Cloning of Higher Plant ω-3 Fatty Acid Desaturases


Arabidopsis thaliana T-DNA transformants were screened for mutations affecting seed fatty acid composition. A mutant line was found with reduced levels of linolenic acid (18:3) due to a T-DNA insertion. Genomic DNA flanking the T-DNA insertion was used to obtain an Arabidopsis cDNA that encodes a polypeptide identified as a microsomal ω-3 fatty acid desaturase by its complementation of the mutation. Analysis of lipid content in transgenic tissues demonstrated that this enzyme is limiting for 18:3 production in Arabidopsis seeds and carrot hairy roots. This cDNA was used to isolate a related Arabidopsis cDNA, whose mRNA is accumulated to a much higher level in leaf tissue relative to root tissue. This related cDNA encodes a protein that is a homolog of the microsomal desaturase but has an N-terminal extension deduced to be a transit peptide, and its gene maps to a position consistent with that of the Arabidopsis fad D locus, which controls plastid ω-3 desaturation. These Arabidopsis cDNAs were used as hybridization probes to isolate cDNAs encoding homologous proteins from developing seeds of soybean and rapeseed. The high degree of sequence similarity between these sequences suggests that the ω-3 desaturases use a common enzyme mechanism.

The ω-6 and ω-3 fatty acid desaturases introduce the second and the third double bonds, respectively, in the biosynthesis of 18:2 and 18:3 fatty acids, which are important constituents of plant membranes. (The ω-3 and ω-6 designations refer to positions of the double bond from the methyl end of fatty acids.) They are also commercially important because the oxidative stability and nutritional value of seed oils is affected by the levels of these fatty acids. In leaf tissue, there are two distinct pathways for polyunsaturated fatty acid biosynthesis, one located in the microsomes and the other located in the plastid membranes. In nongreen tissues and developing seeds, the microsomal pathway predominates. In Arabidopsis thaliana, the microsomal ω-6 and ω-3 fatty acid desaturases are controlled by the fad 2 and fad 3 loci, respectively (Lemieux et al., 1990), and the plastid ω-6 and ω-3 fatty acid desaturations are controlled by the fad C and fad D loci, respectively (Browse and Somerville, 1991). It has been postulated that these loci correspond to structural genes for the desaturase enzymes, which have been recalcitrant to purification and study. Indeed, a cDNA encoding a Brassica napus microsomal ω-3 desaturase was recently cloned by homology to a fragment of Arabidopsis genomic DNA isolated by map-based cloning of the fad 3 locus (Arondel et al., 1992).

Genetic approaches for cloning plant genes encoding biochemically intractable products, such as membrane-associated desaturases, are becoming increasingly more refined and powerful, especially in studies that depend on the small, well-characterized genome of A. thaliana. In addition to map-based cloning, these methods include transposon tagging (Balcells et al., 1991) and T-DNA tagging (Feldmann, 1991; Walden et al., 1991). The T-DNA tagging method, in which insertion of the T-DNA of Agrobacterium tumefaciens into a gene of interest via transformation provides both the mutant phenotype and the means with which to clone the mutant allele, has been most successful to date.

We report here the isolation of the Arabidopsis microsomal ω-3 fatty acid desaturase gene by T-DNA tagging and the subsequent use of its cognate cDNA to manipulate the levels of polyunsaturated fatty acids in transgenic plant tissues as well as to isolate cDNAs from Arabidopsis, soybean, and rapeseed that encode homologs of the microsomal ω-3 desaturase, including putative plastid ω-3 desaturases.

MATERIALS AND METHODS

Screening of an Arabidopsis T-DNA Mutant Population

About 100 T<sub>2</sub> seeds (2 mg) of each of 6000 members of a population of T-DNA transformed lines of Arabidopsis thaliana (ecotype Wassilewskija) (Feldmann and Marks, 1987)

Abbreviations: cM, centimorgan; NOS, nopaline synthase gene; NPTII, neomycin phosphotransferase II; PCR, polymerase chain reaction; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:1, eicosonic acid.
were pooled and the fatty acid content of each of the 6000 pooled samples was determined (Browse et al., 1986).

Nucleic Acid Hybridizations

Unless otherwise noted, nucleic acid hybridizations, including Southern blots, were carried out in 6X SSC, 1% (w/v) SDS, 5% (w/v) dextran sulfate, 10X Denhardt's solution, and 100 μg/mL of denatured salmon sperm DNA at 65°C overnight. They were then washed twice for 10 min in 2X SSC, 0.1% (w/v) SDS, and then for 10 min in 0.2X SSC, 0.1% (w/v) SDS at 65°C. 32P-labeled DNA probes for all hybridizations in this work were prepared by the random primer labeling method (Feinberg and Vogelstein, 1983).

