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Disruption of the *FATB* Gene in Arabidopsis Demonstrates an Essential Role of Saturated Fatty Acids in Plant Growth

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Acyl-acyl carrier protein thioesterases determine the amount and type of fatty acids that are exported from the plastids. To better understand the role of the FATB class of acyl-acyl carrier protein thioesterases, we identified an Arabidopsis mutant with a T-DNA insertion in the *FATB* gene. Palmitate (16:0) content of glycerolipids of the mutant was reduced by 42% in leaves, by 56% in flowers, by 48% in roots, and by 56% in seeds. In addition, stearate (18:0) was reduced by 50% in leaves and by 30% in seeds. The growth rate was reduced in the mutant, resulting in 50% less fresh weight at 4 weeks compared with wild-type plants. Furthermore, mutant plants produced seeds with low viability and altered morphology. Analysis of individual glycerolipids revealed that the fatty acid composition of prokaryotic plastid lipids was largely unaltered, whereas the impact on eukaryotic lipids varied but was particularly severe for phosphatidylcholine, with a >4-fold reduction of 16:0 and a 10-fold reduction of 18:0 levels. The total wax load of *fatb-ko* plants was reduced by 20% in leaves and by 50% in stems, implicating FATB in the supply of saturated fatty acids for wax biosynthesis. Analysis of C_{18} sphingoid bases derived from 16:0 indicated that, despite a 50% reduction in exported 16:0, the mutant cells maintained wild-type levels of sphingoid bases, presumably at the expense of other cell components. The growth retardation caused by the *fatb* mutation was enhanced in a *fatb-ko act1* double mutant in which saturated fatty acid content was reduced further. Together, these results demonstrate the in vivo role of FATB as a major determinant of saturated fatty acid synthesis and the essential role of saturates for the biosynthesis and/or regulation of cellular components critical for plant growth and seed development.

INTRODUCTION

In plants, de novo fatty acid synthesis in plastids can be terminated by the action of plastidial acyltransferases that transfer the acyl group of acyl-acyl carrier protein (acyl-ACP) to produce glycerolipids within the plastid (prokaryotic pathway) or, alternatively, the acyl group from acyl-ACP can be hydrolyzed by acyl-ACP thioesterases (FAT) that release free fatty acids and ACP. After export from the plastid, free fatty acids are re-esterified to CoA to form the cytosolic acyl-CoA pool, which is used primarily for glycerolipid biosynthesis at the endoplasmic reticulum (eukaryotic pathway) (Browse and Somerville, 1991). In Arabidopsis leaves, oleate (18:1) and palmitate (16:0) are the major products of plastid fatty acid synthesis, and ~60% of these products are exported to the cytosol as free fatty acids. In other tissues or plant species, flux through the acyl-ACP thioesterase to the eukaryotic pathway is more predominant, with contributions of ≥90%. Therefore, thioesterases play an essential role in the partitioning of de novo–synthesized fatty acids between the prokaryotic and eukaryotic pathways. Moreover, thioesterase substrate specificity determines the chain length and saturation of fatty acids exported from the plastid (Pollard et al., 1991). Based on amino acid sequence comparisons and substrate specificity, two different classes of acyl-ACP thioesterases have been described in plants (Voelker et al., 1997). The FATA class has highest in vitro activity for 18:1-ACP and much lower activity for saturated acyl-ACP substrates. Members of the second class of thioesterases, FATB, prefer saturated acyl groups but also have activity for unsaturated acyl-ACPs (Doermann et al., 1995; Voelker et al., 1997; Salas and Ohlrogge, 2002).

In the Arabidopsis genome, there are two genes for *FATA* and a single gene for *FATB* (F. Beisson, unpublished data available at http://plantbiology.msu.edu/gene_survey/front_page.htm). All other higher plants that have been examined appear to express both classes of thioesterase (Mekhedov et al., 2000). One salient question is why plants require two classes of acyl-ACP thioesterase and what individual role each plays. The major exported fatty acid in Arabidopsis is 18:1, and based on in vitro activity, it can be predicted that FATA determines the in vivo levels of 18:1 that move out from the plastid (Salas and Ohlrogge, 2002). In the case of FATB, a previous antisense and overexpression study in Arabidopsis demonstrated that this enzyme is involved, at least in part, in the in vivo production of saturates in flowers and seeds (Doermann et al., 2000). Similarly, downregulation of *FATB* expression in soybean also demonstrates partial reduction of seed palmitic acid (Wilson et al., 2001; Buhr et al., 2002). However, the origin of palmitic acid, which remains after gene-silencing procedures, and the extent to which each class of thioesterase contributes in vivo to the production of exportable fatty acids by different tissues remain unresolved.

One possible role for the two thioesterases is to provide control over the saturated/unsaturated balance of membrane fatty

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acids. The composition of almost all plant, animal, and microbial membranes consists of a mixture of saturated and unsaturated fatty acids. Such a mixture is believed to be essential to provide a balance of physical properties (e.g., fluidity) as well as a method to adapt to changes in the environment (e.g., temperature) and to prevent phase transitions or lateral phase separations that are promoted by lipids with uniform fatty acid composition. However, as demonstrated by extensive feeding studies with microbial cells that depend on exogenous fatty acids for membrane synthesis (Walenga and Lands, 1975; McElhaney, 1989), most organisms can accommodate a surprising range of fatty acid structures in their membranes without impairments in growth. Similarly, a wide range of mutations in plant fatty acid desaturases demonstrate that the fatty acid composition of plant membranes can be altered considerably with no apparent phenotype under normal growth conditions. For example, Arabidopsis mutants with elimination of 16:1 *trans*-Δ3, 16:3, 18:3, or large reductions in 18:2 grow normally at 25°C (Wallis and Browse, 2002).

These studies suggest that within a certain range, the composition of fatty acids in membranes is not critical as long as a mixture of acyl chains is provided. However, beyond this range (e.g., under temperature extremes or when major changes in fatty acid composition occur), growth can be affected severely. For example, the *fab2* mutant of Arabidopsis is severely reduced in growth as a result of an increase in 18:0 leaf content to 10 to 15% (Lightner et al., 1994), and in the *fad2 fad6* double mutant, the complete elimination of polyunsaturated fatty acids leads to the loss of photosynthetic ability (Wallis and Browse, 2002). In addition to a role as bulk components of membranes, in which exact structures seem less critical, some unsaturated fatty acids play more specialized roles as precursors for signal molecules (e.g., linolenic acid for jasmonate), and saturated fatty acids are precursors for sphingolipids, surface waxes, and cutin and are involved in protein acylation. Such roles clearly are vital because mutants completely lacking trienoic acids, for example, are male sterile and impaired in pathogen defense (Wallis and Browse 2002), but to date, such critical roles for saturated fatty acids have not been described.

Here, we describe the isolation and analysis of a mutant disrupted in the *FATB* thioesterase gene. Surprisingly, despite only an overall 40 to 50% reduction of saturated fatty acid content, the mutant grew slowly and produced deformed seeds with low viability. These results have revealed an essential role of FATB in the supply of saturated fatty acids for the biosynthesis and/or regulation of components vital for plant growth and seed development.

