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A Mutant of *Arabidopsis* with Increased Levels of Stearic Acid[1]

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A mutation at the *fab2* locus of *Arabidopsis* caused increased levels of stearate in leaves. The increase in leaf stearate in *fab2* varied developmentally, and the largest increase occurred in young leaves, where stearate accounted for almost 20% of total leaf fatty acids. The fatty acid composition of leaf lipids isolated from the *fab2* mutant showed increased stearate in all the major glycerolipids of both the chloroplast and extrachloroplast membranes. Although the stearate content was increased, the *fab2* mutant still contained abundant amounts of 18:1, 18:2, and 18:3 fatty acids. These results are consistent with the expectations for a mutation partially affecting the action of the stromal stearoyl-acyl carrier protein desaturase. Positional analysis indicated that the extra 18:0 is excluded with high specificity from the *sn-2* position of both chloroplast and extrachloroplast glycerolipids. Although stearate content was increased in all the major leaf membrane lipids, the amount of increase varied considerably among the different lipids, from a high of 25% of fatty acids in phosphatidylcholine to a low of 2.9% of fatty acids in monogalactosyldiacylglycerol.

The membrane lipid composition of plants differs from that of animals and fungi primarily in the composition of the chloroplast membranes and in a higher overall level of unsaturation. The chloroplast membranes are composed almost entirely of the uncharged galactolipids MGD and DGD, which make up about 75% of the total chloroplast thylakoid lipid (Webb and Green, 1991). Depending upon the plant species, trienoic fatty acids (18:3 and 16:3) constitute up to 80% of the fatty acids in this organelle (Murphy, 1986). Although these features are common to all higher plants, it remains unclear how these characteristic lipid components are important to the physiology and cell biology of plants.

In plants, de novo fatty acid synthesis occurs exclusively in the plastids (Ohlrogge and Kuo, 1985). The plant fatty acid synthase is composed of individual proteins (Shimakata and Stumpf, 1982), and the primary products of this type II synthase are 16:0-ACP and 18:0-ACP (Roughan et al., 1979). The 18:0-ACP is normally desaturated with high efficiency to 18:1-ACP by the action of a soluble desaturase in the chloroplast stroma (McKeon and Stumpf, 1982; Shanklin and Somerville, 1991; Knutzon et al., 1992) so that 18:0 fatty acids are normally a minor component of plant cell membranes. The 16:0 and 18:1 fatty acids are then utilized by two distinct pathways for the biosynthesis of membrane glycerolipids and the coordinate desaturation of the acyl chains to produce polyunsaturated fatty acids. The basis of the two-pathway hypothesis (Roughan et al., 1980; Roughan and Slack, 1982) is that fatty acids synthesized de novo in the chloroplast (Ohlrogge et al., 1979) may be used either for the production of chloroplast lipids via the prokaryotic pathway (Roughan et al., 1980) or exported as CoA esters (Block et al., 1983) to enter the eukaryotic pathway at extrachloroplast sites, particularly in the ER (Slack and Roughan, 1975; Dubacq et al., 1976; Slack et al., 1976; Simpson and Williams, 1979). The DAG moiety of PC synthesized by the eukaryotic pathway can be returned to the chloroplast, where it contributes to the production of thylakoid lipids (Slack and Roughan, 1975; Williams et al., 1976; Slack et al., 1977). In both of these pathways the only known desaturation point for 18:0 fatty acids occurs immediately after elongation, while they are bound to ACP as thioesters.

In all reported cases the major glycerolipids are first synthesized using 16:0 and 18:1 fatty acids. Further desaturation of the acyl groups to the highly unsaturated forms typical in plant cell membranes occurs via membrane-bound desaturases in the chloroplast and ER. Until recently most information on the function and specificity of these desaturases has come from the characterization of mutants deficient in specific desaturation activities (reviewed by Browse and Somerville, 1991). Recently, the *FAD3* gene has been cloned on the basis of genetic position (Arondel et al., 1992) and the *FAD2* gene has been cloned using T-DNA tagging (Okuley et al., 1994). The cloning of these gene sequences provides the opportunity to examine protein structure and the molecular control of these desaturase genes. Although the isolation of desaturase genes provides important opportunities to extend our knowledge of plant lipid metabolism, the characterization of biochemical mutants can still provide unique insights into

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Abbreviations: ACP, acyl carrier protein; DAG, diacylglycerol; DGD, digalactosyldiacylglycerol; MGD, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SL, sulfoquinovosyl diacylglycerol (sulfolipid); TAG, triacylglycerol; X:Y, a fatty acyl group containing X carbon atoms and Y double bonds (*cis* unless specified).

the role of various membrane components in plant cell biology. Mutations provide a unique means for making relatively precise changes in substrate pools in vivo and for examining the effects of these changes on the metabolic apparatus.

