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# Identification of a Gene that Complements an *Arabidopsis* Mutant Deficient in Chloroplast ω6 Desaturase Activity[1]

**Deane L. Falcone[2], Susan Gibson[2], Bertrand Lemieux, and Chris Somerville***

Carnegie Institution, Department of Plant Biology, 290 Panama Street, Stanford, California 94305 (D.L.F., C.S.); Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251–1892 (S.G.); and Department of Biology, York University, Toronto, Canada M3J 1P3 (B.L.)

**Membrane lipids of the *fad6* (formerly *fadC*) mutant of *Arabidopsis*, which is deficient in chloroplast ω6 desaturase activity, have increased levels of monounsaturated fatty acids and are deficient in trienoic fatty acids. A putative *fad6* cDNA clone was isolated by probing a cDNA library with a degenerate oligonucleotide based on a conserved region within known ω3 desaturase genes. Expression of the cDNA in transgenic plants of a *fad6* mutant restored normal levels of all fatty acids. When used as a hybridization probe, the cDNA identified a restriction fragment-length polymorphism that co-segregated with the *fad6* mutation. Thus, on the basis of a genetic complementation test and genetic map position, the *fad6* gene is encoded by the cDNA. The cDNA encoded a 418-amino acid polypeptide of 47,727 D that displayed a high degree of sequence similarity to a Δ12 desaturase from the cyanobacterium *Synechocystis*. The *fad6* gene exhibited less sequence homology to any known higher plant desaturase, including an endoplasmic reticulum-localized ω6 desaturase corresponding to the *Arabidopsis fad2* gene.**

Fatty acid and glycerolipid synthesis in plant cells occurs through the coordinated participation of enzymes located in both plastid and microsomal membranes (Roughan and Slack, 1982). The initial step in glycerolipid synthesis occurs in the plastid with production of 16:0-ACP by fatty acid synthase. A major portion of 16:0-ACP is elongated to 18:0-ACP and then desaturated to 18:1-ACP by a soluble desaturase. Once formed, these products then enter into one of two interlocked pathways in which glycerolipid synthesis and acyl chain desaturation are completed. The prokaryotic pathway is initiated by the acylation of glycerol-3-P within the plastid. The reactions of the eukaryotic pathway take place in the ER following export of 16:0 and 18:1 fatty acids from the plastid and conversion to CoA esters in the cytoplasm (Roughan and Slack, 1982; Browse and Somerville, 1991). In both pathways, further sequential desaturation occurs via a family of membrane-bound desaturases within the respective cellular compartments (Browse and Somerville, 1991; Schmidt et al., 1993).

The distinct fatty acid compositions found in microsomal and chloroplastic membrane lipids and seed storage oils are the result of an intricate metabolic network that operates to control this composition by regulating fatty acid biosynthesis and flux through the two pathways. Substantial information about the pathways has been obtained from the analysis of a series of *Arabidopsis* mutants that have distinct alterations in the fatty acid compositions of their membrane lipids because of defects in fatty acid desaturation and acylation (Browse et al., 1985; Browse and Somerville, 1991). These mutants have been useful in identifying the number of structural genes involved in the control of fatty acid unsaturation (Browse et al., 1985, 1986, 1989; Kunst et al., 1988, 1989) and in the cloning of these genes by genetic criteria. To date four different classes of genes for membrane-bound desaturases have been cloned and characterized. These include the ER-localized ω3 desaturase (*fad3*; Arondel et al., 1992; Yadav et al., 1993) and ω6 desaturase (*fad2*; Okuley et al., 1994) and two closely related plastid-localized ω3 desaturases (*fad7*; Iba et al., 1993; Yadav et al., 1993; van de Loo and Somerville, 1994; *fad8*; S. Gibson, V. Arondel, K. Iba, C.R. Somerville, unpublished data).

The *fad6* mutant (formerly *fadC*) is deficient in desaturation of 16:1 and 18:1 to 16:2 and 18:2, respectively, on all chloroplast lipids (Browse et al., 1989; Hugly et al., 1989). This mutant appears phenotypically indistinguishable from the wild-type when grown at 22°C, but when grown at low temperature (5°C), the leaves of the mutant become chlorotic and the growth rate is significantly reduced compared to the wild type. Dramatic reductions in chloroplast area and chloroplast membrane content are evident when mutant lines are transferred to low temperature before leaf expansion (Hugly and Somerville, 1992). In the present investigation, we report the isolation and characterization of a cDNA clone encoding the chloroplast ω6 desaturase encoded by the *fad6* gene.

## MATERIALS AND METHODS

### Genetic Material and Growth Conditions

The plant lines used in this study were descended from the Columbia wild type of *Arabidopsis thaliana* (L.) Heynh. and

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[2] Both authors contributed equally to this work.

* Corresponding author; fax 1–415–325–6857.

