The Plant Cell, Vol. 6, 147–158, January 1994 © 1994 American Society of Plant Physiologists

Arabidopsis *FAD2* Gene Encodes the Enzyme That Is Essential for Polyunsaturated Lipid Synthesis

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The polyunsaturated fatty acids linoleate and α-linolenate are important membrane components and are the essential fatty acids of human nutrition. The major enzyme responsible for the synthesis of these compounds is the plant oleate desaturase of the endoplasmic reticulum, and its activity is controlled in Arabidopsis by the *fatty acid desaturation 2* (*fad2*) locus. A *fad2* allele was identified in a population of Arabidopsis in which mutations had been created by T-DNA insertions. Genomic DNA flanking the T-DNA was cloned by plasmid rescue and used to isolate cDNA and genomic clones of *FAD2*. A cDNA containing the entire *FAD2* coding sequence was expressed in *fad2* mutant plants and shown to complement the mutant fatty acid phenotype. The deduced amino acid sequence from the cDNA showed homology to other plant desaturases, and this confirmed that *FAD2* is the structural gene for the desaturase. Gel blot analyses of *FAD2* mRNA levels showed that the gene is expressed throughout the plant and suggest that transcript levels are in excess of the amount needed to account for oleate desaturation. Sequence analysis identified histidine-rich motifs that could contribute to an iron binding site in the cytoplasmic domain of the protein. Such a position would facilitate interaction between the desaturase and cytochrome b_5, which is the direct source of electrons for the desaturation reaction, but would limit interaction of the active site with the fatty acyl substrate.

INTRODUCTION

The polyunsaturated fatty acids linoleate (Δ9, 12–18:2) and α-linolenate (Δ9, 12, 15–18:3) are synthesized by plants but not by most other higher eukaryotes. Both of these fatty acids are essential components of human nutrition, because in mammals they act as precursors not only of membrane lipids but also of families of signaling molecules including the prostaglandins, thromboxanes, and leukotrienes (Smith and Borgeat, 1985). In many higher plants, 18:2 and 18:3 account for more than 70% of the fatty acids in leaf cells and 55 to 70% of the fatty acids in nonphotosynthetic tissues such as roots (Harwood, 1980). In both plants and animals, polyunsaturated acyl structures are considered to be essential membrane components, in part because they are virtually ubiquitous in the membranes of higher eukaryotes. Experimental manipulations designed to reduce membrane polyunsaturation also point to a critical role for these structures in ensuring proper membrane function and organism viability (Hugly and Somerville, 1992; Miquel et al., 1993).

In angiosperms as a whole, the vast majority of polyunsaturated lipid synthesis passes through a single enzyme, the 18:1 desaturase of the endoplasmic reticulum. Although a chloroplast 18:1 desaturase also operates in leaf cells of at least some plants, it is likely that the endoplasmic reticulum enzyme is quantitatively more important even in these cells (Browse and Somerville, 1991; Miquel and Browse, 1992). Furthermore, it is responsible for more than 90% of the polyunsaturated fatty acid synthesis in nonphotosynthetic tissues, such as roots, and in the developing seeds of oil crops, including soybean, sunflower, and canola, in which fatty acids are stored as triacylglycerol oils. Thus, one important function of the endoplasmic reticulum 18:1 desaturase is to provide 18:2 and (following further desaturation) 18:3 required for the correct assembly of cellular membranes throughout the plant. Just as importantly, the enzyme provides the polyunsaturated fatty acids found in vegetable oils that in turn are the major source of essential fatty acids in most human diets.

Mutants of Arabidopsis at the *fatty acid desaturation 2* (*fad2*) locus are deficient in activity of the endoplasmic reticulum desaturase (Miquel and Browse, 1992). Biochemical and genetic studies of these mutants have been important to our understanding of this desaturation step because the enzyme, like most of the plant desaturases, is an integral membrane protein that has been difficult to solubilize and, therefore, to investigate by traditional enzymological methods. In the absence of a purified enzyme, genetic techniques can also

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provide an alternative means to clone the relevant genetic locus. For example, cDNAs corresponding to the *FAD3* gene have been obtained by both map-based cloning (Arondel et al., 1992) and by gene tagging (Yadav et al., 1993). The homology of these cDNAs to other desaturase genes indicates that they correspond to the structural gene encoding the endoplasmic reticulum 18:2 desaturase. The *FAD3* cDNAs have made possible the cloning of at least three distinct, additional desaturase genes. Each of these contained a 5′ peptide sequence with the characteristics of a chloroplast transit peptide and, on this basis, the genes are thought to encode three of the chloroplast desaturases (Iba et al., 1993; Yadav et al., 1993; W. Hitz, J. Okuley, N. Yadav, and J. Browse, unpublished data). Extensive efforts using the four available gene sequences have, to date, failed to isolate a gene encoding the endoplasmic reticulum 18:1 desaturase.

In this study, we describe the identification of a *fad2* allele in a population of Arabidopsis in which mutations have been generated by T-DNA insertion (Feldmann, 1991) and the subsequent cloning and characterization of the wild-type *FAD2* gene. The expansion of the family of genes encoding plant membrane desaturases now permits some discussion of a possible structure for the 18:1 desaturase.

RESULTS

Identification of a *fad2* Allele in a T-DNA Line

To initiate our screen, we grew the 1800 T-DNA insertional lines (T_3 generation) that were then available and directly measured the overall fatty acid composition of leaf tissues by gas chromatography (Browse et al., 1986b; Feldmann et al., 1989). Because the T-DNA–containing lines segregated for the insert (and thus for any resultant mutation), it was necessary for us to sample several individuals from each line. To simplify the procedure, we harvested single leaves from 10 plants of one line and pooled these for analysis. Based on reconstruction experiments using the chemically induced Arabidopsis lipid mutants, we expected to be able to successfully identify any one of seven mutants in a segregating population.

Among the pooled leaf samples analyzed in this way, one sample from line 658 was identified as having an increase in 18:1. The increase was considerably smaller than would be expected from the data on chemically induced *fad2* mutants (Miquel and Browse, 1992)—18:1 in the mutant pool was only 5.5% compared with 2.8% in neighboring, wild-type pools. In principle, this result might have arisen from underrepresentation of homozygous mutants within the small number of leaves that had been pooled for analysis. However, as shown in Table 1, analysis of tissue samples from individual plants of the 658 line demonstrated that whereas the line was segregating for 18:1 content, the most extreme phenotypes (homozygous for the lipid mutation) contained considerably lower proportions of 18:1 than previously described for *fad2* mutants (James and Dooner, 1990; Lemieux et al., 1990). Reciprocal crosses between homozygous individuals from line 658 and the *fad2-1* mutant (Lemieux et al., 1990) produced F_1 progeny with fatty acid compositions intermediate between those of the two parents. The lack of complementation in these crosses demonstrated that the lipid mutation in line 658 is an allele of *fad2*, which we designated *fad2-5*.

When 200 T_3 seeds of line 658 were germinated on agar containing 50 μg/mL kanamycin, only four kanamycin-sensitive individuals were identified. This proportion of kanamycin-sensitive seedlings is a good approximation to a 63:1 ratio and indicated to us that the line contained three unlinked T-DNA inserts. In this and two other experiments, a total of 56 kanamycin-sensitive seedlings were identified. Fifty-three of these were analyzed for fatty acid composition and at least seven displayed 18:1 levels that were higher than those observed in wild-type seedlings grown under these conditions.