RESULTS

Mutant Isolation and Complementation Analysis

In an effort to better understand the in vivo functions of the Arabidopsis *FATB* acyl-ACP thioesterase, a PCR-based strategy was used to screen a population of T-DNA–tagged Arabidopsis plants (Sussman et al., 2000) for disruption of the *FATB* gene. PCR analysis of pooled leaf genomic DNA identified a template from plants containing a T-DNA insertion in the second intron of the *FATB* gene (*fatb-ko*) (Figure 1). Genetic segregation analysis of heterozygote *FATB* T-DNA insertion lines selected one line that segregated with the expected ratio (3:1) for a single T-DNA insertion (280:105 Basta resistant:susceptible) ($\chi^2 = 0.53$, $P > 0.4$). However, because approximately half of the homozygous *fatb-ko* plants were lost during germination (see below), an expected ratio of 2.5:1 (resistant:susceptible) better fit the observed ratio ($\chi^2 = 0.18$, $P > 0.6$). One hundred ten individuals of these 280 resistant plants were grown to full maturity, and of these, 25 had a slow-growth phenotype (see below). Again, the observed phenotypic segregation ratio agreed with the expected 2:0.5 ratio ($\chi^2 = 0.2$, $P > 0.6$) of segregation for a single T-DNA insertion when considering the lower germination rate of the mutant.

A subset of the 110 Basta-resistant plants were randomly selected and subjected to PCR and gas chromatography analysis to determine the genotype and the fatty acid composition, respectively. All plants with a wild-type visual phenotype and fatty acid composition were heterozygous for the *FATB* T-DNA insertion, whereas plants with a mutant visual phenotype and fatty acid composition were homozygous for the same insertion (data not shown). The confirmation that the visual phenotype and the genetic (T-DNA) and biochemical (fatty acid composition) markers all cosegregated suggested that the T-DNA insertion in the *FATB* gene was responsible for both the observed growth and the fatty acid phenotypes. To exclude the possibility that a second-site mutation closely linked to the *FATB* T-DNA insertion was responsible for the phenotype, the wild-type FATB cDNA was inserted under the control of the constitutive 35S promoter of *Cauliflower mosaic virus* (CaMV35S promoter) and

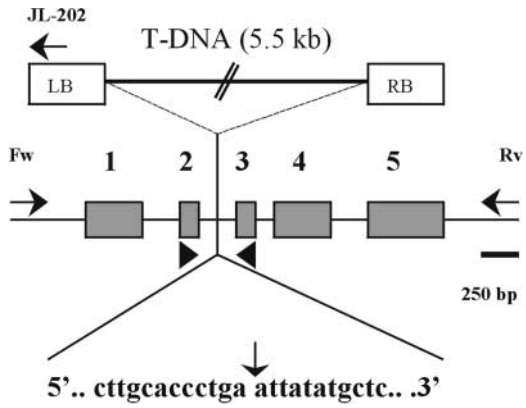

Figure 1. Structure of the Arabidopsis *FATB* Gene Carrying the T-DNA Insertion.

The *FATB* gene is composed of five exons, and the T-DNA is located in the second intron. The arrows above the scheme of the gene represent the primers used for the initial screening of the *fatb* knockout plants. The arrowheads below exons 2 and 3 represent the primers used for mRNA quantification by real-time PCR. The sequence of a portion of intron 2 encompassing the site of integration of the T-DNA (arrow) is shown at bottom. Fw, forward primer; JL-202, T-DNA left border (LB) specific primer; RB, right border; Rv, reverse primer.

expressed in homozygous *fatb-ko* plants. Transgenic lines resistant to both hygromycin B (transgene T-DNA) and Basta (knockout T-DNA) were indistinguishable from wild-type lines and showed normal growth and biochemical characteristics (see below). Therefore, we conclude that disruption of the *FATB* gene is responsible for the phenotypes observed in the mutant plants.

FATB mRNA Expression Analysis

The T-DNA was located in the second intron of the *FATB* gene; therefore, it was possible that the cells could correctly splice out at least a fraction of the precursor FATB RNA to yield mature mRNA. Indeed, by means of reverse transcriptase–mediated PCR with a set of primers that spanned the second intron of the *FATB* gene (Figure 1), small amounts of correctly spliced mRNA were detected (data not shown). To determine the extent of gene disruption, FATB transcript levels were quantified by real-time PCR in wild-type and mutant leaf tissue and found to be >150-fold lower in mutant than in wild-type tissue (Table 1). Therefore, although PCR could detect correctly spliced FATB mRNA, this transcript represented <0.7% of wild-type levels. Furthermore, protein gel blot analysis developed with anti-Arabidopsis FATB antibodies did not detect FATB protein in the insertion mutant (data not shown). These results indicated that the T-DNA insertion generated an essentially complete knockout mutant.

FATB Is Essential for Normal Seedling Growth

The first visual characteristic of *fatb* knockout plants was their size compared with wild-type plants (Figures 2A and 2B). The rosettes of *fatb-ko* plants were approximately half the diameter of wild-type rosettes during the first weeks of growth at 22°C. In addition, the bolting time was delayed in the mutant. More than 90% of the wild-type plants bolted after 4 weeks under our growing conditions, whereas development was delayed in *fatb-ko* such that only after 6 weeks did >90% of *fatb-ko* plants bolt (Table 2). The morphology of the different organs from mutant plants was unchanged compared with that of wild-type plants; however, the stems of the mutant elongated more slowly than wild-type stems. As shown in Figures 3A and 3B, plots of the fresh weight of the aerial parts of wild-type and *fatb-ko* mutant plants indicated that during the first 4 weeks after germination, the plants grew at a constant rate. However, the rate was slower for the mutant. Results shown in Figure 3B (log scale) indicated that wild-type plants increased their fresh weight by 10.6-fold (±0.4) per week, whereas for the *fatb-ko* plants, the increase was only 8.8-fold (±0.6). This 17% reduction in growth rate led to a reduction of >50% in the fresh weight of the mutant after 4 weeks (Figure 3A). The growth of both wild-type and mutant plants slowed after the 4th week, but more so for the former, such that by the 6th week the *fatb* knockout plants differed in size and fresh weight by ~25% (Figure 3A). During the growing phase, the percentage ratio of dry to fresh weight remained at ~9%, whereas during the drying period, it increased to ~14% for both wild-type and mutant plants. The similar morphology but different growth rates suggested that differences in wild-type and mutant plants were the consequence of a reduced growth rate and not altered development of the mutant. Wild-type and *fatb-ko* plants were grown in the presence of 1% sucrose on either plates or liquid culture to determine if the normal growth rate could be recovered. Sucrose availability did not eliminate the slower growth rate of the mutant (data not shown), suggesting that photosynthetic capacity or carbon limitations of the *fatb* knockout plants did not cause the reduced growth rate.

Plant growth rate is modified by temperature, and part of this effect may be associated with variations in the physical properties of cell membranes. To test the possibility that the growth retardation of the *fatb-ko* mutant was a function of temperature, wild-type and *fatb-ko* plants were grown for 2 weeks at 22°C and then transferred to three different temperatures (16, 22, and 36°C). The *fatb-ko* plants showed the same percentage reduction (~50%) of fresh weight per seedling compared with wild-type plants at the three different temperatures. Therefore, the slower relative growth of the mutant plants was not altered within the range of temperatures used. In addition, we tested whether adding exogenous saturated fatty acids by either spraying plants or supplementing seedlings grown in liquid culture could overcome the slower growth of the *fatb-ko* mutant. These procedures were not sufficient to chemically complement the *fatb-ko* phenotype, and the addition of higher amounts of exogenous fatty acids showed deleterious effects on plant growth (data not shown).