We have reported the isolation of a mutant of *Arabidopsis thaliana*, *fab2*, that has increased levels of stearate (18:0) in all plant tissues. This fatty acid composition change has profound ramifications on plant development (Lightner et al., 1994), a possibility unrecognized even though the 18:0-ACP desaturase has been cloned for some time (Shanklin and Somerville, 1991). The *fab2* mutant is a true miniature that completes a normal life cycle but is reduced in the size of almost all organs. Here we report the detailed biochemical characterization of the *fab2* mutant and examine how the biosynthetic apparatus adjusts to accommodate the presence of high levels of 18:0, a fatty acid normally present in very low amounts.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The line *2A11* (*fab2*) was derived by ethylmethane sulfonate mutagenesis from *Arabidopsis thaliana* var Columbia as described (James and Dooner, 1990). The *fab2* mutant was back-crossed four times to Columbia wild type before we performed the experiments reported here. Seeds were sown on potting mix in 4-inch pots and bottom watered with 50% GP Blue (Grace-Sierra Co., Milpitas, CA) fertilizer. Pots were covered with plastic wrap to maintain humidity and placed in a 4°C walk-in cooler overnight for stratification. After chilling treatment the pots were placed in a controlled-environment growth chamber under continuous illumination (100 μmol quanta m^{-2} s^{-1}). After emergence of the cotyledons the plastic overwrap was removed and plants were grown to maturity in the same pots without disturbance.

Fatty Acid and Lipid Analysis

Fatty acid methyl esters were prepared from leaves and other tissues by heating samples at 80°C in 1 mL of 2.5% (v/v) H_2SO_4 in methanol for 60 min in screw-capped test tubes (Browse et al., 1985). After addition of 1.5 mL of H_2O and 0.5 mL of hexane the fatty acid methyl esters were extracted into the organic phase by shaking the tubes and centrifuging at low speed to accelerate separation. One-microliter samples were analyzed by GC on a 15 m × 0.53 mm Supelcowax column (Supelco, Bellefonte, PA) and quantified with flame ionization detection. The chromatograph was programmed for an initial temperature of 140°C for 2 min followed by a 20°C/min ramp to 160°C and a secondary ramp of 5°C/min to 172°C. The final temperature was maintained for 2 min. For comparison of fatty acid composition at varying stages of leaf development, plants were stratified before movement to the growth chamber as described above. At various days after stratification plants were harvested and only the first two true leaves were dissected and trans-methylated as described above.

For leaf lipid extraction whole rosettes, consisting predominantly of leaf tissue, were killed by immersion in liquid N_2 and ground to a fine powder under liquid N_2 in a precooled mortar and pestle. One gram fresh weight of tissue was transferred to a screw-capped test tube with 12 mL of chloroform:methanol:formic acid (10:10:1, v/v) and stored overnight at −20°C (Roughan et al., 1978). After centrifugation the supernatant was transferred to a new tube and stored at −20°C. The pellet was reextracted with 4.4 mL of chloroform:methanol:water (5:5:1, v/v) for 30 min at −20°C. After centrifugation the two extracts were combined and washed with 6 mL of 0.2 M H_3PO_4, 1 M KCl (Hajra, 1974). Lipids were recovered from the chloroform phase, dried under N_2, and redissolved in 0.5 mL of chloroform/g fresh weight of tissue.

Individual polar lipids were purified from the extracts by one-dimensional TLC on $(NH_4)_2SO_4$-impregnated silica gel (Khan and Williams, 1977). Plates were activated by heating for 90 min at 110°C before running in acetone:benzene:water (30:10:2.7, v/v). Nonpolar lipids were separated on plain silica plates after activation by heating for 60 min at 110°C in hexane:diethyl ether:acetic acid (75:25:1, v/v). Lipids were visualized by spraying with a solution of 0.001% primuline in 80% acetone and illuminating with UV light. To quantify the relative amounts of individual lipids and to determine their fatty acid composition the silica from each lipid spot was scraped and transmethylated in a screw-capped tube with an appropriate amount of 17:0 fatty acid to act as an internal standard.

Positional analysis was performed by the method of Siebertz and Heinz (1977) except that 50 mM H_3BO_3 was added to the buffer used for lipase digestion to minimize intramolecular acyl transfer on the lyso-lipids produced. Fatty acid methyl esters were prepared from untreated lipid, lyso derivatives, and free fatty acid derivatives by the method described above.

Labeling Experiments

Sodium [1-^{14}C]acetate (2.0 GBq/mmol, DuPont-New England Nuclear) was diluted to 2.0 MBq/mL with water without the addition of ethanol or surfactants. Each pot of plants (approximately 90 cm^2 soil area) was sprayed with 0.5 mL of the diluted solution with a perfume atomizer. At various times after treatment 1-g (fresh weight) samples were harvested and killed by immersion in liquid N_2. The lipids were then extracted and separated by TLC as described above. Lipids from one chromatographic separation were transferred to scintillation vials containing Biodegradable Counting Scintillant (Amersham) and radioactivity was determined by scintillation counting. Lipids from a second separation were transmethylated as described above. The fatty acid methyl esters were then separated by argentation TLC (Browse et al., 1986) on silica gel G plates that had been dipped in a solution of 5% (w/v) $AgNO_3$ in acetonitrile with the addition of rhodamine. Bands were visualized with UV light. Because argentation TLC does not resolve 16:0 and 18:0 fatty acids, it was necessary to elute the saturated band and separate the saturated fatty acids by reverse-phase TLC. For reverse-phase TLC, Silica Gel 60 plates were activated for 60 min at 110°C and then run in a solution of 5% paraffin oil in hexane until the solvent front reached the top. Plates were allowed to air dry for 15 min before loading the extracted methyl esters.

