Note

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Cloning and Expression of a Gene Encoding a Putative Chloroplast ω6 Fatty Acid Desaturase of Marine *Chlamydomonas*

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Abstract

A cDNA encoding putative chloroplast ω6 fatty acid desaturase was isolated from a cDNA library of marine *Chlamydomonas* sp. strain W-80. The mRNA level of this gene under various conditions of stress was examined by northern blotting analysis, and the transcript level was increased under a cold-stressed (4 ℃) condition.

Low temperature is one of the commonest and the most deleterious environmental stress for living organisms. Under low temperature, the desaturation of fatty acid in membrane lipids plays an important role in the maintenance of fluidity of membranes (Murata and Wada, 1995; Los and Murata, 1998). When the fluidity of membrane lipids is reduced by a low temperature, double bonds are introduced into the fatty acids of lipids, so that the membranes keep the fluid state. Omega(ω)6 fatty acid desaturase catalyzes the desaturation of monoenoic to dienoic fatty acid. In cyanobacteria (Los and Murata, 1998; Sakamoto and Bryant, 1997), the mRNA level of ω6 desaturase (delta 12 desaturase) is dramatically increased when the cells are exposed to a low temperature condition. On the other hand, in soybean (Heppard *et al.*, 1996) and *Arabidopsis thaliana* (Okuley *et al.*, 1994), no increase in ω6 desaturase transcript was observed under low temperature, even though the levels of polysaturated fatty acids in the plant were elevated, and the transcript level of ω3 desaturase was also increased. Regarding algae, there is no study on the regulation of expression of the ω6 desaturase gene under a low temperature condition.

In this study, the cDNA clone of chloroplast ω6 desaturase homologue was isolated from the cDNA library of marine *Chlamydomonas* sp. strain W-80, and the mRNA levels of this gene under various conditions of stress, including cold stress, were examined by northern analysis.

The marine *Chlamydomonas* sp. strain W-80 used in this study was isolated in the coastal area of Wakayama, Japan, and was identified as a *Chlamydomonas* species as described previously (Miyasaka *et al.*, 1998). This algal strain is highly tolerant both to salt stress (up to 2 M NaCl) and to oxidative stress (up to 100 μM methyl viologen; MV). Modified Okamoto medium (MOM; pH 8.0) supplemented with 5 mM NH_4Cl was used for algal cultures (Miura *et al.*, 1986), and the algal cultures were continuously illuminated by fluorescent lamps at a light intensity of 175 $\mu E\ m^{-2}\ s^{-1}$, with aeration by bubbling at a rate of 200 m*l* air/min. The λZAPII (Stratagene, La Jolla CA, USA) cDNA library of *Chlamydomonas* W-80 was constructed as described in a previous paper (Miyasaka *et al.*, 2000). The Luria-Bertani (LB) medium supplemented with 50 mg ml^{-1} of carbenicillin (Cb) was used for *Escherichia coli* (SOLR strain, Stratagene) cultures. The bacterial cell growth was monitored by measuring the OD_{600} of cultures.

A cDNA clone of the ω6 desaturase homologue was isolated from the cDNA library by a functional expression screening method with *E. coli* cells as described previously (Miyasaka *et al.*, 2000). Briefly, the λZAPII cDNA library was mass excised into phagemid DNA, and the host *E. coli* cells carrying the mass excised phagemid DNA were plated onto the selection plate with a high concentration (5%) of NaCl. The plates were incubated at 37 ℃ for 2 days and the salt-stress tolerant bacterial colonies were isolated.

The acquisition of salt-stress tolerance of the *E. coli* cells carrying algal ω6 desaturase gene homologue was further confirmed by back-inoculating the phagemid DNA into the host *E. coli* cells, and by checking the acquisition of stress tolerance of the newly-generated transformants. **Fig. 1** shows the growth curves of the *E. coli* cells with