Table 1. Relative Amounts of FATB mRNA in Leaf Tissue of Wild-Type and *fatb-ko* Arabidopsis Plants

	FATB mRNA		eIF4A1[a] mRNA		
Plant Type	C_t Value	2^{Ct}	C_t Value	2^{Ct}	Ratio of FATB/eIF4A1
Wild type (Ws)	23.5 ± 0.4	1.2×10^7	21.4 ± 0.36	2.8×10^6	0.24
fatb-ko (het)	24.3 ± 0.1	1.0×10^7	21.2 ± 0.15	2.3×10^6	0.23
fatb-ko (hom)	30.4 ± 0.3	1.4×10^9	21.2 ± 0.18	2.4×10^6	0.0016

C_t, threshold cycle; 2^{Ct}, exponential function of base two with variable C_t. het and hom, heterozygous and homozygous for T-DNA insertion; respectively; Ws, Wassilewskija.

[a] Eukaryotic protein synthesis initiation factor A1 (eIF4A1) mRNA was used as an internal control because the levels of this transcript did not differ in leaf tissue of wild-type and *fatb-ko* plants.

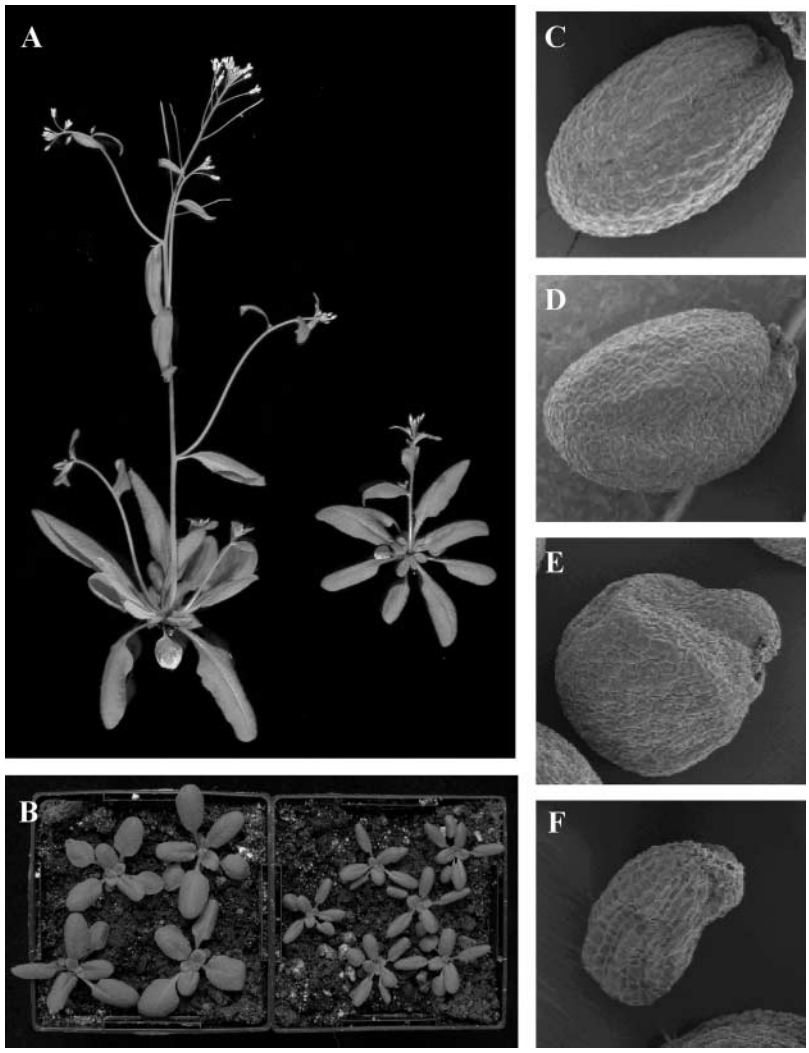

Figure 2. Growth and Morphology of Arabidopsis Wild-Type and *fatb-ko* Plants and Seeds.

(A) Four-week-old wild-type (left) and *fatb-ko* (right) plants.
(B) Two-week-old wild-type (left) and *fatb-ko* (right) plants.
(C) Wild-type Arabidopsis seed.
(D) to **(F)** Wild-type–like, intermediate deformed, and very deformed seeds from *fatb-ko* plants, respectively.

FATB Is Essential for Normal Seed Morphology and Germination

Germination of seeds produced by *fatb-ko* plants was reduced by ~50% on both soil and 1% sucrose (Table 2). Close examination of mature seeds produced by the *fatb-ko* mutant revealed a continuous range of deformity in seed morphology, with wild-type–like seeds at one extreme and very deformed seeds (approximate frequency of 20%) at the other (Figures 2C to 2F, Table 2). The germination rate of very deformed seeds was only 16%. These observations suggested that some stage of seed or embryo development may be substantially affected in the mutant. By analyzing developing siliques, it was not evident that deformed seeds were located in specific segments of that organ. Upon surface sterilization, some mutant seeds also lost the seed coat, suggesting that the structure of this tissue could be altered. Scanning electron microscopy analysis of the seed coat from mutant plants with wild-type–like or intermediate morphology did not show any obvious structural differences compared with wild-type seeds (Figures 2C to 2F). Many deformed seeds from the mutant displayed a shriveled seed coat (Figure 2F).

Fatty Acid Composition of *fatb-ko* Tissues

As indicated in Table 3, palmitate (16:0) in homozygous *fatb-ko* plants was reduced by 42% in leaves, by 56% in flowers, by

48% in roots, and by 56% in seeds compared with wild-type plants. Stearate (18:0) decreased by almost 50% in leaves and by 30% in seeds, with negligible changes in flowers and roots. The *fatb-ko* plants also showed an increase of 150 to 200% in oleate (18:1) and 40 to 60% in linoleate (18:2) in leaves, flowers, and roots. Linolenate (18:3) declined by 15 to 20% in leaves, flowers, and roots. Seed unsaturated fatty acids were less affected. Together, these results demonstrate the in vivo role of FATB as a major determinant of 16:0 in all of the tissues analyzed and also indicate that FATB contributes to the level of 18:0 in leaves and seeds. *fatb-ko* plants transformed with the wild-type FATB cDNA under the control of the CaMV35S constitutive promoter had a fatty acid composition very similar to that of wild-type plants, confirming that the FATB cDNA complemented the biochemical phenotype of the mutant (Table 3).

Fatty Acid Composition of Individual Leaf Glycerolipids

The fatty acid compositions of individual glycerolipids from homozygous *fatb-ko* and wild-type leaves are presented in Table 4. Palmitate reductions occurred mainly in extraplastidial lipids. Although phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol had ~50% reductions in 16:0 compared with the wild type, in phosphatidylcholine, the reduction was almost 80%. The palmitate levels in plastidial lipids were less affected, with a significant reduction (40%) only in sulfoquinovosyldiacylglycerol. All of the extraplastidial glycerolipids except phosphatidylinositol had reduced 18:0. Again, phosphatidylcholine was the most affected, with a 10-fold reduction in 18:0. The characteristic changes in unsaturated fatty acids shown in Table 3 for total leaf lipids—namely, increases in 18:1 and 18:2 and a decrease in 18:3—were most pronounced for the phospholipids and sulfoquinovosyldiacylglycerol. The data in Table 4 also indicate that despite the changes in fatty acid composition, there were no major differences in the relative proportions of leaf glycerolipids between wild-type and *fatb-ko* plants. Finally, the total amount of fatty acid methyl esters produced by the acid-catalyzed transmethylation of Arabidopsis leaves was the same for the wild type (11.5 μmol/g fresh weight) and *fatb-ko* (11.6 μmol/g fresh weight), indicating that *fatb-ko* did not affect net fatty acid accumulation per fresh weight.

