere [1–3]. Besides its importance in the climate, DMSP serves as an osmoprotectant [4,5] and involved in symbiosis [6]. Therefore, DMSP synthesized by marine organisms is an important compound environmentally.

Phytoplankton, marine algae and a few plants are known as abundant sources of DMSP [4,7] although DMSP productions by bacteria [8] and animal [9,10] have also been demonstrated recently. Three DMSP biosynthetic pathways, two for plants and one for algae, have been reported [7,11,12]. Methionine is the common precursor for these three pathways. Algal DMSP biosynthesis occurs via a four-step pathway, L-Met → 4-methylthio-2-oxobutyrate (MTOB) → 4-methylthio-2-hydroxybutyrate (MTHB) → 4-dimethylsulfonio-2-hydroxy-butyrate (DMSHB) → DMSP [12]. In algae, the first step is exerted via transamination whereas L-Met is first methylated in higher plants [13]. While the intermediates involved in these pathways have been identified using bioinformatics, proteome, and gene expression analysis for *Thalassiosira pseudonana* [14,15] and *Fragilariopsis cylindrus* (CCMP 1102) [16], the identities of the genes involved are unknown currently in algae and plants, although the methyltransferase gene for third step, *dsyB,* has been identified in *Alphaproteobacteria* [8].

Diatoms are distributed globally and are a key group of the phytoplankton in the ocean. DMSP is found in high concentrations in some marine diatoms such as *T. pseudonana* [15]. The genome sequence for *T. pseudonana* CCMP1335 is available and used in many genetic analyses [17]. Using genome analysis, *in vivo* activity and functional studies of cDNA products, we have successfully identified the gene encoding the MTHB-methyltransferase (TpMMT) that catalyzes the third step the DMSP biosynthesis pathway in *T. pseudonana*. The isolated gene encodes an 334 amino acid protein, whose of enzymatic properties were examined. Bioinformatics and expression analyses of putative genes for aminotransferase and reductase were also conducted. Regulation of DMSP biosynthesis under salt stress and nitrogen limitation at

Abbreviations: ASW, artificial sea water; DMSHB, 4-dimethylsulfonio-2-hydroxy-butyrate; DMSP, dimethylsulfoniopropionate; GB, glycine betaine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MT, methyltransferase; MTOB, 4-methylthio-2-oxobutyrate; SAM, *S*-adenosyl-L-methionine

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molecular level was also examined.

2. Methods

2.1. Strains, culture conditions and stress treatments

T. pseudonana CCMP1335 in this study was obtained from National centre for Marine Algae and Microbiota (Bigelow, USA). *T. psuedonana* cells were cultured in artificial sea water (ASW) containing 2% of Provasoli's enriched seawater (PES) [12] and 10 mg/L of sodium metasilicate nonahydrate (hereafter ASW) under continuous illuminated condition (120 $\mu molm^{-2}s^{-1}$) at 18 °C. For investigation of salinity effects, the *T. psuedonana* cells were grown to an OD_{420} ~0.4 in the ASW medium containing 0.23 M (x 0.5) or 0.46 M (x 1.0) or 0.68 M (x 1.5) NaCl. Here, x 0.5, x 1.0 and x 1.5 indicate that the concentrations of NaCl were 0.5-, 1.0- and 1.5-fold of sea water, respectively. For the effects of nitrogen deficiency, the cells grown to an OD_{420} ~0.4 in the ASW medium containing 0.23 M (x 0.5) NaCl, were washed with the same medium without sodium nitrate (-N medium) and then cultured. *Escherichia coli* strains *DH5α* and *BL21* (DE3) were grown at 37 °C or 25 °C in LB medium containing appropriate antibiotics. Optical densities were measured at 420 and 620 nm for *T. pseudonana*, and *E. coli*, respectively.

2.2. Extraction of DMSP from T. pseudonana cells

T. pseudonana cells were grown to an OD_{420} of ~0.4, then the cells were collected by centrifugation at 10,000 × g for 10 min. Pellets were suspended in 200 µl of absolute methanol, and then centrifuged again at 22,000 × g for 20 min. The resulting supernatants were dried up by rotary evaporator (TAITEC, Saitama, Japan), and resulting pellets were dissolved in 200 µl of water. Then, 200 µl of chloroform was added, and the mixture was vigorously shaken and further centrifuged at 22,000 × g for 10 min. The upper aqueous phase was transferred to new tubes. DMSP was measured by a time-of-flight mass spectroscopy (TOF-MS) (Kratos AXIMA-CFR,Shimadzu, Kyoto, Japan) [18]. For measurement, 1 µl of the extract, 1 µl of matrix (10 g/L solution of sinapinic acid in 2:3 acetonitrile:0.1% v/v trifluoroacetic acid), and 1 µl of internal standard solution were mixed, dried, and then applied to the instrument. For internal control, the stable isotope d_6-DMSP was synthesized, purified and used as described previously [19]. Another stable isotope internal standard d_{11}-glycine betaine (GB) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), and GB measurement was performed as reported previously [18].