To further test whether the *fad2-5* mutation might be the result of T-DNA insertion, we isolated a derivative line that segregated for a single locus for both kanamycin resistance

Table 1. Fatty Acid Compositions of Root, Leaf, and Seed Tissues of the Wild-Type, *fad2-5*, and *fad2-1* Mutant Arabidopsis Plants[a]

Fatty Acid	Root			Leaf			Seed		
	WT	*fad2-5*	*fad2-1*	WT	*fad2-5*	*fad2-1*	WT	*fad2-5*	*fad2-1*
16:0	24.7	12.7	14.0	13.7	10.0	13.9	10.2	8.5	8.6
16:1	1.2	1.3	2.3	2.4	4.1	2.2	–[b]	–	–
16:3	–	–	–	16.0	15.4	18.5	–	–	–
18:0	3.2	2.0	1.7	0.4	0.5	0.5	2.5	4.0	4.3
18:1	6.8	27.9	55.9	2.3	15.0	20.9	15.4	37.7	53.5
18:2	29.8	20.0	6.4	14.5	4.6	3.8	32.7	8.1	3.2
18:3	29.1	20.4	12.8	50.8	45.0	39.6	20.3	11.3	5.5
20:1	–	–	–	–	–	–	16.6	26.0	23.9

[a] Data for wild type (WT) and *fad2-1* are from Lemieux et al. (1990). Root tissue was harvested from plants grown in liquid medium (Miquel and Browse, 1992). Leaves from 17-day-old plants were analyzed. Seeds were sampled from mature siliques. Data are mol %.
[b] Dashes indicate that the fatty acid was not detected.

and the mutant fatty acid phenotype. Approximately 100 individual T_3 plants were grown to maturity, and seeds were collected. One sample of seed from each T_3 plant was tested for the ability to germinate and grow in the presence of kanamycin. In addition, the fatty acid compositions of 10 additional individual seeds from each line were determined. A T_3 plant, 658-75, was identified whose progeny seeds segregated 28 kanamycin sensitive to 60 kanamycin resistant and seven seeds with low or intermediate 18:1 (15 to 22%) to two with high 18:1 (35 to 38%). Approximately 400 T_4 progeny of the derivative line 658-75 were grown, and their leaf fatty acid compositions were determined. From these, 91 plants were identified as being homozygous for the *fad2-5* mutation. The remaining plants could not be definitively assigned to wild-type and heterozygous classes on the basis of leaf fatty acid composition and, thus, could not be used to test linkage between the T-DNA markers and the fatty acid phenotype. Eighty-three of the 91 homozygous plants were tested for the presence of nopaline, which is a second, easily scored marker of the T-DNA (Errampalli et al., 1991). All 83 plants were positive for the presence of nopaline. Thus, this experiment indicated cosegregation of the *fad2* locus with the T-DNA insert present in the 658-75. Therefore, we set out to isolate plant DNA flanking the site of T-DNA insertion.

Cloning a cDNA That Spans the Site of T-DNA Insertion

The modified T-DNA used to generate the mutant population contains the origin of replication and the ampicillin resistance gene of plasmid pBR322 (Feldmann and Marks, 1987). This feature permits the recovery of T-DNA–plant DNA junction fragments as plasmids in *Escherichia coli* by the method of plasmid rescue (Behringer and Medford, 1992). Genomic DNA from homozygous mutant segregants of the 658-75 line was digested to completion with either the BamHI or SalI restriction enzyme and allowed to religate at a dilute concentration to promote self-ligation. The ligation products were used to transform *E. coli* cells that were then subjected to ampicillin selection. No ampicillin-resistant colony was obtained from the experiment with SalI-digested DNA, but a single ampicillin-resistant colony was identified from the plasmid rescue of BamHI-digested DNA and designated pTF-658. Restriction analysis of pTF-658 with BamHI, SalI, and EcoRI restriction enzymes indicated that in addition to the expected 14.2-kb portion of the T-DNA, it contained a 1.6-kb EcoRI-BamHI fragment of putative plant DNA that would lie adjacent to the left T-DNA border (which contains an EcoRI site). This EcoRI-BamHI fragment was subcloned into a pBluescript SK– vector to yield plasmid pSI658.

When the 1.6-kb fragment was radiolabeled and used to probe gel blots of genomic DNA from wild-type Arabidopsis at high stringency, strongly hybridizing bands were observed, which confirmed that the flanking sequence was plant DNA. For DNA from plants of the segregating 658-75 line, the same bands were present, but, in addition, a set of distinct bands was visible (data not presented). This finding indicated that the 1.6-kb fragment is indeed part of the locus that is interrupted by the T-DNA. The 1.6-kb EcoRI-BamHI fragment was used as a radiolabeled hybridization probe to screen an Arabidopsis λYES cDNA library (Elledge et al., 1991). Four of the positively hybridizing plaques identified were subjected to plaque purification. Plasmids containing the cDNA sequences were then generated by utilizing the cre-lox recombination feature of the λYES library (Elledge et al., 1991). The four cDNAs ranged in length from 1 to 1.4 kb. Restriction enzyme mapping and partial sequence determination indicated that they all represent the same gene. Nucleotide sequencing of two of the cDNAs (pF2a and pF2b) produced the 1372-bp sequence shown in Figure 1A that contains an open reading frame that encodes a predicted protein of 383 amino acids.

A genomic clone corresponding to the cDNA was isolated by screening an Arabidopsis library using the insert from pF2b as a radiolabeled probe. The 6-kb HindIII insert from a purified, positively hybridizing plaque was subcloned into a pBluescript SK– vector. Sequencing of 3 kb of the genomic clone spanning the sequence shown in Figure 1A revealed the presence of a single intron of 1134 bp at a position between nucleotides 88 and 89 of the cDNA. Partial sequencing of the 1.6-kb EcoRI-BamHI genomic border fragment insert in pSI658 from the EcoRI end showed that, for the first 61 nucleotides, it is colinear with the left T-DNA border (except for a deletion of nine contiguous nucleotides at position 42 in the border fragment) and colinear from nucleotides 57 to 104 with that of nucleotides 41 to 88 of the cDNA. This result suggests that the left end of the T-DNA disrupted the gene within a 4-bp (5′-TGTT-3′) region of homology between the T-DNA border and host DNA and shows that the T-DNA disrupted the gene in the transcribed region and 5′ to the intron in the untranslated sequence. The structure of the *fad2-5* locus together with the cloned flanking sequence and three of the cDNA inserts are shown diagrammatically in Figure 1B.

DNA from a genomic phage clone was also used to detect and map a restriction fragment length polymorphism in a gel blot containing HindIII-digested genomic DNA from inbred lines derived from a cross between *Arabidopsis thaliana* (ecotype Wassileskija) and *A. thaliana* marker line W100 (ecotype Landsberg *erecta* background) essentially as described previously by Reiter et al. (1992). A single genetic locus corresponding to this gene was positioned on the upper arm of chromosome 3 between the cosmid c3838 and λAT228 restriction fragment length polymorphism markers and just 4.5 centimorgans proximal to the *fad7* locus previously mapped in the same population (Yadav et al., 1993).