Table 2. Bolting Times and Germination Rates of Wild-Type and *fatb-ko* Arabidopsis Plants

Variable	Wild Type (Ws)	*fatb-ko*
Bolting time[a] (%)		
4th week	92.3	27.6
≥5th week	7.7	72.4
Germination rate (%)		
Soil	96.7	45.6
1% sucrose	94.4	45.8
Deformed seeds in total seeds (%)	0.8	21.7
Germination on soil of deformed seeds (%)	NA[b]	16

[a] Bolting time under a 18-h-light/6-h-dark regimen at 22°C.
[b] NA, not applicable.

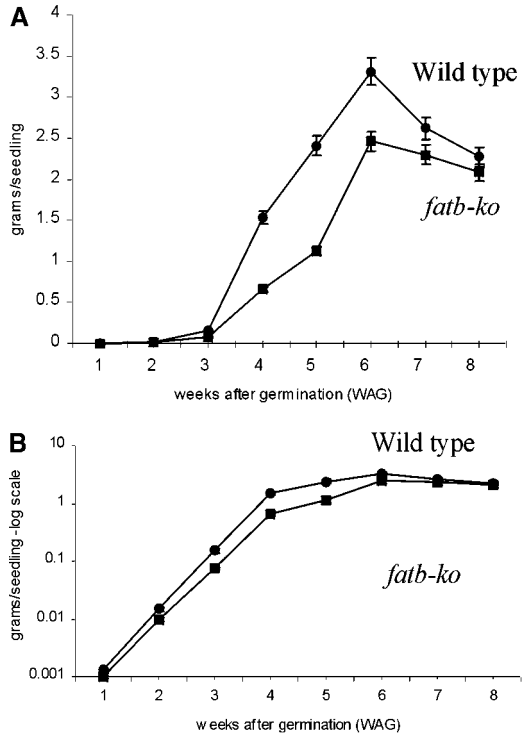

Figure 3. Growth Curves of Arabidopsis Wild-Type and *fatb-ko* Plants.

(A) The fresh weight of the aerial parts of Arabidopsis plants was measured on a weekly basis for a period of 8 weeks. Each time point is the average value of at least seven individual plants.
(B) Logarithm of the fresh weight versus time.

Acyl-ACP Thioesterase Activity

To determine if any compensatory changes occurred in acyl-ACP thioesterase activity, mutant and wild-type plants were assayed for the hydrolysis of 18:1-ACP and 16:0-ACP. The *FATA* gene product has an acyl specificity 18:1 >> 18:0 >> 16:0, whereas the *FATB* gene product has a specificity 16:0 > 18:1 > 18:0 (Doermann et al., 1995; Salas and Ohlrogge, 2002). The activity from the *FATA* gene product dominates acyl-ACP thioesterase activity measurements made with crude extracts. Oleoyl-ACP hydrolytic activity in leaf extracts of wild-type and mutant plants was similar (~125 pmol·min^{-1}·mg^{-1}), and hydrolytic activity on 16:0-ACP was close to background levels and therefore difficult to quantify. These results indicate that measurable acyl-ACP hydrolytic activity does not change in mutant leaf extracts compared with wild-type leaf extracts and, therefore, that endogenous levels of FATA activity are not upregulated in the mutant.

Total Palmitate Content in Arabidopsis Leaf Tissue

Most acid- or base-catalyzed transmethylation methods for fatty acid analysis efficiently convert *O*-acyl groups, such as

Table 3. Fatty Acid Composition of Wild-Type (Ws) and *fatb-ko* Arabidopsis Tissues

Tissue Type	16:0	16:1(9)	16:1(3)	16:3	18:0	18:1(9)	18:2	18:3	Other
Leaf									
Wild type	17.5	0.6	3.7	11.8	1.5	2.6	14.4	47.7	
fatb-ko	10.1	1.3	3.4	12.4	0.8	8.2	23.2	40.1	
35S-FATB[a]	15.5	0.6	4.0	11.9	1.3	4.6	16.8	45.0	
Flower									
Wild type	27.4	1.7		1.2	2.4	3.2	30.6	31.3	
fatb-ko	12.0	4.6		2.6	2.6	8.5	41.9	24.7	
Root									
Wild type	25.3	0.9			6.6	3.6	28.8	21.3	13.2
fatb-ko	13.4	1.1			6.0	9.2	42.5	17.4	10.1
Seed									
Wild type	8.3	0.3			3.5	13.0	27.5	18.6	28.6
fatb-ko	3.6	0.4			2.4	11.9	33.1	16.2	32.1

[a] *fatb-ko* plants transformed with CaMV35S FATB cDNA.

those found in glycerolipids, to fatty acid methyl esters. However, *N*-acyl groups, such as those found in sphingolipids, react very slowly using such methods. To evaluate the total palmitic and stearic acid contents (*O*- and *N*-linked) of Arabidopsis cells, strong alkaline hydrolysis was performed on total leaf tissue, on lipids extracted with multiple chloroform:methanol extractions, and on the solvent-extracted residue. The total 16:0 content in leaves of wild-type plants was 1.87 ± 0.02 μmol/g fresh weight, whereas leaves of *fatb-ko* plants contained 1.14 ± 0.03 μmol/g fresh weight. Thus, a 39% reduction in total 16:0 was observed in the mutant, similar to the 42% reduction of 16:0 in glycerolipids (Table 3). The amount of total stearic acid also was reduced by 50% in leaf tissue, from 0.16 ± 0.01 μmol/g fresh weight in wild-type plants to 0.075 ± 0.005 μmol/g fresh weight in the mutant. The analysis of extracted lipids and solvent-extracted residue indicated that almost all of the 16:0 and 18:0 in the cells was coextractable from leaf tissue, because the lipid fraction contained similar amounts of saturated fatty acids as the total tissue (data not shown). By contrast, the solvent-extracted residue, which may contain acylated proteins and other insoluble lipids, contained 3% of the total 16:0 and no detectable 18:0. Similar reductions of 16:0 and 18:0 were observed in all of the fractions analyzed, indicating that the absence of FATB reduced saturated fatty acid levels in both organic soluble and insoluble components.

Leaf Surface Wax Analysis

The very long chain fatty acids required for wax synthesis are produced by the elongation of 16- and 18-carbon saturated fatty acids (Post-Beittenmiller, 1996). To determine if the 40 to 50% reduction in saturated fatty acids influences total wax load or composition in the *fatb-ko* mutant, leaf and stem epicuticular waxes were analyzed. The results shown in Table 5 indicate that at 5 weeks, total wax load per fresh weight in leaves was reduced by 20% in the *fatb-ko* mutant. However, no novel components or substantial changes in the distribution of wax components were observed at this stage. A 20% reduction in leaf wax load was observed consistently at different stages of plant development (data not shown). Analysis of primary stems indicated a 50% reduction in wax load per fresh weight in the *fatb-ko* mutant compared with the wild type (1.1 ± 0.1 μmol/g fresh weight versus 2.2 ± 0.3 μmol/g fresh weight, respectively), again without changes in the distribution of wax components. These data indicate that the supply of saturated fatty acids by FATB is one factor that limits wax biosynthesis but that reduction of this supply does not result in the replacement of 16:0 by 18:1 or other precursors for surface wax structures. The role of FATB in supplying wax precursors was more evident in stems than in leaves, because the former tissue accumulated higher amounts of epicuticular waxes. Similar tissue-specific wax reductions have been observed in most of the Arabidopsis wax biosynthetic mutants (*eceriferum*), in which reductions in stem waxes were larger than those in leaf waxes (Rashotte et al., 2001).

