J. Biochem. 122, 1224-1232 (1997)

Cloning of a Gene for Chloroplast ω6 Desaturase of a Green Alga, *Chlamydomonas reinhardtii*[1]

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Received for publication, August 7, 1997

A gene for chloroplast ω6 desaturase, which catalyzes the desaturation of monoenoic to dienoic acids in chloroplasts, was isolated from *Chlamydomonas reinhardtii*. We first performed reverse transcriptase-polymerase chain reaction with oligonucleotide primers corresponding to regions conserved among plastid ω6 desaturases of higher plants and Δ12 desaturases of cyanobacteria, using *C. reinhardtii* poly(A)$^+$ RNA. An amplified DNA fragment of 0.5 kb, containing a frame for a protein homologous to these desaturases, was used as a probe for screening cDNA and genomic DNA libraries of *C. reinhardtii*. The cDNA clone of 2.2 kb obtained contained an open reading frame encoding a protein of 424 amino acids with a putative molecular mass of 48.4 kDa, the amino acid sequence of which showed 46-51% homology to those of higher plant plastid ω6 and cyanobacterial Δ12 desaturases. Introduction of the cloned genomic counterpart of this cDNA, designated as *des6*, into a *Chlamydomonas* mutant with defects in chloroplast ω6 desaturation and in the activities of photosystems I and II (PSI and PSII) complemented the desaturation mutation, indicating that the *des6* gene codes for chloroplast ω6 desaturase. The complemented strains did not recover from the photosynthetic lesions, but showed lower PSII activity at 45°C than the desaturation mutant, proving that the photosynthetic lesions in *hf-9* are not caused by the desaturation mutation, and that the lowered unsaturation level of chloroplast lipids in the mutant is responsible for the expression at this high temperature of PSII activity, one of the thylakoid membrane functions.

Key words: *Chlamydomonas reinhardtii*, complementation, desaturation mutation, heat stability, photosystem II.

In higher plants, glycerolipids are synthesized through the cooperation of two organelles, plastids and the endoplasmic reticulum (ER) (*1*). The fatty acid constituents of glycerolipids are first synthesized in the form of acyl-acyl carrier proteins (ACPs) in plastids, mainly as 16:0-ACP and 18:0-ACP, and then most of the latter is converted to 18:1(9)-ACP by Δ9 desaturase in the stroma. The resultant acyl groups, 16:0 and 18:1(9), are incorporated into phosphatidate within chloroplasts for the synthesis of chloroplast lipids, *i.e.*, monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG), and phosphatidylglycerol (PG) (prokaryotic pathway). Otherwise, they are exported to ER for the synthesis of extra-chloroplast lipids such as phosphatidylcholine and phosphatidylethanolamine. A part of the diacylglycerol moiety of phosphatidylcholine is transported from ER to plastids for conversion into chloroplast lipids (eukaryotic pathway).

While Δ9 desaturase is a soluble enzyme, the desaturases that subsequently act are membrane-bound in plastids or ER, requiring glycerolipids as substrates. The membrane-bound ω6 and ω3 desaturases of plastids act on the chloroplast lipids, producing 18:2(9,12) from 18:1(9) and 18:3(9,12,15) from 18:2(9,12), respectively. In a group of higher plants designated as 16:3 plants, the ω9, ω6, and ω3 desaturases of plastid membranes sequentially desaturate 16:0 up to 16:3(7,10,13) in order (*2*). On the other hand, the ω6 and ω3 desaturases of ER convert 18:1(9) into 18:2(9,12), and 18:2(9,12) into 18:3(9,12,15), respectively, in extra-chloroplast lipids (*2*). Owing to the presence of the eukaryotic pathway, *i.e.*, the transport system of the diacylglycerol moiety of PC with C_{18} unsaturated fatty acids from ER to plastids (*1*), the desaturation systems of both plastids and ER are responsible for the unsaturation levels of chloroplast lipids.

The abundance of highly unsaturated fatty acids in chloroplast membrane lipids raises the question of the physiological significance of these fatty acids in chloroplast functions. One way to answer this question is to characterize mutants deficient in highly unsaturated fatty acids of chloroplast lipids. However, mutants as to the respective

[1] This work was supported by Grants-in-Aid for Scientific Research (Nos. 07304082 and 07554068) from the Ministry of Education, Sciences, Sports and Culture of Japan, as well as by the Asahi Glass Foundation, and Marine Biotechnology Institute Co., Ltd.

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Abbreviations: ACP, acyl carrier protein; DGDG, digalactosyl diacylglycerol; ER, endoplasmic reticulum; MGDG, monogalactosyl diacylglycerol; PG, phosphatidylglycerol; RT-PCR, reverse transcriptase-polymerase chain reaction; SQDG, sulfoquinovosyl diacylglycerol; fatty acyl groups are denoted by the numbers of carbon atoms and double bonds, *e.g.*, 18:1(9)=oleoyl.

chloroplast desaturases failed to show a large decrease in desaturation products such as 18:3(9,12,15) in chloroplasts, owing to the import of 18:2(9,12) and 18:3(9,12,15) from ER through the eukaryotic pathway (*3, 4*). Such mutants, in which photosynthesis was little affected, were thus inadequate as tools to deduce the roles of highly unsaturated fatty acids, leaving the necessity of elimination of 18:3(9,12,15) in chloroplast membranes with the combination of plastid and microsomal desaturase mutations. Recently, a triple mutant of *Arabidopsis thaliana* was produced with lesions in both plastid and microsomal $\omega 3$ desaturation, showing only a negligible content (0.1% of total fatty acids) of 18:3(9,12,15) with a concomitantly large increase in the 18:2(9,12) content in all membrane lipids (*5*). Physiological analysis demonstrated no pronounced effect on photosynthesis but revealed the male sterile phenotype, indicating that 18:3(9,12,15) is crucial not for chloroplast functions but for the reproductive process. However, it remains unknown whether dienoic fatty acids such as 18:2(9,12) are also dispensable for chloroplast functions.