Table I. *Fatty acid composition of total leaf lipids from wild-type and fab2 mutant Arabidopsis*

Plants were sampled 14 d after imbibition. Data are mol % ± SE, $n = 5$, except for total fatty acids, which are given in μmol/g fresh weight.

Fatty Acid	Wild Type	*fab2*
16:0	12.8 ± 0.6	12.0 ± 0.2
16:1c	0.5 ± 0.2	
16:1t	2.4 ± 0.1	1.9 ± 0.1
16:2	0.8 ± 0.1	
16:3	12.3 ± 0.8	7.2 ± 0.2
18:0	0.7 ± 0.0	16.6 ± 0.6
18:1	3.0 ± 0.2	1.9 ± 0.5
18:2	15.7 ± 1.4	17.3 ± 0.3
18:3	50.9 ± 0.8	43.1 ± 0.6
Saturates	15.9	30.5
Polyunsaturates	79.7	67.6
Total fatty acids (μmol/g fresh weight)	4.8 ± 0.4	7.4 ± 0.9

Reverse-phase plates were then developed (without activation) in acetonitrile:methanol:water (6:3:1, v/v). Both the argentation plate and reverse-phase plates were autoradiographed, and radioactivity in the individual spots was determined by scintillation counting as above.

Two-dimensional TLC was performed to confirm the identity of some lipids. Silica Gel 60 plates were activated by heating for 60 min at 110°C. After cooling the plate a single lipid spot was made in the lower left corner. Plates were then developed in chloroform:methanol:7 M NH_4OH (65:30:4, v/v). The plate was dried for 10 min under vacuum, rotated 90°, and then developed in chloroform:methanol:acetic acid:water (34:5:5:1.2, v/v).

RESULTS

Biochemical Characterization

The *fab2* mutant was originally isolated in a screen of seed fatty acid composition (James and Dooner, 1990), where it was identified as having a 2- to 3-fold increase in seed stearate. The seed fatty acid composition of the *fab2* mutant showed increases in both 18:0 and 20:0 compared to the wild type (James and Dooner, 1990). Analysis of the seed fatty acid composition of M_2 siblings of the *fab2* mutant showed segregation of the high-stearate character consistent with the expectation for a single recessive nuclear mutation (James and Dooner, 1990). Further examination of the *fab2* mutant has shown that increased stearate is present in all the plant organs examined (Lightner et al., 1994). Because the increased stearate content of the *fab2* mutant is associated with profound alterations of plant growth and development, we have performed a detailed analysis of leaf lipid metabolism in the mutant.

Leaves of the *fab2* mutant contained a considerably increased level of 18:0 compared to the wild type, and decreased levels of 18:3 and 16:3 (Table I). In addition to a change in the relative proportions of different acyl groups in *fab2*, the mutant also contained an increase in total fatty acid content on a fresh weight basis (Table I). The analyses shown in Table I were carried out on 14-d-old plants. Samples from younger *fab2* plants often contained higher levels of 18:0. Therefore, we decided to make a detailed examination of the proportion of stearate in mutant and wild-type leaves at various stages of development by following the fatty acid composition of the first two true leaves of wild-type and mutant plants over time. Figure 1 shows the variation in proportion of 18:0 in these first true leaves as a function of plant age. In our experiment the emergent first true leaves were large enough to sample at 6 d after imbibition (Fig. 1, first time point). The mutant leaves contained almost 20% stearate at this time point, compared to less than 2.5% stearate in the wild-type leaves. The stearate level remained high in the mutant through 10 d and then declined to about 10% of the total fatty acids by 20 d of age, when plants in our growth conditions are nearing the onset of flowering.

Although stearate was significantly increased in the *fab2* mutant, all unsaturated 18-carbon fatty acids (18:1, 18:2, 18:3) were present with only modest reductions in their proportions relative to wild type (Table I). This result suggested that the lesion in the *fab2* mutant resulted from the inability to desaturate a relatively small portion of the 18:0 produced. A likely candidate for such a lesion was the stearoyl-ACP desaturase, which desaturates 18:0-ACP to 18:1-ACP. Although assays for the 18:0 desaturase have been described (Talamo and Block, 1969; McKeon and Stumpf, 1982), in the *fab2* mutant nearly 80% of the 18:0 synthesized is further desaturated, suggesting that any change in enzyme activity is relatively subtle. Furthermore, the profound size differences at the organ, tissue, and cellular level between the mutant and wild-type plants (Lightner et al., 1994) make comparisons based on these parameters dubious, at best. Finally, recent evidence suggests the existence of several isoforms of the 18:0-ACP desaturase gene in the *Arabidopsis* genome. For these reasons it was necessary to take an indirect approach to characterizing the mutation, using detailed com-