Phage Libraries

Libraries of genomic DNA from wild-type A. thaliana (ecotype Wassilewskija) and mutant line 3707 (see "Results") were made by cloning size-fractionated Sau3A partial fragments into BamHI-digested λ Gem-1 phage DNA (Promega). The Arabidopsis cDNA library was made to poly(A)+ RNA isolated from developing soybean seeds (Grimes et al., 1992). The soybean (Glycine max) cDNA library was made to poly(A)+ RNA isolated from developing soybean seeds (Grimes et al., 1992). The rapeseed (Brassica napus) cDNA library was made to poly(A)+ RNA isolated from developing rapeseed seeds (Kieber et al., 1993). The soybean (Glycine max) cDNA library was made to poly(A)+ RNA isolated from developing soybean seeds (Grimes et al., 1992). The rapeseed (Brassica napus) cDNA library was made to poly(A)+ RNA isolated from developing rapeseed seeds (Kieber et al., 1993).

Isolation of T-DNA-Plant DNA Junction Fragments

The left T-DNA-plant DNA junction fragments were isolated from Solt- or BamHI-digested genomic DNA from mutant line 3707 (see "Results") by the method of plasmid rescue (Behringer and Medford, 1992). The right T-DNA-plant DNA junction fragment was isolated by screening a 3700 genomic DNA library with a 32P-labeled 0.6-kb PCR product derived from wild-type Arabidopsis DNA at a region between the site of T-DNA insertion and the region hybridizing to CF3 cDNA (see "Results") (Fig. 1). Southern analysis of phage DNA from a pure, positively hybridizing plaque showed that a 4-kb EcoRI fragment hybridized to the PCR probe. This right junction EcoRI fragment was subcloned into pBluescript SK II vector (Stratagene). Nucleotide sequences of the double-stranded plasmids containing the junction fragments were determined using the Sequenase kit (United States Biochemical).

Isolation of the Wild-Type Arabidopsis ω-3 Desaturase Gene and cDNA

The wild-type Arabidopsis genomic DNA library was screened with a 32P-labeled 1.4-kb EcoRI-BamHI fragment from the left junction fragment (see "Results"). Filters were hybridized overnight at 65°C in 1% (w/v) BSA, 0.5 m NaPi (NaH2PO4 and Na2HPO4), pH 7.2, 10 mM EDTA, and 7% (w/v) SDS and rinsed twice with 0.1X SSC, 1% (w/v) SDS at 65°C for 30 min each. Seven positively hybridizing plaques were purified. Southern analysis of phage DNA from several pure plaques showed that a 5.2-kb HindIII fragment hybridized to the probe. This fragment was isolated and subcloned in pBluescript SK II vector.

The Arabidopsis cDNA library was screened with a 32P-labeled 5.2-kb HindIII genomic DNA fragment in 1 m NaCl, 50 mM Tris-HCl, pH 7.5, 1% (w/v) SDS, 5% (w/v) dextran sulfate, 0.1 mg/mL of denatured salmon sperm DNA at 65°C. Filters were washed twice with 2X SSPE, 0.1% (w/v) SDS at room temperature for 5 min, and finally once with 0.5X SSPE, 0.1% (w/v) SDS at 65°C for 5 min. Positively hybridizing plaques were purified, and their plasmids were excised according to the protocol described in the pBluescript II Phagemid Kit (Stratagene) and subjected to sequencing as described above.

Low-Stringency Screening of Arabidopsis, Soybean, and Rapeseed cDNA Libraries

The Arabidopsis library was screened with 32P-labeled Arabidopsis CF3 cDNA (see "Results"). The soybean library was screened with 32P-labeled CF3 cDNA and a 1.0-kb HhaI fragment from soybean GM3 cDNA (see "Results"). The libraries were screened under low-stringency hybridization conditions in 1 m NaCl, 50 mM Tris-HCl, pH 7.5, 1% (w/v) SDS, 5% (w/v) dextran sulfate, 0.1 mg/mL of denatured salmon sperm DNA at 50°C. Filters were washed twice with 2X SSPE, 0.1% (w/v) SDS at room temperature for 5 min, and finally once with 0.5X SSPE, 0.1% (w/v) SDS at 50°C for 5 min. The rapeseed cDNA library was screened at low stringency with 32P-labeled Arabidopsis CF3 and CFD cDNAs (see "Results"). Filters were hybridized overnight in 50 mM Tris, pH 7.6, 6X SSC, 5X Denhardt's solution, 0.5% (w/v) SDS, 100 μg denatured calf thymus DNA at 50°C and then washed sequentially in 6X SSC, 0.5% (w/v) SDS at room temperature for 15 min; in 2X SSC, 0.5% (w/v) SDS at 45°C for 30 min; and twice with 0.2X SSC, 0.5% (w/v) SDS at 50°C for 30 min. In all cases, several positively hybridizing phases were purified and their cDNA inserts were characterized by nucleotide sequencing, following excision, as described above.

