Arabidopsis Mutants Deficient in Polyunsaturated Fatty Acid Synthesis

BIOCHEMICAL AND GENETIC CHARACTERIZATION OF A PLANT OLEOYL-PHOSPHATIDYLCHOLINE DESATURASE*

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The overall fatty acid composition of leaf lipids in a mutant of Arabidopsis thaliana was characterized by reduced levels of polyunsaturated 18-carbon fatty acids and an increased proportion of oleate as a consequence of a single recessive nuclear mutation. Quantitative analysis of the fatty acid composition of individual lipids demonstrated that all the major phospholipids of the extrachloroplast membranes are affected by the mutation, whereas the chloroplast lipids show fatty acid compositions only slightly different from those of wild type plants. These results are consistent with the parallel operation of two pathways of lipid synthesis in plant leaf cells (the prokaryotic pathway in the chloroplast and the eukaryotic pathway in the endoplasmic reticulum) and with genetic evidence (Browse, J., Kunst, L., Anderson, S., Hugly, S., and Somerville, C. R. (1989) Plant Physiol 90, 522–529) that an independent 18:1/16:1 desaturase operates on chloroplast membrane lipids. Direct enzyme assays confirmed that the mutant plants are deficient in the activity of a microsomal oleoyl-phosphatidycholine desaturase and demonstrated that this desaturase is the major enzyme responsible for the synthesis of polyunsaturated phospholipids. Despite this deficiency in 18:1-desaturase activity, mutant plants contained relatively high levels of 18:3 in their leaf phospholipids. This finding is interpreted as additional evidence that considerable two-way exchange of lipid occurs between the chloroplast and endoplasmic reticulum and that this exchange allows the chloroplast desaturases to provide lipids containing 18:3 to the extrachloroplast compartment, thus partially alleviating the deficiency in 18:1 desaturase activity.

The biochemistry and cellular organization of fatty acid and glycerolipid synthesis in plant cells is quite different from the animal paradigm (Wakil et al., 1983; Dawidowicz, 1987; Bishop and Bell, 1988; Browse and Somerville, 1991). The plant fatty acid synthase is located in the chloroplasts (or other plastids) and is composed of individual proteins (Shi

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makata and Stumpf, 1982). Most of the 16:0-ACP1 produced by this Type II synthase is elongated to 18:0-ACP and then desaturated in the chloroplast stroma by a soluble desaturase (McKeon and Stumpf, 1982; Shanklin and Somerville, 1991), so that 16:0-ACP and 18:1-ACP are the primary products of chloroplast fatty acid synthesis. These products are used by two distinct pathways for the biosynthesis of membrane glycerolipids and the associated production of polyunsaturated fatty acids. The "prokaryotic" pathway (Roughan et al., 1980) located in the chloroplast inner envelope uses 18:1-ACP and 16:0-ACP for the sequential acylation of sn-glycerol-3-phosphate. Because of the substrate specificities of the chloroplast acyltransferases (Frentzen et al., 1983) the phosphatidic acid formed has 18:1 at the sn-1 position and 16:0 at the sn-2 position. This phosphatidic acid is used directly for the synthesis of phosphatidylglycerol and, via diacylglycerol, as a precursor for the synthesis of the other major chloroplast lipids monogalactosyldiacylglycerol, digalactosyldiacylglycerol, and sulfoquinovosyldiacylglycerol (Browse and Somerville, 1991). The "eukaryotic" pathway begins with the hydrolysis of 16:0-ACP and 18:1-ACP followed by the export of these fatty acids to the cytoplasm as CoA thioesters. The CoA esters are used for the synthesis of 16:0/18:1- and 18:1/18:1-phosphatidic acid mainly in the endoplasmic reticulum (Frentzen, 1990). This phosphatidic acid gives rise to the phospholipids, including phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, that are characteristic of the various extrachloroplast membranes (Moore, 1982; Mudd and Datko, 1989). In addition, however, the diacylglycerol moiety of phosphatidylcholine is returned to the chloroplast envelope where it contributes to the production of thylakoid lipids (Roughan and Slack, 1982; Browse et al., 1986b). In oil-accumulating tissues such as the cotyledons of oilseed plants, the eukaryotic pathway provides the lipid precursors of triacylglycerol synthesis (Browse and Somerville, 1991).

In all plant tissues, the major glycerolipids are first synthesized using only 16:0 and 18:1 acyl groups. Subsequent desaturation of the lipids to the highly unsaturated forms typical of the membranes of plant cells is carried out by membrane-bound desaturases of the chloroplast (Roughan et al., 1979; Browse et al., 1985, 1986a, 1989; Kunst et al., 1989; Schmidt and Heinz, 1990) and the endoplasmic reticulum (Slack et al., 1976; Stymne and Appelqvist, 1978; Browse and Slack, 1981). Characterization of these desaturases by traditional biochemical approaches has been very limited, because it has not been possible to solubilize and purify them. Instead, the increased understanding of the mechanisms and regulation of the

1 The abbreviations used are: ACP, acyl carrier protein; EGTA, (ethylenebis(oxyethylenenitriilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
chloroplast desaturases has been accomplished by the character-
ization of four classes of Arabidopsis mutants, each one de-
cicient in a specific desaturation step (Browse et al., 1985,
1986, 1988; Kestler et al., 1989).

In this paper, we report the characterization of mutants
defective in desaturation at 18:1 on the eukaryotic pathway.
The combined use of biochemical and genetic approaches that
is possible with Arabidopsis has permitted elucidation of the
relationship of this desaturase to overall lipid synthesis by
leaf cells and a description of the effect of the mutation in
altering the balance between metabolism in the prokaryotic
and eukaryotic pathways.