In contrast to higher plants, a green alga, *Chlamydomonas reinhardtii*, almost completely lacks the eukaryotic pathway (*6*), which implies that chloroplast desaturases are major determinants of chloroplast lipid unsaturation levels. This coincided with the finding that mutation of chloroplast $\omega 6$ desaturation alone brought about drastic decreases in the unsaturation levels of chloroplast lipids (*7*). So far, cDNAs of desaturases corresponding to chloroplast $\omega 6$ desaturase of *C. reinhardtii*, which is responsible for the synthesis of dienoic fatty acids, have been cloned from higher plants (*8-10*) and cyanobacteria (*11, 12*) but not from eukaryotic algae. The nuclear genome of *C. reinhardtii*, a model of higher plant leaf cells, can be easily transformed by the glass bead method (*13*). In this study, we cloned the cDNA and the genomic counterpart of the chloroplast $\omega 6$ desaturase gene from *C. reinhardtii*, with a view, in the future, to repression of the gene expression in *C. reinhardtii* by genetic manipulation to examine whether dienoic acids are required for normal chloroplast functions.

MATERIALS AND METHODS

Algal Culture—Cells of *C. reinhardtii* 6145c(*nit1*-305) *mt*$^-$, its mutant, *hf-9*, and transformants were grown in oblong glass vessels under constant fluorescent illumination (7 W·m^{-2}) at 28°C, with aeration with air containing 2% CO_2. As the culture medium, 3/10 HSM medium (*14*) was used for the analyses of fatty acids and photosynthetic activities, while SGII medium (*13*) was used for nuclear transformation of *hf-9* by the glass bead method (see below).

DNA and RNA Isolation—*C. reinhardtii* 6145c(*nit1*-305) *mt*$^-$ was grown as described above, and then harvested by centrifugation (8,000 × *g*, 10 min, 4°C). The collected cells were frozen in liquid N_2 and preserved at −80°C until use. DNA and RNA were isolated from these cells, as described in Ref. *15*.

Amplification of the Chloroplast $\omega 6$ Desaturase-Related Sequence of C. reinhardtii—Poly(A)$^+$ RNA was prepared from total RNA with Oligotex-dT30 (Takara), according to the manufacturer's protocol and used as a template for reverse transcriptase-polymerase chain reaction (RT-PCR) with a commercially available kit (Amersham). On the basis of the amino acid sequences, FVIGHDC and HFWMSTF, conserved among higher plant plastid $\omega 6$ and cyanobacterial $\Delta 12$ desaturases (*8-10, 12*), the following two sets of degenerated primers of 20-nucleotide-long oligomers were designed for the RT-PCR: 5′-TTCGT(G/C)-AT(C/T)GG(C/T)CACGA(C/T)TG-3′ and 5′-AAGGT(G/C)GACATCCAGAAGTG-3′ for the sense and antisense primers, respectively. PCR was performed with a DNA Thermal Cycler (Perkin Elmer) with the following thermocycle program: 2 min at 95°C, followed by 45 cycles of 60 s at 95°C and 60 s at 60°C, and finally by extension at 60°C for 7 min. A major product of 0.5 kb was purified from the agarose gel after electrophoresis, blunt-ended with T4 DNA polymerase, then ligated into the *Hinc*II restriction site of M13 mp19. The resultant plasmid was designated as pPR1. The insert of pPR1 was sequenced, as described below.

Screening of Genomic DNA and cDNA Libraries—The insert of pPR1 was purified from the agarose gel after electrophoresis, radiolabeled with [^{32}P]CTP by the random primer method with a commercially available kit (Amersham), and used as a probe for the screening of cDNA and genomic DNA libraries of *C. reinhardtii*. The cDNA library constructed in bacteriophage λgt11 was a gift from Dr. Y. Matsuda (Kobe University). The genomic DNA library of *C. reinhardtii* was constructed in bacteriophage λDASH II (Stratagene), through integration of the genomic DNA partially digested with *Sau*3AI into the phage vector. Plaque hybridization was performed as follows: plaques transferred to nylon membranes (Hybond-N+; Amersham) were hybridized with the radiolabeled probe in a hybridization buffer (Rapid-hyb buffer, Amersham) for 2 h at 65°C, then washed first in 2×SSC (1×SSC consists of 0.15 M NaCl and 0.015 M sodium citrate), 0.1% (w/v) SDS for 20 min at room temperature, then twice in 0.2×SSC, 0.1% (w/v) SDS for 1 h at 65°C. The washed membranes were exposed to X-ray film (LP670C, Konica).

DNA Sequence Determination and Analysis—Small restriction fragments of DNA were blunt-ended, then subcloned into the M13 mp19 vector cut with *Hinc*II for determination of the cDNA and genomic DNA sequences of both strands, and of a single strand, respectively. The single-stranded DNA prepared from the M13 phage was used for dye primer-sequencing reactions, and sequenced with an A.L.F. DNA Sequencer II (Pharmacia Biotech).

Sequence comparisons and alignments were performed with programs in the software package of the DNA Sequence Input and Analysis System (Hitachi Software Engineering). Phylogenetic analyses of amino acid sequences were conducted with the Phylogenetic Analysis Using Parsimony (PAUP) program, as previously described (*16*).