Sequence Analysis

The percent identity between and the multiple sequence alignment of the different deduced amino acid sequences were generated by Gap (by the method of Needleman and Wunsch, 1970) and Lineup, respectively, in the GCG Sequence Analysis Software Package (Genetics Computer, Inc.). These were performed using gap weight and gap length weight values of 3.0 and 0.1, respectively. The phylogenetic tree was generated by the Hein (1990) algorithm using LaserGene Software (DNASTar, Inc.) on an Apple Macintosh computer.
Northern Analyses

The following *Arabidopsis* tissues were harvested and frozen in liquid nitrogen: expanding leaves from the rosette stage of wild-type and line 3707 *Arabidopsis* plants grown side by side in soil in the greenhouse, 2-week-old whole wild-type *Arabidopsis* seedlings (with roots) grown in coarse sand, and in vitro cultured roots (Russell et al., 1992). RNA was isolated as described in Rerie et al. (1991). Poly(A)+ mRNA was isolated using the PolyATtract mRNA isolation system (Promega Corp.). RNA was fractionated on 1.2% Tris-HCl, pH 8.0, 5× Denhardt’s solution, 1.0% (w/v) SDS, 100 μg/mL of salmon sperm DNA, and were washed at 65°C to a stringency of 0.1× SSC. The actin probe, which served to normalize mRNA loadings, was obtained from RNA by reverse transcription and PCR amplification with primers corresponding to nucleotides 938 to 957 and nucleotides 1506 to 1524 of the AAc1 gene (Nairn et al., 1988).

Plant Transformations

For constitutive expression in plants, the 1.4-kb CF3 cDNA (see “Results”) was ligated into the sense orientation between the 35S promoter and the 3′ region of the NOS (Russell et al., 1992) (see Fig. 4). For seed-specific expression, the cDNA insert was cloned in sense orientation between the promoter for the α-subunit of P-conglycinin (Doyle et al., 1990) (see Fig. 4) in plasmid pAW31. Plasmids pAW50 and pAW31 (35S: CF3 cDNA). The plant-selectable markers were the 35S promoter and the 3′ region of the NOS (Russell et al., 1992) to flanking the site of T-DNA insertion. Plant DNA junction fragments from line 3707 were recovered as A. tumefaciens strain LBA4404 (Hoekema et al., 1983) and A. tumefaciens strain R1000 (a C58 strain carrying an Ri-plasmid) (Moore et al., 1979), respectively, by the freeze/thaw method (Holsters et al., 1978).

Complementation of the mutation in line 3707 was carried out by transformation of root explants of line 3707 homozygous for the mutation by *Agrobacterium* strain LBA4404/pAW50 (Russell et al., 1992). Primary transformants (R1) were selected on chlorsulfuron and transferred to individual containers as previously described (Russell et al., 1992). Individual R2 seeds from two independent transgenic plants were analyzed for fatty acid composition.

Carrot (*Daucus carota* L.) cells were transformed by cocultivation of carrot root discs with *Agrobacterium rhizogenes* strains R1000 or R1000/pAW31 (Petit et al., 1986). Inoculated discs were incubated for 2 weeks at 25°C on an agar-solidified water medium and then transferred to a medium containing 500 mg/L of kanamycin sulfate. Hairy roots that formed on the cut surfaces were excised and individually maintained on Murashige and Skoog minimal organics medium (Gibco) with 30 g/L of Suc and 500 mg/L of carbenicillin, with or without 50 μg/mL of kanamycin sulfate, and sampled for fatty acid composition.