MATERIALS AND METHODS

Plant Material and Growth Conditions—The lines of Arabidopsis
thaliana (L.) Heynh. used were all descended from the Columbia wild
type with the exception of the mutant line ALG3, which is in the
Landsberg erecta background. Mutants were isolated from M2 popu-
lations obtained after mutagenesis with ethyl methanesulphonate (Hecht et al., 1985) by spraying plates with 0.001% primuline and min-
ing plates at 22 °C for 1 week. Tissue extracts of appropriate age were analyzed for fatty acid methyl esters as described above.

Chloroplasts were isolated according to Browse et al. (1988) by washing the leaves and other tissues with 0.26 M NaCl containing 1% sucrose. Five sterile seeds were placed in 35 ml of medium in a 125 ml Erlenmeyer flask, and plants were grown on a rotary shaker (100 rpm) at 25 °C under light (40 pmol quanta/m2/s fluorescent illumination, 16-h photoperiod).

We have followed the recommended nomenclature (Browse et al.,
1988) in assigning symbols to the genetic loci identified in this report.
The wild type allele at a locus is indicated by the use of italicized
capital letters in the genotype descriptor.

Fatty Acid and Lipid Analysis—The overall fatty acid composition
of leaves and other tissues was determined by heating samples at
80 °C in 1 ml of 2.5% (v/v) H2SO4 in methanol for 90 min in screw-
capped tubes. After the addition of 1.5 ml of 0.3% NaCl solution and
1 ml of hexane, fatty acids were extracted into the organic phase by
shaking and the tubes were centrifuged at low speed. Samples 1 μl
of the organic phase were separated by gas chromatography on a 15-
m × 0.53-mm Supelcoxwax column (Supelco, Bellefonte, PA) and
quantified using a flame ionization detector. The gas chromatograph
was programmed for an initial temperature of 150 °C for 3 min
followed by an increase of 15 °C/min to 210 °C; this final temperature
was maintained for a further 12 min.

Plant tissues were killed rapidly by immersion in liquid nitrogen
and then ground under liquid nitrogen in a precooled mortar and
pestle. Typically, 1 g of tissue sample was transferred to a screw-
capped centrifuge tube with 12 ml of chloroform/methanol/formic
c acid (10:1:1, by volume) and stored overnight at -20 °C. This pro-
cedure precluded the formation of phosphatidylmethanol which can
be formed by the action of endogenous phospholipase D when plant
material is killed by immersion in liquid nitrogen, and the lipids were
extracted and separated by thin layer chromatography as described
above. The lipids from one chromatographic separation were trans-
fected to scintillation vials containing Biodegradable Counting Scint-
illant (Amersham Corp.), and radioactivity was determined by scin-
tillation counting. Lipids were separated by preparative thin layer
chromatography as described above, and the fatty acid methyl esters
were separated by argentation thin layer chromatography (Browse et al.,
1986) using silica gel G plates that had been dipped in a solution of
5% (w/v) AgNO3 in acetonitrile. Individual bands were identified by
autoradiography and scraped from the plates, and the radioactivity
in them was determined by scintillation counting.

Preparation of Chloroplast and Extrachloroplast Membrane Fra-
tions—Leaf slices (1 mm) were used as the starting material for
protoplast preparation. Production, purification, and rupture of pro-
toplasts were carried out as described by Somerville et al. (1981) with
minor modifications. Chloroplasts were pelleted by centrifugation
for 4 min at 800 × g in a swing-out rotor, and then the essentially
colorless supernatant was centrifuged for 60 min at 105,000 × g to
gel the extrachloroplast membranes. Each pellet was resuspended
in a small volume of buffer and extracted by the method of Bligh
and Dyer (1959). The procedures used in preparing mesophyll protoplasts
resulted in a small amount of migration of fatty acids between
different lipids (Browse et al., 1988). In the results reported here,
correction for this artifact has been made by the procedures we have
used previously (Browse et al., 1986a, 1988).

Desaturase Assays—Roots from 2-week-old plants grown in liquid
 culture were rinsed with ice-cold deionized water and briefly dried
on a paper towel. A sample (0.5–1.0 g, fresh weight) of roots was ground
using a mortar and pestle in 5 ml of homogenization buffer containing
100 mM HEPES/KOH, pH 7.8, 0.4 M sorbitol, 2.5 mM EDTA, 2.5
mM EGTA, 5 mM MgCl2, 1% (w/v) polyvinylpyrrolidone, 0.1%
(w/v) bovine serum albumin, 3 mM glutathione, and 2000 units/ml
 catalase. The crude homogenate was filtered through two layers of
Miracloth (Calbiochem) and then centrifuged at 10,000 × g for 20
min. The resulting supernatant was centrifuged at 100,000 × g for 60
min, and the resulting microsomal pellet was resuspended in the
homogenization buffer in the absence of polyvinylpyrrolidone.

Desaturase assays were carried out at 25 °C in a shaking water bath.
Each reaction mixture (1 ml, final volume) contained microsomal
preparations equivalent to 150 g of phosphatidylcholine, 70 mM
HEPES/KOH, pH 7.8, 1% (w/v) bovine serum albumin, 3 mM glutathione, and 2000 units/ml catalase. The crude homogenate was filtered through two layers of Miracloth (Calbiochem) and then centrifuged at 10,000 × g for 20
min. The resulting supernatant was centrifuged at 100,000 × g for 60
min, and the resulting microsomal pellet was resuspended in the
homogenization buffer in the absence of polyvinylpyrrolidone.