Complementation of the *fad2-1* Mutant

To establish definitively that the cloned gene represents the *fad2* locus, we transformed the cDNA into *fad2-1* mutant plants. The 1.4-kb EcoRI fragment containing the cDNA was isolated

A

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                                                AGAGAGAGATTCTGCGGAGGAGCTTCTTCT   32
TCGTAGGGTGTTCATCGTTATTAACGTTATCGCCCCCTACGTCAGCTCCATCTCCAGAAAC   92

ATGGGTGCAGGTGGAAGAATGCCGGTTCCTACTTCTTCCAAGAAATCGGAAACCGACACC  152
 1 M  G  A  G  G  R  M  P  V  P  T  S  S  K  K  S  E  T  D  T

ACAAAGCGTGTGCCGTGCGAGAAACCGCCTTTCTCGGTGGGAGATCTGAAGAAAGCAATC  212
21 T  K  R  V  P  C  E  K  P  P  F  S  V  G  D  L  K  K  A  I

CCGCCGCATTGTTTCAAACGCTCAATCCCTCGCTCTTTCTCCTACCTTATCAGTGACATC  272
41 P  P  H  C  F  K  R  S  I  P  R  S  F  S  Y  L  I  S  D  I

ATTATAGCCTCATGCTTCTACTACGTCGCCACCAATTACTTCTCTCTCCTCCCTCAGCCT  332
61 I  I  A  S  C  F  Y  Y  V  A  T  N  Y  F  S  L  L  P  Q  P

CTCTCTTACTTGGCTTGGCCACTCTATTGGGCCTGTCAAGGCTGTGTCCTAACTGGTATC  392
81 L  S  Y  L  A  W  P  L  Y  W  A  C  Q  G  C  V  L  T  G  I

TGGGTCATAGCCCACGAATGCGGTCACCACGCATTCAGCGACTACCAATGGCTGGATGAC  452
101 W  V  I  A  H  E  C  G  H  H  A  F  S  D  Y  Q  W  L  D  D

ACAGTTGGTCTTATCTTCCATTCCTTCCTCCTCGTCCCTTACTTCTCCTGGAAGTATAGT  512
121 T  V  G  L  I  F  H  S  F  L  L  V  P  Y  F  S  W  K  Y  S

CATCGCCGTCACCATTCCAACACTGGATCCCTCGAAAGAGATGAAGTATTTGTCCCAAAG  572
141 H  R  R  H  H  S  N  T  G  S  L  E  R  D  E  V  F  V  P  K

CAGAAATCAGCAATCAAGTGGTACGGGAAATACCTCAACAACCCTCTTGGACGCATCATG  632
161 Q  K  S  A  I  K  W  Y  G  K  Y  L  N  N  P  L  G  R  I  M

ATGTTAACCGTCCAGTTTGTCCTCGGGTGGCCCTTGTACTTAGCCTTTAACGTCTCTGGC  692
181 M  L  T  V  Q  F  V  L  G  W  P  L  Y  L  A  F  N  V  S  G

AGACCGTATGACGGGTTCGCTTGCCATTTCTTCCCCAACGCTCCCATCTACAATGACCGA  752
201 R  P  Y  D  G  F  A  C  H  F  F  P  N  A  P  I  Y  N  D  R

GAACGCCTCCAGATATACCTCTCTGATGCGGGTATTCTAGCCGTCTGTTTTGGTCTTTAC  812
221 E  R  L  Q  I  Y  L  S  D  A  G  I  L  A  V  C  F  G  L  Y

CGTTACGCTGCTGCACAAGGGATGGCCTCGATGATCTGCCTCTACGGAGTACCGCTTCTG  872
241 R  Y  A  A  A  Q  G  M  A  S  M  I  C  L  Y  G  V  P  L  L

ATAGTGAATGCGTTCCTCGTCTTGATCACTTACTTGCAGCACACTCATCCCTCGTTGCCT  932
261 I  V  N  A  F  L  V  L  I  T  Y  L  Q  H  T  H  P  S  L  P

CACTACGATTCATCAGAGTGGGACTGGCTCAGGGGAGCTTTGGCTACCGTAGACAGAGAC  992
281 H  Y  D  S  S  E  W  D  W  L  R  G  A  L  A  T  V  D  R  D

TACGGAATCTTGAACAAGGTGTTCCACAACATTACAGACACACACGTGGCTCATCACCTG 1052
301 Y  G  I  L  N  K  V  F  H  N  I  T  D  T  H  V  A  H  H  L

TTCTCGACAATGCCGCATTATAACGCAATGGAAGCTACAAAGGCGATAAAGCCAATTCTG 1112
321 F  S  T  M  P  H  Y  N  A  M  E  A  T  K  A  I  K  P  I  L

GGAGACTATTACCAGTTCGATGGAACACCGTGGTATGTAGCGATGTATAGGGAGGCAAAG 1172
341 G  D  Y  Y  Q  F  D  G  T  P  W  Y  V  A  M  Y  R  E  A  K

GAGTGTATCTATGTAGAACCGGACAGGGAAGGTGACAAGAAAGGTGTGTACTGGTACAAC 1232
361 E  C  I  Y  V  E  P  D  R  E  G  D  K  K  G  V  Y  W  Y  N

AATAAGTTATGAGCATGATGGTGAAGAAATTGTCGACCTTTCTCTTGTCTGTTTGTCTTT 1292
381 N  K  L

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TTTTGGCTGCTCATTATGTT                                           1372
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B

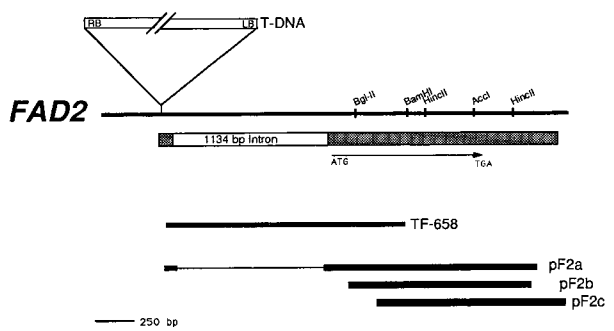

Figure 1. Structure and Organization of the Arabidopsis *FAD2* Gene.

(A) Nucleotide and derived amino acid sequences of a *FAD2* cDNA. The 4-bp sequence that is homologous to a sequence in the T-DNA border and into which T-DNA insertion appears to have occurred is shown in boldface letters. The arrowhead indicates the site of the intron found in the genomic sequence. The three histidine-rich sequences that show homology to other membrane-bound desaturases are underlined. The GenBank accession number of the sequence is L26296.

(B) The genome structure at the *fad2-5* locus showing the T-DNA insert, intron, open reading frame, and partial restriction map. The approximate lengths and locations of the 1.6-kb flanking sequence from the plasmid rescue (TF-658) and three cDNAs (pF2a, pF2b, and pF2c) are shown in the lower part of the figure. RB, right border; LB, left border.

from plasmid pF2a and subcloned into the EcoRI site of the binary vector pGA748. One of the resulting plasmids, designated pGA-Fad2, contained the cDNA in the sense orientation behind the cauliflower mosaic virus 35S promoter. The plasmid pGA-Fad2 was transformed into Agrobacterium strain R1000 carrying the Ri plasmid to generate transformants R1000/pGA-Fad2. These transformants and R1000 cells transformed with pGA748 alone were used to transform *fad2-1* mutant Arabidopsis using a stem-transformation protocol (Arondel et al., 1992). Wild-type Arabidopsis was also transformed with the empty vector control.