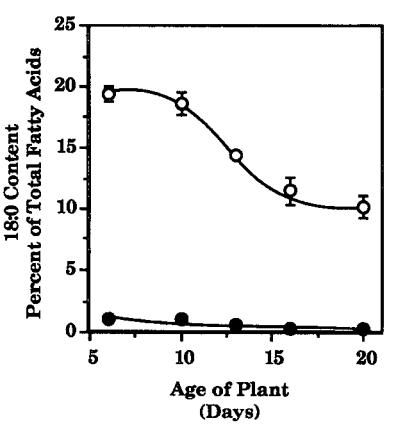

Figure 1. Developmental variation in the 18:0 content of wild-type and *fab2* leaves. The first two true leaves were removed and the lipids were transmethylated as described in "Materials and Methods." (Data shown are the averages [$n = 5$]. Bars show SE).

positional analysis, in vivo labeling, and positional analysis to describe lipid metabolism in the mutant.

Fatty Acid Composition of Individual Leaf Lipids

Analysis of the fatty acid composition of major leaf glycerolipids of the *fab2* mutant showed several important differences from the wild type (Table II). Lipids were prepared from whole rosettes, which consisted almost entirely of leaf tissue, at 21 d after inhibition. There was a significant decrease in the amount of MGD, the major chloroplast lipid, in *fab2* from a wild-type level of 39.5% of total leaf lipids to 29.4% in the mutant. The *fab2* mutant contained increased amounts of PC, which accounted for 22.4% of total lipids in the mutant compared to 14.8% in the wild type, and PE, which accounted for 18.1% of the *fab2* lipids compared to 10.3% in the wild type.

Increased 18:0 levels were found in all the major leaf membrane glycerolipids, a result consistent with the expectation for a change in the activity of the 18:0-ACP desaturase. Although 18:0 content was increased in all the lipids examined, the actual amount of the increase varied considerably among the different lipid classes, with the most extreme changes occurring in the extrachloroplast lipids (Table II). In PC, 18:0 accounted for more than one-quarter of the total fatty acids in *fab2* compared to 2.1% 18:0 in wild type. In PE, 18:0 accounted for 18% of the fatty acids compared to 2.6% in wild type. In both PC and PE the 16:0 content was lower in the *fab2* mutant than in the wild type. Although PC from *fab2* contained only 12.3% 16:0 compared to 20.8% in wild type, the total saturated fatty acid content of *fab2* PC is still more than 1.5 times the wild-type saturate level. On the other hand, PE from *fab2* contains 16.1% 16:0 compared to 28.1% 16:0 in wild-type, making the total saturate content of PE in *fab2* only slightly higher than wild type. Mutant PI, PS, and SL all show increased 18:0 content and decreased 16:0 content, relative to the same lipids in wild-type leaves (Table II).

In contrast to the extrachloroplast lipids PC and PE, the predominant chloroplastic lipid, MGD, contained only 2.9% 18:0 in the mutant (Table II). Despite this low level compared to other *fab2* lipids, this 2.9% level still represented a 10-fold increase over the wild-type 18:0 level of 0.2% of MGD fatty acids. DGD isolated from *fab2* leaves contained 9.8% 18:0 compared to 1% in the wild type. Again, although this level was lower than the 18:0 levels observed in extrachloroplast lipids, it still represented a nearly 10-fold increase compared to the wild type. Despite these large proportional increases in 18:0 content, both MGD and DGD in the mutant remain highly polyunsaturated. Thus, MGD from *fab2* contained 84% trienoic fatty acids (16:3 plus 18:3) compared to 91% trienoic fatty acids in wild-type MGD. Other membrane glycerolipids from the mutant PI, PS, PG, and SL all con-

Table II. *Fatty acid composition of leaf glycerolipids from wild-type (WT) and mutant Arabidopsis*

DBI is the average number of double bonds per glycerolipid molecule.

Glycerolipid	Percent of Total	Fatty Acid Composition (mol %)									
		16:0	16:1t	16:1c	16:2	16:3	18:0	18:1	18:2	18:3	DBI
PC											
WT	14.8	20.8					2.1	5.5	34.3	37.4	3.7
fab2	22.4	12.3					26.4	1.7	21.4	37.1	3.1
PE											
WT	10.3	28.1					2.6	2.3	36.6	30.4	3.3
fab2	18.1	16.1					18.4	0.9	26.8	35.6	3.2
PI											
WT	2.2	39.9					5.3	2.8	26.2	24.4	2.6
fab2	3.5	21.1					17.8	0.9	23.7	36.5	3.2
PS											
WT	3.0	43.4					2.7	2.7	21.3	29.9	2.7
fab2	3.7	30.9					17.3	0.4	15.5	35.8	2.8
SL											
WT	3.0	39.3					2.8	3.3	12.5	42.2	3.1
fab2	3.2	28.3					18.7	1.4	11.6	40.0	2.9
DGD											
WT	10.9	11.6		0.5		3.3	1.0	1.0	4.1	78.5	5.1
fab2	9.7	10.2		0.2		2.8	9.8	2.6	3.9	70.4	4.6
PG											
WT	11.2	26.5	16.5			1.3	1.3	4.5	8.6	41.3	3.0
fab2	9.5	25.6	15.7			1.9	14.5	3.5	8.3	30.5	2.3
MGD											
WT	39.5	2.0	0.7	0.4	1.5	30.3	0.2	1.1	2.9	61.0	5.7
fab2	29.4	4.3	0.6	2.0	0.8	20.6	2.9	1.3	3.9	63.6	5.3
TAG											
WT	0.8	16.3	0.0	0.0	0.0	2.7	0.9	10.5	23.0	46.1	4.1
fab2	2.6	15.6	0.0	0.0	0.0	0.8	30.7	7.9	12.6	32.4	2.7