Sphingoid Base Analysis

Because sphingoid base and sphingolipid synthesis are initiated by Ser palmitoyltransferase (Lynch, 1993), an analysis of sphingoid bases was conducted. Leaves of wild-type (0.54 ± 0.09 μmol/g fresh weight) and *fatb-ko* (0.50 ± 0.08 μmol/g fresh weight) plants did not differ significantly in the total amount of sphingoid bases. However, differences were observed in the relative abundance of the individual sphingoid bases (Table 6). Most significantly, trihydroxy-18:0 (t18:0) increased by almost fourfold in the total sphingoid bases of the *fatb-ko* mutant. The total sphingoid base composition was similar to that reported by Sperling et al. (1998): the most abundant sphingoid base in Arabidopsis leaf tissue was t18:1(8E), followed by t18:1(8Z). Monoglucosylceramide is considered one of the most abundant sphingolipids; therefore, the sphingoid base composition in leaf monoglucosylceramide was analyzed. The base composition in monoglucosylceramide of wild-type and mutant plants was indistinguishable and was similar to that reported by Imai et al. (2000) (Table 6). The difference in composition between total and monoglucosylceramide sphingoid base composition indicates that monoglucosylceramide is not the predominant sphingolipid in Arabidopsis leaves. As suggested by Imai et al. (2000), complex sphingolipids such as phosphoinositolceramides could be more abundant than monoglucosylceramide in Arabidopsis leaf tissue. Sphingoid bases in extracted lipids and solvent-extracted residue also were analyzed. The latter fraction can contain sphingoid bases from highly glycosylated phosphoinositolceramides and glycosylphosphatidylinositol moieties from glycosylphosphatidylinositol-anchored proteins. Again, no major changes in sphingoid base composition between wild-type and *fatb-ko* plants were observed except in t18:0 (Table 6).

fatb-ko act1 Double Mutant

The lipid analysis demonstrated that despite the absence of FATB in the plastids, mutant plants contained ~50% of the saturated fatty acids found in wild-type plants. What is the origin

Table 4. Fatty Acid Composition of Leaf Glycerolipids of Wild-Type (Ws) and *fatb-ko* Arabidopsis Plants

Glycerolipid	Percent of Total	16:0	16:1(3)	16:3	18:0	18:1(9)	18:1(11)	18:2	18:3
Phosphatidylcholine									
Wild type	11.2 ± 0.3	21.1			2.4	6.1		34.7	35.5
fatb-ko	13.6 ± 0.7	4.5			0.2	17.8	1.3	46.1	30.1
Phosphatidylethanolamine									
Wild type	10.0 ± 0.2	29.0			2.1	2.8		35.1	30.8
fatb-ko	7.9 ± 0.6	11.6			1.1	11.3	1.2	50.8	24.9
Phosphatidylserine									
Wild type	0.9 ± 0.1	30.8			6.8	2.9		27.1	32.3
fatb-ko	0.8 ± 0.1	17.2			2.9	10.2		44.3	25.2
Phosphatidylinositol									
Wild type	5.5 ± 0.1	40.0			2.0	2.2		25.3	30.3
fatb-ko	4.4 ± 0.1	21.2			1.8	9.2		36.9	29.3
Phosphatidylglycerol									
Wild type	12.0 ± 0.3	29.2	22.0		1.1	4.5		10.8	32.1
fatb-ko	12.6 ± 0.3	24.3	20.7			8.5	0.9	16.9	27.6
Sulfoquinovosyldiacylglycerol									
Wild type	4.3 ± 0.1	34.6			1.5	3.6	0.7	21.5	37.8
fatb-ko	4.2 ± 0.6	20.4			0.5	10.5	1.2	36.4	30.8
Digalactosyldiacylglycerol									
Wild type	20.2 ± 0.3	12.8		4.2	1.0	1.1		6.7	73.9
fatb-ko	19.4 ± 0.1	12.0		5.4	0.5	1.2	0.5	7.9	70.5
Monogalactosyldiacylglycerol									
Wild type	35.5 ± 0.1	1.6		38.1		0.8		3.0	53.4
fatb-ko	36.7 ± 0.2	1.3		40.3		1.6		3.2	50.7

Except where indicated, values shown are mol %.

of these remaining saturated fatty acids? Palmitate and stearate, as 16:0-ACP and 18:0-ACP, respectively, may be used directly by acyltransferases in the plastid for prokaryotic lipid synthesis (Somerville et al., 2000). To evaluate how much of the saturated fatty acids remaining in the *fatb-ko* plants derive from acyl group fluxes through plastidial acyltransferases, a cross between *fatb-ko* plants and the Arabidopsis *act1* (*ats1*) mutant was performed. The *act1* mutant has reduced plastidial glycerol-3-phosphate:acyl-ACP acyltransferase activity, the first step in the plastid pathway of glycerolipid biosynthesis. Although *act1* plants contain reduced amounts of 16:3 and increased amounts of 18:1, the growth of this mutant is normal (Kunst et al., 1988). However, the *fatb-ko act1* double mutant was severely impaired in growth compared with wild-type, *fatb-ko*, and *act1* plants (Figure 4). Leaf fatty acid analysis of the double mutant indicated that this tissue contained ~3 to 4 mol % 16:0, representing a 70% reduction compared with the wild type (Tables 3 and 7). The levels of 18:1 in leaves were increased in the double mutant compared with those in *fatb-ko*, whereas 18:2 and 18:3 levels remained almost unchanged (Tables 3 and 7). Interestingly, leaf levels of 18:0 in the double mutant were similar to those in *fatb-ko*, indicating no further decrease in overall 18:0 by the second mutation. Analysis of individual lipid classes from leaf tissue of the double mutant revealed that the C_{16} acyl compositions of extraplastidial glycerolipids and phosphatidylglycerol did not differ substantially from those in *fatb-ko* leaves (data not shown). Moreover, the relative abundance of each glycerolipid was similar. These results demonstrated that the second mutation (*act1*) affected primarily 16:0 accumulation in plastidial glycerolipids, with minor effects on extraplastidial lipids compared with *fatb-ko*.

In summary, double mutant plants blocked in both FATB and ACT1 were reduced further in saturated fatty acid content to levels ~30% of those in wild-type plants. These plants displayed a smaller size; thus, this more severe growth phenotype associated with a greater reduction in saturated fatty acids further demonstrates the essential role of saturated fatty acids in maintaining normal rates of plant growth.