Hairy roots emerging from explants were maintained for 4 days on medium containing cefotaxime and carbenicillin for counterselection of the Agrobacterium and then transferred to the same medium containing kanamycin to select roots that had been cotransformed with the kanamycin resistance gene from the pGA748 vector. Six days after this transfer, the 12 kanamycin-resistant root cultures from the experiment employing the pGA-Fad2 construct were sampled together with three cultures derived from *fad2-1* and one culture derived from wild-type Arabidopsis control transformations. The fatty acid composition of each sample was determined. Figure 2 shows the desaturase products, 18:2 and 18:3, plotted as a percentage of the total fatty acids in each sample. It is clear that the fatty acid phenotype in roots from *fad2-1* (11 to 21% 18:2 + 18:3) was very substantially complemented in all the kanamycin-resistant transformants with the exception of one (number 5), which showed a proportion of 18:2 + 18:3 that was only a little higher than the mutant controls. In some transformants, the proportion of 18:2 + 18:3 was slightly higher than that in the wild-type control.

Limited Transcript Levels in *fad2-5* Allow Considerable Desaturation

The comparison of the fatty acid compositions of roots, shoots, and seeds of *fad2-5* mutant plants with similar tissues from the *fad2-1* mutant in Table 1 demonstrates the leaky nature of the mutation found in the T-DNA line. If the proportions 18:2 plus 18:3 (the two products of the 18:1 desaturase) are used as an approximate, relative measure of desaturase activity, then *fad2-5* is estimated to have 53 ([40.4−19.2]/[58.9−19.2]), 52, and 24% of wild-type activity in roots, leaves, and seeds, respectively. We examined the steady state levels of *FAD2* RNA in wild-type, *fad2-5*, and *fad2-1* tissues. As shown in Figure 3, the level of *FAD2* RNA in both shoots and roots of *fad2-1*

was indistinguishable from that detected in corresponding wild-type tissues, which suggests that the *fad2-1* allele produces a mutant protein without affecting the size or stability of the mRNA. In contrast, the *fad2-5* plants showed greatly reduced levels of an RNA of a similar length to the wild-type molecule. However, this transcript was barely detectable in shoot tissue and was only at a slightly higher level in roots. Densitometry of the bands shown in Figure 3 suggested that the transcript level in roots of *fad2-5* plants is no more than 12 to 15% of the wild type. This very low transcript level was nevertheless able to effect more than half of the 18:1 desaturation attributable to the *FAD2* gene product in wild-type Arabidopsis roots. Because the bands shown in Figure 3 were the only regions of the gel blot to hybridize to the radioactive cDNA probe, it is probable that they represent the total level of transcript available for synthesis of the desaturase.

FAD2 Transcript Level Is Not Increased in Response to Chilling

An active *FAD2* gene product is essential for the survival of Arabidopsis at low temperatures (Miquel et al., 1993). Furthermore, observations in plants and other organisms suggest that the level of membrane lipid unsaturation increases with decreasing temperature, possibly as a regulated response to chilling treatment (Somerville and Browse, 1991; Thompson, 1993). To determine whether transcriptional regulation of *FAD2* occurs in Arabidopsis in response to chilling treatment, we transferred wild-type Arabidopsis plants from 22 to 6°C. At the time of transfer and at different times after transfer, samples of leaf material were harvested, and the level of *FAD2* transcript within the total RNA was determined by gel blot analysis. The data in Figure 4 indicate that the steady state level of *FAD2* transcript, relative to both the estimated amount of total RNA and the level of 28S RNA transcript, remained approximately constant for at least 3 days after transfer to the cold. Thus, transcriptional regulation of the *FAD2* gene apparently plays no role in acclimation of Arabidopsis to low temperatures. However, our results leave open the possibility that 18:1 desaturase activity could be regulated at either the translational or enzyme levels.

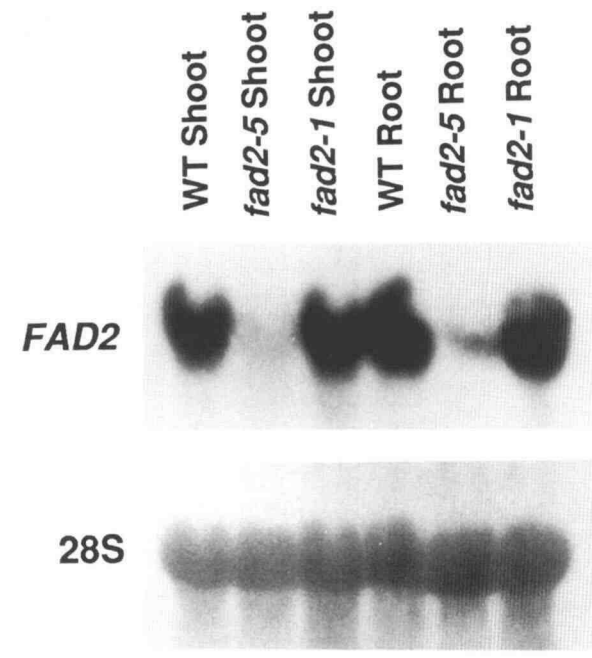

Figure 3. Transcript Levels in Wild-Type and *fad2* Mutant Arabidopsis.

Ten micrograms of total RNA per lane from shoots and roots of wild-type (WT), *fad2-5*, and *fad2-1* Arabidopsis plants was subjected to gel blot analysis using the pF2a insert as a probe (*FAD2*). Blots were stripped and reprobed with the Arabidopsis 28S rRNA gene (28S) as a loading control.

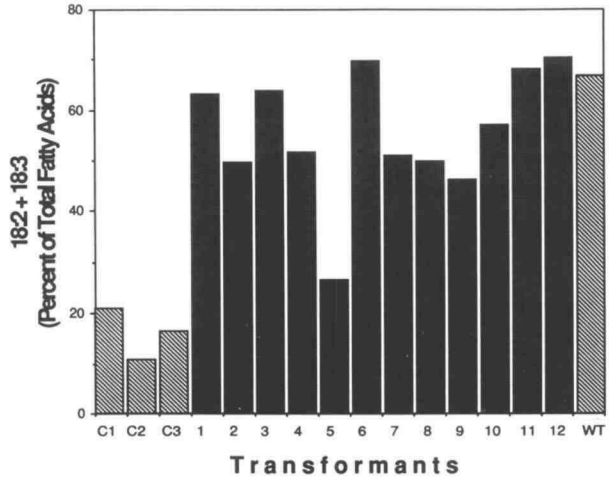

Figure 2. Polyunsaturated Fatty Acid Content of Transgenic *fad2-1* Plants.

The results shown are 18:2 + 18:3 (as percent of total fatty acids) for samples of hairy-root cultures from 12 independent transformants of the Arabidopsis *fad2-1* mutant with the pF2a cDNA insert. Control transformants (empty vector) of *fad2-1* (C1 to C3) and wild-type (WT) plants are included for comparison.