tained 15 to 20% 18:0 compared with values of 1 to 5% for wild type.

Transmission electron micrographs (Lightner et al., 1994) of *fab2* chloroplasts showed an increase in the number of lipid-containing plastoglobuli compared to wild type. For this reason we used TLC to separate neutral leaf lipids and compare the levels of these lipids in wild-type and mutant plants. The most abundant neutral lipid class, TAG, made up 2.6% of the total leaf glycerolipid in *fab2* compared to <1% stearate in wild-type TAG. This TAG contained more than 30% 18:0 in the mutant compared to <1% 18:0 in wild-type TAG. This level of 18:0 in *fab2* TAG represents both the highest stearate content of any of the examined glycerolipids and the largest proportional increase in stearate, 30-fold, compared to wild-type leaf TAG. Diacylglycerols were present at less than one-fourth the level of TAG in both wild-type and *fab2* leaves (data not shown).

Positional Analysis of Fatty Acids

In *fab2* leaves, 18:0 accounted for almost one-fourth of the fatty acids on extrachloroplast lipids. In fact, almost 40% of the fatty acids in PC (the major structural lipid of extrachloroplast membranes) were fully saturated. Because disaturated phospholipid molecules (including molecules that contain 16:1-*trans*) have been shown to profoundly affect membrane phase behavior in model systems (Murata and Yamaya, 1984), we considered it important to find out whether desaturated molecular species of glycerolipids were present in *fab2* leaf extracts. Leaf PG from the mutant contains 56% 16:0 + 16:1-*trans* + 18:0 (Table II), indicating that at least 12% of the PG molecules must be desaturated. For other lipids, which contain less than 50% saturated fatty acids, additional information can be obtained by determining the fatty acid composition at the *sn*-2 position of the glycerol using lipase digestion (Siebertz and Heinz, 1977). In addition, the chain length of the fatty acid at the *sn*-2 position of glycerolipids has been shown to be an excellent predictor of whether a particular lipid molecule was synthesized by the prokaryotic or eukaryotic pathway (Roughan and Slack, 1982; Browse et al., 1986). Lipids synthesized on the prokaryotic pathway typically contain 16-carbon fatty acids at *sn*-2, whereas lipids synthesized on the eukaryotic pathway have an 18-carbon fatty acid at this position. Therefore, we carried out positional analyses on four of the major glycerolipids from wild-type and mutant plants.

Table III shows the fatty acid composition of the lyso derivatives of MGD, DGD, PC, PE, and PG purified from leaf tissue of wild-type and *fab2 Arabidopsis*. The most striking observation from these data was the very low amounts of 18:0 fatty acids at the *sn*-2 position of all the examined *fab2* lipids. In the chloroplast lipids MGD and DGD and the extrachloroplast lipids PE and PC, this positional specificity results in an *sn*-2 that lacks both 16:0 and 18:0, indicating that disaturated species of these lipids are rare in both wild-type and *fab2* mutant leaves. A second observation from the positional analysis was that a larger proportion of the total MGD in the *fab2* mutant was synthesized by the eukaryotic pathway. In the wild type 33% (sum of 18-carbon fatty acids) of the MGD was derived from the eukaryotic pathway, but in *fab2* almost 45% of the MGD was produced by this route. By contrast, there is little or no difference in the proportions of DGD, PC, or PG made by the two pathways.

Labeling of Leaf Lipids in Vivo

Our compositional analysis of *fab2* plants suggested a complexity in the metabolism of 18:0 fatty acids and their incorporation into glycerolipids that is not apparent in the wild-type background. In vivo labeling of fatty acids with [^{14}C]acetate allows the analysis of both the flow of carbon through the various leaf glycerolipids and the simultaneous analysis of the desaturation state of the fatty acids esterified to these glycerolipids. This labeling also allows examination of the possibility of increased turnover of specific lipids in the mutant. Such an increase in lipid turnover has been demonstrated in the *fad5* (previously *fadB*) mutant of *Arabidopsis* (Kunst et al., 1989), a mutant deficient in a chloroplast 16:0 desaturase activity. To examine how the differences in 18:0 distribution between different leaf glycerolipids occurred