**RESULTS**

**An *Arabidopsis* Mutant Defective in ω-3 Desaturation Due to T-DNA Insertion**

Since the T3 seeds of T-DNA-transformed lines are segregating for the T-DNA insert, we combined approximately 100 T3 seeds (2 mg) of each of 6000 members of the population of T-DNA-transformed lines of *A. thaliana* and determined the fatty acid content of each of the 6000 pooled samples (Browse et al., 1986). Based on our knowledge of chemically induced lipid mutants (James and Dooner, 1990; Lemieux et al., 1990), we expected to be able to identify mutants in a segregating line using this approach. Three lines with reduced 18:3 were identified, and each of these was shown to be segregating for the mutant phenotype. The first of these lines identified (line 3707) produced homoygous seeds that contained 3.1% 18:3 (Table I), suggesting that the mutation in this line is “leaky” compared with the previously described *fad* 3–2 mutant, which contains 1.9% 18.3 (James and Dooner, 1990). Individual plants of line 3707 were selfed, and 262 of the resultant T1 progeny were grown and assayed for the presence of nopaline in leaf extracts (Errampalli et al., 1991). In addition, T2 seeds from each of the T1 plants were analyzed in bulk for fatty acid composition and for their ability to germinate in the presence of kanamycin (Feldmann et al., 1989). The analysis showed that the T1 progeny of line 3707 fell into three classes (Table II), indicating that a single T-DNA insertion conditioned the low 18.3 phenotype in line 3707. The co-segregation of the mutant phenotype and T-DNA markers indicates with 95% certainty that the T-DNA and the mutation are no further than 1.2 cM apart. On the basis of this estimation of linkage, we proceeded to isolate plant DNA flanking the site of T-DNA insertion.

**Arabidopsis ω-3 Desaturase Gene and cDNA**

Since the modified T-DNA we used contains the origin of replication and the ampicillin resistance gene of plasmid pBR322 (Feldmann and Marks, 1987), the left T-DNA-plant DNA junction fragments from line 3707 were recovered as plasmids in *Escherichia coli* by the method of plasmid rescue.
Yadav et al. tested for the presence of nopaline, and their selfed, progeny seeds (T3) were tested for the mutant fatty acid phenotype and kanamycin resistance.

<table>
<thead>
<tr>
<th>Number of T4 Individuals</th>
<th>Phenotype</th>
</tr>
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<tbody>
<tr>
<td>64</td>
<td>Nopaline absent in leaves; progeny (T3) seeds show wild-type fatty acid composition and are all kanamycin sensitive</td>
</tr>
<tr>
<td>134</td>
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(Behringer and Medford, 1992). For this, line 3707 genomic DNA was isolated, digested with either Sall or BamHI restriction enzyme, self-ligated, and used to transform E. coli cells. This resulted in the isolation of plasmids pS1 and pB1 from the Sall- and BamHI-digested DNAs, respectively. Restriction analysis of these plasmids showed that, in addition to the expected fragments of the T-DNA, pS1 contained a 2.9-kb EcoRI-Sall fragment and pB1 contained a 1.4-kb EcoRI-BamHI fragment (in each case, the EcoRI site being in the left T-DNA border), and that the 1.4-kb EcoRI-BamHI fragment in pB1 was contained within the 2.9-kb EcoRI-Sall fragment in pS1. Southern analysis using a radiolabeled 1.4-kb EcoRI-BamHI fragment as the hybridization probe showed that it hybridized to specific fragments of genomic DNA from both wild-type and line 3707 plants (data not shown). The nucleotide sequence of approximately 0.8 kb of the junction fragment starting from the EcoRI site in plasmid pS1 was determined; it was co-linear with the sequence of the T-DNA up to nucleotide position 65 in the left T-DNA border repeat (Yadav et al., 1982). The sequence beyond this point of divergence showed no significant identity to the T-DNA and revealed no extended open reading frame.

To isolate a cDNA corresponding to the site of T-DNA insertion, we isolated the corresponding genomic DNA from wild-type plants. For this we used the 32P-labeled 1.4-kb EcoRI-BamHI fragment as the hybridization probe to screen a phage library made to wild-type Arabidopsis genomic DNA. Southern analysis of DNA isolated from several positively hybridizing clones showed that a 5.2-kb HindIII fragment (Fig. 1) hybridized to the 1.4-kb EcoRI-BamHI fragment. The 5.2-kb HindIII fragment was isolated and used, in turn, as a probe to screen an Arabidopsis cDNA library. Several positively hybridizing plaques were purified and their plasmids were excised. One plasmid contained a 1.4-kb cDNA, designated CF3, which was shown by Southern analysis to hybridize to a region of wild-type Arabidopsis genomic DNA present to the right of the site of T-DNA insertion as shown in Figure 1.

The nucleotide sequence of CF3 cDNA revealed a large open reading frame (nucleotides 46–1206) that encodes a 386-amino acid polypeptide, designated A3 (Fig. 2). Comparison of the deduced amino acid sequence A3 to that of the polypeptide encoded by the structural gene (des A) for a cyanobacterium fatty acid desaturase (Wada et al., 1990) revealed an overall identity of 26% and higher identity over shorter stretches of amino acids. This strongly suggested that CF3 cDNA encoded Arabidopsis microsomal ω-3 fatty acid desaturase. Arabidopsis polypeptide A3 showed 93 and 68% overall identity to the subsequently published polypeptide sequences of rapeseed ω-3 fatty acid desaturase (Arondel et al., 1992) and a mung bean cDNA, ARG1, made to IAA-induced mRNA (Yamamoto et al., 1992), respectively. However, it showed overall identities of only 21 and 17% to the microsomal stearoyl-CoA desaturases from rat (Thiede et al., 1986) and yeast (Stukey et al., 1990), respectively. No significant homology was observed with the soluble stearoyl-acyl