DISCUSSION

Acyl-ACP thioesterases are responsible for the export from the plastid of fatty acids produced by the de novo fatty acid synthesis system. In this study, an Arabidopsis insertion mutant of the *FATB* gene was isolated, and we describe its effects on plant growth and on the production and utilization of saturated fatty acids. In a previous study, antisense of FATB in Arabidopsis using the CaMV35S promoter resulted in a substantial reduction of 16:0 only in flowers and seeds and minimal differences in leaves and roots and did not show any visual phenotype (Doermann et al., 2000). The lack of FATB antisense impact on leaves suggested either that FATB was not the major controller of 16:0 in leaves or that the reduction of FATB mRNA was not sufficient to reduce 16:0 levels in certain tissues. The characterization of an Arabidopsis *fatb* knockout mutant in this study demonstrates that the second of these interpretations is correct

Table 5. Major Components of Epicuticular Leaf Waxes from Wild-Type (Ws) and *fatb-ko* Arabidopsis Plants

Carbon Chain Length	Wild Type	*fatb-ko*
Alkanes		
27	1.4 ± 0.1	1.2 ± 0.05
29	21.9 ± 1.2	22.1 ± 0.1
30	1.2 ± 0.1	1.0 ± 0.03
31	39.4 ± 1.1	40.5 ± 0.5
32	0.9 ± 0.1	0.9 ± 0.03
33	11.7 ± 0.4	12.7 ± 0.03
Free fatty acids		
24	3.5 ± 0.2	3.3 ± 0.06
26	5.7 ± 0.2	5.3 ± 0.09
28	2.2 ± 0.4	1.8 ± 0.1
30	0.7 ± 0.1	0.5 ± 0.1
Primary alcohols		
26	3.1 ± 0.4	2.3 ± 0.3
28	3.6 ± 0.5	3.6 ± 0.3
30	2.2 ± 0.1	2.2 ± 0.02
32	2.3 ± 0.1	2.2 ± 0.02
Total (μg/g fresh weight)	104.1 ± 6.6	83.9 ± 4.5
Total (μmol/g fresh weight)	0.24 ± 0.01	0.19 ± 0.01

Except where indicated, values shown are mol %.

and that FATB is a major control point for saturated fatty acid fluxes in all tissues. The more extensive biochemical phenotype and the reduced growth rate and seed viability observed in the mutant compared with antisense plants suggest that the FATB enzyme or its mRNA may be in large excess and difficult to reduce by antisense to sufficient levels to produce a growth phenotype.

Seedling Growth and Seed Development

A large number of mutants with diverse changes in fatty acid composition have been isolated in Arabidopsis (Wallis and Browse, 2002). Most of these are not readily distinguishable from wild-type plants when grown at standard temperatures (15 to 25°C). One of the few examples in which a mutation affecting fatty acid composition has consequences for plant growth is the *fab2* mutant in Arabidopsis, in which increased levels of 18:0 in the membrane lipids result in dwarf plants (Lightner et al., 1994). However, this phenotype can be ameliorated partially by growing the mutant at high temperature, suggesting that membrane fluidity or a related physical property reduces the growth of the mutant at 22°C.

The *fatb* knockout is the first example of an Arabidopsis mutant with reduced levels of saturated fatty acids in which a reduction in vegetative growth occurred under standard growth conditions. Low temperature did not alleviate or high temperature exacerbate the slow-growth phenotype of *fatb-ko* plants, suggesting that effects other than changes in bulk membrane physical properties limited the growth of the mutant. Hence, in contrast to *fab2*, in which high 18:0 levels may disrupt the proper function of membranes, reduced saturate levels in *fatb-ko* plants may alter the biosynthesis and function of critical cell components. However, we cannot exclude the possibility that decreased amounts of saturates may be associated with more subtle changes in the physical properties of cellular membranes that could affect functions such as transport and vesicle formation. The *fatb-ko* mutant, with an ~50% reduction in palmitate, also contrasts with the *fab1* mutant, which is increased by ~50% in palmitate but displays normal growth (Wu et al., 1994). The *fatb* knockout mutant also is distinguished from other fatty acid mutants in its effect on seed development and germination (Figures 2C to 2F, Table 2). However, in contrast to the slow-growth phenotype, which occurred in all seedlings, the penetrance of the seed phenotype was incomplete. At this stage, it is not clear whether the seed defects are a consequence of alterations during specific seed developmental phases or an indirect effect caused by deficiencies in the supply of nutrients from maternal tissues.

Reduced Export of Palmitate in *fatb-ko* Plants and Other Sources of Palmitate and Stearate in the Cell

Palmitoyl-ACP pools in the plastid are subject to three major reactions: acyltransfer to glycerol, elongation to 18:0-ACP, and

Table 6. Sphingoid Base Content of Leaf Tissue from Wild-Type (Ws) and *fatb-ko* Arabidopsis Plants

Variable	t18:1(8E)	t18:1(8Z)	t18:0	d18:1(8E)	d18:1(8Z)	d18:2(4,8)
Total tissue						
Wild type	60	32	2	4	t	2
fatb-ko	57	32	8	2	1	1
Monoglucosylceramide						
Wild type	24	59	t	11	t	5
fatb-ko	25	59	t	10	2	5
Solvent-extracted lipids						
Wild type	62	33	2	2	1	t
fatb-ko	57	36	5	2	t	t
Solvent-extracted residue						
Wild type	85	7	3	t	3	1
fatb-ko	76	7	13	t	3	1

Values shown are mol %. Dihydroxy bases are as follows: 8-sphingenine (d18:1[8E or Z]) and 4,8-sphingadienine (d18:2[4E,EZ or 4E,8Z]); trihydroxy bases are as follows: 4-hydroxysphinganine (t18:0) and 4-hydroxy-8-sphingenine (t18:1[8E or 8Z]). t, trace.

Figure 4. Growth and Morphology of Arabidopsis Wild-Type, *fatb-ko*, *act1*, and *fatb-ko act1* Plants.

Four-week-old wild-type (Wassilewskija) (bottom right), *fatb-ko* (bottom left), *act1* (top right), and *fatb-ko act1* double mutant (top left) plants. The size of the *act1* mutants was similar to the size of wild-type Columbia plants (data not shown). The size of wild-type plants derived from the cross between *fatb-ko* (Wassilewskija) and *act1* (Columbia) was intermediate to the size of the parental ecotypes (data not shown).

hydrolysis by FATB. Mutations that block or reduce all three of these fates now are available. In the *fab1* mutant (Wu et al., 1994), reduction in 16:0-ACP elongation results in increased 16:0 in both prokaryotic and eukaryotic lipids, suggesting that flux into both of these pathways can be increased by the increased availability of 16:0-ACP. Moreover, in both *fatb-ko* and *fab1* leaves, there is an increase in 16:1(9) levels, suggesting that 16:0-ACP pools are increased within chloroplasts of these two mutants. By contrast, in the *act1* mutant, the loss of the acyltransferase pathway results in increased 16:0-ACP elongation to 18:0 rather than increased flux to the eukaryotic path via the FATB thioesterase (Kunst et al., 1989). Similarly, in *fatb-ko*, the reduction in flux via the thioesterase also primarily increased elongation to C_{18} rather than increased the flux of C_{16} into prokaryotic lipids. These contrasting responses suggest that the elongation rate of 16:0-ACP is regulated primarily by the availability of substrate but that the contributions of the FATB and acyltransferase reactions to 16:0 flux likely have additional levels of control.

Lipid analysis (Table 3) demonstrated that despite the homozygous *fatb-ko* insertion, mutant plants still produced ~50% of the palmitate found in wild-type plants. How and where is the remaining palmitate balance in a plant cell produced? Similar statements and questions can be made for stearate. Palmitate is both an intermediate and an end product of de novo fatty acid synthesis in the plastid. Palmitate, as 16:0-ACP, may be used directly by acyltransferases in the plastid for prokaryotic lipid synthesis, in particular by the lysophosphatidic acid *sn*-2 acyltransferase (Frentzen et al., 1983). Alternatively, after hydrolysis by acyl-ACP thioesterases, free fatty acids, including palmitic acid, may be exported from the plastid, a proposed mechanism now substantiated by in vivo labeling (Pollard and Ohlrogge, 1999). Finally, plant mitochondria have the capacity for de novo synthesis of fatty acids (Wada et al., 1997), and although it is considered a minor pathway, this organelle could partially compensate for low 16:0 levels in the *fatb* mutant. Could (1) the transfer of palmitate from prokaryotic lipids to eukaryotic lipids, (2) the FATA acyl-ACP thioesterase, and/or (3) mitochondrial fatty acid synthesis account for the remaining exported palmitate production?

