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A Mutant of *Arabidopsis* Deficient in Desaturation of Palmitic Acid in Leaf Lipids[1]

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ABSTRACT

The overall fatty acid composition of leaf lipids in a mutant of *Arabidopsis thaliana* was characterized by elevated amounts of palmitic acid and a decreased amount of unsaturated 16-carbon fatty acids as a consequence of a single nuclear mutation. Quantitative analysis of the fatty acid composition of individual lipids suggested that the mutant is deficient in the activity of a chloroplast ω9 fatty acid desaturase which normally introduces a double bond in 16-carbon acyl chains esterified to monogalactosyldiacylglycerol (MGD). The mutant exhibited an increased ratio of 18- to 16-carbon fatty acids in MGD due to a change in the relative contribution of the prokaryotic and eukaryotic pathways of lipid biosynthesis. This appears to be a regulated response to the loss of chloroplast ω9 desaturase and presumably reflects a requirement for polyunsaturated fatty acids for the normal assembly of chloroplast membranes. The reduction in mass of prokaryotic MGD species involved both a reduction in synthesis of MGD by the prokaryotic pathway and increased turnover of MGD molecular species which contain 16:0.

The leaves of higher plants contain a family of fatty acid desaturases that are located in the chloroplast and the endoplasmic reticulum (9, 18). The first step in desaturation of 18-carbon fatty acids is catalyzed by a soluble chloroplast desaturase that inserts a double bond at the ω9 position of stearic acid bound to ACP[2] (14). Subsequent desaturation of 18-carbon acyl groups takes place on microsomal PC (15, 18, 20) and on the plastid lipids MGD, DGD, SL, and PG (1, 11, 16, 17). By contrast, the first step in 16-carbon fatty acid desaturation appears to take place on lipid substrates. One enzyme specifically introduces a *trans*-double bond in 16:0 esterified to the sn-2 position of chloroplast PG (2). A separate enzyme appears to be responsible for introducing a *cis*-double bond in the 16-carbon acyl group on the sn-2 position of MGD. Several lines of evidence indicate that sequential desaturation of 16:1 to 16:3 also takes place on position sn-2 of chloroplast MGD (17, 19). However, genetic analysis of mutants deficient in plastid ω3 and ω6 desaturases indicates that the enzymes that convert 16:1 to 16:2 and the enzyme that converts 16:2 to 16:3 are not specific for MGD or for the length or position of the acyl group (4, 6). A small amount of unsaturated 16-carbon acyl groups in DGD may be due to conversion of MGD to DGD (7).

Except for the 18:0-ACP desaturase, all the desaturases are thought to be membrane-bound enzymes that lose activity during membrane solubilization. Indeed, activity of chloroplast desaturases is lost upon chloroplast rupture or exposure of intact chloroplasts to mild hypotonic conditions (1). The difficulties associated with solubilization and reconstitution of desaturase activity *in vitro* have hindered traditional biochemical investigations of these enzymes. Therefore, we have pursued a genetic approach to study the desaturation process in plant membrane lipids by the isolation of a number of mutants with specific changes in unsaturation of leaf fatty acids. We have previously characterized mutants deficient in *trans*-16:1 synthesis (2) and mutants deficient in the synthesis of trienoic fatty acids (4). Here we describe the isolation and characterization of a mutant deficient in desaturation of 16:0 to *cis*-16:1.

MATERIALS AND METHODS

Plant Material

The mutant line JB67 was isolated from the Columbia wild type of *Arabidopsis thaliana* (L.) Heynh. following mutagenesis with ethyl methane sulfonate (2). It was backcrossed to the wild type four times before being used for the experiments reported here. Plants were grown at 22°C with continuous fluorescent illumination (100–150 μE m^{-2} s^{-1}) on a perlite:vermiculite:sphagnum (1:1:1) mixture irrigated with a mineral nutrient solution.

Lipid Analysis

Leaf material was frozen in liquid N_2, and lipids were extracted with chloroform:methanol:formic acid (10:10:1 by volume) as previously described (5). Individual lipids were isolated by TLC on silica gel G coated plates (13) or $(NH_4)_2SO_4$-impregnated silica gel G plates (12) and were transmethylated with methanolic-HCl (Supelco) after the addition of 14:0 or 17:0 methyl ester as internal standard. The resulting methyl esters were then quantified by gas chromatography (3). Fatty acid positional distribution of MGD,

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[2] Abbreviations: ACP, acyl carrier protein; DAG, diacylglycerol; DGD, digalactosyldiacylglycerol; *fadB*, symbol for a gene controlling fatty acid desaturation; MGD, monogalactosyldiacylglycerol; *n:x*, fatty acid containing *n* carbons and *x* double bonds; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SL, sulfolipid.