Figure 1. A, Restriction map of the region of wild-type Arabidopsis DNA containing the region hybridizing to the entire CF3 cDNA (open bar). T-DNA marks the site corresponding to position of the T-DNA insertion in line 3707. B, Southern analysis of genomic DNA from wild-type (W) and 3707 (T) Arabidopsis plants using 32P-labeled CF3 cDNA as hybridization probe. The arrows show the novel, right junction fragments in line 3707 due to T-DNA insertion. Enzymes used are Sall (S), HindIII (H), and EcoRI (E). Lane M contains 1-kb ladder DNA size markers.

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Table II. Co-segregation of mutant fatty acid phenotype and T-DNA markers in line 3707

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Figure 2. Comparison of the deduced amino acid sequences of higher plant \( \omega-3 \) fatty acid desaturase homologs using standard one-letter amino acid codes. Identical and similar residues are shown on backgrounds of black and gray, respectively. Ap, Cp, Sp, A3, C3, and S3 refer to the deduced amino acid sequences encoded by Arabidopsis CFD cDNA, rapeseed BND cDNA, soybean GMD cDNA, Arabidopsis CF3 cDNA (Fad 3), rapeseed BN3 cDNA, and soybean GM3 cDNA, respectively.

Southern analysis of genomic DNA from wild-type and line 3707 plants using \( ^{32} \)P-labeled CF3 cDNA showed that the T-DNA integrated 5' to the desaturase coding sequence (Fig. 1). To accurately determine the site of T-DNA integration, the right T-DNA-plant DNA junction fragment was isolated by screening a library made to genomic DNA from line 3707 with \( ^{32} \)P-labeled CF3 cDNA. Comparison of the nucleotide sequence of the wild-type \( \omega-3 \) desaturase gene with those of the T-DNA-disrupted gene in the left and right junction fragments showed that the insertion in line 3707 resulted in a 56-bp deletion at the site of integration that occurred 612 bp 5' to the initiation codon for the desaturase coding sequence.

Northern analysis of poly(A)* RNA isolated from leaf tissues of wild-type Arabidopsis and line 3707 homozygous for the low 18:3 phenotype showed that, relative to the wild-type tissue, the mutant tissue contained about one-fifth to one-tenth the amount of an apparently full-length \( \omega-3 \) desaturase mRNA (Fig. 3). The above data, taken together with the leaky mutant phenotype in line 3707, suggest that the T-
DNA insertion altered the quantitative expression of the microsomal ω-3 fatty acid desaturase without physically interrupting its mRNA.

Overexpression of Arabidopsis ω-3 Fatty Acid Desaturase in Transgenic Tissues

To confirm the identity of the gene product encoded by CF3 cDNA, the complete cDNA was introduced in the sense orientation behind a seed-specific promoter into line 3707. Five of six R1 seeds from each of two independent transgenic plants tested showed more than a 10-fold increase in 18:3 level (Table III). The remaining seed from each transformant showed the mutant fatty acid phenotype.

CF3 cDNA was also introduced into carrot roots in the sense orientation behind the 35S promoter in binary vector pAW31 via the binary Ri plasmid transformation method. By this method, only a fraction (about half) of the hairy roots grown in the absence of kanamycin, 6 showed the mutant fatty acid phenotype.

CF3 cDNA was also introduced into carrot roots in the sense orientation behind the 35S promoter in binary vector pAW31 via the binary Ri plasmid transformation method. By this method, only a fraction (about half) of the hairy roots grown in the absence of kanamycin, 6 showed the mutant fatty acid phenotype.

An Arabidopsis cDNA Encoding a Homolog of the Microsomal ω-3 Desaturase

32P-labeled CF3 cDNA was used as a hybridization probe at low stringency to screen the Arabidopsis etiolated hypocotyl cDNA library (Kieber et al., 1993). Several weakly hybridizing plaques were purified and their plasmids were excised and partially sequenced. The nucleotide sequence of 1550 bp of the cDNA insert, designated CFD, in one plasmid revealed an open reading frame encoding a 446-amino acid polypeptide, designated Ap, with an estimated molecular mass of 51 kD. Alignment of polypeptide Ap sequence with that of the Arabidopsis microsomal ω-3 desaturase showed an overall 22, 23, and 25), one root of the intermediate class (number 36), one root with normal 18:3 (number 20), and one R1000 control root were tested for their ability to grow on kanamycin and for the presence of the chimeric gene in their genomic DNA. Roots 4, 19, 22, and 25 were kanamycin resistant, root 36 was weakly kanamycin resistant, and roots 20 and 23 and the R1000 control root were kanamycin sensitive. Southern analyses using 32P-labeled 35S:CF3 cDNA:3'NOS chimeric gene showed that all roots of the high and intermediate classes contained the chimeric gene, whereas the root with normal 18:3 (number 20) and the R1000 control root did not (Fig. 4). It is unclear if the intermediate content of 18:3 in root 36 is related to the reduced intensity of hybridization to the 1.4-kb fragment in this root. Root 23 has an apparent deletion of approximately 0.5 kb in the 4.4-kb HindIII fragment, and its kanamycin sensitivity suggests that the deletion is in the 35S:NPTII:3'NOS chimeric gene.

