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Cloning of a Higher-Plant Plastid ω-6 Fatty Acid Desaturase cDNA and Its Expression in a Cyanobacterium

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Oligomers based on amino acids conserved between known plant ω-3 and cyanobacterium ω-6 fatty acid desaturases were used to screen an *Arabidopsis* cDNA library for related sequences. An identified clone encoding a novel desaturase-like polypeptide was used to isolate its homologs from *Glycine max* and *Brassica napus*. The plant deduced amino acid sequences showed less than 27% similarity to known plant ω-6 and ω-3 desaturases but more than 48% similarity to cyanobacterial ω-6 desaturase, and they contained putative plastid transit sequences. Thus, we deduce that the plant cDNAs encode the plastid ω-6 desaturase. The identity was supported by expression of the *B. napus* cDNA in cyanobacterium. *Synechococcus* transformed with a chimeric gene that contains a prokaryotic promoter fused to the rapeseed cDNA encoding all but the first 73 amino acids partially converted its oleic acid fatty acid to linoleic acid, and the 16:1(9c) fatty acid was converted primarily to 16:2(9c,12) in vivo. Thus, the plant ω-6 desaturase, which utilizes 16:1(7c) in plants, can utilize 16:1(9c) in the cyanobacterium. The plastid and cytosolic homologs of plant ω-6 desaturases are much more distantly related than those of ω-3 desaturases.

In leaf tissue there are two distinct pathways for the biosynthesis of the polyunsaturated fatty acids 18:2 and 18:3, one located in cytosolic membranes and the other located in plastid membranes. In *Arabidopsis thaliana*, cytosolic and plastid ω-6 fatty acid desaturations that result in the production of diene fatty acids are controlled by the *FAD 2* and *FAD 6* loci, respectively. Cytosolic and plastid ω-3 desaturations that result in the production of triene fatty acids are controlled by *FAD 3* and *FAD 7*, respectively (Lemieux et al., 1990; Browse and Somerville 1991). It has been postulated that these loci correspond to structural genes for the desaturase enzymes. For *FAD 3*, proof of this postulate has come from the cloning of a cDNA encoding a desaturase corresponding to the *FAD 3* locus (Arondel et al., 1992; Yadav et al., 1993).

Cytosolic ω-3 fatty acid desaturase cDNAs have been used to isolate their plastid homologs by screening plant cDNA libraries under low-stringency hybridization conditions, and the amino acid sequences of the higher-plant ω-3 desaturases show significant similarity (68% or greater) both among different plant species and between the microsomal and plastid homologs within each species (Iba et al., 1993; Yadav et al., 1993). However, this screening did not result in the isolation of cDNAs encoding other than ω-3 desaturases.

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Recently, the T-DNA tagging method was used to isolate the *Arabidopsis* microsomal ω-6 fatty acid desaturase (*FAD 2*) and it showed only 36% similarity at the amino acid level with the ω-3 desaturases (Okuley et al., 1994). In contrast to the results with ω-3 desaturases, low-stringency screening of an *Arabidopsis* cDNA library with the *Arabidopsis* microsomal ω-6 desaturase cDNA did not result in the identification of its plastid homolog. Although plant ω-3 desaturases show poor similarity (less than 27%) to the *Synechocystis* PCC6803 *DES A* gene, which encodes a ω-6 desaturase (Wada et al., 1990), there are short stretches of amino acids that are conserved between them. We used degenerate oligomers, which were made to one such stretch of amino acids, as a hybridization probe to screen an *Arabidopsis* cDNA library for sequences encoding the plastid ω-6 desaturase. We report here the cloning of cDNAs encoding a novel higher-plant desaturase that appears to be a plastid ω-6 desaturase, and we confirm its identity by its functional expression in cyanobacterium *Synechococcus* sp. strain PCC7942.

MATERIALS AND METHODS

Cloning of an *Arabidopsis thaliana* Desaturase cDNA Using Degenerate Oligomers as a Hybridization Probe

Unless otherwise noted, nucleic acid hybridizations, phage library screening, sequencing, and sequence analysis were as described previously (Yadav et al., 1993).