Desaturase assays were carried out at 25 °C in a shaking water bath.
Each reaction mixture (1 ml, final volume) contained microsomal
membranes equivalent to 5 μmol of phosphatidylcholine, 70 mM
HEPES/KOH, pH 7.8, 1% (w/v) bovine serum albumin, 3,000 units/ml
catalase, 8 mM ATP, 0.25 mM CoA, 2.5 mM NADH, and 5.6 kBq
14C]CoA 2.2 GBq/mmol; Du Pont-New England Nuclear). Reactions
were started by the addition of labeled substrate and terminated by
adding 6 ml of chloroform/methanol (1:2, v/v). Lipids were
extracted following the addition of 0.2 M H2PO4, 1 M KCl (Hajra,
1974). Lipids were recovered in the chloroform phase, dried under
N2 and redissolved in 0.5 ml of chloroform. For large tissue samples,
appropriately larger volumes of extraction solvents were used.

Individual lipids were purified from the extracts either by two-
dimensional thin layer chromatography on silica gel G (Nichols, 1964)
or by one-dimensional thin layer chromatography on (NH4)2SO4-
impregnated silica gel G by the method of Khan and Rhoads (1977).
Silica gel G plates (Sil250) were from J. T. Baker Inc. The (NH4)2SO4-
impregnated plates were prepared by dipping the plates in 0.15 M
(NH4)2SO4 and drying them at room temperature before activation
(75–90 min at 110 °C). Lipids were located by staining with I2 or by
spraying the plates with solution of 0.001% prunin in 80% acetonitrile,
followed by visualization under ultraviolet light. In order to
determine the fatty acid composition and the relative amounts of
individual lipids, the silica gel from each lipid spot was transferred to
a screw-capped tube, an appropriate amount of 17:0 fatty acid was
added as an internal standard, and fatty acid methyl esters were
prepared and analyzed as described above.

Fatty acid composition at the sn-1 and sn-2 positions of individual
lipids were determined by lipase digestion. After thin layer
chromatography, lipids were extracted from the silica gel by the
method of Bligh and Dyer (1959). The protocol for digestion with
Rhizopus lipase (Sigma), including purification of the lyso derivatives
and fatty acids was that described by Siebertz and Heinz (1977),
except that 50 mM H2SO4 was added to the buffer used for lipase
digestion to minimize intramolecular acyl transfer on the lyso-lipids
produced. Fatty acid methyl esters were formed from untreated lipids,
lyso-lipids, and fatty acids as described above, and the fatty acid
composition of each compound was determined by gas chromato-
graphic analysis.

RESULTS

Genetic Analysis—The mutant lines JB9 and JB12 were isolated
without selection by screening independently muta-

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...lipids were derivatized using 2.5% H2SO4 in methanol, and the fatty acid methyl esters were analyzed by gas chromatography of fatty acids derived from small tissue samples (Browse et al., 1985). These lines were retained because leaves exhibited an increased amount of 18:1 and decreased amounts of 18:2 and 18:3 fatty acids, as compared with wild type leaves (Table I). The mutant plants were essentially indistinguishable from the wild type when grown under standard environmental conditions (22 °C, 150 μmol quanta/m2/s continuous fluorescent illumination, 60–70% relative humidity). Two additional mutant lines, 4A5 and AL63, which exhibited similar fatty acid profiles, were kindly provided by Drs. D. James and H. Dooner (DNA Plant Technologies, Oakland, CA) and Dr. L. Kunst (Plant Biotechnology Institute, Saskatoon, Canada), respectively. Reciprocal crosses between the four available lines all produced progeny whose fatty acid composition was the same as both parents (data not shown). The lack of genetic complementation in these experiments indicates that the four lines contain mutations at the same locus. Therefore we characterized only one of the lines, JB9, in detail.

To determine the genetic basis of the alteration in lipid fatty acid composition, reciprocal crosses were performed between JB9 plants and wild type Arabidopsis. Leaves of the F1 progeny showed a slightly increased level of 18:1 as compared with the wild type (Table I), suggesting that the wild type allele is incompletely dominant. The frequency of individuals with the mutant phenotype in the F2 population resulting from self-fertilization of F1 plants was also measured by gas chromatography of leaf samples. Of 131 F2 plants analyzed, 37 had fatty acid compositions similar to the original JB9 line, whereas the remaining 94 individuals had leaf fatty acid compositions similar to those of the wild type or the F1 hybrid. This pattern of segregation is a good fit (χ² = 0.57, p > 0.4) to the 3:1 hypothesis and indicates that the altered fatty acid composition is due to a single nuclear mutation at a locus we have designated fad2. Consequently, the four lines JB9, 4A5, JB12, and AL63 were designated, respectively, fad2-1, fad2-2, fad2-3, and fad2-4.

Biochemical Characterization—In leaves of the mutant, decreased levels of 18:2 and 18:3 fatty acids are accompanied by an increase in 18:1 (Table I). In plants, 18:2 and 18:3 are synthesized by sequential desaturation of 18:1. Thus, the simplest explanation of these observations is that the mutant is deficient in the activity of a fatty acid desaturase which, in the wild type, introduces a double bond at the ω-6 position of 18-carbon acyl groups. We have previously described an Arabidopsis mutant fadC deficient in the activity of a chloroplast ω-6-fatty acid desaturase that acts on both 18:1 and 16:1 acyl groups esterified to glycerolipids of the chloroplast membranes (Browse et al., 1989). The fatty acid composition of the fad2 mutants is quite different from that of fadC plants. In particular, the relative proportions of 16:1 and 16:3 fatty acids are largely unaltered in the JB9 line when compared with wild type Arabidopsis (Table I). From these data, it is more likely that the fad2 gene controls desaturation of membrane lipids at sites outside the chloroplast. This conclusion is consistent with previous findings that the roots and seeds of fad2 plants also have decreased levels of 18:2 and 18:3 fatty acids, as compared with wild type and concomitant increases in the level of 18:1 (Lemieux et al., 1990).