Genomic Southern and Northern Analyses—Total RNA (5 μg) was denatured and then run on a 1% agarose gel containing formaldehyde for Northern analysis, while genomic DNA (5 μg) was digested with the respective enzymes and run on a 1% agarose gel for Southern analysis, as described in Ref. *15*. These gels were blotted onto nylon membranes (Hybond-N+; Amersham) according to the manufacturer's protocol, and the membranes were independently hybridized with the probe labeled with [^{32}P]-CTP, washed, then exposed to X-ray film under identical

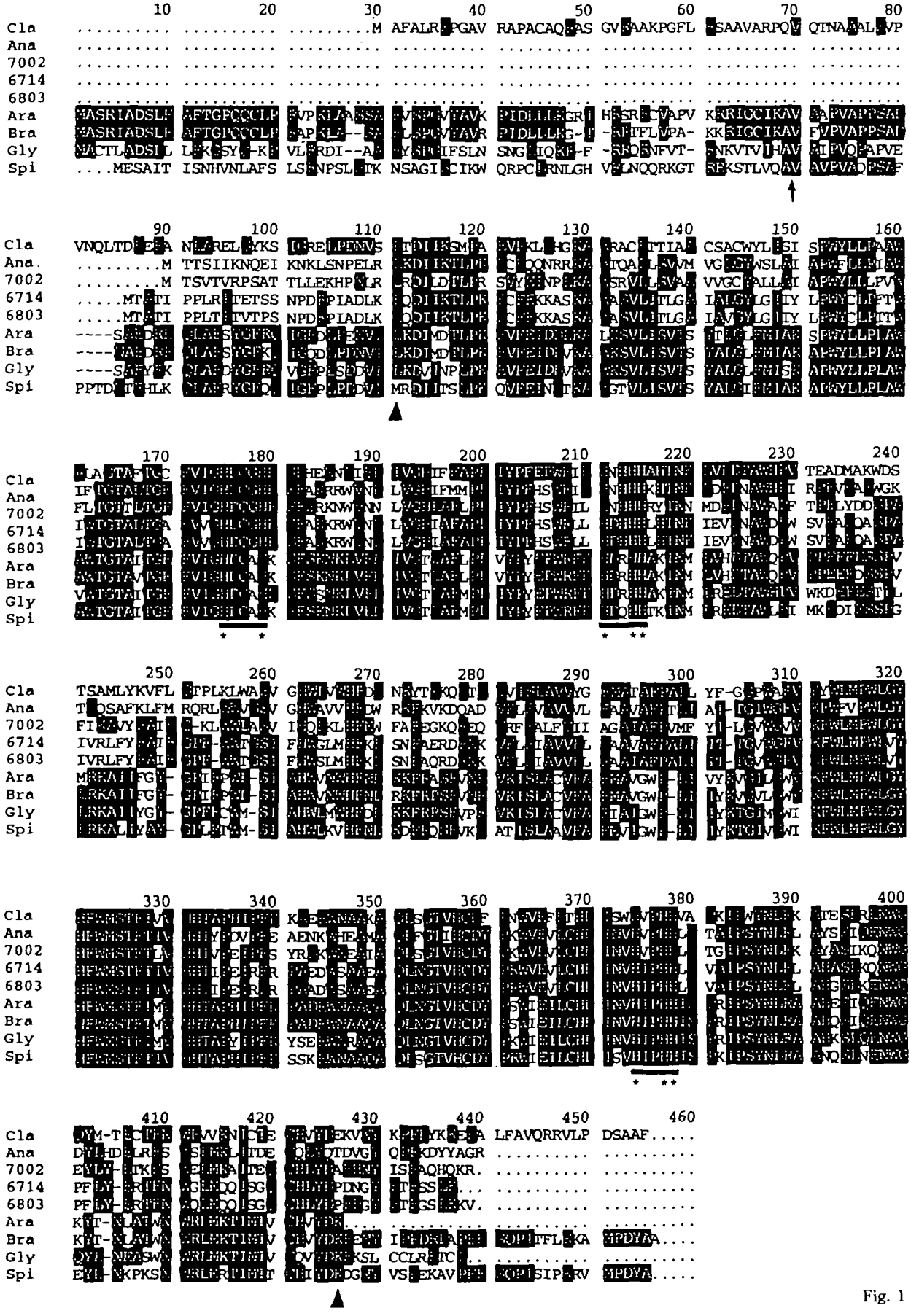

Fig. 1

conditions, as described under "*Screening of Genomic DNA and cDNA Libraries.*"

Transformation of C. reinhardtii—A mutant of *C. reinhardtii*, *hf-9* (*7*), grown in SGII-NH_4 medium to a density of *ca.* 1×10^6 cells·ml^{-1}, was transformed by the glass bead method (*13*). The cells, treated with autolysin to remove cell walls, were mixed with a polyethylene glycol solution, glass beads and plasmid DNA for subsequent agitation with a vortex mixer for 30 s. The cell suspension was then spread on SGII-NO_3 plates containing 1% agarose. The plates were air-dried, then incubated for *ca.* 10 days under continuous light at 28°C, until colonies of transformants appeared.