Two sets of 36-nucleotide-long oligomers, each 16-fold degenerate, were designed based on the amino acid sequence FVLGHDCGHGSF, which is conserved among all the known plant ω-3 desaturase sequences (Yadav et al., 1993) and to the corresponding region FVVGHDCGHGRF in the *DES A* sequence (Wada et al., 1990). The degenerate oligonucleotides used inosines (I) at all third positions of codons with 4-

Abbreviations: pfu, plaque-forming unit; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. The double bond configuration and position in isomeric unsaturated fatty acids is designated in parentheses behind the chain length:double bond number and is the number from the carboxyl end for the first carbon in the double bond, e.g. 16:2(7,10) is 7,10-hexadecadienoic acid. When determined or known from the literature, the stereoconfiguration is abbreviated c for *cis* or t for *trans* configuration; if unknown, no designation is given. The ω designations refer to positions of the double bond from the methyl end of fatty acids.

fold degeneracy, as well as at the first position of Leu/Val at the third amino acid position, and used the TTC codon for Phe (the preferred codon usage in *Arabidopsis*). The two sets have the following sequences:

5′-TTCGTIITIGGICA(C/T)GA(C/T)TG(C/T)GGICA (C/T)GGIAGITTC-3′

5′-TTCGTIITIGGICA(C/T)GA(C/T)TG(C/T)GGICA (C/T)GGITCITTC-3′

These oligonucleotides were mixed in equimolar amounts and the mixture was designated FCR-1. Twenty-seven picomoles of the probe FCR-1 were end labeled with [γ-^{32}P]ATP by T_4 polynucleotide kinase, and unincorporated [γ-^{32}P]ATP was removed by passing the reaction mixture through a 0.5-mL Bio-gel P2 column (Bio-Rad) that had been equilibrated with 6× SSC (20× SSC = 175.3 g of NaCl, 88.2 g of Na citrate in 1 L at pH 7.0).

To exclude *Arabidopsis* cDNAs encoding cytosolic and plastid ω-3 desaturases as well as cytosolic ω-6 desaturase, the library was also screened with radiolabeled fragments specific for these sequences. These specific fragments were amplified by PCR (Perkin-Elmer Cetus, AmpliTaq kit) using primers specific for ω-3 and ω-6 desaturase cDNAs encoding each of the desaturases. The resulting 600-bp PCR products from each desaturase cDNA were gel purified and mixed in equimolar amounts. These mixed fragments, designated FM-1, were labeled with [α-^{32}P]dCTP using the Random Priming Labeling Kit (BRL).

An *Arabidopsis thaliana* cDNA library (Kieber et al., 1993) was mass excised by addition of 4 μL of the cDNA library (containing about 4 × 10^5 pfu) and 4 μL of R408 helper phage (10^{10} pfu mL^{-1}) (Stratagene) to 0.8 mL of *Escherichia coli* XL-1 cells (1.0 A_{600}) grown in NZY broth (5 g of NaCl, 2 g of $MgSO_4$, 5 g of yeast extract, 10 g of casein hydrolysate/L at pH 7.5). Phage and cells were incubated for 15 min at 37°C followed by the addition of 20 mL of 2× YT (10 g of NaCl, 10 g of yeast extract, 16 g of bacto-typtone/L) and incubation for 3 h at 37°C. After incubation at 70°C for 20 min, cells and debris were removed by centrifugation and the supernatant was saved. To each of three 0.2-mL aliquots of XL-1 cells (1.0 A_{600}) 1 μL of the above supernatant was added and the cultures were incubated at 37°C for 15 min. Infected cells were plated onto three 25 × 150-mm 2× YT agar plates containing 150 μg mL^{-1} ampicillin and incubated at 37°C for 15 h. Approximately 36,000 colonies from three plates were transferred onto nitrocellulose filters (Schleicher & Schuell, BA85). Cells were regrown at 37°C until colonies were near their original size, a second transfer to nitrocellulose filters was made, and the colonies were regrown a third time. Filters were air dried and successively soaked for 1 min each in 0.5 M NaOH/1.5 M NaCl, 0.5 M Tris (pH 8.0)/1.5 M NaCl, and 2× SSC/0.2 M Tris, pH 8.0. Filters were air dried and then vacuum dried at 80°C for 1 h.

Filters from the first transfer were prehybridized in 5% dextran sulfate, 5× SSC, 100 μg mL^{-1} denatured salmon sperm DNA, 50 mM Tris, pH 8.0, 0.1% SDS, and 5× Denhardt's solution (50× Denhardt's solution = 5 g of Ficoll, 5 g of PVP, and 5 g of BSA in 500 mL of H_2O) for 3 h at 37°C. They were hybridized with denatured, radiolabeled probe FCR-1 in the same buffer for 40 h at 37°C and then washed twice for 10 min each with 0.6× SSC, 0.1% SDS at 37°C and once at 42°C. Filters from the second transfer were prehybridized in the same solution at 65°C, hybridized with denatured, radiolabeled probe FM-1 for 18 h at 65°C, and washed twice for 15 min each with 0.2× SSC, 0.1% SDS at 65°C. Both sets of filters were autoradiographed on Kodak X-Omat-AR film with an intensifying screen at −80°C for 15 h.