Fatty Acid Composition of Individual Lipids—Considerably more information about the desaturation step controlled by the fad2 gene product can be deduced from an analysis of the fatty acid composition of individual lipids extracted from leaf tissue of wild type and mutant plants (Table III). The data show that there are no major changes in the proportions of the various polar lipids in the mutant. However, the three phospholipids that are components of the extrachloroplast membranes of plant cells (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol) all show a marked increase in the level of 18:1 and a concomitant decrease in the amount of 18:2 + 18:3. In both phosphatidycholine and phosphatidylethanolamine, for example, 18:1 is increased more than 12-fold over wild type levels. Those lipids that are found predominantly in the chloroplast (monogalactosyldiglycerol, digalactosyldiglycerol, sulfoquinovosyldiglycerol, and phosphatidylglycerol) show very much smaller increases in 18:1.

In wild type Arabidopsis, almost 50% of the chloroplast lipids are derived from phosphatidycholine of the endoplasmic reticulum via the eukaryotic pathway (Browse et al., 1986b). The fact that the chloroplast lipids are still highly unsaturated in the mutant can be attributed to the presence of the independent 18:1/16:1 desaturase in the chloroplast. Genetic and biochemical evidence indicates that the chloroplast desaturase can act on 18:1 esterified to either the sn-1 or sn-2 position of all the major chloroplast glycerolipids (Browse et al., 1989). Thus, in wild type and fadC plants, the extrachloroplast desaturase(s) are responsible for 18:2 synthesis on the eukaryotic pathway (Browse et al., 1986b, 1989), but it appears that in fad2 plants 18:1 containing lipids that re-enter the chloroplast can be converted to 18:2 by the desaturase regulated by the FADC locus. Recently, it has been demonstrated that the chloroplast 18:1/16:1 desaturase is located in the chloroplast envelope (Schmidt and Heinz, 1990).

To directly measure the consequences of the fad2 mutation on chloroplast and extrachloroplast fractions, we prepared mesophyll protoplasts from leaves of wild type and JB9 plants. Gently ruptured protoplasts were used as the source of highly pure chloroplast and extrachloroplast membrane fractions. The extrachloroplast membranes from the mutant contained 49% 18:1 compared with 39% in the wild type. In the chloroplast fraction, 18:1 increased from 2.5% in the wild type to 10.6% in the mutant. These data are clearly in general agreement with the results shown in Table II. A further important observation made from the data in Table II is that phosphatidycholine (and phosphatidylethanolamine and phosphatidylinositol) from leaves of fad2 plants contained moderately high levels of 18:3. This was true of the fad2–2 and fad2–3 alleles also (data not presented). In leaf mesophyll cells, approximately 40% of the total cell phosphatidycholine is found in the chloroplasts (Browse et al., 1986b), where it is

| Table I | Fatty acid composition of total leaf lipids from wild type (WT) and mutant Arabidopsis |
|---------|-----------------------------------------------------|
| Homozygous (fad2-1) and heterozygous (F1) of the cross fad2-1 X WT | mutant Arabidopsis |
| | Fatty acid | WT | F1 (fad2-1 X WT) | fad2-1 |
| | | | | pmol quanta/m2/s |
| 16:0 | 15.0 ± 0.13 | 15.0 ± 0.10 | 12.0 ± 0.08 |
| 16:1-cis | 1.1 ± 0.04 | 1.2 ± 0.04 | 1.3 ± 0.09 |
| 16:1-trans | 2.7 ± 0.03 | 2.9 ± 0.13 | 3.1 ± 0.18 |
| 16:2 | 1.1 ± 0.03 | 1.2 ± 0.05 | 1.0 ± 0.11 |
| 16:3 | 13.8 ± 0.19 | 15.5 ± 0.25 | 16.8 ± 0.49 |
| 18:0 | 1.0 ± 0.04 | 1.1 ± 0.08 | 0.6 ± 0.04 |
| 18:1 | 3.5 ± 0.14 | 4.9 ± 0.15 | 20.9 ± 0.95 |
| 18:2 | 15.7 ± 0.17 | 13.4 ± 0.13 | 3.8 ± 0.11 |
| 18:3 | 46.0 ± 0.2 | 44.7 ± 0.16 | 40.5 ± 0.67 |

* = S.E. (n = 24).
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TABLE II
Fatty acid composition of individual leaf lipids from wild type (WT) and mutant Arabidopsis grown at 22°C

Lipids were extracted from leaf material of 15-day-old plants frozen in liquid nitrogen. The polar lipids derived from 0.1 g, fresh weight, of tissue were separated by thin layer chromatography, and fatty acid compositions were determined by gas chromatography of methyl esters obtained by derivatization of individual lipids using 2.5% H2SO4 in methanol. Lipids were quantified by adding 5 μg of 17:0 as an internal standard prior to derivatization. For details, see “Materials and Methods.” Data are mole %.