The cross of *fatb-ko* and *act1* plants demonstrated that the prokaryotic pathway provides ~60% of the saturated fatty acids in leaves of *fatb-ko*. Approximately half of the saturates that are still produced in the double mutant can be attributed to

Table 7. Leaf Fatty Acid Composition of Wild-Type (Columbia), *act1*, and *fatb-ko act1* Double Mutant Arabidopsis Plants

Plant Type	16:0	16:1	16:1(3)	16:3	18:0	18:1(9)	18:2	18:3
Wild type	16.2	1.4	3.0	11.3	1.5	3.0	13.8	49.8
act1	14.3	1.1	3.1	0.4	1.6	11.9	24.1	43.1
fat b-ko act1	3.1	1.2	3.2	0.5	0.8	23.4	24.8	41.5

Values shown are mol %.

plastidial phosphatidylglycerol (produced with prokaryotic character by an unknown path) and to FATA activity. The Arabidopsis FATA-encoded thioesterase has a small but measurable in vitro activity toward 16:0- and 18:0-ACP (~2 and 16% of the activity toward 18:1-ACP, respectively), and our results suggest that these enzymes have in vivo hydrolytic activity toward 16:0- and 18:0-ACP, a conclusion also drawn by Nadev et al. (1992). The mutants studied here demonstrate that FATA, mitochondrial fatty acid synthesis, or other sources of 16:0 are minor contributors to leaf saturated fatty acid flux compared with FATB and the plastid acyltransferases.

As summarized in Figure 5, the results of this study allow a better estimate of the relative contributions of alternative pathways for saturated fatty acid supply in plants. In wild-type plants, the total C_{16} fatty acids incorporated into membrane glycerolipids is 33.3 mol % units, which includes 16:1(3)-*trans* in phosphatidylglycerol and 16:2 and 16:3 in monogalactosyldiacylglycerol. Approximately 23 units are used for prokaryotic lipid synthesis, assuming that all phosphatidylglycerol and monogalactosyldiacylglycerol are derived from this pathway and that the proportion of 16:0-containing digalactosyldiacylglycerol that is prokaryotic is one-third, based on its *sn*-2 distribution (Kunst et al., 1989). Assuming that half of the sulfoquinovosyldiacylglycerol is of prokaryotic origin, of the remaining 10.3 units that are exported from the plastid, ~2.5 units return to this organelle as sulfoquinovosyldiacylglycerol and digalactosyldiacylglycerol and the remaining 7.8 units are used for phospholipid synthesis (Figure 5). In the *fatb-ko* line, we do not know what proportion of the 16:0-containing digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol pools are now of prokaryotic origin, so our estimates are ranges. Approximately 2.6 to 4.2 units of palmitate (25 to 45% of the wild type) are exported and 2.6 units (33% of the wild type) are retained for phospholipid synthesis, whereas 0 to 1.6 units (0 to 65% of the wild type) are returned for plastid lipid synthesis. Palmitate used for prokaryotic lipid synthesis is barely affected by the mutation, increasing from 23 units to 23.6 to 25.6 units. In the *fatb-ko act1* double mutant, ~3.5 units of palmitate (35% of the wild type) are exported and 2.5 units (33% of the wild type) are retained for phospholipid synthesis, whereas 0 to 1 unit (0 to 40% the wild type) is returned to the plastid.

Partition of Palmitate and Stearate to Nonglycerolipid Products

Palmitate constitutes the primary saturated fatty acid exported from plastids and incorporated into membrane glycerolipids. In addition to glycerolipids, several other cellular components derived from the exported palmitate and/or stearate play essential structural and perhaps signaling roles for cell growth. First, sphingoid bases in plants are 18-carbon amino alcohols that are synthesized outside of the plastid from palmitoyl-CoA and Ser by the action of Ser palmitoyltransferases (Lynch, 1993). In addition, 16:0 or its hydroxylated derivatives can be *N*-linked to sphingoid bases to form ceramides and sphingolipids. Second, in epidermal cells, saturates (16:0 and 18:0) are precursors of the cutin/suberin monomers and wax components (Post-Beittenmiller, 1996). Third, myristoylation (14:0) and palmitoylation (16:0) of proteins are critical for the localization and regulation of protein activity (Yalovsky et al., 1999).

In wild-type leaves, the total 16:0 content is ~1.9 μmol/g fresh weight. Deducting the contribution from prokaryotic lipid synthesis, ~1.2 μmol/g fresh weight of 16:0 must be exported from the plastid to fuel glycerolipid synthesis. Sphingolipid base synthesis requires another 0.5 μmol/g fresh weight of palmitate export. In addition, there are significant levels of 16:0 and 2-hydroxy-16:0 *N*-acyl groups in sphingolipids, so the total flux of palmitate into sphingolipids actually is greater. Thus, sphingolipid synthesis may consume 30 to 40% of the total cytosolic palmitate pool. However, despite a reduction of ~50% in extraplastidial 16:0 in the *fatb* mutant, the total amount per fresh weight of sphingoid bases in leaf tissue was similar to that in the wild type. One interpretation of the constancy of sphingolipid production is that sphingoid base synthesis is maintained tightly at the expense of acyl composition changes in other glycerolipids. Furthermore, because sphingolipids are essential lipids for cell growth (Wells and Lester, 1983), the slow growth of *fatb-ko* plants could result from a slower supply of the critical 16:0 component to sphingolipids.

Although in leaf mesophyll cells, the major fraction of fatty acids is used for the biosynthesis of membrane glycerolipids, in epidermal cells, most newly produced fatty acids are directed toward the biosynthesis of cutin and epicuticular waxes. In Arabidopsis leaves, an epicuticular wax load of 0.2 μmol/g fresh weight represents only ~10% of the total leaf pool of palmitate plus stearate, but within the epidermal cells, the proportions are much higher. Analysis of leaf and stem epicuticular waxes in the *fatb-ko* mutant showed 20 and 50% reductions in total wax load, respectively, indicating that the FATB thioesterase is one source for the production of wax precursors in epidermal cells.

In leaf tissue of the *fatb* mutant, all extraplastidial phospholipids showed a reduction of ~50% in the relative 16:0 levels (Table 4), except for phosphatidylcholine, which showed a 78% reduction. The fact that phosphatidylcholine was most affected suggests that this phospholipid is a key pool in the delivery or partition of palmitate in the cell, for example, as an indirect donor of saturated groups to sphingolipid biosynthesis. In addition, the 10-fold reduction in the relative 18:0 levels also indicated that phosphatidylcholine may play a role in 18:0 partitioning. Phosphatidylcholine plays a major role in the flux of glycerol and fatty acids during membrane glycerolipid biosynthesis; therefore, an intriguing question is whether the larger changes in the fatty acid composition of phosphatidylcholine could affect its function and be responsible, at least in part, for the slower growth rate of the *fatb* knockout plants.