Abbreviations: ACP, acyl-carrier protein; eIF4a, initiation factor 4a; CaMV, cauliflower mosaic virus; RFLP, restriction fragment-length polymorphism; 16:0, palmitic acid; 16:1, *cis*-palmitoleic (ω9); 16:3, *cis*-hexadecetrienoic (ω3,6,9); 18:1, *cis*-oleic (ω9); 18:2, *cis*-linoleic (ω6,9); 18:3, *cis*-linolenic (ω3,6,9).

are available from the Arabidopsis Resource Center at Ohio State University. The *fad6* (formerly *fadC*) mutant line LK3 was isolated as described previously from ethyl methanesulfonate-treated seeds (Browse et al., 1989) and was backcrossed to the wild-type line four times before used in this study. The *Arabidopsis* λYES cDNA library used here was generously provided by Ron Davis (Stanford University) and was described by Elledge et al., (1991). Plasmid p313–33 is available from the Arabidopsis Resource Center at Ohio State University, and the sequence is available from GenBank as accession No. U09503.

Plants were grown at 22°C under continuous fluorescent illumination (100–150 $\mu$mol $m^{-2}$ $s^{-1}$) on a sphagnum, vermiculite, perlite (1:1:1) potting soil irrigated with commercial fertilizer (Peters 20:20:20).

### RFLP Mapping

Seed from individual $F_2$ plants, from a cross between the Niederzenz ecotype and the *fad6* mutant line LK3, was harvested separately to form a series of $F_3$ seed pools. The *fad6* phenotype of the $F_2$ parent of each $F_3$ seed pool was determined by measuring the fatty acid composition of at least 10 plants from each $F_3$ pool by GC of leaf extracts. Total DNA from the $F_3$ pools was isolated using a cetyltrimethylammonium bromide extraction procedure (Rogers and Bendich, 1988) and digested with *Eco*RV, and the fragments were resolved by agarose gel electrophoresis. Southern blots were probed with the insert from plasmid p313–33.

### Nucleic Acid Hybridizations

Nylon filter replicas of phage libraries were screened with $^{32}$P-labeled DNA probes by carrying out hybridizations with one of the following regimes: For probing with the degenerate oligonucleotide GGNCA(CT)GA(CT)TG(CT)GGNCA, the oligomer was end labeled with $^{32}$P using T4 polynucleotide kinase (Sambrook et al., 1989). A solution containing 3 M tetramethylammonium chloride, 10 mM sodium phosphate (pH 6.8), 1.3 mM EDTA, 0.5% (w/v) SDS, and 0.5% (w/v) nonfat dry milk was used. Hybridizations took place overnight at 44°C, and washings were performed four times for 20 min each in 6× SSC, 0.15% (w/v) SDS at room temperature and then once for 30 min in 4× SSC, 0.1% (w/v) SDS at room temperature. High-stringency hybridizations using randomly primed gene fragments as probes took place overnight in 30% formamide, 0.2 M NaCl, 20 mM sodium phosphate (pH 7.7), 2 mM EDTA, 1% (w/v) SDS, 0.5% (w/v) nonfat dry milk, and 0.1% (w/v) sodium PPi at 65°C. Washing was performed in 2× SSC, 0.15% SDS at room temperature. Intermediate-stringency hybridizations were performed for 14 h in 0.2 M NaCl, 20 mM sodium phosphate (pH 7.7), 2 mM EDTA, 1% (w/v) SDS, 0.5% (w/v) nonfat dry milk, 10% (w/v) dextran sulfate, and 0.1% (w/v) sodium PPi at 65°C, then washed four times with 2× SSC, 0.15% (w/v) SDS at room temperature for 30 min, and then washed at 65°C for 45 min with 1× SSC, 0.1% (w/v) SDS. Southern hybridizations were carried out in 6× SSC, 1% (w/v) SDS, 5% dextran sulfate, 10× Denhardt's solution at 65°C. After overnight hybridization, membranes were washed twice for 10 min in 2× SSC, 0.1% (w/v) SDS at room temperature and then twice for 15 min in 0.2 ×SSC at 65°C.

### Northern Analysis

Total RNA was isolated from *Arabidopsis* plants grown under continuous illumination in environmental chambers at 22°C. At the times indicated, the rosette portion of the plants were harvested, immediately frozen in liquid nitrogen, and then stored at −80°C until used. RNA was isolated using a guanidine isothiocyanate procedure (Puissant and Houdebine, 1990).

RNA was separated on 1.2% (w/v) agarose gels containing 0.66 M formaldehyde, then transferred to nylon membranes, and cross-linked by UV irradiation. Membranes were hybridized with the insert from p313–33 that was labeled with $^{32}$P by random priming (Feinberg and Vogelstein, 1983). The blots were stripped and reprobed with a 300-bp fragment of the *Arabidopsis* eIF4a cDNA (Metz et al., 1992), which is known to be constitutively expressed in *Arabidopsis* (Taylor et al., 1993). Hybridization was performed as described by Fourney et al. (1991). Membranes were washed under moderately stringent conditions using 1× SSC, 1% (w/v) SDS for 20 min, followed by two 20-min washes in 0.1× SSC, 0.1% SDS at 50 to 55°C. The membranes were visualized by autoradiography and quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The level of expression of the *fad6* gene was expressed as a ratio of the intensity of hybridization of the *fad6* probe to the intensity of hybridization of the eIF4a probe.