Table III. *Fatty acid composition at the sn-2 position of leaf glycerolipids*

Glycerolipid	Fatty Acid Composition (mol %)					
	16:0	16:X	18:0	18:1	18:2	18:3
MGD						
Wild type	1.4	64.9	0.3	0.6	3.8	27.9
fab2	1.2	53.3	0.6	0.2	2.1	42.0
DGD						
Wild type	11.3	6.3	0.8	1.2	2.7	77.8
fab2	10.8	6.7	1.9	0.7	2.7	77.2
PE						
Wild type	4.0		0.5	2.6	54.1	39.4
fab2	1.5		0.8	2.1	40.9	54.7
PC						
Wild type	2.0		0.7	7.6	42.7	46.2
fab2	1.8		4.1	2.4	31.8	58.9
PG						
Wild type	47.8	33.9	0.9	1.1	7.4	8.9
fab2	46.7	34.2	2.0	0.6	7.2	9.3

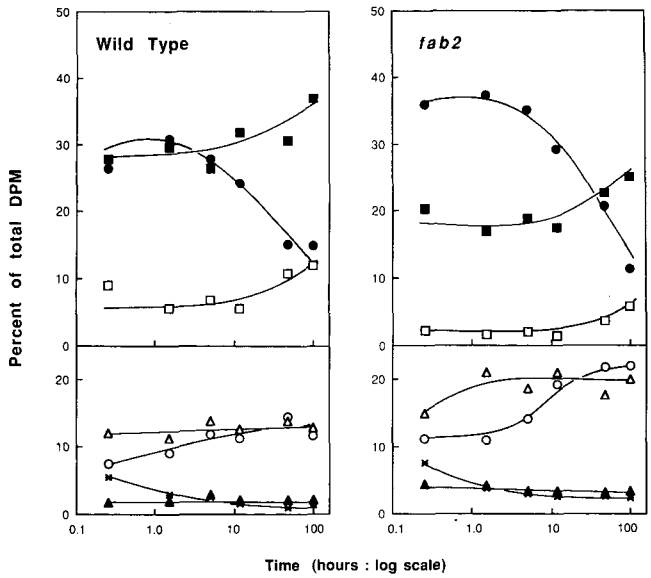

Figure 2. Redistribution of radioactivity in leaf lipids of wild-type and *fab2 Arabidopsis* after labeling with [1-^{14}C]acetate. ●, PC; ■, MGD; △, PG; ○, PE; □, DGD; *, DAG; ▲, TAG.

and to extend our comparison between the mutant and wild type, we supplied plants with [1-^{14}C]acetate and followed redistribution of radioactivity in lipid acyl groups during the subsequent 100 h.

The labeling kinetics of wild-type *Arabidopsis* were similar to those found previously (Browse et al., 1986) and were consistent with the parallel operation of two pathways of lipid synthesis. Thus, the high initial level of label in MGD (Fig. 2) in both the wild type and mutant represented MGD derived from the prokaryotic pathway. Between 10 and 100 h, the proportion of label in MGD and DGD of both wild-type and mutant plants increased as the eukaryotic pathway transferred label to these lipids from PC. The proportional rise in MGD label was greater for the mutant than for the wild type. This observation is consistent with the data obtained from lipase analysis on the relative contributions of the two pathways to MGD synthesis. PE in *fab2* also labeled to a larger extent than in the wild type, consistent with data from compositional analysis. The label in PG of the mutant was significantly higher than the 10% value predicted by compositional analysis. To ensure that this radioactivity had in fact been incorporated into PG, we eluted the PG spot from one-dimensional NH_4SO_4 TLC and analyzed the compounds with two-dimensional TLC. All the radioactivity migrated in a single spot to a position consistent with the identification of this compound as PG. At present we do not have any explanation for this increased label in PG of *fab2* plants. The *fab2* mutant had higher incorporation of radioactivity into TAG and DAG than the wild type.

Labeling of individual fatty acids on various lipids was followed by separating lipids and then trans-esterifying the fatty acids to produce fatty acid methyl esters. The methyl esters were separated in two chromatographic steps to resolve all the fatty acids. The first step, argentation TLC, separated all fatty acids except the 16:0 and 18:0 acids, which co-migrated in the chromatographic system. Because the lesion in *fab2* is directly related to the desaturation of 18:0 fatty acids, it was necessary to resolve these two fatty acids. Fatty acid methyl esters from the regions of the argentation plates containing 16:0 and 18:0 were eluted and then separated by reverse-phase TLC.

The majority of the label in wild-type PC at early times was in monoenoic fatty acids (Fig. 3A). This label was turned over as radioactivity in dienoic and then trienoic fatty acids increased. At early times, 16:0 of wild-type PC contained 18% of the total label and remained at this level through the length of the experiment. The absence of turnover of 16:0 resulted from the inability to desaturate 16:0 in the eukaryotic pathway where PC is produced (Browse and Somerville, 1991). In *fab2*, PC also labeled quickly with monoenoic fatty acids (Fig. 3B). An observable lag was seen, when compared with wild type, in the incorporation of label into trienoic fatty acids in *fab2*. The radioactivity in 16:0 of *fab2* PC was considerably less than that found in wild-type PC. Label in 18:0 of *fab2* PC appeared at very early time points, and the proportion of radioactivity in 18:0 reached a value comparable to those suggested by mass analysis at 48 h before declining at 100 h.