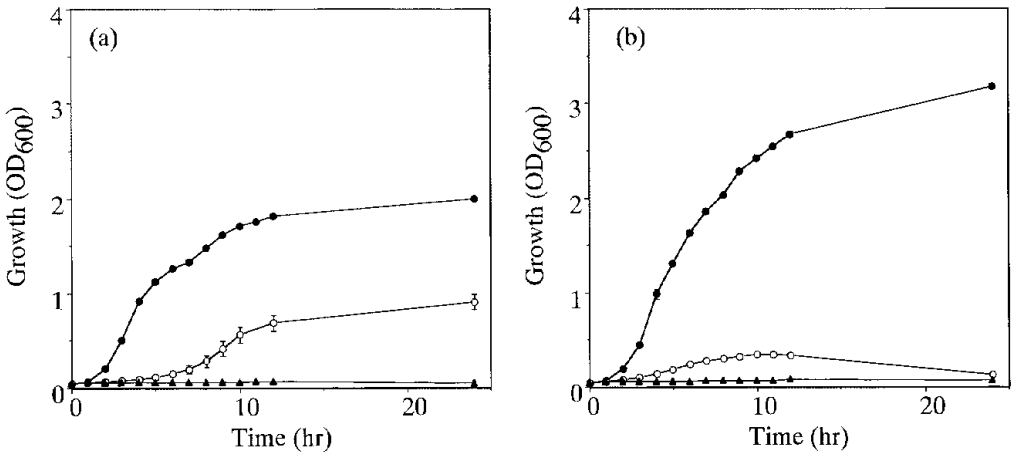

Fig. 1 NaCl salt-stress tolerance of *E. coli* cells carrying algal ω6 desaturase homologous gene. The *E. coli* cells with algal ω6 desaturase homologous gene (a), and with pBluescript vector (b) were cultured in LB-Cb medium with 1(●), 5(○), and 7% (▲) NaCl. One percent is the standard NaCl concentration in LB medium. Cell growth was monitored by measuring the OD_{600} of cultures. Values are the means ± standard error for three cultures.

Chlamydomonas W-80 ω6 desaturase homologue (a) and with pBluescript vector (b) in the LB-Cb medium with 1, 5, and 7% NaCl. In the 1% NaCl (standard concentration in LB medium) medium, the growth of the *E. coli* cells with ω6 desaturase homologue was considerably lower compared to that of the control, suggesting that the expression of this gene has some suppressive effects on the cell growth. In the 5% NaCl medium, the control cells showed only a slight increase in OD_{600} value for 10 hours after the inoculation of the cells, but no growth was observed after 24 hours, while the cells with ω6 desaturase homologue kept growing and reached approximately 45% of the 1% NaCl culture after 24 hours, indicating that the expression of the ω6 desaturase homologue has a protective function against the NaCl salt stress in *E. coli* cells. In the 7% NaCl medium, no cell growth was observed in either the cells with ω6 desaturase homologue or with the pBluescript vector. The reason the *E. coli* cells with the algal ω6 desaturase gene acquired the tolerance against the salt-stress is, however, not clear, and further studies are required in the future to examine the physiological significance of the expression of algal ω6 desaturase homologue in *E. coli* cells.

The DNA sequence of cDNA clone of ω6 desaturase homologue of *Chlamydomonas* W-80 was determined completely from both strands. The cDNA clone (DDBJ accession No. AB031546, sequence data not shown) was 1,565 bp long, with 31 bp of poly(A) tail, and had a 1,263 bp coding region (421 amino acids, calculated molecular mass of 47,930), and 122 bp and 149 bp 5' and 3' non-coding regions, respectively. The coding region of this gene was located in the proper reading frame forming the fusion protein with the β-galactosidase gene of the pBluescript vector. The sequence TGTAA, a putative polyadenylation signal, was found 13 bp upstream of the poly(A)-tail. The deduced amino acid sequence of ω6 desaturase homologue of *Chlamydomonas* W-80 showed 67 % homology to that of fresh water green alga *Chlamydomonas reinhardtii* (Sato *et al.*, 1997), 50 to 55 % to higher plant ω6 desaturases (Hitz *et al.*, 1994; Falcone *et al.*, 1994), and 45 to 52 % to cyanobacterial delta 12 desaturases (Schmidt *et al.*, 1994; Sakamoto *et al.*, 1994). Although the ω6 desaturase homologue of *Chlamydomonas* W-80 showed a relatively low homology to other known ω6 desaturase genes, it had three His-clusters which were reportedly essential for the desaturation reaction (Shanklin *et al.*, 1994). The amino acid sequence, around these His-clusters, of the ω6 desaturase homologue of *Chlamydomonas* W-80 were also well conserved (**Fig. 2**); we therefore concluded that the isolated gene was the ω6 desaturase homologous gene of *Chlamydomonas* W-80. The ω6 desaturase homologue of *Chlamydomonas* W-80 had a potential chloroplast transit peptide (Franzen *et al.*, 1990) in its N-terminal region, and was expected to be a chloroplast enzyme.