### DNA Sequence Determination and Analysis

DNA sequencing was performed with an ABI Catalyst-8000 robot and an ABI373A DNA sequencer (Applied Biosystems, Foster City, CA) using dye terminator or dye primer-sequencing reactions. Sequence comparisons and alignments were performed with programs in the GCG Sequence Analysis software package (Genetics Computer Group, Madison, WI).

### Plant Transformation

The cDNA insert in p313–33 was excised by digestion with *Xho*I, filled in with the Klenow fragment of DNA polymerase I, and ligated to the *Sma*I site of pUC18, generating plasmid pUC33. The 1.7-kb insert in pUC33 was excised as an *Xba*I-*Sst*I fragment and ligated into the same sites of the binary Ti plant transformation vector, pBI121 (Clontech, Palo Alto, CA), placing the cDNA insert under the control of the CaMV 35S promoter. This plasmid, designated pBI121–33, was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Arabidopsis fad6* plants were transformed by inoculating freshly cut stems with overnight cultures of *A. tumefaciens* harboring pBI121–33 or pBI121 without insert (Chang et al., 1994). Seeds from twice-inoculated plants were plated on Murashige and Skoog basal salt (GIBCO) agar plates containing 0.5 g $mL^{-1}$ Mes buffer (pH 5.6) and 50 $\mu$g $mL^{-1}$ kanamycin sulfate to select for plants that had acquired the T-DNA inserts.

### Fatty Acid Analysis

Rosette leaves were taken from plants and either used immediately for extraction and methyl ester preparation of fatty acids (Browse et al., 1986) or stored at −80°C in Teflon-capped tubes until needed. Typically, samples were taken in triplicate for each plant. Gas chromatographic analysis was performed as described by Browse et al. (1986) using a Hewlett-Packard 5800 series gas chromatograph equipped with Supelco 0.75-mm × 20-m SP2330 glass capillary columns.

### Nomenclature

For convenience we have referred to the various desaturases as ω6, ω3, or Δ12 desaturases to indicate where they act on fatty acid substrates. It is not our intention to convey any information about the mechanism by which these enzymes achieve this apparent product specificity.

## RESULTS

### Isolation of a Putative *fad6* cDNA Clone

Initial attempts to clone the *fad6* gene from *Arabidopsis* by using cDNA clones encoding several different ω3 desaturases (Arondel et al., 1992; Iba et al., 1993) or a cyanobacterial Δ12 desaturase (Wada et al., 1990) as heterologous probes failed to identify a clone. Therefore, a mixed oligonucleotide probe was designed based on a comparison of the deduced amino acid sequences of the *Brassica* ω3 desaturase *fad3* (Arondel et al., 1992), the cyanobacterial Δ12 desaturase *desA* (Wada et al., 1990), and the *Arabidopsis fad7* (Iba et al., 1993) and *fad8* (S. Gibson, unpublished data) genes. The amino acid sequence GHDCGH was found to be conserved in all four desaturase sequences available at the time. A 17-bp, 128-fold degenerate oligonucleotide encoding the conserved motif was used to probe approximately $10^6$ plaques from an *Arabidopsis* cDNA library. Approximately 2% of the phage hybridized to the consensus oligomer, and 60% of these also hybridized at high stringency to *fad3*, *fad7*, *fad8*, or one of four anonymous clones identified in a preliminary screen. The nucleotide sequence of these four anonymous clones did not exhibit any apparent deduced amino acid sequence homology to known desaturase genes (results not presented), and the nucleotide sequence homology was, therefore, considered spurious. Fifty-eight phage that hybridized to the consensus oligomer but not to the desaturase genes or the four anonymous clones were plaque purified. These phage were sorted into one of eight homology classes by hybridizing various members of the group to all of the other members. Approximately 400 bp of nucleotide sequence were then obtained from each end of the largest clone in each of the eight classes. One clone p313, which exhibited a high degree of deduced amino acid sequence similarity to a cyanobacterial 18:1 desaturase (*desA*), was used as a probe to rescreen the cDNA library for a putative full-length cDNA clone. The isolate with the longest cDNA insert, p313–33, was completely sequenced on both stands and used in all further studies. Since, as noted below, this clone complemented the *fad6* mutation, we refer to it throughout as the *fad6* gene. None of the other seven classes of clones exhibited significant deduced amino acid sequence homology to any known desaturase.