| % of total polar lipids | 16:0 | 16:1 | 16:2 | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 |
|-------------------------|------|------|------|------|------|------|------|------|
| Phosphatidylcholine      |      |      |      |      |      |      |      |      |
| WT                      | 17.2 | 20.6 | 0.6  | —    | —    | 2.7  | 4.4  | 38.8 |
| fad2-1                  | 19.9 | 12.6 | 1.5  | —    | —    | 0.9  | 55.2 | 8.3  |
| Phosphatidylethanolamine|      |      |      |      |      |      |      |      |
| WT                      | 10.3 | 31.2 | —    | —    | —    | 3.4  | 3.3  | 43.0 |
| fad2-1                  | 9.1  | 22.9 | —    | —    | —    | 1.5  | 43.0 | 12.0 |
| Phosphatidylglycerol    |      |      |      |      |      |      |      |      |
| WT                      | 3.5  | 43.5 | —    | —    | —    | 5.2  | 4.3  | 27.0 |
| fad2-1                  | 3.1  | 37.6 | 1.1  | —    | —    | 3.1  | 31.2 | 7.1  |
| Sulfoquinovosyldiacylglycerol | 2.5 | 43.2 | —    | —    | —    | 3.7  | 5.3  | 10.4 |
| fad2-1                  | 2.0  | 44.7 | —    | —    | —    | 3.0  | 10.3 | 6.8  |
| Phosphatidylglycerol    |      |      |      |      |      |      |      |      |
| WT                      | 10.1 | 20.7 | 33.5 | —    | —    | 1.8  | 6.0  | 12.5 |
| fad2-1                  | 11.4 | 22.3 | 31.5 | —    | —    | 1.8  | 10.2 | 8.8  |
| Digalactosyldiacylglycerol |     |      |      |      |      |      |      |      |
| WT                      | 14.2 | 13.6 | 0.3  | 0.6  | 2.1  | 1.1  | 1.3  | 5.0  |
| fad2-1                  | 12.9 | 15.3 | 0.5  | 1.0  | 3.7  | 1.0  | 5.0  | 6.6  |
| Monogalactosyldiacylglycerol | 42.3 | 1.5  | 1.5  | 1.3  | 30.6 | 0.2  | 1.5  | 2.4  |
| fad2-1                  | 41.6 | 1.6  | 2.3  | 1.9  | 37.5 | 0.2  | 3.9  | 2.5  |

* Dashes indicate that the acyl group was not detected.
* Δ3-trans isomer.

TABLE III
Fatty acid compositions of phosphatidylcholine from different sources

Chloroplast and extrachloroplast fractions were derived from leaf mesophyll protoplasts of wild type, fadC and fad2 plants, and the lipids were extracted from them as described under “Materials and Methods.” Extraction of root tissue, purification, and fatty acid analysis of phosphatidylcholines were carried out as described in Table II. (WT, wild type; data are mole %).

| Leaf mesophyll cells | Root tissue |
|---------------------|-------------|
|                     | Chloplast    | Extrachloroplast | WT | fad2 |
|                     | fadC | fad2 | fadC | fad2 |     |
| 16:0                | 21.4 | 28.1 | 13.3 | 27.3 | 32.8 | 15.6 | 26.8 | 9.7  |
| 18:0                | 28.0 | 4.6  | 0.9  | 4.4  | 3.9  | 1.2  | 2.9  | 0.9  |
| 18:1                | 5.7  | 12.2 | 58.2 | 7.5  | 11.6 | 62.7 | 3.0  | 81.5 |
| 18:2                | 55.5 | 38.4 | 6.0  | 34.2 | 41.3 | 5.0  | 35.4 | 1.6  |
| 18:3                | 33.1 | 16.6 | 20.0 | 25.6 | 10.5 | 15.5 | 30.8 | 4.9  |

localized exclusively to the outer envelope membrane (Dorne et al., 1990). In order to determine if this chloroplast phosphatidylcholine in particular is being desaturated by the chloroplast ω6-desaturase, we measured the fatty acid composition of phosphatidylcholine from chloroplast and extrachloroplast fractions obtained from protoplasts of wild type, fadC, and fad2-1 plants (Table III). In both the fadC and fad2-1 mutants, the chloroplast phosphatidylcholine is only slightly more unsaturated than phosphatidylcholine of the extrachloroplast membranes. The phosphatidylcholine of both the mutants contains higher levels of 18:1 than phosphatidylcholine of the wild type, but the inhibition of 18:1 desaturation is 5-fold higher in fad2-1 than in the fadC mutant. These data indicate that the ω6-desaturase, which is controlled by the fad2 locus, is the predominant enzyme involved in providing polyunsaturated phosphatidylcholine to the chloroplast envelope but that the fadC gene product also contributes in determining the final fatty acid composition of phosphatidylcholine both in the chloroplast envelope and in other membranes of the leaf mesophyll cells. Because the overall fatty acid composition of roots from fad2 plants contained only very low levels of 18:3 (Lemieux et al., 1990), we also purified phosphatidylcholine from lipid extracts of root tissue of wild type and fad2 plants (Table III). The fatty acid composition of phosphatidylcholine from roots of wild type plants was generally similar to that found for the same lipid in wild type leaves. However, the phosphatidylcholine from roots of fad2 plants contained less than 5% 18:3, as compared with 18-20% in phosphatidylcholine from leaf tissue (Tables II and III). The source of the additional 18:3 in the phosphatidylcholine of leaf cells is discussed below.

In phosphatidylcholine of leaf and root tissue of fad2 plants, the decrease in polyunsaturated fatty acids is accompanied by a 40-60% reduction in the level of 16:0, as compared with
wild type (Tables II and III). It is not clear at present how this pleiotropic effect is related to the deficiency in 18:1 desaturation. However, a similar decrease in the proportion of 16:0 in phosphatidylcholine was observed in all four independently derived fad2 alleles, indicating that both of the changes in fatty acid composition are the result of a single mutation.