DGD, PC, and PE was established following degradation with *Rhizopus arrhizus* lipase (triacylglycerol acylhydrolase EC 3.1.1.3; Boehringer Mannheim GmbH) as described (8).

The labeling of intact *Arabidopsis* plants with [^{14}C]acetate and the analysis of distribution of radioactivity in the various lipids were done essentially as described previously (5). The proportion of MGD synthesized by the prokaryotic and eukaryotic pathways was estimated by measuring the proportion of label in 16-carbon fatty acids esterified to MGD (5). MGD isolated from leaves at various times after application of [^{14}C] acetate was transmethylated (3). The methyl esters were hydrogenated to 16:0 and 18:0 which were separated by reverse phase TLC (5).

RESULTS AND DISCUSSION

Genetic Analysis

The mutant line JB67 was recovered from among 2000 M_2 plants descended from mutagenized seed by direct assay of leaf fatty acid composition (2). This line was retained because it accumulated elevated levels of palmitic acid and was deficient in unsaturated 16-carbon fatty acids (Table I). In addition to this change in the level of unsaturation of its leaf lipids, the mutant had a slight (15%) reduction in Chl content but was otherwise indistinguishable from the wild type in appearance and growth characteristics under standard conditions.

The genetic basis of the altered lipid composition was established by crossing the mutant with the wild type as maternal parent. The leaves of F_1 progeny showed levels of 16-carbon fatty acids which were similar to that of the wild type parent, indicating that a single functional copy of the *fad*B gene product was almost sufficient to ensure normal fatty acid composition. The frequency of the homozygous mutant phenotype in the F_2 generation was also analyzed by gas chromatography. Of 308 F_2 plants, 78 had substantially increased 16:0 levels and were deficient in unsaturated 16-carbon fatty acids whereas the remaining 230 individuals had a leaf fatty acid composition which was similar to that of the wild type or the F_1 hybrid. The 3:1 segregation pattern (χ^2 = 0.013, $P \geq 0.9$) indicated that the altered fatty acid composition is due to a single nuclear mutation at a locus we designate *fad*B.

Table I. *Fatty Acid Composition of Total Lipids of Mutant and Wild-Type (WT) Arabidopsis*

Fatty Acid	WT	F_1 (WT[a] × JB67)	JB67
		mol % ± sd[a]	
16:0	13.0 ± 0.8	16.4 ± 0.8	24.1 ± 0.9
16:1 *cis*	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.3
16:1 *trans*	3.6 ± 0.4	2.8 ± 0.3	2.8 ± 0.4
16.2	1.7 ± 0.5	1.0 ± 0.3	0.3 ± 0.1
16.3	15.7 ± 1.0	12.5 ± 0.7	0.3 ± 0.2
18:0	0.7 ± 0.2	1.2 ± 0.2	1.1 ± 0.4
18:1	2.4 ± 0.4	3.0 ± 0.4	2.5 ± 0.5
18:2	12.2 ± 0.6	13.6 ± 0.3	17.1 ± 1.0
18:3	49.0 ± 1.4	48.2 ± 1.4	50.2 ± 1.5
Total C16	35.5	34.2	29.0

[a] $n = 10$.

Biochemical Characterization

Leaves of the mutant contained essentially no 16:3 but did show increased levels of 16:0 (Table I), which is a precursor of 16:3 during the desaturation of prokaryotic MGD (17). Furthermore, there was no significant accumulation of 18:0 in the mutant, indicating that the mutation specifically affects desaturation of 16-carbon fatty acids. Thus, the simplest explanation for the altered lipid composition of the mutant is that the *fad*B locus controls the activity of an ω9 fatty acid desaturase which specifically desaturates 16:0 esterified to MGD. Since this desaturase activity has not yet been demonstrated by an *in vitro* assay, the enzymic lesion in the mutant could not be demonstrated directly.

The loss of 16:3 from the mutant was not entirely compensated for by the increase in 16:0. For this reason, 16-carbon acyl groups account for only 29% of the total fatty acids in the mutant compared with 35.5% in the wild type (Table I).