### Sequence Analysis

Analysis of the nucleotide sequence of the 1.7-kb cDNA insert present in p313–33 revealed a full-length open reading frame of 1254 nucleotides encoding a 418-amino acid polypeptide with a predicted molecular mass of 47,727 D (GenBank accession No. U09503). The deduced amino acid sequence was 54% identical with and 71% similar to the *desA* Δ12 desaturase gene from the cyanobacterium *Synechocystis* PCC6803 (Wada et al., 1990, 1993) (Fig. 1). The amino-terminal portion of the cDNA sequence exhibited no homology to the *desA* amino-terminal region. This is consistent with the possibility that this region of the gene product might be involved in targeting the polypeptide to the chloroplast as suggested by results from genetic and biochemical analyses indicating that the *fad6* gene product is localized in the plastid (Browse et al., 1989; Schmidt and Heinz, 1990, 1993). However, as was also noted in the sequence analysis of the *Arabidopsis fad8* gene (S. Gibson, V. Arondel, K. Iba, C.R. Somerville, unpublished data), the amino-terminal sequences showed no recognizable features characteristic of chloroplast transit peptides (von Heijne et al., 1989). Furthermore, both of the chloroplast desaturases (*fad6*, *fad7*) have unusually long amino-terminal extensions compared to the corresponding ER-localized proteins (*fad2*, *fad3*). This may reflect the fact that the chloroplast desaturases appear to localize in the envelope membrane (Schmidt and Heinz, 1990), whereas most previously described chloroplast proteins have been located in the thylakoid membranes, the lumen, or the stroma. Alternatively, these results may be additional support for the emerging view that chloroplast transit peptides are characterized by a structure that is devoid of a regular secondary or tertiary structure (von Heijne and Nishikawa, 1991).

Comparison of the *fad6* sequence to the recently isolated *Arabidopsis fad2* gene, which encodes an ER-localized ω6 desaturase (Okuley et al., 1994), revealed an unexpectedly low level of sequence homology (24% identical and 49% similar; Fig. 1). This is in contrast to the high level of homology seen among ω3 desaturases (Iba et al., 1993; Yadav et al., 1993; van de Loo and Somerville, 1994) in which sequence identities range from 55 to 84% even between different plant species.