DISCUSSION

Cosegregation Analysis

The *fad2-5* allele that we isolated in the T-DNA insertion line 658 exhibited a leaky fatty acid phenotype (Table 1; Lemieux et al., 1990). More importantly, some of the seedlings that died on kanamycin-containing medium were homozygous or heterozygous for the *fad2-5* mutant phenotype. Both of these results argue against the *fad2-5* mutation being caused by

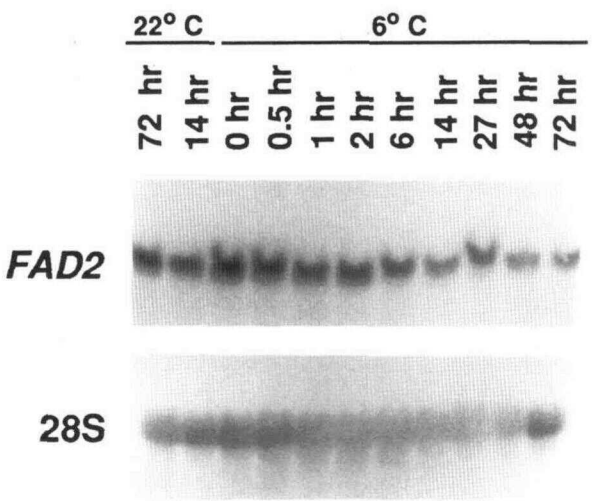

Figure 4. Transcript Levels in Wild-Type Plants after Transfer to 6°C.

Ten micrograms of total RNA per lane from plants maintained at 22°C or transferred to 6°C for up to 72 hr was subjected to gel blot analysis using the pF2a insert as a probe (*FAD2*). Blots were stripped and reprobed with the Arabidopsis 28S rRNA gene (28S) as a loading control.

T-DNA insertion. In particular, the identification of the mutant fatty acid phenotype in plants that died during kanamycin selection would normally be taken as strong evidence that the *fad2* gene was not tagged. However, our successful cloning of the *FAD2* locus demonstrated that these dead seedlings probably contained the T-DNA insert. It must be assumed that either the neomycin phosphotransferase II gene, which is responsible for kanamycin resistance in the T-DNA, was inactivated in these individuals (perhaps as a result of DNA methylation) or that the seedlings were inviable for unrelated reasons. It should also be noted that our second cosegregation analysis—the identification of nopaline in all 83 homozygous *fad2-5* individuals that were tested—provides only a very weak test of linkage. Even if the T-DNA and the *fad2* locus were as much as 5 centimorgans apart, there would still be a 40% probability of our obtaining the observed result. These results illustrate some of the difficulties involved in trying to decide whether to clone the T-DNA flanking regions in the search for a mutant locus.

Leaky *fad2-5* Allele Results from T-DNA Insertion in the 5′ Untranslated Region of the *FAD2* Gene

The comparison of the fatty acid compositions of *fad2-5* tissues with those of *fad2-1* and the wild type (Table 1; Lemieux et al., 1990) indicated that the homozygous T-DNA mutant contained considerable 18:1 desaturase activity. Despite the location of the T-DNA in the transcribed (but untranslated) region of the *FAD2* gene, a low level of *FAD2* transcript was detected in roots and shoots of *fad2-5* plants. Presumably, this low level of transcript is responsible for the increased 18:1 desaturation relative to other alleles of *fad2*.

The T-DNA from the 3850:1003 plasmid is large (>16 kb), and the presence of a 14.2-kb BamHI-EcoRI fragment in our rescued plasmid, although not conclusive, suggests that at least one copy of the T-DNA was incorporated into the genome at the site of insertion. For this reason, it is highly unlikely that the endogenous *FAD2* promoter is responsible for the synthesis of *FAD2* mRNA that we observed. Although we cannot rule out this possibility or the possibility that a promoter exists within the 1134-bp intron (Figure 1B), our findings strongly indicate that there is a weak but functional promoter within the left border region of the T-DNA. The promoter controls the synthesis of an RNA which, when processed, is of a similar length to the transcript produced in wild-type and *fad2-1* plants. Interestingly, a T-DNA insertion in the *fad3* gene of line 3707 of the T-DNA population also produced a leaky mutation (Yadav et al., 1993). In this case, the site of insertion is 612 bp 5′ to the initiation codon, but it is the right border of the T-DNA that is proximal to the *FAD3* open reading frame. From these results, we inferred that the residual activity of the endoplasmic reticulum 18:2 desaturase observed in homozygous mutants from line 3707 is mediated by a truncated form of the endogenous promoter confined to the 612-bp region 5′ to the initiation codon of the *FAD3* gene.

The ability of the insert from pF2a to complement the fatty acid phenotype of the *fad2-1* mutant confirmed the identity of the cloned gene. Given the low transcript level associated with the leaky *fad2-5* phenotype, it is not surprising that all but one of the transgenic hairy-root cultures produced more than 60% of the level of 18:2 plus 18:3 found in wild-type plants (Figure 2). At least two of the transgenic cultures exhibited slightly higher proportions of 18:2 plus 18:3 and lower 18:1 than the wild-type control, suggesting the possibility that *FAD2* overexpression can lead to more extensive desaturation of 18:1. However, the nearly complete (>90%) desaturation of 18:1 that occurred in wild-type Arabidopsis root cultures limits our ability to detect additional synthesis of polyunsaturated fatty acids as a result of *FAD2* overexpression. We would anticipate that the effects of overexpression might be more dramatic in plant species that contain higher levels of 18:1.

In both root and shoot tissues of wild-type Arabidopsis, the levels of *FAD2* transcript appeared to be high, considering that much lower transcript levels will support 50% of the desaturation attributable to the endoplasmic reticulum 18:1 desaturase in the wild type (Figure 3 and Table 1). This result suggests that in wild-type plants, the *FAD2* transcript may be present severalfold in excess of the amount needed to account for 18:1 desaturase activity and that there may be considerable translational and post-translational control of expression. Because the desaturation of 18:1 is the critical step in polyunsaturated lipid synthesis, it is possible that this excess is maintained to ensure that the enzyme activity is never limited by availability of transcript. Such a concept is consistent with the observation that the level of *FAD2* transcript does not increase following

transfer to low temperatures even though the membrane lipids of Arabidopsis, like those of many plants, became more unsaturated with decreasing temperature (Browse et al., 1986a).

FAD2 Sequence Shows Low Homology to Other Fatty Acid Desaturases

The homologies between the open reading frame of *FAD2* and previously described desaturases are low at both the nucleotide and protein levels. Comparisons of the predicted protein sequences of eight different fatty acid desaturases are included in Table 2. Only the comparisons with the Arabidopsis FAD3 and FAD7 (formerly FADD) sequences indicate moderate homologies (35 to 40% identity; 55 to 60% similarity), whereas the Brassica FAD6 homolog and cyanobacterial DesA sequences show weak homology (24% identity). As shown in Table 2, the FAD2 sequence does not show any more homology to the other membrane desaturase sequences than it does to the very divergent, soluble enzyme that catalyzes the desaturation of 18:0-ACP.