Positives were scored as hybridizing to probe FCR-1 but not to probe FM-1. Since the proximity of other colonies prevented a clonal inoculation, the area surrounding each positive colony was used as inoculum and grown in liquid 2× YT media containing 150 μg mL^{-1} ampicillin for 15 h at 37°C. Two colonies scored as hybridizing to both probes were treated in the same way to serve as positive controls in further screening. Plasmid DNA was purified from each of the 11 mixed cultures using the Magic Minipreps DNA Purification System (Promega). Inserts were released from the pBluescript vector (Stratagene) by digestion with *Eco*RI restriction endonuclease (New England Biolabs), separated on a 0.8% agarose gel, stained with ethidium bromide, and blotted for Southern analysis. The blots were hybridized with probe FCR-1 as above except that all incubations were at 42°C. The hybridized and washed blots were exposed at −80°C for 1 h.

Cultures that contained clones with inserts that hybridized in the above Southern analysis were streaked onto 15 × 100 mm 2× YT agar plates containing 150 μg mL^{-1} ampicillin and incubated for 15 h at 37°C. Twelve colonies from each plate were individually grown in 2× YT for 15 h at 37°C and the Southern analysis of plasmid DNA from each colony was repeated as above.

Cloning of Rapeseed and Soybean cDNAs Encoding Homologs of the *Arabidopsis* Desaturase-Related Sequence

The 1.1-kb *Arabidopsis* cDNA insert from pTCAC1 (see "Results") was gel purified, radiolabeled with ^{32}P as described above, and used to screen approximately 150,000 pfu from cDNA libraries made to developing seeds of *Brassica napus* (rapeseed) and *Glycine max* (soybean) (Yadav et al., 1993). The *B. napus* library was hybridized and washed at 62°C and the soybean library was hybridized and washed at 50°C. Several positively hybridizing plaques were purified and the cDNAs were excised as plasmids. Plasmid DNA from overnight cultures was purified, digested with *Eco*RI, and size analyzed on a 0.8% agarose gel.

Transformation of *Synechococcus* sp. Strain PCC7942 with a Chimeric Gene for the Expression of the Rapeseed Desaturase

Plasmid pTCCA1 containing the rapeseed cDNA was cut with *Pvu*II, which has a restriction site located near the putative mature protein N terminus (Ser^{73} of the rapeseed amino acid sequence shown in Fig. 1) and ligated to an *Eco*RI linker, 5′-pGGAATTCC-3′. Following *Eco*RI digestion of the ligated DNA, a 1.3-kb fragment containing the coding region

of the mature plant desaturase and 3′ noncoding sequences from pTCCA1 was isolated and cloned into the *Eco*RI site of bacterial expression vector pKK388–1 (Clontech) in the sense orientation with respect to the *trc* promoter, resulting in plasmid pKKCC.

Plasmid pAM854 (Bustos and Golden, 1991) is a *Synechococcus* recombinational transformation vector comprising pBR328 containing a 2.8-kb *Bam*HI fragment of *Synechococcus* genomic DNA inserted at a unique *Bam*HI site in the vector. A modified spectinomycin/streptomycin resistance cassette was inserted into a *Xho*I site within the *Synechococcus* DNA. To form plasmid pAG1, a unique *Sma*I site adjacent to the resistance marker cassette and within the *Synechococcus* DNA of pAM854 was modified by digestion and linker addition to produce a *Bgl*II site.

A *Bam*HI fragment containing the chimeric *trc* promoter:plant mature desaturase gene was isolated from pKKCC and cloned into the *Bgl*II site of plasmid pAG1 to result in plasmid pAG1CC. Plasmid pAG1CC was sequenced across the *Eco*RI site between the *trc* promoter and the rapeseed cDNA. The deduced amino acid sequence of the peptide produced by expression of pAG1CC is then MAAEFP followed by the sequence beginning at amino acid 74 of the rapeseed sequence in Figure 1.

Synechococcus sp. strain 7942 (previously referred to as *Anacystis nidulans* R2) was grown and transformed with plasmids pAG1 and pAG1CC as described by Golden et al. (1987). Transformants were plated on BG-11 plates containing 40 mg L^{-1} spectinomycin and 5 mg L^{-1} streptomycin (Bustos and Golden, 1991) and incubated at 30°C under light. After 1 week, several resistant transformants appeared as green colonies. Several of these colonies were picked, restreaked on fresh medium containing antibiotics, and cultured as described above.