One feature of desaturase sequences that has been previously noted (Schmidt et al., 1993; Okuley et al., 1994) is the three His-rich motifs that are implicated as possible iron coordination centers in the enzyme active site (Fox et al., 1993). Although structural studies have yet to be accomplished with membrane-bound desaturase proteins, the degree of conservation of the His's in the motifs among the divergent desaturase sequences presented here is evidence that these residues are likely to be important for enzyme activity. Since the *fad6* amino acid sequence extends the list of known sequences for membrane-bound desaturases, an analysis of fully conserved residues can be made. The number of amino acid residues identical in all desaturases present in Figure 1 is only 36, of which 6 are the invariant residues

```
      1                                                  50
fad6  MASRIADSLF AFTGPQQCLP RVPKLAASSA RVSPGVYAVK PIDLLLKGRT
desa  .......... .......... .......... .......... ..........
fad3  .......... .......... .......... .......... ..........
fad7  ........MA NLVLSECGIR PLRIYTTTPR SNFLSNNNKF RPSLSSSSYK
fad2  .......... .......... .......... .......... ..........

      51                                                 100
fad6  HRSRRCVAPV KRRIGCIKAV AAPVAPPSAD SAEDREQLAE SYGFRQIGED
desa  .......... ......MTAT IPPLTPTVTP SNPDRP.... ..........
fad3  .......... .......... .MVVAMDQRT NVNGDPGAGD RKKEERFDPS
fad7  TSSSPLSFGL NSRDGFTRNW ALNVSTPLTT PIFEESPLEE DNK.QRFDPG
fad2  .......... .......... ...MGAGGRM PVPTSSKKSE TDTTKRV.PC

      101                                                150
fad6  LPENVTLKDI MDTLPKEVFE IDDLKALKSV LISVTSYTLG LFMIA.....
desa  .IADLKLQDI IKTLPKECFE KKASKAWASV LITLGAIAVG YLGII.....
fad3  AQPPFKIGDI RAAIPKHCWV KSPLRSM.SY VVRDIIAVAA L.AIAAVYVD
fad7  APPPFNLADI RAAIPKHCWV KNPWKSL.SY VVRDVAIVFA L.AAGAAYLN
fad2  EKPPFSVGDL KKAIPPHCFK RSIPRSF.SY LISDIIIASC FYYVATNYFS

      151                                                200
fad6  ....KSPWYL LPLAWAWTGT AITGFFVIGH DCAHKSFSKN KLVEDIVGTL
desa  ....YLPWYC LPITWIWTGT ALTGAFVVGH DCGHRSFAKK RWVNDLVGHI
fad3  ......SWFL WPLYWAAQGT LFWAIFVLGH DCGHGSFSDI PLLNSVVGHI
fad7  ......NWIV WPLYWLAQGT MFWALFVLGH DCGHGSFSND PKLNSVVGHL
fad2  LLPQPLSYLA WPLYWACQGC VLTGIWVIAH ECGHHAFSDY QWLDDTVGLI

      201                                                250
fad6  AFLPLVYPYE PWRFKHDRHH AKTNMLVHDT AWQPVPPEEF E.........
desa  AFAPLIYPFH SWRLLHDHHH LHTNKIEVDN AWDPWSVEAF Q.........
fad3  LHSFILVPYH GWRISHRTHH QNHGHVENDE SWVPLPERVY KKLPH.....
fad7  LHSSILVPYH GWRISHRTHH QNHGHVENDE SWHPMSEKIY NTLDK.....
fad2  FHSFLLVPYF SWKYSHRRHH SNTGSLERDE VFVPKQKSAI KWYGKYLNNP
                    *  **
      251                                                300
fad6  .........S SPVMRKAIIF GY.GPIRPWL SIAHWVNWHF NLKKFRASEV
desa  .........A SPAIVRLFYR AIRGPFWWTG SIFHWSLMHF KLSNEAQRDR
fad3  STRMLRYTVP LPMLAYPLYL CYRSPGKE.. ..G..SHFNP YSSLFAPSER
fad7  PTRFFRFTLP LVMLAYPFYL WARSPGKK.. ..G..SHYHP DSDLFLPKER
fad2  LGRIMMLTVQ FV.LGWPLYL AFNVSGRPYD GFA..CHFFP NAPIYNDRER

      301                                                350
fad6  NRVKISLACV FAFMAVGWPL IVYKVGILGW VKFWLMPWLG YHFWMSTFTM
desa  NKVKLSIAVV FLFAAIAFPA LIITTGVWGF VKFWLMPWLV YHFWMSTFTI
fad3  KLIATSTTC. WSIMFVSLIA LSFVFGPLAV LKVYGVPYII FVMWLDAVTY
fad7  KDVLTSTAC. WTAMAALLVC LNFTIGPIQM LKLYGIPYWI NVMWLDFVTY
fad2  LQIYLSDAG. ILAVCFGLYR YAAAQGMASM ICLYGVPLLI VNAFLVLITY

      351                                                400
fad6  VHHTAPH..I PFKPADEWNA AQAQLNGTVH CDYPSWIEIL CHDINVHIPH
desa  VHHTIPE..I RFRPAADWSA AEAQLNGTVH CDYPRWVEVL CHDINVHIPH
fad3  LHHHGHDEKL PWYRGKEWSY LRGGL.TTID RDYGIFNNIH HDI.GTHVIH
fad7  LHHHGHEDKL PWYRGKEWSY LRGGL.TTLD RDYGLINNIH HDI.GTHVIH
fad2  LQH..THPSL PHYDSSEWDW LRGAL.ATVD RDYGILNKVF HNITDTHVAH
                                                    *  *
      401                                                450
fad6  HISPRIPSYN LRAAHESIQE NWGKYTNLAT WNWRL.MKTI MTVCHVYDK.
desa  HLSVAIPSYN LRLAHGSLKE NWGPFLYERT FNWQL.MQQI SGQCHLYDPE
fad3  HLFPQIPHYH LVDATKAAKH VLGRYYREPK TSGAIPIHLV ESLVASIKKD
fad7  HLFPQIPHYH LVEATEAAKP VLGKYYREPD KSGPLPLHLL EILAKSIKED
fad2  HLFSTMPHYN AMEATKAIKP ILGDYYQFDG TPWYVAMYRE AKECIYVEPD
      *
      451                                  479
fad6  .......... .......... .........
desa  HGYRTFGSLK KV........ .........
fad3  HYVSDTGDIV FYETDPDLYV YASDKSKIN
fad7  HYVSDEGEVV YYKADPNLYG EVKVRAD..
fad2  R.EGDKKGVY WYNNKL.... .........
```

**Figure 1.** Comparison of the deduced amino acid sequences of various desaturases. Asterisks indicate conserved His motifs proposed to be important in iron binding. Bold face residues indicate amino acids conserved among all sequences, and shaded areas indicate homologous residues in one or more of the different desaturase sequences. Dots indicate spaces introduced to maximize alignments. desa, Amino acid sequence encoding a Δ12 desaturase from *Synechocystis* P6803 (Wada et al., 1990); fad7, fad3, fad2, amino acid sequences of *Arabidopsis* genes encoding, respectively, plastid ω3, ER ω3, and an ER 18:1 desaturase.

located within the His-rich motifs. The multiple alignment also reveals that the ω6 desaturases have characteristics that readily distinguish them from ω3 desaturases. In particular, by comparison with the ω3 desaturases, the ω6 desaturases contain seven or eight additional amino acid resicues beginning at residue 139, lack two amino acid residues at positions 326 to 327, and have four to six additional amino acids beginning at residue 250.