The overall low identity in these comparisons explains why *FAD3*, *FAD7*, and other cDNAs failed to identify the *FAD2* gene when they were used as heterologous probes. A second strategy that we and others employed in the search for *FAD2* was to design oligonucleotide probes to a nine–amino acid sequence (FVL/VGHDCGH; residues 97 to 105 in Arabidopsis FAD3) that is conserved between the higher plant 18:2 desaturases and the *desA* gene of *Synechocystis* strain PCC 6803 (Wada et al., 1990; Yadav et al., 1993). The corresponding sequence in FAD2 (WVIAHECGH; residues 101 to 109) is sufficiently divergent at the nucleotide level to preclude the possibility of successfully using such oligonucleotide probes to identify *FAD2* cDNA clones.

A considerable difference between the *FAD2* and *FAD7* genes is also seen in the structures of the genomic clones. The *FAD7* gene contains seven introns that vary in length from 79 to 301 bp and that are all located within the coding sequence (Iba et al., 1993). By contrast, the only intron in the *FAD2* sequence is large (1134 bp) and located 5′ to the open reading frame but still in the transcribed region of the gene. Unlike the Arabidopsis *FAD3* gene product but like the soybean and mung bean FAD3 homologs (Yadav et al., 1993), the predicted sequence of the FAD2 protein lacks either of the lysine-rich, carboxy-terminal motifs that have been suggested to represent the retention signal for integral membrane proteins in the endoplasmic reticulum (Jackson et al., 1990). The predicted FAD2 protein also lacks a recognizable signal sequence for targeting to the endoplasmic reticulum. This indicates that the protein may insert into the membrane post-translationally as does the rat 18:9 desaturase (Thiede et al., 1985).

Table 2. Homology between the Deduced Amino Acid Sequences of Fatty Acid Desaturases

Sequence	Identity[a]	Similarity[a]
	%	%
Arabidopsis FAD3	37.2	58.2
Arabidopsis FAD7	34.4	58.5
Brassica FAD6 homolog	24.3	48.6
Synechocystis Des A	23.8	50.5
Synechocystis Δ6	18.0	48.8
Brassica 18:0-ACP	19.1	41.7
Yeast-18:0-CoA	19.1	43.3
Rat-18:0-CoA	17.0	44.8

[a] Comparisons are with the FAD2 sequence employing the Gap program of the GCG package (Devereux et al., 1984) using a gap weight of 3.0 and length weight of 0.1.

Identification of Putative Iron Binding Motifs

The fatty acid desaturases fall into a general class of enzymes that contain iron that is not incorporated within a heme prosthetic group (Heinz, 1993). Recently, spectroscopic analysis has been used to demonstrate that the soluble 18:0-acyl-carrier-protein (18:0-ACP) desaturase is a member of a class of diiron-oxo proteins in which the two atoms of iron, bridged by an oxygen, are coordinated into the protein by histidine and carboxylic acid (aspartate and glutamate) residues (Fox et al., 1993). Because of the very close similarity between the reactions catalyzed by the membrane-bound desaturases and the desaturation of 18:0-ACP, it is reasonable to consider the possibility that the integral membrane proteins also contain a diiron-oxo reaction center, even though some data suggest that the mammalian membrane-bound 18:0–Coenzyme A desaturase may contain only one molecule of iron per polypeptide (Strittmatter et al., 1974).

Two quite different coordination structures are known from higher resolution x-ray crystallography of soluble diiron-oxo proteins. In the free radical protein of ribonucleotide reductase, the diiron cluster is ligated to two histidines and four acidic amino acids (Nordlund et al., 1990). The primary sequence of this protein contains two EXXH motifs (the critical residues are on the same face of an α-helix) that provide four of these six ligands. The same replicate EXXH motifs are present in unrelated enzymes (including the 18:0-ACP desaturase) that are recognized from their spectroscopic characteristics as diiron-oxo proteins (Fox et al., 1993). The structure of hemerythrin (Stenkamp et al., 1984) reveals a diiron cluster that is ligated through five histidines and two acidic residues. Three pairs of residues in the primary coordination sphere (H54 E58, H73 H77, and H106 D111) are three or four residues apart in the primary sequence, but the detailed structure of this active site is quite distinct from that found in ribonucleotide reductase (Nordlund et al., 1990). It should be noted that the EXXH motifs are found in enzymes that catalyze reactions for which high-valent iron-oxo structures have been proposed as catalytic intermediates, whereas hemerythrin is an O_2 binding protein with no catalytic capacity.

With these considerations in mind, we started our analysis of the putative sequences of FAD2 and the other desaturases

by attempting to identify possible iron binding motifs. Comparisons of the higher plant, membrane-bound desaturases FAD2, FAD3, FAD7 (Figure 5), and FAD6 (W. Hitz, personal communication) revealed sizable blocks of homology and similarity but only relatively short stretches where there was perfect agreement across all four sequences. The FAD2 sequence contains only one EXXH motif (residues 106 to 109; in the other plant desaturases this is present as DXXH) so that the protein does not meet the criteria of a putative diiron-oxo protein of the ribonucleotide reductase type. The other three plant sequences each contain a second D/EXXH motif, but these second copies are at very different positions in the various proteins. This suggests that they are not likely to be part of a conserved active site.

As a second stage of comparison, we assembled a total of eight distinctly different sequences including the four higher plant sequences together with the yeast OLE1 (Stukey et al., 1990), *Synechocystis* Δ6 and Δ12 (Wada et al., 1990; Reddy et al., 1993), and the rat 18:0-CoA (Thiede et al., 1986) desaturases. The wide evolutionary and biochemical divergence represented by these eight sequences allowed the overall homology to degenerate to a point at which it was possible to identify just three regions of strong conservation among

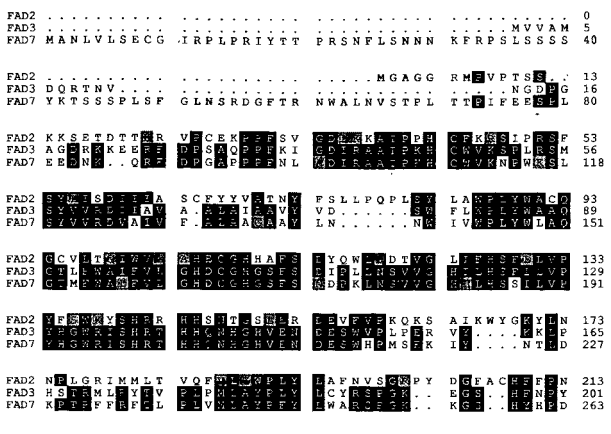

Figure 5. Comparison of the Deduced Amino Acid Sequences of Three Arabidopsis Fatty Acid Desaturases.

Identical and similar residues are shown on backgrounds of black and gray, respectively. The *FAD3* gene encodes the endoplasmic reticulum 18:2 desaturase; the *FAD7* gene (previously *FADD*) encodes a chloroplast 18:2 desaturase.

these integral membrane desaturases. The most strongly conserved motif is represented in FAD2 by WKYSHRRHH (residues 137 to 145). The tryptophan and all three histidine residues are present with the same spacing in all eight sequences, except that in the *Synechocystis* Δ12 desaturase, the spacing in the HXXHH box is increased by one residue (WRYRHNYLHH; residues 119 to 128) (Reddy et al., 1993). Interestingly, the tryptophan and the second histidine (with the same spacing as FAD2) are present in the two soluble plant acyl-ACP desaturases (Shanklin and Somerville, 1991; Cahoon et al., 1992) where the histidine is a critical residue in one-half of the proposed iron binding site (Fox et al., 1993).