Growth and Analysis of *Synechococcus* Transformants

Two independent transformants each of pAG1, vector only control (A1 and A3), and pAG1CC, vector containing the plant chimeric gene (C1 and C3), were maintained under continuous light in liquid BG-11 medium containing 20 mg L^{-1} spectinomycin and 5 mg L^{-1} streptomycin. In one experiment (see Table I, Set I), transformants A1 and C1 were inoculated into 10 mL of the medium and grown under continuous light in a roller drum at 37°C for 4 d. In two other experiments, transformants A3 and C3 were grown in 50 mL of the medium in a shake flask under continuous light for 5 d either at 30°C (Set II) or at 25°C (Set III). The 25°C growth condition was repeated and analyzed three times.

Fatty acid methyl esters were either made by direct methanolysis of pelleted cells (Browse et al., 1986) or by extracting pelleted cells with acetone, removing the solvent by evaporation under N_2, and trans-esterifying the extracted lipids with 1% sodium methoxide in methanol. Fatty acid methyl esters were separated by GC on a Hewlett-Packard 5890 with an Omegawax 320 column (0.32 mm × 30 m, Supelco) run from 200°C to 220°C at 2°C min^{-1}. GC-MS analysis was done on a Hewlett-Packard 5890 gas chromatograph with DB-1 capillary column and a series 5971 mass-selective detector.

Dimethyl disulfide adducts of the fatty acid methyl esters were prepared (Yamamoto et al., 1991) for analysis of the double bond positions in fatty acids. GC-MS analysis was done using an SP2330 column (0.32 mm × 30 m, Supelco) run at 150°C for 5 min followed by a 5°C min^{-1} gradient to 240°C on the Hewlett-Packard GC-MS system.

RESULTS

Cloning of an *Arabidopsis* cDNA Encoding a Desaturase-Related Sequence

Stearoyl-acyl carrier protein desaturase (McKeon and Stumpf, 1982), the cyanobacterial ω-6 desaturase (Wada et al., 1993), and the safflower cytosolic ω-6 desaturase (Kearns et al., 1991) all require O_2 and a source of reductant for activity, and they all perform very similar functions. Therefore, it seems probable that there may be primary sequence conservation around their active sites. Alignment of the deduced amino acid sequences for the cytosolic and plastid ω-3 desaturases from three plant species shows several regions that are identical, although their overall similarity is only about 67% (fig. 2 in Yadav et al., 1993). One particularly well-conserved region is 13 amino acids in length at positions 97 to 110 in the *Arabidopsis* microsomal ω-3 desaturase (Yadav et al., 1993). Ten residues of this sequence are also identical in a similarly placed region of the *Synechocystis* ω-6 desaturase (Wada et al., 1990).

This region contains a motif (DCGH) that is similar to the EXXH motif that has been demonstrated by crystallographic structure to be a part of the complex, di-iron-binding site in ribonucleotide reductase (Nordlund et al., 1990) and has been shown by spectroscopy and sequence homology to be present in the soluble, higher-plant fatty acid desaturase stearoyl-acyl carrier protein desaturase (Fox et al., 1993). Despite this similarity, other elements of the di-iron-binding site of ribonucleotide reductase, such as a repeat of the EXXH sequence about 100 amino acids carboxyl to the first and two additional acidic residues (Nordlund et al., 1990), are not obviously present in the known membrane-bound desaturases (Yadav et al., 1993; Okuley et al., 1994).

The sequence from the region near amino acid 100 also has similarity to the zinc-binding domain of a class of metalloproteins known as matrix metalloproteinases. The plant representative of this class is a 170-amino acid protein from soybean leaves (McGeehan et al., 1992) with the sequence VAVHEIGHLLGL identified as its probable zinc-binding domain. The essential motif H(D/E)XGH is conserved among the known membrane-bound fatty acid desaturases from plants but not from yeast or mammals (Okuley et al., 1994). Although the possible function of this domain is not clear, we focused on it as the basis for the design of probes for cDNAs encoding other desaturases due to its conservation.