To examine the regulation of ω6 desaturase homologous gene expression in *Chlamydomonas* W-80 cells, the mRNA levels of this gene under various conditions of stress were examined by northern blotting analysis (**Fig. 3**). For the northern blotting experiment, approximately 1.5 liter of algal cultures in the early logarithmic phase (OD_{680}=0.8) in 2 liter flat culture bottles were exposed to various

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C. W-80          ------------------------MAMAMPKLGGLRMRPAAPSAAGVPVLG--ARRCAV   60
C. reinhartdii   ------------------------MAFALRSPGAVRAPACAQRASGVRAAKPGFLRSAA
SynPCC7002       ------------------------------------------------------------
Arabidopsis      MASRIADSLFAFTGPQQCLPRVPKLAASSARVSPGVYAVKPIDLLLKGRTHRSRRCVAPV

C. W-80          KVRTAAPAMTVSDPTKAGFMSDEDRAALAKELGYRQLGKELPDHVTLNTIVQSMPKEVFE  120
C. reinhartdii   VARPQVQTNAAALSVPVNQLTDEERANLARELGYKSIGRELPDNVSLTDIIKSMPAEVFK
SynPCC7002       --------MTSVTVRPSATTLLEKHP----------------NLRLRDILDTLPRSVYE
Arabidopsis      KRRIGCIKAVAAPVAPPSADSAEDREQLAESYGFRQIGEDLPENVTLKDIMDTLPKEVFE
                                         *                    * *   * *

C. W-80          IDHGKAWRAVLTSITAMAGCLYLISVSPWYLLPFAWALAGTAFTGFFVVGHDAGHRSFHK  180
C. reinhartdii   LDHGKAWRACLTTIAACSACWYLISISPWYLLPAAWALAGTAFTGCFVIGHDCGHRSFHE
SynPCC7002       INPLKAWSRVLLSVAAVVGCYALLAIAPWYLLLPVWFLTGTTLTGFFVIGHDCGHRSFSR
Arabidopsis      IDDLKALKSVLISVTSYTLGLFMIAKSPWYLLPLAWAWTGTAITGFFVIGHDCAHKSFSK
                     **    *                *****   *   **  ** ** *** * **

C. W-80          NNLIEDIVGTIMFAPLIYPFEPWRIKHNHHHAHTNKLEEDTAWVPIEQEKMKDWNGVTSA  240
C. reinhartdii   NNLIEDIVGHIFFAPLIYPFEPWRIKHNHHHAHTNKLVEDTAWHPVTEADMAKWDSTSAM
SynPCC7002       KNWVNNLVGHLAFLPLIYPFHSWRILHNHHHRYTNNMDEDNAWAPFTPELYDDSPAFIKA
Arabidopsis      NKLVEDIVGTLAFLPLVYPYEPWRFKHDRHHAKTNMLVHDTAWQPVPPEEFESSPVMRKA
                       **   * ** **   **  * **  **    * ** *

C. W-80          LFKFFLGTPLKLWASVGHWAIWHFDLNKYTEKQRPRVIVSLLACAAFACTVLPWLVINHG  300
C. reinhartdii   LYKVFLGTPLKLWASVGHWLVWHFDLNKYTPKQRTRVVISLAVVYGFMATAFPALLYFGG
SynPCC7002       VYRAIRGKLWWLAS-VIHQLKLHFNWFAFEGKQREQVRFSALFVIIAGAIAFPVMFYTLG
Arabidopsis      IIFGYGPIRPWLS--IAHWVNWHFNLKKFRASEVNRVKISLACVFAFMAVGWPLIVYKVG
                           *    *   **          * *           *     *

C. W-80          VWGLVKYWLMPWLGYHFWMSTFTVIHHTAPHIPFKPAAEWNAAKAQLSGTVHCDFPAWVE  360
C. reinhartdii   PWAFVKYWLMPWLGYHFWMSTFTVVHHTAPHIPFKKAEEWNAAKAQLSGTVHCDFPNWVE
SynPCC7002       VWGVVKFWLMPWLGYHFWMSTFTLVHHTVPEIPFSYRDKWNEAIAQLSGTVHCDYPKWVE
Arabidopsis      ILGWVKFWLMPWLGYHFWMSTFTMVHHTAPHIPFKPADEWNAAQAQLNGTVHCDYPSWIE
                    ** ****************  *** * ***     ** * *** ****** * * *

C. W-80          FLTHDISVHVPHHVSSKIPWYNLRKAHASLKENWGEHMCETTFNWRMLKNIFTELHVYDE  420
C. reinhartdii   FLTHDISWHVPHHVAPKIPWYNLRKATESLRENWGQYMTECTFNWRVVKNICTECHVYDE
SynPCC7002       VLCHDINVHVPHHLSTGIPSYNLRKAYASIKQNWGEYLYETKFSWELMKAITEQCHLYDA
Arabidopsis      ILCHDINVHIPHHISPRIPSYNLRAAHESIQENWGKYTNLATWNWRLMKTIMTVCHVYDK
                  * ***  * ***    ** **** * *  *  ***         *   * *    * **

C. W-80          KTFGYKPFDWKKEEPLFAAQRAAYPNSM--
C. reinhartdii   KVN-YKPFDYKKEEALFAVQRRVLPDSAAF
SynPCC7002       EHNYISFAQHQKR-----------------
Arabidopsis      ------------------------------
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Fig. 2 Alignment of amino acid sequences of ω6 desaturase of *Chlamydomonas* W-80, *Chlamydomonas reinhartdii, Synechococcus* PCC7002 (SynPCC7002; delta 12 desaturase) and *Arabidopsis thaliana*. Asterisks indicate the amino acid conserved in all the sequences. The three His-clusters essential for the desaturation reaction are underlined. The alignment was computed with the CLUSTAL w program (Thompson *et al.*, 1994).