Fatty Acid Composition of Individual Lipids

The consequences of the deficiency in 16:0 desaturation are shown more clearly by an analysis of individual lipids extracted from leaf tissue of wild-type and mutant plants (Table II). The proportions of the various polar lipids in the leaves showed no major change between the mutant and wild type. Although 16:3 could not be detected in MGD of the mutant, the amount of 16:0 present in this lipid was substantially less than the amount of 16:3 in wild-type MGD. All together, there is a 60% decrease in the proportion of 16-carbon fatty acids in MGD of the mutant compared with wild-type MGD. This is consistent with a reduced level of 16-carbon fatty acids in the mutant (Table I), but it is noteworthy that both DGD and PC showed increased levels of 16:0 in the mutant compared with the wild type (Table II). Positional analysis of MGD using *Rhizopus* lipase confirmed that 16:0 in the mutant was predominantly at the *sn*-2 position as was 16:3 in the wild type (Table III). In contrast, PC and DGD of the mutant showed increased levels of 16:0 at both *sn*-1 and *sn*-2 positions.

Taken together, these observations suggest that the failure to desaturate 16:0 at position sn-2 of MGD is accompanied by a reduction in the proportion of prokaryotic MGD in the mutant. The total percentage of MGD in the leaf polar lipids remains the same as wild type, implying increased synthesis of MGD by the eukaryotic pathway. In principle, this reduced accumulation of prokaryotic MGD might result from a decreased flux of carbon into the prokaryotic pathway or an increased turnover of 16:0-containing MGD species. Such a turnover could provide the extra 16:0 found in PC and DGD. The observation that a proportion of the extra 16:0 in PC of the mutant is at the *sn*-2 position of this lipid, suggests that diacylglycerol formed by the prokaryotic pathway may be involved in PC synthesis in the mutant. Other mechanisms, such as enhanced utilization of certain molecular species (*e.g.* dilinoleoyl-PC) for MGD synthesis by the eukaryotic pathway

Table II. *Fatty Acid Composition of Leaf Lipids from Wild-Type (WT) and Mutant Arabidopsis grown at 22 °C*

The values are the means obtained in three independent experiments.

Acyl Group	MGD		DGD		SL		PG		PC		PE		PI	
	WT	JB67	WT	JB67	WT	JB67	WT	JB67	WT	JB67	WT	JB67	WT	JB67
							mol %							
16:0	2.0	13.8	13.6	26.0	38.5	39.3	28.0	30.8	24.4	30.2	30.0	31.9	43.5	44.1
16:1	0.5	0.1	—[a]	—	—	—	21.1	19.5	—	—	—	—	—	—
16:2	1.8	0.1	0.4	0.1	—	—	—	—	—	—	—	—	—	—
16:3	30.9	—	2.6	—	—	—	—	—	—	—	—	—	—	—
18:0	0.1	0.6	1.0	2.5	2.4	2.4	1.0	2.0	2.1	2.4	2.2	2.1	3.0	5.6
18:1	0.6	1.0	1.5	0.9	2.8	3.1	4.8	5.1	5.3	5.5	3.3	3.5	3.6	4.4
18:2	3.7	3.1	5.7	2.0	14.7	10.4	10.9	10.8	32.4	30.2	34.4	33.5	22.5	19.8
18:3	60.0	80.7	75.1	67.9	41.3	44.7	33.1	30.0	35.0	31.6	29.8	26.1	27.1	25.0
Proportion of leaf polar lipids (%)														
	38.9	38.3	16.1	13.7	4.0	3.3	10.0	11.7	16.6	17.7	11.2	12.3	3.9	3.4

[a] Acyl group was not detected.

Table III. *Fatty Acid Distribution in Lipids from Wild Type (WT) and Mutant*

The symbol 16:X represents the combined amount of 16:1, *trans*-16:1, 16:2, and 16:3.

Acyl Group	*sn*-1		*sn*-2	
	WT	JB67	WT	JB67
		mol %		
MGD				
16:0	0	1.0	1.0	12.1
16:X	1.2	0	34.5	0.4
18:0	0	1.2	0	0
18:1	0.5	0.5	0.1	0.1
18:2	1.8	1.9	0.6	1.6
18:3	46.4	45.4	13.8	35.8
DGD				
16:0	10.0	18.0	6.3	14.3
16:X	0	0	2.3	0
18:0	1.3	3.0	0	0.4
18:1	2.4	1.3	0	0.3
18:2	2.4	0	3.1	2.0
18:3	33.9	27.7	38.3	33.0
PC				
16:0	26.0	29.6	2.1	4.4
18:0	3.0	2.3	0.4	2.0
18:1	3.2	3.4	4.9	5.4
18:2	11.0	10.2	26.3	25.2
18:3	6.4	5.4	15.9	13.0