Table III. Fatty acid composition of seeds of line 3707 transformed with β-conglycinin promoter:CF3cDNA:3'NOS chimeric gene

Wild-type and mutant 3707 compositions are each an average of three individual seeds. Samples A-1 to A-6 are individual R1 seeds from one 3707 transformant, and samples B-1 to B-6 are individual R2 seeds from an independent 3707 transformant. The level of each fatty acid is shown as a percentage of all five.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percent Fatty Acid</th>
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<tbody>
<tr>
<td></td>
<td>16:0</td>
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<tr>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>0.20</td>
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<tr>
<td>Mutant 3707</td>
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</tr>
<tr>
<td>SE</td>
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<tr>
<td>A-1</td>
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<tr>
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tissue CF3 mRNA was much more abundant than CFD mRNA (Fig. 3).

CFD cDNA was hybridized to genomic DNA from A. thaliana (ecotype Wassileskija and marker line W100 ecotype Landesberg background) digested with EcoRI. A restriction fragment length polymorphism was identified and mapped as previously described (Reiter et al., 1992). A single genetic locus corresponding to this cDNA was positioned on the upper arm of chromosome 3 at a position 8 ± 2 cM proximal to cosmid c838 restriction fragment length polymorphism marker, 9 ± 2 cM distal to the AT228 marker, and 39 ± 7 cM distal to the glabrous (gl-1) locus (Reiter et al., 1992).

Soybean and Rapeseed cDNAs Encoding Homologs of Arabidopsis CF3 and CFD cDNAs

cDNA libraries representing the mRNA population of soybean and rapeseed cotyledons actively engaged in oil biosynthesis were screened at a low stringency for cDNAs related to Arabidopsis CF3 and CFD cDNAs. The rapeseed library was screened with 32P-labeled CF3 and CFD cDNAs. Several cross-hybridizing clones were purified in each case and subjected to nucleotide sequence analyses. Rapeseed cDNA, designated BN3, was 1336 bp and contained a large open reading frame that encodes a 377-amino acid polypeptide, designated C3. Rapeseed cDNA, designated BND, contained a 1416-bp sequence with an incomplete open reading frame that encodes a 404-amino acid polypeptide, designated Cp. Comparison of the amino acid sequences of rapeseed polypeptides C3 and Cp and Arabidopsis polypeptides A3 and Ap showed that C3 and Cp polypeptides are homologs of the Arabidopsis microsomal and putative plastid ω-3 desaturases, respectively. This conclusion is based on percent identity at the amino acid level (Table IV) and the presence or absence of an N-terminal amino acid sequence (Fig. 2), even though the deduced amino acid in BND cDNA contains only part of the putative transit peptide sequence.

The soybean cDNA library was screened with 32P-labeled CF3 cDNA and one of the purified clones was shown to contain a cDNA insert, designated GM3, with a large open reading frame that encodes a 380-amino acid polypeptide, designated S3. A 1-kb HhaI fragment of GM3 cDNA was isolated and used to rescree the soybean cDNA library at low stringency. This resulted in the isolation of a distinct cDNA, designated GMD, that contained an open reading frame that encodes a 454-amino acid polypeptide, designated Sp. Comparison of the soybean polypeptides S3 and Sp with rapeseed polypeptides C3 and Cp and Arabidopsis polypeptides A3 and Ap revealed that S3 and Sp polypeptides are homologs of the Arabidopsis microsomal and putative plastid ω-3 desaturases, respectively. This conclusion is based on percent identity at the amino acid level (Table IV) and the presence or absence of an N-terminal amino acid sequence (Fig. 2), even though the deduced amino acid in BND cDNA contains only part of the putative transit peptide sequence.