We designed two sets of 16-fold degenerate 36-mers (probe FCR-1) that encode the above-mentioned conserved amino acid sequences FV(L/V)GHDCGHGF. Probe FCR-1 hybridizes to the cDNAs encoding both the cytosolic and plastid *Arabidopsis* ω-3 desaturase sequences. To remove these sequences from consideration in the search for additional desaturase cDNAs, we differentially screened the *Arabidopsis*

cDNA library with a mixed probe consisting of 0.6-kb fragments specific for *Arabidopsis* cytosolic and plastid ω-3 desaturases and for the cytosolic ω-6 desaturase. Initial screening of the *Arabidopsis* library was not successful due to a high background with probe FCR-1. In an effort to improve the signal-to-noise ratio when using FCR-1, we chose to mass excise the phage library to create a plasmid library and screen a limited number of individuals in that library as bacterial colonies.

With this method we identified several colonies that hybridized to oligomer probe FCR-1, many of which also hybridized to probe FM-1. However, some colonies hybridized to probe FCR-1 but not to probe FM-1. Nine such colonies were purified from the mixed cultures by Southern analysis of plasmid DNA using probe FCR-1. Strongly hybridizing clones were obtained from three of nine primary picks. The two largest of these, pTCAC1 and pTC-FC3.4, were partially sequenced. Over a 500-bp region, pTC-FC3.4 showed no homology to other fatty acid desaturase sequences except for a 24-bp sequence that has 23 bases that match FCR-1. The deduced amino acid sequence obtained from the partial sequence of pTCAC1 is shown in Figure 1 and includes the sequence FVIGHDCAHKSFS, which contains 10 of the 12 residues that were the basis of FCR-1. Although the 162-amino acid sequence deduced from the partial sequence was only about 30% identical to the same region of the other higher-plant fatty acid desaturases, it was found to be 56% identical to the deduced amino acid sequence of *Synechocystis des* A desaturase. Based on this similarity, we postulated this cDNA to encode the plastid ω-6 desaturase.

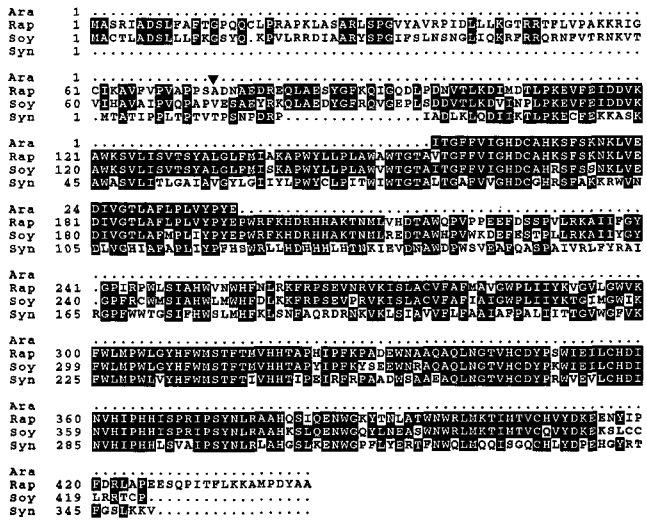

Figure 1. Comparison of the deduced amino acid sequences of the plastid ω-6 fatty acid desaturases from rapeseed (Rap), soybean (Soy), and the partial sequence of *Arabidopsis* (Ara) with that of the *Synecocystis* ω-6 fatty acid desaturase (desA) (Syn). Identical residues are shown on black backgrounds. The rapeseed sequence is marked with an arrow at the putative mature peptide N terminus used in the *Synechococcus* expression studies.

Isolation of Rapeseed and Soybean Homologs of the *Arabidopsis* Putative ω-6 Desaturase

Because of our interest in oilseed crops, further characterization of the putative plastid ω-6 desaturase was done on rapeseed and soybean homologs of the *Arabidopsis* clone. For this, the cDNA insert in pTAAC1 was used as a heterologous probe to isolate related clones from cDNA libraries made to developing seeds of rapeseed and soybean. From several pure, positively hybridizing clones, one clone from rapeseed (designated pTCCC1) and one clone from soybean (designated pSF-236S) that contained the largest, single cDNA inserts were sequenced. The deduced amino acid sequences from those clones are given and compared to the *DES A* sequence in Figure 1. Although their overall percent similarity with *DES A* desaturase is 52%, that with cytosolic ω-3, plastid ω-3, and cytosolic ω-6 desaturases of higher plants is less than 27%.

Functional Expression of the Rapeseed Putative Plastid ω-6 Desaturase in *Synechococcus*

Since cyanobacteria and higher-plant plastids contain similar lipid and 18:1 fatty acid species, and since their desaturases both utilize Fd as the immediate electron donor (Schmidt and Heinz, 1990; Wada et al., 1993), it was reasonable to test the biological function of the cDNA insert in pTCCC1 in a cyanobacterium.