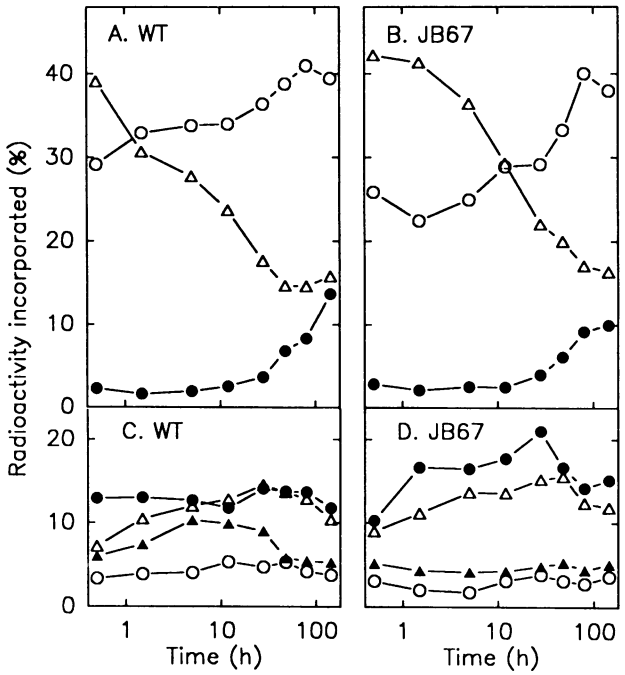

Figure 1. Redistribution of radioactivity in leaf lipids of wild type and JB67 mutant of *Arabidopsis* after labeling with [^{14}C]acetate. Symbols: (A and B) △, PC; ○, MGD; ●, DGD; (C and D) ○, SL; ●, PG; △, PE: ▲, PI.

might also result in a higher proportion of 16:0 within PC of the mutant.

Labeling of Leaves

To elucidate the mechanisms involved in reducing the level of 16:0-containing MGD in the mutant, we labeled wild-type and mutant plants with [^{14}C]acetate, and then followed redistribution of radioactivity in lipids during the subsequent chase period (Fig. 1). The kinetics of labeling in the wild type were similar to those found previously (5) and are consistent with the parallel operation of the prokaryotic and eukaryotic pathways of lipid synthesis. We have demonstrated elsewhere (5, 13) that the label found in MGD at the beginning of the experiment is derived from the prokaryotic pathway, whereas the increased amount of label in MGD at longer times reflects the movement of lipids synthesized by the eukaryotic pathway in the endoplasmic reticulum into the chloroplast where conversion to MGD takes place. The *fad*B mutant had only about 80% as much label as the wild type in MGD at the two earliest times (Fig. 1). Since this does not reflect the magnitude of the reduction in the proportion of prokaryotic MGD in the mutant evident from the results in Table II, it appears

that reduced synthesis of MGD does not by itself account for the difference between mutant and wild type.

To examine the possibility that accelerated turnover of prokaryotic MGD could account for the reduced ratio of prokaryotic to eukaryotic MGD in the mutant, we measured the labeling of eukaryotic and prokaryotic MGD during a pulse-chase with [^{14}C]acetate. Prokaryotic MGD is primarily composed of 18-C/16-C molecular species, while eukaryotic MGD is primarily composed of 18-C/18-C species. It is, therefore, possible to estimate the relative contribution of the two pathways of MGD synthesis from the radioactivity in 16-carbon fatty acids of this lipid (5). In Figure 2, the label in prokaryotic, eukaryotic, and total MGD is expressed as percentages of the label in all polar lipids during a 100 h pulse-chase. Throughout the course of the experiment there was no net loss of ^{14}C from prokaryotic species in the wild type. By contrast, in the mutant there was a steady decline in the amount of label in prokaryotic MGD throughout the experiment, indicating accelerated turnover of prokaryotic MGD in the mutant. This was largely compensated for by increased labeling of MGD via the eukaryotic pathway in the mutant so that by the end of the experiment, the total radioactivity in MGD of the mutant was comparable to that in the wild type.

DISCUSSION

Comparison of the fatty acid composition of MGD from the mutant and wild type supports the conclusion that the mutant line JB67 is able to synthesize prokaryotic MGD, but is deficient in activity of a chloroplast ω9 desaturase. Our results are consistent with a proposal that the substrate for this enzyme is a 16-carbon acyl group esterified to MGD (18, 19). This feature distinguishes the 16:0 desaturase from the ω3 and ω5 desaturases (4, 6) which do not exhibit specificity with respect to the length of the acyl chain, its point of attachment to the glycerol backbone (*sn*-1 or *sn*-2), or the lipid head group. In this respect, the desaturase controlled by