Table IV. Percent identity at the amino acid level between different higher plant ω-3 fatty acid desaturase homologs

<table>
<thead>
<tr>
<th></th>
<th>Ap</th>
<th>C3</th>
<th>Cp</th>
<th>S3</th>
<th>Sp</th>
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<tr>
<td>A3</td>
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<tr>
<td>S3</td>
<td>68</td>
<td>68</td>
<td>68</td>
<td>74</td>
<td>68</td>
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</table>

The nomenclature of the homologs is the same as in Figure 2.
tides A3 and Ap showed that S3 and Sp polypeptides are homologs of the Arabidopsis microsomal and putative plastid ω-3 desaturases, respectively. This conclusion is based largely on the presence or absence of an N-terminal amino acid sequence (Fig. 2) but also partly on the percent identity at the amino acid level (Table IV). The N-terminal sequence in the deduced amino acid sequence of GMD cDNA is authentic, because the open reading frame in GMD cDNA has an in-frame termination codon 5' to the initiation codon and its deduced amino acid sequence begins at the only Met residue in it. Since it also shares characteristics of transit peptides of nuclear-encoded chloroplast proteins, including the N-terminal dipeptide Met-Ala, we deduce that it is the transit peptide.

**DISCUSSION**

Although T-DNA tagging has been useful in cloning plant genes, the genes that have been isolated have been those that affect visible, easily scored multigenic traits (Feldmann, 1991). This report provides support for the utility of T-DNA tagging in cloning an arbitrary gene for which there is only a specific assay for gene function. Statistical analysis would suggest that, assuming random insertion of the T-DNA into the Arabidopsis genome (100 Mbp), screening the present population of approximately 12,000 Arabidopsis transformants containing approximately 16,000 T-DNA inserts (Feldmann, 1991) would provide a 30 to 40% chance of uncovering a mutant at an arbitrary locus of 2.5 to 3 kb. If, as some surmise (Walden et al., 1991), the T-DNA insertion is biased toward transcriptionally active regions of the genome, then the probability of uncovering active genes increases correspondingly.

The identification of the polypeptide encoded by CF3 cDNA as the microsomal ω-3 fatty acid desaturase is based on its complementation of the mutation in line 3707. Expression of the Arabidopsis enzyme in line 3707, under the control of a seed-specific promoter, resulted in a 12-fold increase in 18:3 content when compared with the untransformed mutant 3707, and a 2-fold increase in 18:3 content when compared with the wild-type control. Overexpression of the Arabidopsis enzyme in carrot hairy roots resulted in a more than 7-fold increase in the 18:3 content, and almost all endogenous 18:2 was converted to 18:3. Overexpression of the rappedese enzyme in wild-type Arabidopsis roots was previously reported to result in a 1.6-fold increase in 18:3 content (Arondel et al., 1992). Thus, the reaction catalyzed by ω-3 desaturase appears to be a rate-limiting step in the biosynthesis of 18:3 in Arabidopsis seeds as well as in Arabidopsis roots and carrot hairy roots. This observation is supported by genetic studies withfad 3 mutants that indicate gene dosage-dependence of thefad 3 phenotype (Lemieux et al., 1990). If, as seems likely, the ω-3 desaturase enzyme is also rate limiting in agronomically important oilseeds such as rapeseed or soybean, then the alteration of the 18:3 content in the triacylglycerols of these plants by transgenic approaches should prove practicable.

Mutants of Arabidopsis with specific alterations in membrane lipid composition have provided considerable information about the effects of lipid structure on membrane function (Somerville and Browse, 1991). However, these mutants have invariably exhibited decreases in unsaturation relative to wild-type plants. The isolation of the ω-3 desaturase gene and the demonstration that overexpression of its coding sequence can result in very high 18:3 content in transgenic plants will now enable the study of the physiology and cell biology of plants in which the levels of membrane unsaturation are higher than normal.

CFD cDNA, which was isolated using CF3 cDNA as a hybridization probe at low stringency, encodes polypeptide Ap, which is a structural homolog of the microsomal ω-3 fatty acid desaturase, but with an N-terminal extension (Fig. 2). This N-terminal sequence is deduced to be a transit peptide because it shares several characteristics of transit peptides of nuclear-encoded chloroplast proteins. These include a high content of hydroxylated residues, a low content of acidic residues, and the N-terminal dipeptide Met-Ala (de Boer and Weisbeek, 1991; von Heijji and Nishikawa, 1991). In addition, it is co-linear with, and shares limited homology to, the deduced transit peptide described for the soybean putative plastid ω-3 desaturase. mRNA corresponding to CFD cDNA accumulates at very high levels in leaf but not in root tissue (Fig. 3). Finally, CFD cDNA maps 39 ± 7 cm distal to the gl-1 locus. Two plastid fatty acid desaturase mutations, fad D and fad B, were mapped 40 ± 6 and 28 ± 6 cm, respectively, to the gl-1 locus (Hugly et al., 1991). Thus, the map position for the gene encoding CFD cDNA is consistent with that of the Arabidopsis fad D locus, which controls plastid ω-3 desaturation. Based on the above discussion, we postulate that the CFD cDNA is derived from the fad D locus and encodes the plastid ω-3 fatty acid desaturase. This conclusion will be confirmed by the biological expression of the CFD cDNA.