We chose to transform *Synechococcus* sp. strain PCC7942, which produces saturated and monounsaturated fatty acids but lacks all polyunsaturated fatty acids. A chimeric gene was constructed comprising a bacterial *trc* promoter fused to the coding sequence of the rapeseed putative mature fatty acid desaturase; this was cloned adjacent to a spectinomycin/streptomycin selectable marker and within a *Synechococcus* genomic fragment of a cyanobacterial recombinational transformation vector (Bustos and Golden, 1991).

The independent cyanobacterial transformants C1 and C3, which contain the higher-plant chimeric gene, produced fatty acids identified as 16:2 and 18:2 by the retention times of their fatty acid methyl esters, whereas the independent control transformants A1 and A3, which contain only the vector, did not (Table I). Fatty acids of the transformants A3 and C3 were further analyzed by GC-MS following the formation of dimethyl disulfide adducts of the fatty acid methyl esters. This revealed that two 18:1 isomers, 18:1(9c) and 18:1(11), are present in the control transformant A3, and that only the 18:1(9c) was utilized by transformant C3 to produce 18:2(9,12).

The appearance of an apparent 16:2 product was surprising because in higher plants the substrate for the ω-6 desaturase is 16:1(7c) (Nichols, 1965), whereas cyanobacteria produce primarily 16:1(9c) (Higashi and Murata, 1993). Partial purification of fatty acid methyl esters made from leaf lipids of *B. napus* by reverse-phase liquid chromatography followed by dimethyl disulfide derivatization and GC-MS analysis confirmed that the higher-plant leaf 16-carbon fatty acids are 16:1(7), 16:2(7,10), and 16:3(7,10,13) (data not shown). Interpretation of the mass spectra from a similar analysis of

Table I. *Fatty acid composition of transformed Synechococcus*

C3-1, -2, and -3 analyses are from three separate subcultures of transformant C3. The 18:2 and 16:2 produced were 18:2(9,12) and 16:2(9,12) as determined from the mass spectra of their methyl ester/dimethyl disulfide derivatives.

	Percent of Total Fatty Acids						
	16:0	16:1	16:2	18:0	18:1(9)	18:1(11)	18:2
Set I, 37°C							
A1, control	45.0	32.0		6.9	9.5	6.5	
C1	56.0	22.0	3.6	9.1		4.3	8.0
Set II, 30°C							
A3, control	45.0	44.0		1.7	3.9	2.9	
C3	45.0	44.0	7.5	1.9		1.9	2.2
Set III, 25°C							
A3, control	41.8	51.5		1.7	2.4	3.1	
C3-1	45.5	36.6	12.1	1.7	0.8	3.0	1.3
C3-2	45.4	37.9	10.5	0.8	2.1	1.6	1.6
C3-3	43.4	40.5	10.6	0.9	1.6	1.9	1.0

fatty acids from both *Synechococcus* transformants A3 and C3 confirmed that the main 16:1 isomer is 16:1(9) along with a small amount of 16:1(11).

The total ion trace through the region of derivatives of unsaturated fatty acids is shown for transformant C3 in Figure 2A. Peaks corresponding to the methyl ester/dimethyl disulfide derivatives of the monoene acids are identified on the chromatogram. The mass spectra for the peaks marked B and C are shown in Figure 2, B and C, respectively. As reported by Yamamoto et al. (1991) and confirmed using 18:2 standards, fully *S*-methylated derivatives of diene fatty acids are not formed. Rather, a mixture of the two possible di *S*-methyl species is formed. From the mass spectrum shown in Figure 2B, we conclude that the *S*-methyl derivative has the structure shown at the top of the spectrum. This follows from the small molecular ion at m/z 360 and the prominent fragment at m/z 217, which is indicative of cleavage between the dimethyl disulfide adduct formed from the double bond closest to the carboxyl end of the fatty acid. The fragment at m/z 143 may correspond to the ion produced by the remainder of the fatty acid derivative in this fragmentation. This is the pattern expected from derivatization of the ω-7 double bond in a 16:2(9,12) precursor.

From the mass spectrum and assignment of prominent ion fragments shown in Figure 2C, we conclude that the structure of the *S*-methyl derivative is that shown at the top of the spectrum. The molecular ion and fragmentation pattern is consistent with that expected from the derivative formed across the ω-4 double bond of a 16:2(9,12) precursor.