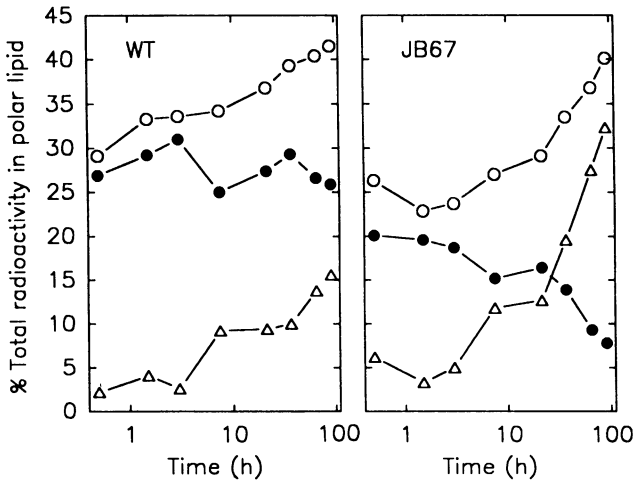

Figure 2. Radioactivity in MGD derived from prokaryotic and eukaryotic pathways of lipid synthesis in wild-type and mutant *Arabidopsis* after labeling leaves with [^{14}C]acetate. Symbols: ●, Prokaryotic MGD; △, eukaryotic MGD; ○, total MGD.

the *fadB* gene is similar to the desaturase which acts only on 16:0 esterified to the *sn*-2 position of PG (2). The observation that the mutant accumulates MGD containing 16:0 at position *sn*-2 indicates that introduction of the ω9 double bond is a prerequisite for further desaturation of 16-carbon acyl chains by the other chloroplast desaturases. Similar conclusions were reached for the desaturases of *Chlorella* presented with various monoenoic fatty acids as substrates (10).

The accumulation of 16:0 at position *sn*-2 of MGD is consistent with the expectation for a mutant unable to desaturate 16:0 to 16:1. However, the *fadB* mutation also caused a decrease of more than 60% in the amount of MGD containing 16-carbon fatty acids which was compensated for by increased synthesis of MGD containing 18-carbon fatty acids. The implication is that MGD containing 16:0 at *sn*-2 is either turned over more rapidly and, thus, does not accumulate to normal levels, or that accumulation of this lipid species inhibits the synthesis of MGD by the prokaryotic pathway located in the chloroplast envelope. A similar phenomenon was previously observed (6) in a *fadC* mutant of *Arabidopsis*, which is deficient in desaturation of 16:1 and 18:1 fatty acids on chloroplast lipids. In this case we observed a 35% reduction in the mass of prokaryotic MGD species that could be largely accounted for by decreased synthesis of MGD by the prokaryotic pathway.

The relative importance of accelerated turnover *versus* inhibition of synthesis of prokaryotic MGD in the *fadB* mutant was estimated by measuring the amount of label in prokaryotic and eukaryotic MGD during a 100 h pulse-chase with [^{14}C]acetate. Reduced incorporation of label into prokaryotic MGD at the earliest time points indicated that synthesis of prokaryotic MGD was reduced in the mutant. In addition, the amount of label in prokaryotic MGD declined by about 65% during the pulse chase, whereas there was no decline in the amount of label in prokaryotic MGD in the wild type. These effects were compensated for by increased accumulation of label in eukaryotic MGD at later time points so that, by the end of the experiment, the mutant and wild type had accumulated the same amount of label in MGD. Therefore, the reduced mass of prokaryotic MGD species in the mutant is due to both a reduced flux of fatty acids into the prokaryotic pathway and an increased turnover of MGD molecular species which contain 16:0. It seems likely that, in the mutant, a significant proportion of the 16:0-ACP, which might otherwise enter the prokaryotic pathway, is converted to 18:1-ACP and directed toward synthesis of lipids by the eukaryotic pathway. Such a reallocation was observed in the *act1* mutant of *Arabidopsis* in which the prokaryotic pathway is blocked by a deficiency in the chloroplast ACP:glycerol-3-phosphate acyl transferase (13). In addition, substantial increases in the amount of 16:0 in polar lipids, especially PC and DGD, suggests that the turnover of prokaryotic MGD is accompanied by the reutilization of the fatty acids for synthesis of other lipids.

It is particularly noteworthy that the decreased accumulation of prokaryotic MGD in the mutant does not lead to a decrease in the absolute amount of this lipid, but rather is compensated for by increased synthesis of eukaryotic MGD. Similar results were obtained with a mutant deficient in

plastid ACP:glycerol-3-phosphate acyltransferase (13). Therefore, it appears that the relative flux through the two pathways of lipid synthesis is regulated, by an unknown mechanism, to offset the potentially deleterious effects of a major change in lipid unsaturation in the mutant which might otherwise result from the *fadB* mutation.

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