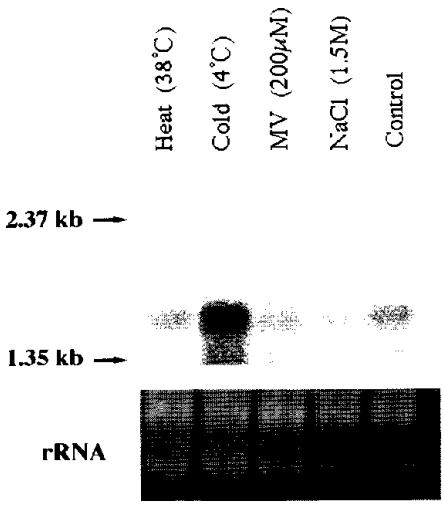

Fig. 3 ω6 desaturase homologue mRNA accumulation in *Chlamydomonas* W-80 grown under various conditions of stress. Algal cells were exposed to various kinds of stress (heat, cold, oxidative, and high salt stresses) for 6 hours under a continuous illumination (175 μE m^{-2} s^{-1}). Each lane contains 12 μg of total RNA isolated from the stress-treated algal cells. rRNA shows the RNA sample stained with ethidium bromide.

kinds of stress, such as heat, cold, oxidative, and high salt stresses, for 6 hours under continuous illumination (175 μE m^{-2} s^{-1}). For salt stress, the NaCl concentration in the medium was elevated from 0.5 M (standard concentration of MOM) to 1.5 M by adding solid NaCl. For oxidative stress (200 μM MV), 1.5 m*l* of the 0.2 M stock MV solution was added to the culture medium; MV can be reduced by the photosynthetic apparatus yielding a monocation radical, which rapidly generates superoxide (O_2^-) (Rabinowitch *et al.*, 1987). For heat and cold stresses, the cells were cultured at 38 ℃ and at 4 ℃, respectively. The effects of NaCl and MV on algal cell growth were examined in advance by culturing the cells in the liquid medium with various concentrations of these substances (data not shown), and the concentrations which caused approximately 30~50 % growth inhibition have been chosen for the northern blotting experiment.

After the treatment with various conditions of stress for 6 hours, total RNA was extracted from the

algal cells with the ISOGEN RNA extraction kit (Nippon Gene, Toyama, Japan). Twelve micro grams of total RNAs were electrophoresed on a 1.2 % agarose/2.1% formaldehyde gel, and transferred to a Nytran membrane (Schleicher & Schuell, Keene NH, USA) by capillary blotting with 10 x SSC (1.5 M sodium chloride/0.15 M sodium citrate, pH 7). The RNA was immobilized by UV crosslinking, and the northern blot was hybridized overnight at 42 ℃ in 50% formamide (v/v)/0.2% SDS (w/v)/2 x Denhardt/6 x SSC/(100 mg ml^{-1} calf thymus DNA) with a ^{32}P-labeled probe. The ^{32}P-labeled probe was prepared by random priming using the PCR-generated template of *Chlamydomonas* W-80 ω6 desaturase homologue. After hybridization, the membrane was washed with 2 x SSC/0.1% SDS for 15 min at room temperature, 2 x SSC / 0.1% SDS for 15 min at 65 ℃, and 0.2 x SSC/0.1% SDS for 20 min at 65 ℃ twice. The membrane was exposed to a Fuji imaging plate (Fuji Film, Tokyo, Japan), and the hybridization signals were quantified with a BAS 2000 reader (Fuji Film).

The single mRNA was detected to be approximately 1.6 kb, indicating that our ω6 desaturase homologous cDNA clone (1,565 bp with 31 bp poly (A) tail) is full-length. The level of transcript of the ω6 desaturase homologue increased 2.3-fold in the cold-treated cells compared to non-stressed control cells, while the transcript levels under the other stressed-conditions were 62% (NaCl salt stress), 80% (MV oxidative stress), and 87% (heat stress) of the control (no stressed condition), respectively. Thus the mRNA level was increased specifically under a cold-stressed condition in the algal cells, suggesting that in algal cells the expression regulation of ω6 desaturase plays an important role, the same as in cyanobacteria, in the membrane lipid desaturation process under a low temperature condition.

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