## RFLP Mapping of the *fad6* Gene

Since *Arabidopsis* is known from genetic analysis to have eight different desaturases (Somerville and Browse, 1991), including two closely related isoforms of chloroplast ω3 desaturases (S. Gibson, V. Arondel, K. Iba, C.R. Somerville, unpublished data), we sought to establish that the insert in p313–33 genetically mapped to the same location as the *fad6* mutation. This was performed by examining the progeny of crosses made between the *fad6* mutant and the wild-type line of the Niederzenz ecotype. An RFLP was found between the two lines when *Eco*RV-digested total DNA from the two lines was hybridized with the insert from p313–33. The p313–33 cDNA was then used to probe total DNA isolated from $F_3$ families, resulting from a cross between the lines, which were previously scored as homozygous or heterozygous for the *fad6* mutation on the basis of leaf fatty acid composition. In each of the 41 $F_3$ families examined, the RFLP pattern agreed with the results of the fatty acid analysis (data not shown). Because of the relatively small numbers of $F_3$ families used, our results would not be expected to distinguish two genes that were separated by less than about 1.5 map units. However, since the other known *fad* mutations are located at least 13 map units from the *fad6* mutation (Hugly et al., 1991; Arondel et al., 1992), these results were adequate to exclude the possibility that p313–33 encoded any of the other known *fad* loci. Thus, within the limits of resolution of this experiment, the cDNA insert encoded in p313–33 co-segregated with the *fad6* mutation, providing additional evidence that the 313–33 cDNA encodes the *fad6* gene.

## Complementation Analysis

Direct evidence that the open reading frame contained in the p313–33 cDNA encoded an ω6 desaturase was provided by complementation of the *fad6* mutant line of *Arabidopsis*. The biochemical phenotype of the *fad6* mutant is characterized by the virtual absence of trienoic fatty acids derived from the prokaryotic pathway and an increased accumulation of 16:1 and 18:1 monounsaturated species (Browse et al., 1989). To test for complementation, the 1.7-kb full-length cDNA insert in p313–33 was subcloned into the binary Ti vector pBI121 under transcriptional control of the 35S promoter and used to produce transgenic plants of the *fad6* mutant line using the in vivo method of transformation (Chang et al., 1994). A total of 17 kanamycin-resistant transgenic seedlings were obtained from approximately 148,000 seeds plated. The fatty acid composition of the rosette leaves of these plants showed a partially or fully restored accumulation of 16:3 fatty acids, indicating complementation of the primary biochemical deficiency (Fig. 2). The extent of com-

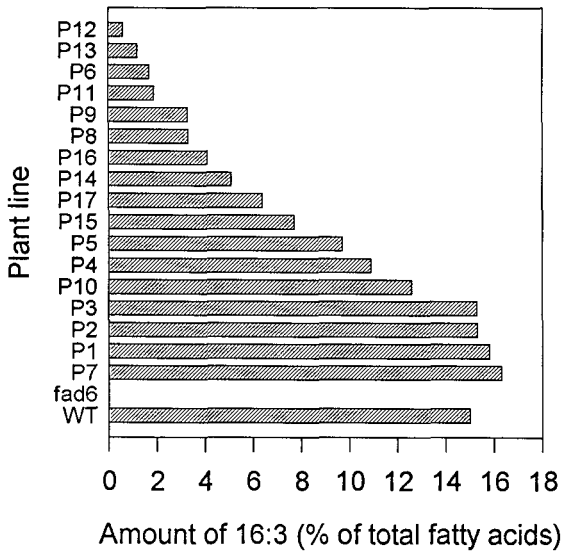


**Figure 2.** Amount of 16:3 in rosette leaves of complemented transgenic *Arabidopsis* mutant lines. WT, Wild type.

plementation could be readily assessed because the level of 16:3 in the *fad6* mutant line is below the limit of detection, thereby permitting trace amounts of 16:1 desaturase activity to be detected in the transgenics. Each of the 17 transformants revealed a significant and readily measurable amount of 16:3, with levels ranging from those found in the wild type (about 15% of total fatty acids) to a low extreme of less than 1% of total fatty acids. Four of the transformants containing the *fad6* gene showed wild-type levels of 16:3, whereas the remaining 13 had lower amounts.

Significant leaf-to-leaf variation in fatty acid composition was frequently observed among different rosette leaves from the same transgenic plants. This contrasts with the high degree of reproducibility in fatty acid composition found in nontransformed plants. The basis for this was examined by comparing the fatty acid compositions of single leaves from fully expanded rosettes, the first set of cauline leaves, and the top-most cauline leaves of single 26-d-old plants (Table I). The various stages of leaf material from the wild type showed a slight decrease in 16:3 and a small elevation in the percentages of 16:1 and 18:1 fatty acids when younger leaves were compared to more mature leaves. However, in the transgenic *fad6* plants, the developmental stage of the leaf had a pronounced effect on both 16:1 and 18:1 accumulation and in the amount of 16:3 detected. The distinct leaf stages exhibited the most apparent influence in diminishing the 16:3 fatty acid proportion in the weakly complemented transgenic lines P5 and P13.

Plant-to-plant variation in the fatty acid composition of the progeny of the transgenic lines was also observed (results not presented). This is thought to be due to segregation of multiple T-DNA inserts or to phenomena related to co-suppression. Analyses of these effects must await the development of lines that are homozygous for the T-DNA insertions.