The HXXHH motif is repeated toward the carboxy terminus of each sequence (in FAD2 as HVAHHLFS, residues 315 to 322). Again, all three histidine residues and the spacing are conserved in all eight membrane desaturases, except that the *Synechocystis* Δ12 desaturase once again has one extra residue in the spacing (HQVTHHLFP, residues 302 to 310). Finally, the FAD2 sequence HECGHHSF (residues 105 to 112) showed conservation of the first two histidines in all the higher plant and cyanobacterial enzymes as well as aspartate (glutamate in FAD2) as the second residue. In both the yeast and rat sequences, the spacing between the two histidines in this region is increased (HRLWSH in both), and there is no D/EXXH sequence present.

As discussed above, we failed to identify conserved, paired D/EXXH motifs within the eight sequences that were compared. It is possible that the positioning of the motifs varies between the different enzymes for functional reasons and/or that the ligands required to coordinate a diiron cluster are recruited from distinct individual sites throughout each protein sequence. However, the large number of highly conserved histidines among the blocks of homology that were identified suggest that if the membrane desaturases are diiron-oxo proteins, then a histidine-rich primary coordination shell for the iron cluster should be considered as a possibility, even though such a structure has, to date, only been identified in a protein that does not have a catalytic function. Alternatively, the membrane-bound desaturases may not be diiron-oxo proteins and the conserved histidine motifs may represent a different type of iron binding site. Of the three histidine-rich motifs that we have identified, the second and third in the protein sequence show the greatest degree of conservation. The spacing would place all three histidines on the same face of an α-helix (except for the *Synechocystis* Δ12 desaturase), although there is no evidence that the proteins are helical in these regions.

The Problem of Getting the Substrate Acyl Chain to the Active Site

The hydropathy profile calculated for the predicted FAD2 protein and displayed in Figure 6 is similar in several respects to those of other membrane-bound desaturases (Stukey et al., 1990; Wada et al., 1990; Reddy et al., 1993; E. Lark and J. Browse, unpublished data). Two long stretches (>45 residues

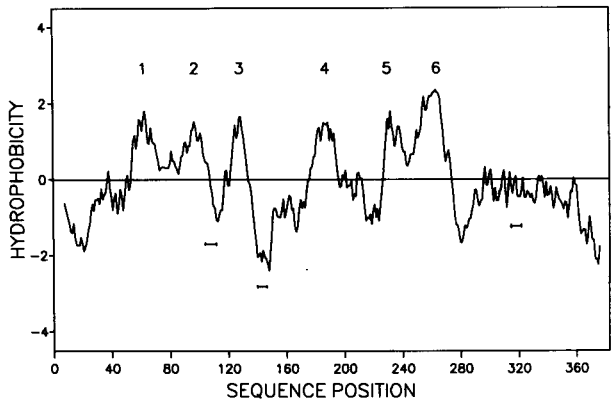

Figure 6. Hydropathy Plot of the Arabidopsis FAD2 Sequence.

The plot was made according to the method of Kyte and Doolittle (Devereux et al., 1984). Six candidate membrane-spanning sequences are numbered. The short lines indicate regions corresponding to the three histidine-rich sequences that show homology to other membrane-bound desaturases.

each) of hydrophobic residues are present (55 to 107 and 228 to 273 in FAD2) together with shorter hydrophobic sequences near the center of the molecule.

From comparisons of the yeast and rat proteins, Stukey et al. (1990) proposed a model in which the long hydrophobic sequences form two membrane-traversing loops, each comprised of two membrane-spanning, α-helical segments. In this structure, the bulk of the protein (240 of 334 residues for the rat 18:0-CoA desaturase) is on the cytosolic side of the endoplasmic reticulum membrane. However, in the FAD2 hydropathy profile, there are two additional hydrophobic stretches that are very good candidates for single-pass membrane-spanning segments (regions 3 and 4 in Figure 6). The hydropathy plots of other plant desaturases also show one or more potential (single-pass) membrane-spanning sequences in addition to the proposed loops (data not presented). One of these (region 3 shown in Figure 6) corresponds to a comparable peak in the profiles of the predicted yeast and rat proteins (Stukey et al., 1990). However, acceptance of all six potential membrane-spanning regions in the FAD2 structure would make it impossible to orient all the conserved histidine-containing motifs on the cytosolic side of the membrane. The most highly conserved motif (residues 137 to 145) would always be on the side of the membrane away from the other two. One possible explanation that would retain the proposed rat/yeast structure for the plant and cyanobacterial enzymes is that the additional hydrophobic regions are involved in other aspects of protein structural determination or interaction with the substrate membrane lipids. Potentially, it will be possible to resolve these questions for one or more of the desaturase proteins using direct measurements of membrane topology (Jennings, 1989).

Regardless of the actual topology of the 18:1 desaturase, it is clear from Figure 6 that all the putative active site histidines are located in relatively hydrophilic regions of the protein. Presumably, such exposed locations would facilitate interactions of the active site with cytochrome b_5 (the immediate electron donor to the desaturase) on the cytoplasmic surface of the membrane (Heinz, 1993). However, the enzyme will use the two electrons from cytochrome b_5 to abstract two hydrogens of an 18:1 acyl chain that are on carbons 12 and 13 and, thus, normally 25 Å or so below the membrane surface. Moving the electrons through the protein toward the center of the bilayer and drawing a glycerolipid molecule out of the membrane to effect desaturation are both energetically challenging options, although a precedent for partial removal of a glycerolipid substrate from the bilayer is well established in the case of phospholipase A_2 enzymes (Scott et al., 1990). The most straightforward explanation for this apparent conflict may lie in the extremely dynamic nature of the membranes' fatty acid core (Venable et al., 1993). The flexibility of the acyl chains allows the C12–C13 region of the desaturase substrate (where the double bond is to be placed) a small but significant probability of being located close to the membrane surface at any given time. The rate of oleate turnover by desaturation in Arabidopsis and other higher plants is slow, with a half-time, calculated from in vivo labeling experiments (Miquel and Browse, 1992), of 2 to 4 hr. Consequently, the frequency with which the C12–C13 region of any particular 18:1 acyl chain would be located close to the membrane surface might not limit the rate of desaturation (Heinz, 1993).

Conclusion

The cloning of the Arabidopsis *FAD2* gene and the unusual structure and expression pattern of the *fad2-5* insertional allele have provided information on the molecular-genetic control of the 18:1 desaturation step carried out by the product of the *FAD2* gene. Sequence analysis of the predicted FAD2 protein and other membrane-bound fatty acid desaturases has permitted useful speculation about the structure and location of a putative active site. In addition, the availability of genes encoding the endoplasmic reticulum 18:1 and 18:2 desaturases (Arondel et al., 1992; Yadav et al., 1993) should now permit the manipulation of tissue fatty acid compositions through overexpression and the use of antisense techniques. Such approaches will contribute to our understanding of how membrane lipid composition affects plant function and may lead to the useful manipulation of seed storage lipids to produce modified vegetable oils.