Measurement of Photosynthetic Activity—Cells at the logarithmic growth phase were harvested by centrifugation at $3,000\times g$ for 5 min, then resuspended in 50 mM Tricine/KOH (pH 7.5) at the concentration of 60 μg Chl·ml^{-1}. PSI and PSII activities in these cells were determined as described in Ref. *17*, except that the cells were disrupted with glass beads (425-600 μm, Sigma) instead of a sonicator, as follows: 5 ml of cell suspension was mixed with an equal volume of glass beads in a test tube, cooled on ice, then agitated seven times with a vortex mixer for 30 s with 30 s intervals. PSI activity was measured in these disrupted cells with the reduced form of 2,6-dichlorophenol-indophenol (DCIP) and methylviologen as the electron donor and acceptor, respectively. PSII activity was measured in whole cells with *p*-benzoquinone as the electron acceptor. O_2 evolution or uptake was measured with a Clark-type electrode (Rank Brothers). The electrode chamber was filled with 5 ml of reaction mixture, kept at the designated temperature for 5 min in the dark, then illuminated with a tungsten projector lamp (128 W·m^{-2}).

Analyses of Lipids—The total lipids were extracted from cells by the method of Bligh and Dyer (*18*). Fatty acid methyl esters were prepared from total lipids by treatment

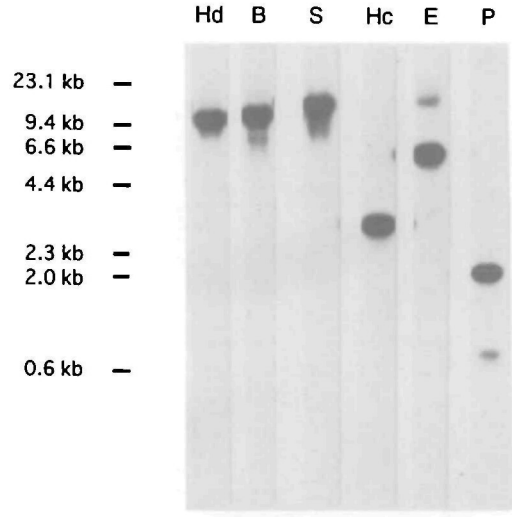

Fig. 3. **Genomic Southern analysis of the *des6* gene.** Genomic DNA (5 μg) digested with the indicated enzymes was electrophoresed, blotted onto a menbrane, and then probed with the ^{32}P-labeled cDNA fragment. B, *Bam*HI; E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; P, *Pst*I; S, *Sal*I.

Fig. 1. **Alignment of the amino acid sequences of chloroplast ω6 desaturase of *C. reinhardtii*, Δ12 desaturases of cyanobacteria, and plastid ω6 desaturases of higher plants.** The sources of the sequences are as follows: Cla, *C. reinhardtii* (this study); Ana, *Anabaena variabilis* (*12*); 7002, *Synechococcus* PCC7002 (*12*); 6714, *Synechocystis* PCC6714 (*12*); 6803, *Synechocystis* PCC6803 (*11*); Ara, *A. thaliana* (*10*); Bra, *B. napus* (*8*); Gly, *G. max* (*8*); Spi, *S. oleracea* (*9*). Arrowheads indicate the first and last amino acid of the regions used to construct a molecular phylogenic tree of chloroplast ω6 desaturase and corresponding ones (Fig. 5). An arrow indicates the cleavage site for the transit peptide of plastid ω6 desaturase of *S. oleracea* (*9*). Eight His residues conserved among membrane-bound desaturases are denoted by asterisks, and the resultant His-motifs are underlined.

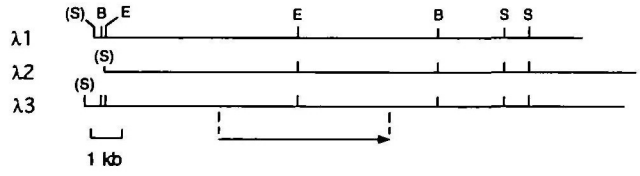

Fig. 2. **Restriction maps of genomic clones.** Restriction sites are designated as B (*Bam*HI), E (*Eco*RI), and S (*Sal*I). (S) at the left side of each clone indicates the *Sal*I site from multi-cloning site of the λ Dash II vector. The arrow indicates the genomic region covering *des6* cDNA and the direction of transcription.

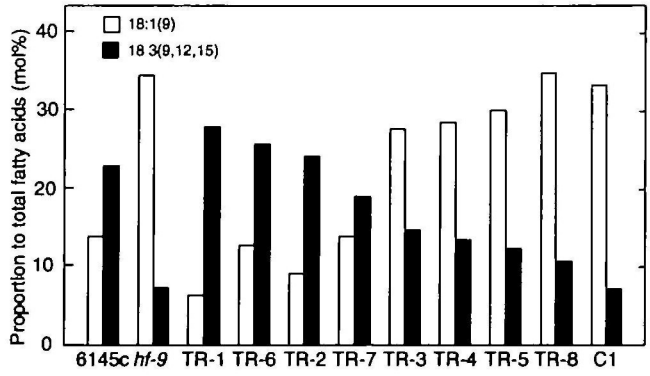

Fig. 5. **Fatty acid compositions of the transformants.** The contents of 18:1(9) and 18:3(9,12,15) are indicated for the parent (6145c), its desaturation mutant (*hf-9*), and transformants with pTR-1 (Tr-1 to -5), pTR-2 (Tr-6 to -8), and pNI1 (C1). The values for the Tr-series are the means of two independent experiments, while the others are for one experiment, respectively.