## Expression Analysis

To determine whether significant control of *fad6* gene expression occurs at the transcriptional level, a northern analysis was performed. Total RNA was prepared from leaf tissue of wild type and the LK3 mutant at various times after germination and probed with the insert from p313–33. To compare the level of expression of the *fad6* gene in different samples, the blots were stripped and reprobed with the constitutively expressed eIF4a gene (Taylor et al., 1993),

**Table I.** *Fatty acid analysis of transgenic plants*

Leaves at different stages of development were individually assayed to determine the effect on *fad6* expresion. Values are the means ± SD of three leaves for each stage.

| Sample | Fatty Acid | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 16:0 | 16:1(c+t) | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 | Other |
| | *mol%* | | | | | | | |
| Wild-type Columbia | | | | | | | | |
| Rosette | 15.3 ± 1.7 | 5.3 ± 1.7 | 16.1 ± 2.4 | 2.8 ± 0.5 | 5.3 ± 1.5 | 9.0 ± 0.7 | 35.2 ± 2.8 | 13.8 |
| Bottom cauline | 16.8 ± 1.6 | 5.5 ± 2.3 | 13.4 ± 4.2 | 3.6 ± 1.2 | 5.0 ± 0.8 | 8.8 ± 0.4 | 36.6 ± 4.4 | 10.0 |
| Top cauline | 17.1 ± 1.4 | 8.4 ± 1.2 | 11.7 ± 3.7 | 4.4 ± 1.0 | 6.4 ± 1.9 | 9.2 ± 0.5 | 34.8 ± 3.9 | 13.3 |
| LK3 (*fad6*) | | | | | | | | |
| Rosette | 18.1 ± 0.4 | 14.6 ± 1.5 | 0.0 | 3.1 ± 0.7 | 15.5 ± 0.4 | 10.5 ± 0.9 | 32.7 ± 1.4 | 8.6 |
| Top cauline | 18.7 ± 0.6 | 14.1 ± 0.7 | 0.0 | 3.4 ± 0.6 | 14.9 ± 0.7 | 11 ± 1.2 | 32.4 ± 1.6 | 8.9 |
| Transgenic P1 | | | | | | | | |
| Rosette | 17.1 ± 1.8 | 7.7 ± 0.6 | 9.8 ± 6.0 | 3.7 ± 0.3 | 7.9 ± 4.9 | 8.1 ± 0.1 | 37.3 ± 3.5 | 12.0 |
| Bottom cauline | 17.1 ± 0.9 | 8.2 ± 2.4 | 10.1 ± 6.3 | 2.8 ± 0.3 | 8.5 ± 3.6 | 8.4 ± 1.2 | 39.1 ± 2.3 | 8.6 |
| Top cauline | 15.2 ± 1.2 | 12.6 ± 0.8 | 4.4 ± 0.7 | 4.0 ± 0.1 | 12.1 ± 0.7 | 7.3 ± 1.0 | 32.5 ± 0.2 | 15.9 |
| Transgenic P5 | | | | | | | | |
| Rosette | 15.8 ± 1.5 | 13.6 ± 1.0 | 4.5 ± 2.5 | 2.6 ± 0.2 | 12.3 ± 1.2 | 8.2 ± 0.3 | 37.6 ± 0.3 | 8.0 |
| Top cauline | 17.4 ± 0.6 | 14.8 ± 1.2 | 0.3 ± 0.5 | 3.3 ± 0.5 | 17.0 ± 0.5 | 8.5 ± 0.2 | 33.2 ± 0.9 | 8.8 |
| Transgenic P13 | | | | | | | | |
| Rosette | 16.8 ± 1.4 | 14.9 ± 1.0 | 3.6 ± 1.0 | 2.1 ± 0.0 | 13.8 ± 0.6 | 9.5 ± 0.8 | 35.3 ± 0.7 | 6.1 |
| Top cauline | 16.5 ± 0.9 | 13.7 ± 1.0 | 0.0 | 4.1 ± 0.4 | 15.8 ± 1.6 | 8.6 ± 0.9 | 30.7 ± 2.7 | 14.7 |

which served as a standard. The level of expression of *fad6* was then expressed as the ratio of the intensity of the hybridization signal from RNA probed with the *fad6* gene to the intensity of the hybridization signal from the same filter probed with the eIF4a gene (Table II).

No significant difference was observed in the amount of *fad6* mRNA in the mutant or the wild type. The presence of normal levels of *fad6* mRNA in leaves of the *fad6* mutant line indicates that the mutation leading to the deficiency in plastid ω6 activity does not influence the level of *fad6* mRNA accumulation. Therefore, there does not appear to be a mechanism that senses the degree of unsaturation of the chloroplast membranes and alters the expression of the gene in an attempt to compensate for the effects of the mutation. Although no major changes were detected in *fad6*-specific mRNA levels in wild-type tissues in this analysis, this does not rule out the possibility of transcriptional controls acting under other conditions or of other modes of regulation acting at the posttranscriptional level that could play a role in modulating *fad6* gene expression.

Comparison of the levels of *fad6* mRNA to those of the constitutively expressed control gene eIF4a indicated that in both mutant and wild type there was a progressive decline in the amount of *fad6* mRNA during the later stages of growth (Table II). This presumably reflects reduced demand for membrane biogenesis in fully expanded leaves compared with expanding leaves.