METHODS

Screening of the Arabidopsis T-DNA Population

Approximately 30 plants from each of 1800 lines in a population of *Arabidopsis thaliana* (ecotype Wassilewskija) that were transformed

with a modified T-DNA (Feldmann and Marks, 1987) were grown in 3-in-diameter pots in controlled environment chambers. Ten leaves (each from a different plant) were harvested from a pot and pooled for analysis (Browse et al., 1986b) to provide a single, average fatty acid composition for each segregating line. Once line 658 had been identified as segregating for increased 18:1, homozygous mutant segregants of this line were used in reciprocal crosses with plants containing the *fatty acid desaturation 2-1* (*fad2-1*) allele (Lemieux et al., 1990; Miquel and Browse, 1992) and the F_1 progeny analyzed for fatty acid composition. The *fad2-1* allele is derived from the Columbia ecotype. However, wild-type plants of Columbia and Wassilewskija exhibit very similar leaf fatty acid compositions so that comparisons of mutants in these two genetic backgrounds are entirely valid.

Two markers were used to follow segregation of the T-DNA inserts. Activity of the nopaline synthase gene was determined by assaying for the presence or absence of nopaline in extracts of single leaves using the method of Errampalli et al. (1991). Activity of the neomycin phosphotransferase II gene was determined by germinating seeds on agar medium containing 50 μg/mL kanamycin (Feldmann et al., 1989). The derivative line, 658-75, was identified as the T_4 progeny of a single T_3 plant that was segregating for the fatty acid phenotype and for a single kanamycin resistance locus.

Isolation of a T-DNA–Plant DNA Junction Fragment

Genomic DNA was prepared from leaves of homozygous mutant individuals of the 658-75 line by the method of Rogers and Bendich (1985). A sample (1.5 μg) of this DNA was digested in a 50-μL volume with 20 units of BamHI (Bethesda Research Laboratories) according to the manufacturer's instructions. Following purification by phenol extraction and ethanol precipitation, the digested DNA was resuspended at 2.5 μg/mL in a ligation buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 4 units of T4 DNA ligase and incubated for 16 hr at 16°C. Competent cells (Bethesda Research Laboratories) were transfected with 10 ng of ligated DNA per 100 μL according to the manufacturer's specifications. Transformants were selected on Luria–Bertani plates (10 g Bacto Tryptone, 5 g Bacto yeast extract 5 g NaCl, 15 g agar per liter, pH 7.4) containing 100 μg/mL ampicillin. After overnight incubation at 37°C, the plates were scored for ampicillin-resistant colonies.

A single, ampicillin-resistant colony was used to start an overnight culture in Luria–Bertani medium containing 25 mg/L ampicillin. Plasmid DNA from pTF-658 was isolated from harvested cells by an alkaline lysis method (Ausubel et al., 1991) to provide DNA for restriction analysis and for the synthesis of labeled probes. The 1.6-kb EcoRI-BamHI fragment from the pTF658 plasmid was also subcloned into the pBluescript SK– vector (Stratagene) as pSI658.

Isolation of cDNA and Genomic Clones Corresponding to the *FAD2* Gene

The EcoRI-BamHI fragment from plasmid pTF-658 was purified and labeled with α-^{32}P-dCTP using a random priming kit (Bethesda Research Laboratories) according to the manufacturer's recommendations. Approximately 17,000 plaques of an Arabidopsis cDNA library in the λYES vector (Elledge et al., 1991) were screened essentially as described by Ausubel et al. (1991). Fifteen positively hybridizing plaques were identified, and five of these were subjected to plaque purification. The cDNA clones were converted to plasmids by incubation in *Escherichia coli* BNN-132 cells (Elledge et al., 1991), and ampicillin-resistant plasmid clones were grown and used as a source of plasmid DNA as described above. Four cDNA clones (pF2a, pF2b, pF2c, and pF2d) that were confirmed as hybridizing to the EcoRI-BamHI fragment from pTF-658 were subjected to restriction analysis and partial sequencing. Because the four cDNAs contained sequences in common, the longest cDNA clone pF2a (1.4 kb) was sequenced in both directions using a DNA polymerase (Sequenase T7; U.S. Biochemical Corp.) according to the manufacturer's instructions, and primers were designed from newly acquired sequences as the experiment progressed.

The pF2b cDNA insert was used as a radiolabeled probe to screen an Arabidopsis genomic DNA library (Yadav et al., 1993). DNA from several pure, strongly hybridizing phage were analyzed by gel blot hybridization to the cDNA probe to identify a 6-kb HindIII insert fragment that contained the entire coding region of the gene. This fragment was subcloned into the pBluescript SK– vector and partially sequenced as described above. Analysis of the predicted protein sequences of *FAD2* and other desaturase gene products was performed using the GCG sequence analysis software package (Devereux et al., 1984).

Plant Transformation

The 1.4-kb cDNA fragment of pF2a was subcloned into the EcoRI site of the binary vector pGA748 (kindly supplied by G. An, Washington State University, Pullman). This vector contains the cauliflower mosaic virus 35S promoter, the nopaline synthase 3′ terminator sequence, and the *NPTII* gene that confers kanamycin resistance (An et al., 1988). A vector construct (pGA-Fad2), cloned in *E. coli* and identified as being correctly inserted in the sense orientation by restriction enzyme analysis, was transformed into *Agrobacterium tumefaciens* R1000 (a C58 strain carrying the Ri plasmid) using the freeze–thaw method (Holsters et al., 1978) to produce strain R1000/pGA-Fad2. This strain and a corresponding control (no insert) were used to transform *fad2-1* and wild-type Arabidopsis using a stem-transformation protocol (Arondel et al., 1992).

Measuring Levels of *FAD2* Transcript

Shoot material of wild-type, *fad2-1*, and *fad2-5* Arabidopsis plants was harvested from plants as the first flowers were opening. Root material was harvested from 2-week-old plants grown in liquid culture (Miquel and Browse, 1992). For the chilling experiment, plants were grown at 22°C, 100 to 120 μmol quanta m^2 sec^{-1} constant light and 60 to 70% relative humidity for 17 days before transfer to 6°C with the same light and humidity. Samples of shoot material were taken at intervals after transfer and from control plants left at 22°C. Harvested plant tissue was rapidly frozen in liquid nitrogen and the RNA extracted from it by a phenol–SDS method (Ausubel et al., 1991). In the separations shown here, 10 μg of total RNA was fractionated by electrophoresis on 1.2% (w/v) agarose 2.2 M formaldehyde gels and blotted onto nitrocellulose membranes (Schleicher & Schuell). The blots were probed sequentially with ^{32}P-labeled probes from the cDNA insert of pF2a and from the Arabidopsis 28S rRNA gene as described by Ausubel et al. (1991).

ACKNOWLEDGMENTS

This work was supported by grants from the U.S. Department of Agriculture (No. 92-37301-7728) and the National Science Foundation (No. DCB-9105550) to J.B. We are grateful to those who helped to screen the T-DNA population, particularly Florence Garlick and Mary Aegerter. We thank Elmer Heppard, Russell Booth, and Pablo Scolnik for help with sequencing and mapping the clones. We would also like to thank Keith Davis (Ohio State University) for generous use of facilities. Sequence analysis was performed using the facilities of the VADMS Center at Washington State University. We are also grateful to Bill Hitz and John Shanklin for discussing ideas about possible protein structures. The λYES Arabidopsis cDNA library was a generous gift from the laboratory of Ron Davis.

Received October 26, 1993; accepted November 29, 1993.

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