2.3. Molecular phylogenetic analysis

Candidate genes responsible for *Thalassiosira* DMSP biosynthetic pathway were searched in JGI and NCBI databases. Based on the enzyme names, the candidate genes were searched among *T. pseudonana* genome in JGI database. Homology search for corresponding gene was performed by using BLAST program. Amino acid sequences were aligned using ClustalW. Phylogenetic tree was constructed using ClustalW and TreeViewX software. The robustness of the tree was assessed by bootstrap analysis (1000 replicates).

2.4. Isolation, expression, and purification of target MTHB-methyltransferase protein

Two candidate genes for MTHB methyltransferase, *TpMT1* (XM_002296942) and *TpMT2* (Tp23128), were used in this study. These candidate genes were amplified from cDNA of *T. pseudonana* by PCR using specific primer sets (Table S1). For *TpMT1*, the ORF was amplified without putative transit peptides at N-terminus. PCR reactions were initiated with KOD plus neo polymerase (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Obtained DNA fragments were added deoxyriboadenocine (dA) using with Mighty TA-cloning Kit for PrimeSTAR (Takara, Otsu, Japan), cloned into pMD20-T vector (Takara), and then transformed into *E. coli DH5α*. Resulting plasmids were purified by Miniprep kit (QIAGEN, Hilden, Germany) and then sequenced by ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, USA). The pMD20-T vectors containing TpMT1 or TpMT2 were digested with the restriction enzymes, and the resulting DNA fragments were ligated into the expression vector pGEX6P-1(GE Healthcare Life Science, Little Chalfont, United Kingdom). Resulting plasmids, pGEX6P-1_TpMT1 or pGEX6P-1_TpMT2 were transformed into *E. coli BL21* (DE3).

E. coli BL21 (DE3) cells harboring expression vectors were grown in 1 ml of LB medium with 50 µg/ml of ampicillin at 37 °C for overnight. These cultures were added to 200 ml of LB medium with 50 µg/ml of ampicillin, and subcultured for several hours. When their optical density reached to ~0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures to a final concentration of 100 µM, and the cultures were cooled to 25 °C. After culturing for 15 h, the cells were collected by centrifugation at 7000 × g for 20 min, and suspended in 5 ml of Buffer A (50 mM Tris-HCl, pH 8.0, 5 mM DTT, 1 mM $MgCl_2$) containing 1% Triton-X. The cells were broken by sonication (output: 7, repeat of 30 s on and 30 s off, total 4 min), and the supernatants after centrifugation at 22,000 × g for 15 min were used for protein purification step.

For purification of GST-fused protein from pGEX6P-1 based system, 100 µl (bed volume) of Glutathione Sepharose 4B beads (GE Healthcare Life Science) equilibrated with Buffer A was added to the supernatants, and then incubated at 4 °C for 30 min. After incubation, the beads were collected and washed with 1 ml of Buffer A three times, and then 1 unit of PreScission Protease (GE Healthcare Life Science) was added into the beads suspension in 100 µl of Buffer A. After incubation at 4 °C for overnight, the processed proteins were collected by centrifugation. The purified protein concentrations were determined by Bradford method using bovine serum albumin as a standard [18].

2.5. Gel filtration chromatography

Gel filtration analyses were carried out at 4 °C using an AKTA prime liquid chromatography system (GE Healthcare Life Science, Little Chalfont, United Kingdom). The purified methyltransferase or soluble protein extract of *T. pseudonana* was subjected to HiLoad 16/600 Superdex 75 pg gel filtration column chromatography (GE Healthcare Life Science) using Buffer A as mobile phase with flow rate at 2.0 ml/min. Ovalbumin and Bovine Serum Albumin were used as molecular mass markers. The eluted fractions were subjected to SDS-PAGE analysis and stained by Coomassie Brilliant Blue or further used for activity measurement. Before the SDS-PAGE analysis, the proteins in each fraction were precipitated with trichloroacetic acid. The intensity of the band on the gel was quantified by ImageJ software and was normalized by setting the signals from maximum intensity to 100.

2.6. Enzyme assays

DMSP biosynthetic activities for aminotransferase, reductase, methyltransferase and decarboxylase were examined according to the methods of Summers et al. [12] with slight modifications. *T. pseudonana* cells were collected by centrifugation and resuspended in 100 mM of Tris-HCl (pH 8.0). Cell suspensions were disrupted by sonication (output: 7, repeat of 15 s on and 15 s off, total 1 min on time, model VP-5s, TITEC). After centrifugation at 22,000 × g for 10 min at 4 °C, 20 µl of resulting supernatants were used for enzyme assays. Purified recombinant enzymes were prepared as described above. Protein concentration was measured by Bradford method [18].

Aminotransferase activity was determined in the reverse reaction. For this, the conversion of MTOB to L-methionine was measured by coupling the reduction of 2-oxoglutarate by NADH in a reaction