## DISCUSSION

The identity of the cDNA clone p313–33 as corresponding to the *Arabidopsis fad6* gene was demonstrated by the ability of the cDNA to fully complement the *fad6* mutant and by the fact that it genetically mapped to the same region of chromosome 4 as the *fad6* locus. In addition, the high degree of sequence similarity between *fad6* and the *Synechocystis desA* gene, which has been shown to be an 18:1 desaturase (Wada et al., 1990, 1993), substantiates the identity of the *fad6* gene product as a desaturase. The high degree of similarity of the *fad6* sequence to the cyanobacterial *desA* gene presumably reflects the evolutionary origin of the *fad6* desaturase gene. The unexpectedly low level of sequence homology to the recently isolated microsomal *fad2* ω6 desaturase, which catalyzes an 18:1 desaturation reaction analogous to the *fad6* desaturase, indicates that at least some equivalent steps in the desaturation pathway are catalyzed by proteins that appear to have diverged early in plant evolution (Okuley et al., 1994). This contrasts with the discovery of a family of ω3 desaturases that are very similar in their primary sequence despite the different cellular locations in which they are presumed to reside (Arondel et al., 1992; Iba et al., 1993; Yadav et al., 1993). There is no obvious functional explanation for the comparatively low degree of sequence similarity between *fad6* and *fad2*.

Previous genetic studies with heterozygotes resulting from a cross between the *fad6* mutant and wild-type lines indicated that the proportion of 16:3 in leaves of the heterozygote was very similar to the level found in the wild type (Browse et al., 1989). This was interpreted as evidence that the level of desaturase activity in the wild type is normally present in excess or that some mechanism exists to up-regulate the desaturase gene to maintain a constant level of 16:3 and 18:3 in chloroplast membranes. The factors that regulate fatty acid desaturation are not known in higher plants. However, when the degree of unsaturation of cyanobacterial membranes is experimentally decreased by catalytic hydrogenation, the cyanobacterial cells respond by rapid induction of transcription of a desaturase gene (Vigh et al., 1993). This suggests that these species possess a mechanism that senses some aspect of the physical properties of the membrane and transduces that information to a mechanism that controls gene expression. If a similar system existed in higher plants to control expression of the *fad6* gene, we would expect to observe increased levels of *fad6* mRNA in the mutant as compared to the wild type. The fact that we do not see any difference in expression of the *fad6* gene in the mutant and wild type indicates that a comparable mechanism is not operative for the *fad6* gene.

Measurements of gene expression at different stages of plant development revealed that the steady-state level of *fad6* mRNA decreases during the later stages of plant development. This is consistent with the idea that desaturase expression is highest in expanding leaves where the rate of lipid synthesis is probably elevated to accommodate synthesis of new membranes. Indeed, transgenic *fad6* mutant plants containing the *fad6* cDNA under the transcriptional control of the CaMV 35S promoter exhibited subnormal levels of trienoic fatty acids in young, rapidly growing leaf tissue but had normal levels of fatty acids in fully expanded tissues. This probably reflects insufficient levels of CaMV 35S promoter activity in the young tissues where the rate of membrane synthesis may outpace the rate of FAD6-catalyzed desaturation.

The characterization of the *fad6* desaturase gene extends the rapidly growing number of cloned genes encoding plant membrane-bound desaturases. While this article was in review, Hitz et al. (1994) reported the isolation of ω6 desaturase genes from soybean and rape using a partial cDNA clone from *Arabidopsis*. Expression of the rape cDNA in *Synechocystis* resulted in synthesis of 16:2 and 18:2. The perfectly deduced amino acid sequence homology between the partial

**Table II.** *Steady-state level of fad6 mRNA in leaves of wild type (WT) and the fad6 mutant at different stages of development*

| Sample/Age (d) | *fad6*/eIF4a Ratio[a] |
|---|---|
| WT/13 | 0.96 |
| WT/20 | 0.81 |
| WT/26 | 0.37 |
| WT/29 | 0.40 |
| LK3/13 | 1.11 |
| LK3/20 | 1.09 |
| LK3/26 | 0.36 |
| LK3/29 | 0.41 |

[a] For each lane on a northern blot, the amount of radioactivity that hybridized to the *fad6* transcript was divided by the amount of radioactivity that hybridized to the eIF4a transcript, as measured by a phosphorimager. The blots were probed first with *fad6*, quantitated, and stripped and then reprobed with the eIF4a cDNA.

*Arabidopsis* cDNA and the sequence reported here indicates that they are the same genes. The fatty acid desaturase encoded by the *fad6* cDNA represents the fifth gene from *Arabidopsis* encoding a distinct desaturase. Since the hydrophobic nature of the membrane-bound desaturases has hampered direct biochemical investigations of these enzymes, the collection of genes encoding a variety of desaturases provides useful, new experimental tools for understanding this important class of enzymes.

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