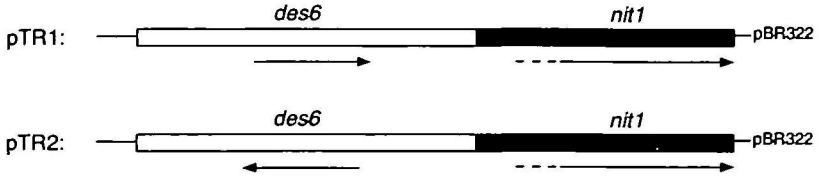

Fig. 4. **Plasmid constructs for the transformation of *hf-9*.** pTR1 and pTR2 were constructed with pBR322 by insertion of the genomic DNA regions of *des6* (open box) and the nitrate reductase gene (*nit1*, shadowed box) in the same and opposite directions with respect to transcription, respectively. The arrows indicate the length and direction of transcription of the genes.

TABLE I. **PSI and PSII activities of the transformants.** The values are the means ± SD of three independent experiments. PSI and PSII activities were measured in the parent (6145c), its desaturation mutant (*hf-9*), and four individual transformants that had recovered from the desaturation mutation (TR-1, -2, -6, and -7). PSI activity was assayed with the reduced form of DCIP and methylviologen as the electron donor and acceptor, respectively. PSII activity was examined with H_2O and *p*-benzoquinone as the electron donor and acceptor, respectively.

	Activity (μmol O_2·(mg Chl)$^{-1}$·h^{-1})					
	6145c	*hf-9*	TR-1	TR-2	TR-6	TR-7
PSI	408±21	269±15	322±32	304±42	313±42	323±13
PSII	313±36	74±3	82±7	80±4	70±4	79±2

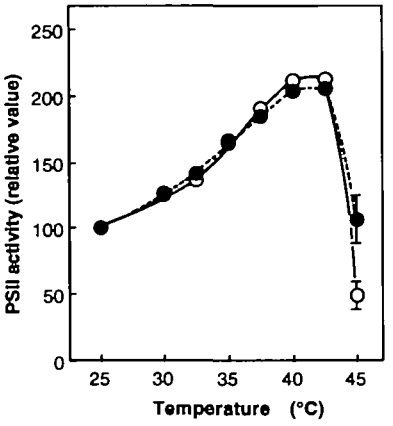

Fig. 6. **Effect of unsatuartion levels of chloroplast lipids on thermal property of PSII activity.** PSII activity was measured in the cells of the strain transformed with pNI1 (●) and the strain complemented for chloroplast ω6 desaturation (○), with *p*-benzoquinone as the electron acceptor at the designated temperatures after 5 min equilibration in the dark. The temperature-dependent curves of PSII activity were normalized against the values at 25°C (97 and 82 μmol O_2·mg Chl^{-1}·h^{-1} in the mutant and complemented strains, respectively). The values are the means of two (25 to 32.5°C) or three independent experiments (35 to 45°C). Standard error was within ± 8 from 35 to 42.5°C for both strains, and thus the bar indicating the error is omitted in the figure.

with 5% anhydrous methanolic-HCl, then analyzed by capillary gas-liquid chromatography as described previously (*19*).

RESULTS

Isolation of Genomic DNA and cDNA Encoding a Putative Chloroplast ω6 Desaturase—From the enzymatic similarity, chloroplast ω6 desaturase of *C. reinhardtii* is considered to be a homolog of cyanobacterial Δ12 and higher plant plastid ω6 desaturases. On the basis of conserved regions of known sequences of these desaturases, *i.e.* FVIGHDC and HFWMSTF, we obtained a 0.5 kb DNA fragment by the RT-PCR method with *C. reinhardtii* poly(A)$^+$ RNA. This fragment was subcloned in the M13 mp19 vector cut with *Hin*cII to yield plasmid pPR1. The nucleotide sequence of pPR1 contained a frame for an amino acid sequence homologous to those of higher plant plastid ω6 and cyanobacterial Δ12 desaturases (data not shown). The insert of pPR1, radioactively labeled with ^{32}P, was then used as a probe for the screening of cDNA and genomic DNA libraries.

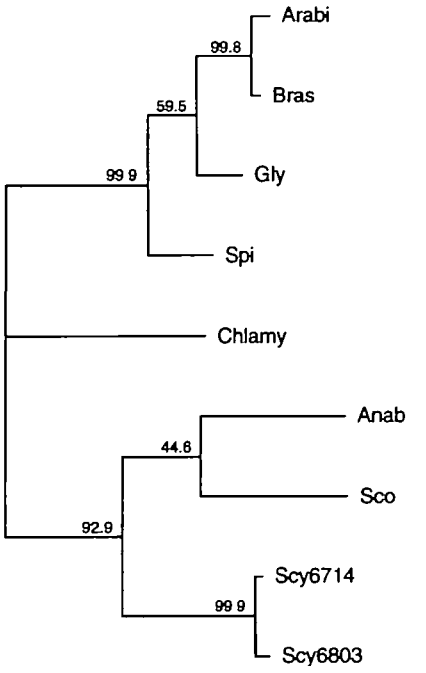

Fig. 7. **The phylogenic tree of chloroplast ω6 desaturase of *C. reinhardtii*, Δ12 desaturases of cyanobacteria, and plastid ω6 desaturases of higher plants.** The region between the two amino acids indicated by arrowheads in Fig. 1 was used for construction of the tree by PAUP. The horizontal length of each branch is proportional to the number of amino acid substitutions. The bootstrap confidence levels of internal branches based on 10,000 replicates are also shown. The sources of the sequences are as follows: Anab, *Anabaena variabilis* (*12*); Sco, *Synechococcus* PCC7002 (*12*); Scy-6714, *Synechocystis* PCC6714 (*12*); Scy6803, *Synechocystis* PCC-6803 (*11*); Chlamy, *C. reinhardtii* (this study); Arabi, *A. thaliana* (*10*); Bras, *B. napus* (*8*); Gly, *G. max* (*8*); Spi, *S. oleracea* (*9*).