**Assay of the Microsomal 18:1-Phosphatidylcholine Desaturase**—The indirect evidence presented above strongly suggests that the fad2 gene controls the activity of a constitutively expressed 18:1 desaturase that is probably located in the endoplasmic reticulum. To confirm this conclusion, we set out to measure the desaturase by direct enzyme assay. An 18:1-phosphatidylcholine desaturase has been demonstrated in microsomal preparations from pea leaves (Slack et al., 1976) and from developing oilseed cotyledons (Stynme and Appelqvist, 1978; Slack et al., 1979). When leaf tissue from wild type Arabidopsis plants was used as a source of microsomal membranes, we were unable to demonstrate any 18:1 desaturase activity, even though [14C] 18:1 was readily incorporated into phosphatidylcholine by these preparations (data not shown). This is consistent with our experience with leaf tissue from several species. Furthermore, the active microsomal desaturase from pea leaves was completely inhibited in the presence of a cell-free homogenate derived from Arabidopsis leaf tissue. These results suggest that compound(s) released during leaf homogenization may be powerful inhibitors of 18:1-phosphatidylcholine desaturation.

To circumvent this problem, we prepared microsomal membranes from root tissue of wild type and mutant plants. Under conditions determined in previous studies of microsomal 18:1-phosphatidylcholine desaturases (see "Materials and Methods"), microsomes from wild type plants were able to convert 14% of the [14C]18:1 incorporated into phosphatidylcholine to 18:2 during a 30-min assay (Fig. 1). In contrast, the 14C label associated with 18:2 in microsomes of the fad2-1 mutant was no higher than 1% of the background levels observed in control assays that were extracted with chloroform/methanol before addition of the [14C]18:1-CoA. These results indicate that the mutant contains no more than 3% of the desaturase activity measured in microsomes from wild type plants. The wild type desaturase activity was not affected by incubation with homogenates or microsomes derived from fad2-1 root tissue (data not shown), demonstrating that the fad2 mutation involves a specific loss of desaturase activity and is not due to the accumulation of an inhibitor in fad2 plants.

**Labeling of Leaf Lipids in Vivo**—Fatty acid analyses of monogalactosyldiacylglycerol from plants of the JB9 line consistently showed a 15–20% increase in the level of 16:3 relative to wild type (Table II). A similar increased level of 16:3 was found in monogalactosyldiacylglycerol of the other fad2 alleles also (data not shown). The 16:3 fatty acid is only produced by the prokaryotic pathway (Browse et al., 1986b), and its increased abundance in the fad2 mutants thus suggests that the prokaryotic pathway is responsible for a greater proportion of monogalactosyldiacylglycerol synthesis in the mutant than in the wild type. In order to investigate this possibility and to extend the comparison between lipid biosynthesis in the mutant and that in the wild type, we labeled plants with [14C]acetate and then followed redistribution of radioactivity in lipid acyl groups during the subsequent 144 h.

The kinetics of labeling in the wild type (Fig. 2) are similar to those found previously (Browse et al., 1986b) and are consistent with the parallel operation of the prokaryotic and eukaryotic pathways of lipid synthesis. We have demonstrated elsewhere (Browse et al., 1986b) that the label found in

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**Fig. 1.** Desaturation of 18:1 phosphatidylcholine by microsomes from wild type and mutant Arabidopsis. Microsomes prepared from root tissues were incubated with [1-14C]oleoyl-CoA in the presence or absence (control) of 2.5 mM NADH as described under "Materials and Methods." After a 30-min incubation at 25°C, the reactions were terminated by the addition of 6 ml of CHCl3/CH3OH (1:2, v/v) and the lipids extracted. Phosphatidylcholine (PC) purified by thin layer chromatography of an aliquot of the lipid extract was derivatized using 2.5% H2SO4 in methanol, and the fatty acid methyl esters were separated by argentation thin layer chromatography, as described under "Materials and Methods." The substrate (18:1) and product (18:2) of the desaturase were visualized by autoradiography and identified on the basis of their comigration with authentic standards. The relative incorporation of [14C]18:1 into phosphatidylcholine and the subsequent desaturation to [14C]18:2 were quantified by scintillation counting of the individual methyl ester bands.

**Fig. 2.** Redistribution of radioactivity among the polar leaf lipids of wild type (WT) and fad2-1 mutant Arabidopsis. Plants were grown at 22°C under continuous illumination (150 μmol quanta/m2/s). Eighteen-day-old plants were labeled with [14C]acetate at zero time. At the times indicated, 1-g leaf samples were harvested and frozen in liquid nitrogen, and the lipids were extracted. After separation by thin layer chromatography, individual lipid bands were scraped from plates, and the radioactivity in them was measured by scintillation counting. For details see "Materials and Methods." 

monogalactosyldiacylglycerol soon after the start of the experiment represents monogalactosyldiacylglycerol derived from the prokaryotic pathway, whereas the increase in monogalactosyldiacylglycerol at longer times reflects synthesis of this lipid from phosphatidylcholine via the eukaryotic pathway. On this basis, the data for fad2-1 (Fig. 2) do indeed demonstrate a marked increase, as compared with the wild type, in monogalactosyldiacylglycerol synthesis by the prokaryotic pathway and a corresponding decrease in synthesis.
of this lipid by the eukaryotic pathway. Both synthesis and turnover of \[^{14}C\]phosphatidylcholine are reduced in the mutant (Fig. 2), reflecting a decreased flux through the eukaryotic pathway. Radioactivity only accumulates in digalactosyldiacylglycerol toward the end of the experiment, indicating that digalactosyldiacylglycerol is synthesized mainly by the eukaryotic pathway in the fad2-1 mutant, as it is in the wild type.