In wild-type MGD (Fig. 3C) the majority of label at early times was in monoenoic and dienoic fatty acids. About 10%

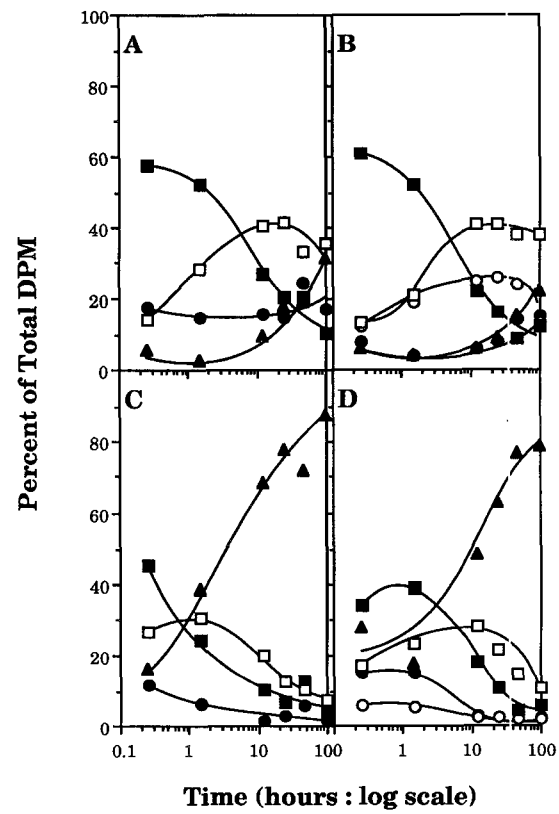

Figure 3. Redistribution of radioactivity in fatty acids of PC (A and B) and MGD (C and D) of wild type (A and C) and *fab2* (B and D) after [1-^{14}C]acetate labeling. ●, 16:0; ○, 18:0; ■, monoenoic; □, dienoic; ▲, trienoic.

of the label at 30 min was present in 16:0 and this level steadily decreased, consistent with the desaturation of 16:0 on MGD (Kunst et al., 1989). The label in dienoic fatty acids peaked by 90 min in wild-type MGD, and by 10 h trienoic fatty acids accounted for more than 60% of the label in wild-type MGD. In *fab2* (Fig. 3D) the dienoic fatty acids did not peak until after 10 h and the increase in trienoic fatty acids was also delayed. Stearate accounted for about 5% of the MGD label in *fab2* at early times and slowly declined to less than 2% of the total label.

DISCUSSION

The isolation and characterization of biochemical mutants has allowed for significant advances in our understanding of metabolism. In plant lipid metabolism there exists a large collection of mutants, each of which is altered in a specific step in the production of membrane glycerolipids (Browse and Somerville, 1994). The *fab2* mutant is the first of these mutants that contains an increased level of stearate, a particularly low-abundance fatty acid in almost all plants (Hilditch and Williams, 1964). The *fab2* mutant is unique among the *Arabidopsis* fatty acid mutants in that it has an extreme morphological phenotype. The plants are miniatures under normal growing conditions, producing leaves and rosettes that are less than one-thirtieth of the wild-type size. We have previously shown that this morphological phenotype is the result of the increased stearate content of the mutant and is reversible by growth of the plants at high temperature. A second site suppressor mutation restores normal fatty acid composition and normal growth in the mutant background (Lightner et al., 1994). Here we have extended our comparison of wild-type and mutant plants to describe the distribution of this increased 18:0 throughout the cellular lipids of *fab2* leaves and to examine the alterations in lipid metabolism that arise from the presence of 18:0 in unusually large amounts.

Seeds of the *fab2* mutant contain increases in both 18:0 and 20:0, a product of the elongation of 18:0 fatty acids (Stumpf and Pollard, 1983; James and Dooner, 1990). In leaves, the mutant also shows substantial increases in 18:0 content. Interestingly, the 18:0 content of *fab2* varies in an age-dependent manner (Fig. 1). The highest 18:0 levels (approximately 20%) occurred in the youngest tissue and the proportion of 18:0 declined as the tissue aged. One explanation for this developmental variation in the effects of the mutation could be that during the period of rapid membrane synthesis, with its attendant high rates of lipid metabolism, the partial block in 18:0 desaturation is most pronounced. When membrane biogenesis slows in mature tissues, the lower rate of 18:0 desaturation in the mutant more nearly matches the overall rate of lipid synthesis. In this respect, it would have been useful to carry out separate labeling experiments on young and mature tissues of *fab2* plants. However, the very small size of mutant plants precluded such an approach. In addition to changes in the proportions of different fatty acids, the *fab2* mutant contains an absolute increase in leaf fatty acids on a fresh weight basis (Table I). This result is consistent with anatomical evidence, which shows a dramatic decrease in leaf cell size and thus an increase in the ratio of membrane surface to volume contained (Lightner et al., 1994).