The screening of the cDNA library constructed in bacteriophage λgt11 resulted in the isolation of 18 independent positive clones from 300,000 plaques. We arbitrarily selected 5 clones and revealed that all contained an identical insert of 2.2 kb (data not shown). The 2.2 kb insert excised with *Eco*RI from 1 clone was then subcloned in pUC119 cut with *Eco*RI, yielding the plasmid pCD1. Small restriction fragments of pCD1 insert were blunt-ended, then integrated into the *Hin*cII site of the M13 mp19 vector for determination of the nucleotide sequences of both strands. The sequence contained an insert of 2,247 bp containing an ORF encoding a protein of 424 amino acid residues with a putative molecular mass of 48.4 kDa (Fig. 1, the DNA sequence data was deposited in the DDBJ, EMBL, and GeneBank nucleotide sequence databases with the following accession number AB007640). The amino acid sequence was 45-46% identical to those of Δ12 desaturases of cyanobacteria, and 48-51% to those of plastid ω6 desaturases of higher plants, but showed only limited identity with other known desaturase sequences (data not shown). This protein, together with cyanobacterial Δ12 and higher plant plastid ω6 desaturases, contained eight His residues that form three His-clusters (Fig. 1, underlined). It is postulated that these His-motifs, conserved in all known membrane-bound desaturase sequences, act as ligands for iron atom(s) for the desaturation

reaction, as judged by site-directed mutagenesis of rat Δ9 desaturase (*20*) and Δ12 desaturase of *Synechocystis* PCC6803 (*21*). The ORF was preceded by a 5′ untranslated region of 39-bp and followed by a 3′ untranslated region of 936-bp with a trace of poly(A)-tail, 7-bp (data not shown). The sequence TGTAA, a putative polyadenylation signal of *C. reinhardtii*, was localized 14 bp upstream of the trace of poly(A)-tail (data not shown). Since the protein encoded by the pCD1 insert was shown to be chloroplast ω6 desaturase not only by sequence analysis but also by the expression of its function (see below), we designated this gene as *des6*.

By screening 15 genome equivalents (100,000 plaques) of the genomic DNA library constructed in bacteriophage λDASH II, we obtained three independent positive clones (λ1, λ2, and λ3). λ1, λ2, and λ3 contained inserts of 16.2, 17.4, and 17.6 kb, respectively, and restriction mapping revealed a region of overlap in these clones (Fig. 2). The genomic DNA region covering *des6* cDNA was determined in the λ3 clone by Southern analysis with the pCD1 insert as a probe, and the sequencing revealed that *des6* cDNA is interrupted by introns on the genome (data not shown).

Genomic Southern and Northern Blotting—We performed genomic Southern and Northern analyses with the pPR1 insert as a probe. In the genomic Southern hybridization, only one main signal was detected when genomic DNA was digested with the respective enzymes (Fig. 3). The minor signal in the case of *Eco*RI- or *Pst*I-digestion was caused by the restriction sites in the genomic region corresponding to the probe (data not shown). These results indicate that the *des6* gene is present in one copy on the genome. Northern analysis exhibited a signal at 2.4 kb, which indicates that the *des6* cDNA cloned, the size of which is 2.2 kb without the poly(A)-tail, is of full-length (data not shown).

Transformation of the Chlamydomonas Mutant—The *Chlamydomonas* mutant, *hf-9*, has a lesion in chloroplast ω6 desaturation, accumulating 16:1(7) and 18:1(9) with decreases in more highly unsaturated fatty acid contents in chloroplast lipids. We investigated whether or not the *des6* gene can complement the desaturation mutation, by introducing the *des6* gene into *hf-9* by the glass bead method (*13*). Since *hf-9* was derived from *C. reinhardtii* 6145c *nit-1*, which is deficient in nitrate reductase activity, we constructed plasmids in which the *des6* gene is connected to the nitrate reductase gene, a selectable marker. Figure 4 shows the plasmid constructs for the complementation test: the 13.7 kb *Sal*I fragment of λ3 was integrated, in either direction, into plasmid pNI1, composed of the *Sal*I-*Bam*HI fragment of pBR322 and the 10 kb *Sal*I-*Bam*HI insert of pMN24 (*13*) containing the nitrate reductase gene, generating plasmid pTR-1 or pTR-2. Five independent transformants with pTR-1 and three with pTR-2, which are able to grow with NO_3^- as a sole nitrogen source, were selected. Analysis of the fatty acid compositions of total lipids showed that transgenic lines TR-1, -2, -6, and -7 (TR-1 and -2 from pTR-1, and TR-6 and -7 from pTR-2) had increased 18:3(9,12,15) contents comparable to the level in the parent, at the expense of 18:1(9) (Fig. 5). The ratio of 16:4(4, 7,10,13) to 16:1(7) in these lines was higher than that in *hf-9*, and similar to the parent level (data not shown). However, the other four lines (TR-3 to -5 and TR-8) did not show remarkable recovery from the desaturation defect. In each of six control lines transformed with pNI1 only (C1 to C6), no restoration of the unsaturation level was observed (only C1 is shown in Fig. 5). These results indicate that the *des6* gene complements the chloroplast ω6 desaturation mutation in TR-1, -2, -6, and -7, and thus encodes chloroplast ω6 desaturase. The minimal change in the unsaturation levels in transformants TR-3 to -5 and TR-8 may reflect incomplete insertion for full expression of the desaturation gene or the effect of the position at which the desaturase gene was inserted.