Rapeseed polypeptide C3 was identified as the microsomal ω-3 desaturase by its high (93%) identity at the amino acid level to Arabidopsis microsomal ω-3 desaturase. The rapeseed ω-3 desaturase reported in this study had a 96% amino acid sequence identity with the previously reported rapeseed ω-3 desaturase. It seems likely, therefore, that the two rapeseed polypeptides are isozymes. Soybean polypeptide S3 has 68% and 67% identity with Arabidopsis microsomal and putative plastid ω-3 desaturases, respectively. Since it lacks the N-terminal extension transit peptide, we postulate that it encodes the microsomal ω-3 desaturase.

Soybean polypeptide Sp contains an N-terminal extension deduced to be a transit peptide. The length of the deduced transit peptide in Sp is similar to that in Ap, the putative plastid ω-3 desaturase of Arabidopsis. Although there is little amino acid sequence identity with the Arabidopsis transit peptide, the extension has characteristics similar to those of transit peptides of nuclear-encoded chloroplast proteins. Thus, it is likely that soybean polypeptide Sp is a plastid ω-3 fatty acid desaturase. The rappedese BND cDNA encodes a polypeptide, Cp, that was identified to be the plastid ω-3 desaturase based on a 90% identity to the Arabidopsis putative plastid desaturase, but the rappedese cDNA is incomplete and encodes only a part of a putative transit peptide.

Our identification of the rappedese and soybean polypeptides is supported by the phylogenetic analysis based on Hein’s alignment algorithm (Hein, 1990). This algorithm
assumes that the sequences are related in some way and constructs a phylogeny based on evolutionary parsimony (Fig. 5). The analysis shows that the earliest divergence in ancestral relationships is between the group of sequences we have identified as microsomal ω-3 fatty acid desaturases and the group we have identified as putative plastid ω-3 fatty acid desaturases. Based on these homologies, it is also apparent that the previously unidentified mung bean cDNA (ARG1) (Yamamoto et al., 1992) encodes a mung bean microsomal ω-3 desaturase.

Microsomal ω-3 desaturases from Arabidopsis and rapeseed, both in this and the previously published report (Arondel et al., 1992), share the motif of two Lys residues positioned three and five residues from the C terminus that is believed to be sufficient for retention of transmembrane ER proteins (Jackson et al., 1990). This motif is absent from the putative plastid homologs from all three species. However, its significance is unclear because the soybean homolog S3 lacks it altogether, and the mung bean homolog (encoded by ARG1 cDNA) (Yamamoto et al., 1992) shows a Lys-Ser-Lys tripeptide at the C terminus. Additional soybean homologs of polypeptide S3 are being investigated.

Comparison of the deduced amino acid sequences of the different ω-3 desaturase homologs, of both the microsomal and the putative plastid types, shows that they have overall identities of 66% or greater at the amino acid levels (Table IV). It also shows that the percent identity between the microsomal and the putative plastid desaturases within each species is similar to that between the soybean and Arabidopsis microsomal (68%) or plastid (69%) homologs.

We were not successful in cloning the microsomal ω-6 fatty acid desaturase from the Arabidopsis cDNA library using 32P-labeled CF3 and CFD cDNAs as probes under low stringency hybridization conditions in which CF3 and CFD cDNAs cross-hybridize. This suggests that the microsomal ω-3 desaturase is more closely related to the putative plastid ω-3 desaturase than it is to the microsomal ω-6 desaturase. There is evidence that microsomal desaturations use phosphatidylcholine as the lipid substrate and Cyt b5 as the immediate electron donor (Smith et al., 1990), whereas plastid desaturations (Schmidt and Heinz, 1990) and cyanobacterial desaturations (Wada et al., 1993) use galactolipids as the lipid substrate and reduced Fd as the electron donor. Because microsomal and plastid desaturations use different lipid substrates and immediate electron donors, the high degree of similarity between the primary structures of the microsomal ω-3 desaturase and its putative plastid homolog suggests a conserved enzyme mechanism and common structural motifs that recognize the 18:2 fatty acyl moiety.

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LITERATURE CITED


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Figure 5. Phylogenetic tree of ω-3 desaturase homologs based on the gene conversion algorithm (Hein, 1990). The length of the branches is proportional to the evolutionary divergence. ARG1 is the auxin-induced mung bean cDNA (Yamamoto et al., 1992); other nomenclature is the same as in Figure 2.


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