A more accurate estimate of the contribution of each pathway to the synthesis of monogalactosyldiacylglycerol and digalactosyldiacylglycerol can be made by determining the fatty acids present on the sn-2 position of these lipids using a position-specific lipase (Siebertz and Heinz, 1977). Several lines of evidence (Roughan and Slack, 1982; Browse et al., 1986b; Browse and Somerville, 1991) indicate that the chain length of the fatty acid at the sn-2 position is an accurate predictor of whether a particular molecule has been synthesized by the prokaryotic (16-carbon fatty acid at sn-2) or eukaryotic (18-carbon fatty acid at sn-2) pathway. The fatty acid compositions of lysylipids obtained after lipase digestion of lipids from wild type and mutant plants are shown in Table IV. The composition of these lysylipids reflects the fatty acid composition at sn-2 of the parent glycerolipid. The fatty acid compositions at sn-1 of the parent lipids were calculated by difference using the data in Tables II and IV, and the results of these calculations agreed within 5% with the analysis of unesterified fatty acids derived from the lipase digestion (results not shown). The data in Table IV indicate that the proportion of total leaf monogalactosyldiacylglycerol made by the eukaryotic pathway was 17.2% (sum of all 18-carbon fatty acids) in the mutant, as compared with 26.7% in wild type plants. On the other hand, the eukaryotic pathway was responsible for 81.4 and 69.5% of digalactosyldiacylglycerol synthesis in wild type and mutant plants, respectively. The characterization of three other Arabidopsis lipid mutants (Kunst et al., 1988, 1989; Browse et al., 1989) has demonstrated that the balance between the two pathways of glycerolipid synthesis may be regulated in response to mutations in specific alterations in the fatty acid composition of their leaf lipids. Investigations of five classes of mutants have contributed to our knowledge of the biochemistry and regulation of chloroplast lipid synthesis (Browse and Somerville, 1991) and to our understanding of the importance of lipid structure to the function of the photosynthetic membranes (Browse et al., 1985; McCourt et al., 1987; Kunst et al., 1989; Hugly et al., 1989). In this report, we describe the characterization of an Arabidopsis mutant deficient in polyunsaturated fatty acids of the extrachloroplast membranes. The desaturation of 18:1 fatty acids is synthesized mainly by the eukaryotic pathway in the fad2-1 mutant, as it is in the wild type.

### Table IV

Fatty acid composition at the sn-2 position of glycerolipids from leaves of wild type (WT) and mutant Arabidopsis

| Lipid                   | 16:0 | 16:X | 18:0 | 18:1 | 18:2 | 18:3 |
|-------------------------|------|------|------|------|------|------|
| Monogalactosyldiacylglycerol |     |      |      |      |      |      |
| WT                      | 1.9  | 71.4 | 0.6  | 0.6  | 1.5  | 24.0 |
| fad2-1                  | 2.5  | 80.2 | 0.5  | 3.1  | 1.1  | 12.5 |
| Digalactosyldiacylglycerol |     |      |      |      |      |      |
| WT                      | 13.0 | 5.6  | 0.3  | 0.3  | 0.9  | 3.5  |
| fad2-1                  | 21.0 | 9.6  | 0.3  | 5.7  | 8.3  | 89.7 |
| Phosphatidylcholine     |      |      |      |      |      |      |
| WT                      | 5.7  | 1.0  | 9.3  | 48.3 | 35.5 |
| fad2-1                  | 5.2  | 0.7  | 70.7 | 9.2  | 14.3 |

The genetic approach has been extremely useful in studies of metabolic pathways, including the pathways of lipid biosynthesis, in Escherichia coli and yeast (Raetz, 1986; Vanden Boom and Cronan, 1989; Carman and Henry, 1989). One reason for the success of mutational analysis in elucidating lipid metabolism lies in the fact that many of the enzymes are membrane-bound proteins that in many cases have been refractory to purification and characterization by traditional biochemical techniques. Since the same barriers exist to the investigation of membrane lipid synthesis in higher plants, we have isolated a series of Arabidopsis mutants that contain specific alterations in the fatty acid composition of their leaf lipids. Investigations of five classes of mutants have contributed to our knowledge of the biochemistry and regulation of chloroplast lipid synthesis (Browse and Somerville, 1991) and to our understanding of the importance of lipid structure to the function of the photosynthetic membranes (Browse et al., 1985; McCourt et al., 1987; Kunst et al., 1989; Hugly et al., 1989). In this report, we describe the characterization of an Arabidopsis mutant deficient in polyunsaturated fatty acids of the extrachloroplast membranes. The desaturation of 18:1 fatty acids is synthesized mainly by the eukaryotic pathway in the fad2-1 mutant, as it is in the wild type.

TABLE IV

Fatty acid composition at the sn-2 position of glycerolipids from leaves of wild type (WT) and mutant Arabidopsis

Individual lipids were purified by silica gel thin layer chromatography as described in Table II, eluted from the gel, and incubated with Rhizopus lipase, as described under "Materials and Methods." The resulting lyso lipids (representing the sn-2 position of the parent lipid) were purified by thin layer chromatography and derivatized using 2.5% H_2SO_4 in methanol, and the resulting fatty acid methyl esters were analyzed by gas chromatography. 16:X represents the sum of 16:1 + 16:2 + 16:3. Data are mole %.
Although the enzyme is assayed and described as an 18:1-phosphatidylcholine desaturase, analysis of the fatty acid compositions of individual leaf lipids (Table II) showed that all the major extrachloroplast phospholipids are affected to a similar extent in the mutant. This indicates that the desaturase is responsible for the synthesis of polyunsaturated fatty acids for most of the phospholipids of the cell. It is possible that 18:2 and 18:3 fatty acids synthesized on phosphatidylcholine are transferred to other phospholipids by acyl exchange or by head group exchange. However, we have previously shown that the chloroplast desaturases controlled by fadC and fadD show little or no specificity for particular lipid head groups, but instead desaturate fatty acids on all the major lipids of the chloroplast membranes (Browse et al., 1986a, 1989). By analogy, it is possible that the FAD2 gene product desaturates 18:1 esterified to any of the endoplasmic reticulum phospholipids. If this is the case, then the enzyme probably acts on 18:1 at both positions of the glycerol backbone, since the fad2 mutation results in the accumulation of 18:1 at both positions of phosphatidylcholine (Tables II and IV) and phosphatidylethanolamine (data not shown).