PSI and PSII Activities in Transformants—Thylakoid membrane lipids mainly comprise chloroplast lipids with high unsaturation levels such as MGDG and DGDG, and this gives rise to the question of the roles of highly unsaturated fatty acids in this membrane system. *hf-9*, with much lower unsaturation levels of chloroplast lipids than the parent, was impaired in PSI and PSII activities, which raised the possibility that high unsaturation levels of chloroplast lipids are responsible for these thylakoid membrane functions (*22*). Since tetrad analysis of *hf-9* was hampered by the inability of *hf-9* to mate, owing to its shorter and paralyzed fragella (*7*), we could not determine whether the mutant phenotypes of desaturation and photosynthesis were due to the same mutation. To clarify this point, we measured the PSI and PSII activities of TR-1, -2, -6, and -7, the unsaturation levels of which were restored on *des6* gene introduction (Table I).

The PSI and PSII activities of *hf-9* decreased to 24 and 66%, respectively, of those of the parent. All transgenic lines with recovered unsaturation levels showed essentially unaltered PSI and PSII activities compared with those of *hf-9*, indicating that the impaired photosynthetic activities of *hf-9* are caused by mutation(s) in gene(s) other than *des6*.

We previously showed that PSII activity was lower in *hf-9* than in the parent in the range between 7 and 40°C, but higher in *hf-9* at 45°C, which suggested that the lowered unsaturation level of thylakoid membranes in *hf-9* was correlated with the maintenance of PSII activity at high temperature such as 45°C (*23*). To examine this correlation, the temperature-dependency of PSII activity was determined in a mutant and a complemented strain as to chloroplast ω6 desaturation (Fig. 6). The strain transformed only with the nitrate reductase gene (C1, Fig. 5) showed a peak of PSII activity at around 40°C, which is similar to the profile of *hf-9* (*23*). PSII activity of the complemented strain (TR-1, Fig. 5) was almost indistinguishable from that of the mutant strain from 25 to 42.5°C, but remarkably repressed at 45°C. The results thus confirmed that the elevated thermal tolerance of PSII activity at the high temperature is conferred by the lowered unsaturation level.

DISCUSSION

Structure of the Chloroplast ω6 Desaturase of C. reinhardtii—In higher plants, plastid ω6 desaturase catalyzes the synthesis of dienoic acids from monoenoic acids with chloroplast lipid substrates, and is thereby involved in the synthesis of the highly unsaturated fatty acids such as trienoic ones. In this study, we cloned cDNA and its genomic counterpart for *C. reinhardtii* chloroplast ω6 desaturase, using RT-PCR method, on the basis of amino acid sequences conserved among higher plant plastid ω6 desaturases (*8-10*) and cyanobacterial Δ12 desaturases

(*11, 12*). The *Chlamydomonas* gene designated as *des6* encodes a protein of 424 amino acid residues showing 46-51% homology with cyanobacterial Δ12 and higher plant ω6 desaturases, and complete conservation of the His-motifs, putative Fe-ligands, of membrane-bound desaturases (*20, 21*). *des6* was functionally confirmed to be the chloroplast ω6 desaturase gene by the complementation of the chloroplast ω6 desaturation mutation in *hf-9* (Fig. 5).

Alignment of the desaturases suggested N-terminal extensions of *ca.* 60 and 80 amino acid residues in *C. reinhardtii* and higher plants, respectively, from the first Met residue in cyanobacteria (Fig. 1). The ω6 desaturases, encoded by the nuclear genome in higher plants as well as in *C. reinhardtii*, are considered to be targeted from the cytoplasm to chloroplast envelope membranes (*24*). The premature plastid ω6 desaturase of *Spinacia oleracea* possesses a stretch of 65 amino acid residues with some features of the chloroplast transit peptide upstream of the N-terminus of the mature form (*9*). Three other higher plant desaturases also shared such features in the N-terminal 60 amino acids, the probable region of the transit peptide: the abundance of basic amino acids such as Arg and Lys (9 to 12), and few acidic amino acids (only two). The shorter N-terminal extensions in *C. reinhardtii* than in higher plants may be correlated with the observation that the transit peptides of chloroplast-destined proteins in *C. reinhardtii* are generally shorter than the corresponding ones in higher plants (*25*). The N-terminal region of 40 amino acid residues of the *Chlamydomonas* desaturase may cover the region of its transit pepetide, exhibiting some characteristics of *Chlamydomonas* chloroplast transit peptides (*26*), *i.e.*, high contents of Ala (30%) and Arg (15%) with no acidic residues, and the potential to form an amphiphilic α-helix (data not shown).

A phylogenic tree of *Chlamydomonas* chloroplast ω6 desaturase and the corresponding enzymes of cyanobacteria and higher plants was constructed with amino acid sequences of 312-315 residues (Fig. 7, the first and last amino acid residues of the regions are indicated by arrowheads in Fig. 1). The tree shows that, in view of the number of amino acid substitutions, the cyanobacterial and higher plant desaturases can each be classified into a monophyletic group and that the *Chlamydomonas* desaturase is positioned at an almost equal distance from each group.

The presence of two isozymes has been reported for plastid ω3 desaturase of *A. thaliana* (*27*) and microsomal ω6 desaturase of *G. max* (*28*). A high homology score for the amino acid sequence was obtained in both cases: 80 and 73% for the ω3 and ω6 desaturases, respectively. A single signal was detected on genomic Southern hybridization of *C. reinhardtii*, probed with a part of *des6* cDNA (Fig. 3), indicating the absence of other genes highly homologous to *des6*. It is probable that the chloroplast ω6 desaturase gene is present in one copy on the genome of *C. reinhardtii*.