The two peaks marked 18:2 (Fig. 2A) were similarly identified as the two positional isomers derived from 18:2(9,12).

DISCUSSION

Screening the *Arabidopsis* cDNA library with degenerate oligonucleotides based on a 12-amino acid sequence conserved between higher-plant cytosolic and plastid ω-3 desaturases and a cyanobacterial ω-6 desaturase allowed the cloning and identification of a desaturase-related sequence. Alignment of the deduced amino acid sequences for the plant desaturase-related sequences from rapeseed and soybean showed about 72% similarity (Fig. 1). Similar alignment with other glycerolipid desaturases shows that these new desaturase-related proteins have N-terminal extensions that begin with the dipeptide MetAla and are net positively charged, both of which are characteristics of plastid transit peptides (von Heijne and Nishikawa, 1991). Similar alignment of the rapeseed and soybean homologs to the *Synechocystis* ω-6 desaturase shows similarities of 48 and 52%, respectively, whereas the similarity to both plastid and microsomal linoleate desaturases is less than 27%. These lines of evidence indicated that the new sequence might be a plastid ω-6 desaturase.

The cyanobacterium *Synechococcus* sp. strain PCC7942 is a good organism in which to test for the biological function of an ω-6 desaturase, since it lacks all polyunsaturates and has previously been used to demonstrate expression of a cyanobacterial ω-6 desaturase (Wada et al., 1990). Expression of the rapeseed desaturase in *Synechococcus* resulted in the production of two new fatty acid species. These were identified as 16:2 and 18:2, confirming the sequence to be a diene-producing desaturase. Although both 16- and 18-carbon fatty acids acted as substrates for the higher plant enzyme, 18:1 is apparently the preferred substrate. Depending on growth temperature, between 50% and nearly all of the available 18:1(9c) was converted to 18:2 (Table I). In contrast, conversion of 16:1 to 16:2 was slightly temperature dependent, with greater conversion at lower growth temperatures. Conversion was much less complete as well, ranging from 11% of the apparently available 16:1 at 37°C to about 23% at 25°C. Similar ratios of conversion were observed when *Synechococcus* was transformed with *DES A* (Wada et al., 1990).

In higher plants, the double bond in 16:1 is at position 7 and the 16-carbon di- and triene fatty acids are 16:2(7,10) and 16:3(7,10,13) based on our analysis of dimethyl disulfide derivatives and that of Nichols (1965). In cyanobacterium, the bond in 16:1 is at position 9 (Higashi and Murata, 1993; our data not shown). These results indicate that the plastid