The defect in phospholipid desaturation does not alter the proportions of individual lipids found in leaves of the fad2 plants relative to the wild type (Table II). Nevertheless, the pattern of lipid synthesis is affected. The 14C-labeling experiment (Fig. 2) and positional analysis of leaf lipids (Table IV) both indicate that in leaves of the mutant, there is a 36% reduction, as compared with wild type, in the amount of monogalactosyldiacylglycerol synthesized by the eukaryotic pathway and a corresponding increase in the synthesis of monogalactosyldiacylglycerol by the prokaryotic pathway. Previous studies of the fadB (Kunst et al., 1989), fadC (Browse et al., 1989), and act1 (Kunst et al., 1988) mutants of Arabidopsis have provided evidence that lipid metabolism is regulated to ameliorate the consequences of each mutant lesion by altering the flux through the two pathways of lipid synthesis. However, it is not clear why the balance between the two pathways should be altered in the fad2 mutants, since 18:1 entering chloroplast lipids on the eukaryotic pathway appears to be efficiently desaturated by the chloroplast ω6-desaturase. A possible explanation is that 18:1-containing lipids are not efficiently transferred between the endoplasmic reticulum and the chloroplast.

Although fad2 plants do not contain any detectable microsomal 18:1 desaturase activity (Fig. 1), the leaf phospholipids contain levels of 18:3 that are as high or nearly as high as those found in phospholipids from wild type leaves (Table II). A likely explanation for this observation is that synthesis of 18:3 from 18:1 is mediated by the chloroplast desaturases controlled by the fadC and fadD loci. The similarity in fatty acid composition of phosphatidylcholine of the chloroplast and extrachloroplast membranes (Table III) suggests that reversible exchange of lipids takes place between the different compartments of leaf cells. We have shown previously that the fadD locus exerts partial control over the 18:3 content of the extrachloroplast membranes of leaf cells (Browse et al., 1989). The labeling kinetics of individual fatty acids derived from phosphatidylcholine of mutant plant leaves shows the appearance of radioactivity in 18:3 only at longer incubation times (Fig. 3), when it is known that flux through the eukaryotic pathway is carrying lipid back to the chloroplast (Fig. 2, Browse et al., 1986b). The low level of label in 18:2 of phosphatidylcholine from fad2 plants (Fig. 3) also mitigates against the direct involvement of phosphatidylcholine in the desaturation of 18:1 through 18:2 to 18:3. Finally, root tissues...
of the mutant contain levels of 18:3 that are considerably lower than those found in roots from wild type plants. Root tissues do not contain significant activities of the chloroplast enzymes (Browse et al., 1986a; Browse et al., 1989), and thus it would not be anticipated that the fad2 mutation could be circumvented in roots as it appears to be in leaf cells. Taken together, these results demonstrate a strong cooperation between the desaturases of the chloroplast and endoplasmic reticulum in determining the final complement of fatty acids found in the different membranes of leaf cells. This conclusion is in agreement with and extends the observations made from our previous studies of the fadD mutant (Browse et al., 1986a).

The nearly wild type levels of 18:3 found in phospholipids of the fad2 leaves raise the possibility that in wild type Arabidopsis, the chloroplast desaturases are largely responsible for the synthesis of 18:3 (but not 18:2) in the extrachloroplast membranes. However, we and others have recently presented genetic evidence for a second endoplasmic reticulum desaturase that also adds 18:2 to 18:3 (Lemieux et al., 1990; James and Dooner, 1990). Preliminary analysis of the mutants deficient in 18:2 desaturation indicates that in the absence of the wild type allele at FAD2 (i.e. normal synthesis of 18:2), the endoplasmic reticulum 18:2-desaturase is responsible for at least half of the 18:3 present in the phospholipids of the extrachloroplast membranes. On this basis, we suggest that synthesis of polyunsaturated phospholipids in leaves of wild type Arabidopsis takes place largely via 18:1- and 18:2-desaturation of the endoplasmic reticulum but that in the fad2 mutants the chloroplast desaturases provide an alternative route for desaturation.

The two pathways of lipid synthesis contribute almost equally to the production of chloroplast membrane lipids in wild type Arabidopsis (Browse et al., 1986b), and the balance of fluxes through these pathways may be altered to ameliorate the effects of mutations that block steps in one of the pathways (Browse and Somerville, 1991). Because of this metabolic flexibility, the four classes of mutants with defects in chloroplast desaturation that have been characterized to date (Browse et al., 1985, 1990a, 1989; Kunst et al., 1989) retain levels of polyunsaturated lipids that range from 68 to 100% of those found in leaves of wild type Arabidopsis. However, the fad2 locus controls the major (or only) endoplasmic reticulum desaturase. The isolation of the fad2 mutants described here, and our previous characterization of a fadD mutant deficient in the chloroplast 16:1/18:1 desaturase (Browse et al., 1989), will allow us to generate a fadC-fad2 double mutant that is predicted to be almost completely unable to synthesize polyunsaturated lipids. This work is now under way. Such double mutants, together with the existing single mutants, will provide a series of plant lines with specific alterations in the fatty acid composition of their membrane lipids and will therefore provide the means to investigate, at the whole plant level, the metabolic and physiological consequences of these changes in membrane structure.

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