Function of Unsaturated Fatty Acids in Biological Membranes—Highly unsaturated fatty acids are abundant in chloroplast membrane lipids, suggesting the involvement of the high unsaturation levels in the expression of chloroplast membrane functions. However, a triple mutant of *A. thaliana* with defects in plastid and microsomal isozymes of ω3 desaturases, containing no 16:3(7,10,13) and only negligible 18:3(9,12,15), with accumulation of 16:2(7,10) and 18:2(9,12), unexpectedly showed a deleterious effect not on photosynthesis, but on pollen fertility, which led to the male-sterile phenotype. This damage was complemented by a supply of 18:3(9,12,15) or its derivative, jasmonate, indicating that 18:3(9,12,15) is not crucial for photosynthesis, at least under the experimental growth condition, but is required for the reproductive process by the jasmonate signaling pathway (*5*).

On the other hand, no evidence has been presented as to whether dienoic acids are indispensable for chloroplast functions. Mutants of chloroplast ω6 desaturation have been isolated from *A. thaliana* (*4*) and *C. reinhardtii* (*7*). The *Arabidopsis* mutant retained large amounts of 18:3 (9,12,15) in chloroplast lipids, since its chloroplasts were supplied with 18:2(9,12), which is subsequently desaturated to 18:3(9,12,15) by plastid ω3 desaturase, and 18:3 (9,12,15) from ER through the eukaryotic pathway. Thus, this mutant is not usable for assessment of the roles of dienoic acids in chloroplast functions. The *Chlamydomonas* mutant, *hf-9*, showed remarkable decreases in the contents of dienoic and trienoic acids of chloroplast lipids, since chloroplast ω6 desaturase is the predominant source of 18:2(9,12) in the chloroplasts in *C. reinhardtii*, which is almost completely devoid of the eukaryotic pathway (*6*). Although the defects of the PSI and PSII activities in *hf-9* suggested the involvement of dienoic fatty acids in the expression of these thylakoid membrane functions (*22*), no correlation of these photosynthetic lesions with the desaturation mutation was observed in this study, indicating that gene defect(s) other than that of the desaturase brought about the photosynthetic damage in *hf-9* (Table I). However, these results still do not rule out the possibility of the involvement of dienoic fatty acids in the expression of photosynthetic activity. Since *hf-9* can not be backcrossed with wild-type strains, owing to its short and paralyzed flagella, it is desirable to produce new mutants with a defect only in the *des6* gene in order to clarify the relationship of unsaturated fatty acids with chloroplast functions. Disruption of the *des6* gene on the nuclear genome by means of homologous recombination is one way to produce such mutants (*29*).

Some higher plants acclimated to high temperatures show enhanced heat stability of PSII activity and concomitant decreases in the unsaturation levels of chloroplast lipids, which suggested the role of the lowered unsaturation levels of thylakoid membranes in the thermal tolerance of PSII activity (*30, 31*). This is compatible with the acquisition of the heat tolerance of PSII activity in some mutants of *A. thaliana* and *C. reinhardtii* as to chloroplast desaturases with lowered unsaturation levels of chloroplast lipids (*23, 32*), and also in pea chloroplasts with more saturated thylakoid membrane lipids through catalytic hydrogenation (*33*). However, these reports show enhancement of PSII stability at high temperatures only in parallel with the decreased unsaturation levels. The reduction in PSII activity at 45°C caused by the recovery of chloroplast ω6 desaturation through the introduction of desaturase gene, which was revealed in the present study (Fig. 6), is direct evidence of the involvement of lowered unsaturation level in the enhanced heat tolerance of PSII activity. The decreased unsaturation level may support the PSII activity at the high temperature through the repression of the phase-separation of non-bilayer lipid, MGDG, as was postulated in Ref. *32*, or through the lipid-protein interac-

tion to change the conformation of PSII complex to exert high activity even at the extreme temperature (*23*).

In contrast to our report, the heat stability of PSII was found to be indistinguishable between a mutant of Δ12 desaturase of a cyanobacteria, *Synechocystis* PCC6803, with decreased unsaturation levels of thylakoid membrane lipids, and a transformant recovered from the mutation through the introduction of the desaturase gene, which revealed no correlation of the lowered unsaturation level of thylakoid membrane lipids with the stability of PSII activity at high temperatures (*34*). This inconsistency in the effect of the lowered unsaturation levels on the thermal stability of PSII may be explained by the fact that thylakoid membrane lipids of the cyanobacteria, the unsaturation levels of which are lower than those of *C. reinhardtii*, are originally suited for the photosynthesis at high temperatures (*7, 34*).

The expression of some desaturase genes is under regulation by environmental stimuli such as temperature and wounding (*27, 35, 36*), tissue-specificity (*28*), or the developmental stage (*28*). Future work should be performed to determine whether or not the *des6* gene expression in *C. reinhardtii* is contolled by environmental factors or the life cycle, *e.g.*, cell differentiation from vegetative cells to gametes. Such work will provide clues for elucidation of the roles of dienoic or higher unsaturated fatty acids under particular conditions.

We wish to thank Drs. Y. Matsuda (Kobe University) and Y. Nakamura (National Institute of Agrobiological Resources) for kindly giving us the cDNA library of *C. reinhardtii*. We also thank Dr. S. Yoshida (Kirin Brewery Co.) for giving us the advice on molecular biological techniques.

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