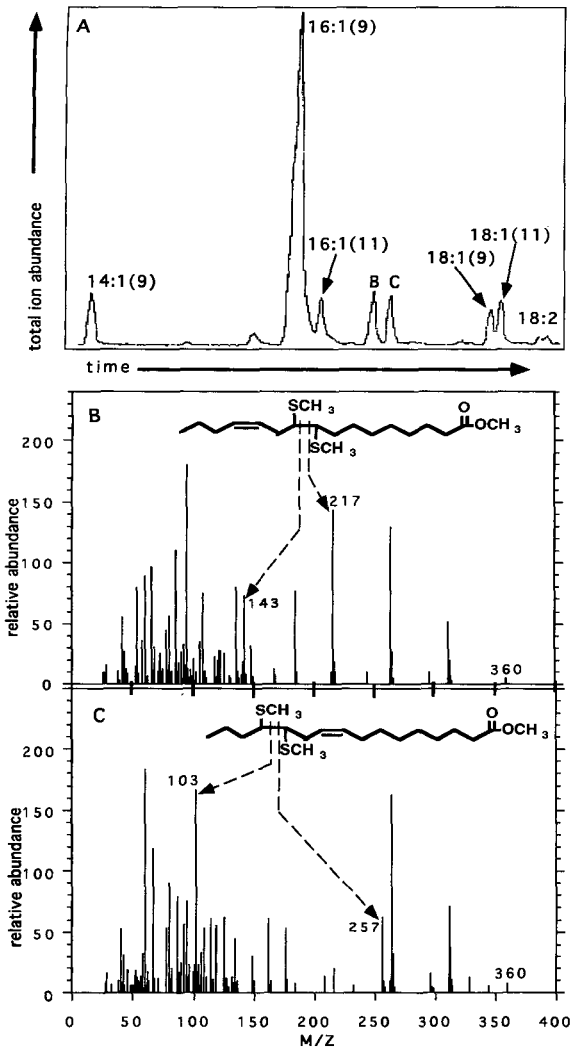

Figure 2. The total ion trace and mass spectra of the methyl ester/dimethyl disulfide derivatves of the unsaturated fatty acids produced by *Synechococcus* transformant C3. A, Total ion trace through the region of the chromatogram in which the derivatized unsaturated fatty acids elute. Monoene fatty acids were identified by their mass spectra and are identified on the chromatogram. B is the spectrum of peak B and its interpretation. The molecular ion at mass 360 and the prominent fragments at 217 and 143 are consistent with the fragmentation pattern (shown) expected from the dimethyl disulfide adduct of the ω-7 double bond in 16:2(9,12). C is the spectrum of peak C and its interpretation. The molecular ion at mass 360 and the fragments at 103 and 257 are consistent with the fragmentation pattern (shown) expected from the dimethyl disulfide adduct of the ω-4 double bond in 16:2(9,12). The two small peaks labeled 18:2 (A) are the analogous 18-carbon compounds.

ω-6 desaturase from higher plants can utilize at least three substrates: 18:1(9c), 16:1(7c), and 16:1(9c). This observation is similar to that made in studies on the desaturation of fatty acids supplied to *Chlorella vulgaris* (Gurr, 1971; Howling et al., 1972) except that those authors suggested the existence of two desaturases, one for the 9c substrates and one for the 7c substrate.

The main product arising from desaturation of 16:1(9c) in transformant C3 appears to be the 16:2(9,12) isomer and is thus an ω-4 desaturation product rather than an ω-6 desaturation product. We cannot rule out the production of small amounts of the 16:2(9,10) isomer, which would be the product of ω-6 desaturation; however, the separations of both the fatty acid methyl esters and of their dimethyl disulfide adducts is reasonably good between positional isomers [i.e. 16:1(9) and 16:1(11)]. Thus, the production of other 16:2 isomers must be small in comparison with that of 16:2(9,12). The production of the ω-4 product when 16:1(9c) is the available substrate suggests that the enzyme introduces the second double bond in a methylene-interrupted configuration measuring from the first double bond rather than from the methyl end of the fatty acid. The same pattern of activity was observed when *Synechococcus* was transformed with *Des A*. Desaturation at ω-6 was observed when 18:1(9) was the substrate, and ω-4 desaturation was observed when 16:1(9) was the substrate (Murata et al., 1990). As stated by Higashi and Murata (1993) for the cyanobacterial enzyme, the higher-plant enzyme is also not an ω-6 desaturase. Only when the enzyme acts on its normal, 16:1(7c) or 18:1(9c) substrates does it behave as an ω-6 desaturase.

Although the results obtained from the transformation of *Synechococcus* do not definitively prove that *B. napus* cDNA is the product of *FAD 6*, they do prove that it catalyzes the same reaction. The suggestion from deduced amino acid sequence and sequence alignment that the cDNA contains a transit peptide indicates that it is probably expressed in the same cellular compartment as the *FAD 6* gene product.

The evolutionary relationship of the fatty acid desaturases from soybean and rapeseed and of the cyanobacterial ω-6 desaturase as determined by the Hein method (Hein, 1990) is shown in Figure 3. One striking feature is the high sequence similarity of the *FAD 3* and *FAD 7* peptides compared with the widely differing putative *FAD 6* and *FAD 2* peptides, such that there are three distinct families of enzymes. One interpretation of the ontogeny of the higher-plant fatty acid desaturases concerns the plastid and cytoplasmic progenitors of the higher plant cell. Only one was competent to produce triene fatty acids, whereas both could produce diene fatty acids. One of the ω-3 desaturases may have evolved postsymbiosis from the gene expressed in the other compartment, whereas the two distantly related diene-producing enzymes remain in their original compartments.

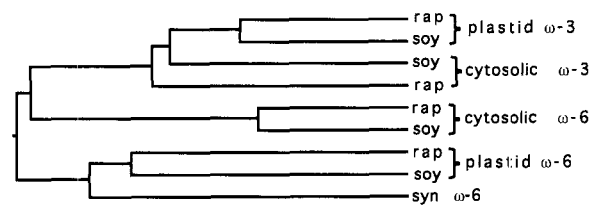

Figure 3. Phylogenetic relations among the derived amino acid sequences of the higher-plant polyene fatty acid desaturases and the ω-6 fatty acid desaturase of *Synecocystis*. rap, soy, and syn are the abbreviations for rapeseed, soybean, and *Synecocystis*, respectively. Comparison was by the Hein method (Hein, 1990) with the relative divergence indicated by the bracket length.

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