Although the *fab2* mutant contains large increases in 18:0 fatty acids, it is important to note that 18:1, 18:2, and 18:3 fatty acids are all present in the mutant with only modest reductions from their wild-type levels. Thus, the substantive alteration in *fab2* is not the absence or large reduction of a particular acyl group, as has been found in many of the *Arabidopsis* lipid mutants, but rather the overabundance of a particular acyl group, in this case 18:0. Two other mutants with high levels of saturated fatty acids have been described, *fad5* and *fab1*. The *fad5* mutant contains increased 16:0 fatty acids as a result of a deficiency in the activity of the chloroplast 16:0-MGD desaturase. The *fad5* mutant contains 24% 16:0 in leaves and 25% total saturates (16:0 + 18:0), but has no apparent morphological phenotype in normal growth temperatures (Kunst et al., 1989). The *fab1* mutant is reduced in the activity of 3-ketoacyl-ACP synthase II, contains 22% 16:0 in leaves and 23% total saturates (Wu et al., 1994), and also has no visible phenotype at normal growth temperatures (Wu et al., 1994). These data compare to 31% total saturates in the *fab2* mutant, 12% of which is contributed by 16:0 fatty acids (Table I). The *fab2* mutant, however, has profoundly altered growth and morphology (Lightner et al., 1994).

The lipid composition of the *fab2* mutant differs in two important ways from that of the wild type. First, the *fab2* mutant contains larger overall proportions of extrachloroplast lipids compared to wild type. PC makes up 22% of *fab2* lipids compared with only 15% in the wild type. There are similar proportional increases in PE, PI, and PS in the mutant. In contrast, MGD constitutes only 31% of *fab2* leaf lipids compared to 41% in wild type. This increase in extrachloroplast lipids relative to chloroplast lipids is consistent with anatomical observations, indicating that *fab2* has lower numbers of chloroplasts per cell in the leaf (Lightner et al., 1994). The *fab2* mutation causes increased levels of stearate in all glycerolipids examined, which is consistent with a mutation that affects desaturation of 18:0 bound to ACP. 18:1 derived from ACP is used by both the prokaryotic and eukaryotic pathways (Browse and Somerville, 1991), and a mutation that reduces the activity of the 18:0-ACP desaturase should result in 18:0 increases in both chloroplast and extrachloroplast lipids. Although the 18:0 content of all leaf lipids is increased, the relative amount of increase varied considerably between different lipids. PC, a major extrachloroplast lipid, contains more than 26% 18:0 in *fab2*. In contrast, *fab2* MGD contains only 3% stearate. In general, our data show that the absolute level of stearate is most affected in the extrachloroplast lipids, but chloroplast lipids in *fab2* contain large proportional increases in stearate compared to their wild-type counterparts.

High levels of polyunsaturated fatty acids are present in all the major lipids of *fab2* leaves so that the mutation generally has a small to moderate effect on the number of double bonds per glycerolipid molecule. Other mutations that affect the later desaturation steps have larger effects on the number of double bonds even though these mutations do not have the strong pleiotropic effects on plant growth that are found in the *fab2* line. For example, the *fad6* (previously *fadC*) mutation reduced the average number of double bonds in MGD from 5.8 in wild type to 3.7 in the mutant (Browse

et al., 1989), a decrease very much larger than that caused by the *fab2* mutation, without affecting plant morphology. The *fab2-1* mutation reduced the number of double bonds in PC from 3.6 in the wild type to 2.7 in the mutant (calculated from Miquel and Browse, 1992), again a larger change than in the *fab2* mutant, and again without changing the growth characteristics of the *fad2-1* mutant plant under normal temperatures. These observations indicate that changes in membrane lipid unsaturation are not meaningfully assessed by simple measurements such as the average number of double bonds.

Our positional analysis indicates that for all of the lipids examined except PG, disaturated molecular species are relatively rare in the *fab2* mutant. In fact, the total saturated fatty acid content (16:0 + 18:0) at the *sn-2* position in *fab2* lipids was very similar to the wild type for all the lipids examined (Table III). Our results also show that a larger proportion of chloroplast MGD and DGD is derived from the eukaryotic pathway than is normally found in wild type. This decrease in prokaryotic MGD may also result from the exclusion of 18:0 by the prokaryotic acyl transferase, reducing the overall flux through this pathway. Altered flux through the two-pathway scheme has been shown in other *Arabidopsis* mutants (Kunst et al., 1989; Miquel and Browse, 1992).

Recent findings suggest the possibility that several isoforms of the 18:0-ACP desaturase gene occur in the *Arabidopsis* genome (John Shanklin, personal communication). Using genetic and physical mapping techniques, it should be possible to establish if the *fab2* mutations reside in (or near) one of these structural genes. We have previously reported the isolation of a semi-dominant intergenic suppressor mutation that restores the 18:0 level to near the wild-type level (Lightner et al., 1994). One possible explanation for this suppression would be a mutation increasing the expression of one of the 18:0-ACP desaturases in a manner that compensates for the *fab2* lesion. The existence of multiple isoforms of the desaturase genes, the *fab2* mutation, and a second site suppressor of this mutation provide powerful genetic tools with which to examine the effects of stearate content on plant cell biology.

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