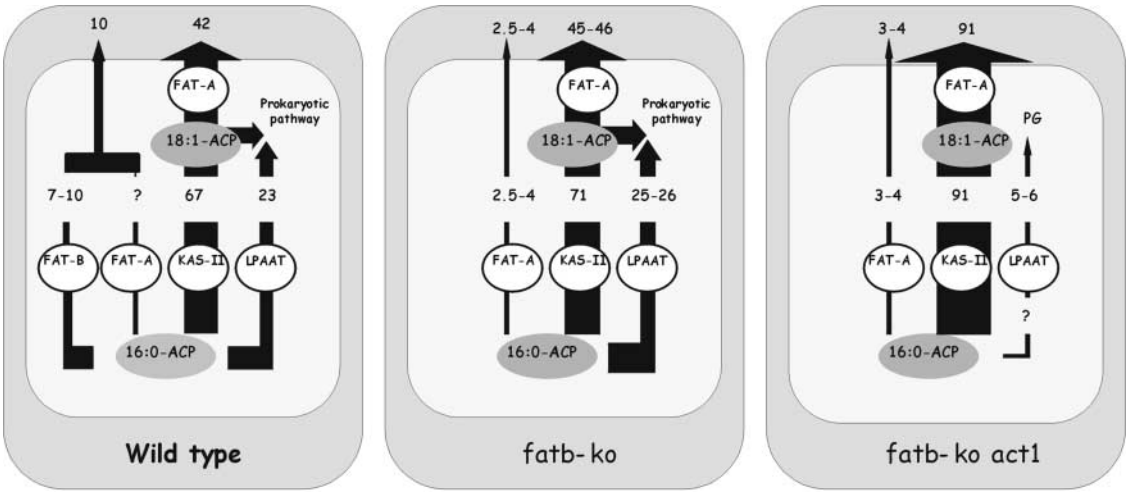

Figure 5. Simplified Scheme of Predicted Fluxes of C_{16} and C_{18} Fatty Acids in Membrane Leaf Glycerolipids of Arabidopsis Wild-Type, *fatb-ko*, and *fatb-ko act1* Mutant Plants.

The numbers in the figure represent average values of mol % units of C_{16} and C_{18} fatty acids accumulated in membrane glycerolipids. A range of values is given when alternative biosynthetic pathways were considered (see text for details). The average mol % units were calculated based on the mol % abundance of a particular fatty acid in a specific lipid, the relative abundance of the lipid, and the contribution of each pathway (prokaryotic and eukaryotic) to the synthesis of that lipid (Browse et al., 1986) (see Discussion for more details). Arrows represent the flux of acyl molecules through FATB and FATA, 3-ketoacyl-ACP synthase II (KAS-II), and lysophosphatidate *sn*-2 acyltransferase (LPAAT), which are either used for lipid biosynthesis in the plastid (prokaryotic pathway) or exported from the same organelle. ACT1 (glycerol-3-phosphate acyltransferase) transfers 18:1 to glycerol-3-phosphate in the prokaryotic pathway. Question marks indicate that (1) the actual flux of 16:0 through FATA in wild-type leaves is not known and an upper limit of 4 mol % units can be estimated based on *fatb-ko* data (however, flux through FATA in *fatb-ko* could be a compensatory mechanism) and (2) alternative pathways for the biosynthesis of phosphatidylglycerol in the *fatb-ko act1* double mutant can be considered.

Conclusions

The *fatb-ko* line shows a reduction in saturated fatty acids exported to the cytosol, a 17% reduction in the rate of growth, and altered seed morphology and germination. Although this study clearly demonstrates the requirement for the *FATB* gene and saturated fatty acids for normal rates of Arabidopsis growth and viable seed formation, the specific function(s) supplied by saturated fatty acids to sustain normal growth remain uncertain. Other than the reduction in saturated fatty acid content and a decrease in wax load, alterations in glycerolipids and sphingoid base composition were minor. Future work will focus on whether the growth rate of the mutant is linked to the biosynthesis of specific cellular components, subtle variations in membrane properties, or changes in fatty acid synthase or lipid turnover/degradation rates or a combination of these effects. The recent development of new isotope labeling techniques to investigate lipid synthesis and turnover/degradation will be critical to answer these questions (Pollard and Ohlrogge, 1999; Bao et al., 2000).

However, it is important to note that a lack of change in a critical component for growth may indicate its essential nature more than change does. Thus, the slower production of an essential lipid in the *fatb* mutant could slow growth to a balance point between the synthesis of that component and growth; therefore, no change in the level of the key component per plant weight would be expected in the mutant. For example, although we observed no overall reduction in sphingoid base accumulation, the essential nature of sphingolipid synthesis for growth in other systems (Wells and Lester, 1983) suggests that compositional changes might not be expected. Similarly, if rates of protein acylation, cutin biosynthesis, or the synthesis of other saturate-derived components are essential to growth, a biochemical phenotype in these components may not be observed. Thus, the isolation of suppressor mutants for the *fatb-ko* phenotype may also provide insights into the underlying mechanisms that connect the supply of saturated fatty acids to the biosynthesis and/or regulation of essential plant growth components.

METHODS

Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana* and *fatb-ko* mutant plants (ecotype Wassilewskija) were grown at 80 to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 22°C under an 18-h-light/6-h-dark photoperiod. Seeds were always stratified for 3 days at 4°C. Selection of T-DNA–tagged plants was performed by soaking the soil with 50 μg/mL commercial Basta (Finale; AgrEvo, Montvale, NJ). Surface-sterilized seeds of Arabidopsis were germinated on 0.8% (w/v) agar-solidified Murashige and Skoog (1962) medium supplemented with 1% (w/v) sucrose. For the experiments at different temperatures, the plants were grown for 2 weeks at 22°C (16 h of light) and then transferred to 16 and 36°C or kept at 22°C under identical lighting conditions.

Arabidopsis *fatb-ko act1* double mutants were generated using Arabidopsis *fatb-ko* plants as the pollen donor and Arabidopsis *act1* (Kunst et

al., 1988) as the pollen recipient. Seeds obtained from the crosses were sown on soil in the presence of 50 μg/mL Basta to select for heterozygous *act1*/+ *fatb-ko*/+ seedlings. F2 seedlings from F1 heterozygous plants were screened by gas chromatography (GC) and PCR to detect homozygous, heterozygous, and wild-type plants for *act1* and *fatb-ko* loci.

Mutant Isolation

A T-DNA (5.5-kb) insertion into the *FATB* gene was identified by screening pooled genomic DNA prepared from a T-DNA–tagged Arabidopsis (ecotype Wassilewskija) collection (Sussman et al., 2000). The gene-specific primers used for the screening of insertions into the *FATB* gene were 5′-CTCATATCCACATATATCTCTCTCTCACC-3′ (forward) and 5′-CAAGCAAGCAAGGTGGTAGTAGCAGATAT-3′ (reverse), and the T-DNA–specific primer matching the left end of the T-DNA (JL-202) was 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′. A *FATB* genomic fragment was labeled using random priming and used to detect specific PCR products by DNA gel blot hybridization (Sambrook et al., 1989). The T-DNA/*FATB* junctions from both ends of the insertion were amplified by PCR, subcloned, and sequenced from both ends to determine the insertion point for the 5.5-kb T-DNA.

Complementation Analysis

The binary vector pBINAR-Hyg (Becker, 1990) carrying the wild-type FATB cDNA was a gift from P. Doermann (Max Planck Institute, Golm, Germany). The vector was used to transform Arabidopsis *fatb* knockout plants by *Agrobacterium tumefaciens* vacuum infiltration (Bechtold et al., 1993). The transformation of homozygote plants for the *FATB* T-DNA insertion did not render transgenic seedlings; therefore, heterozygote *fatb-ko* plants were transformed. Transgenic seedlings were first selected on agar plates in the presence of 25 μg/mL hygromycin B. After 7 days, seedlings were transferred to soil presoaked with 50 μg/mL Basta to select against wild-type plants for the *fatb* knockout T-DNA insertion. DNA was extracted from hygromycin B–resistant (transgene T-DNA) and Basta-resistant (knockout T-DNA) plants using the Qiagen Plant DNA Extraction Kit (Chatsworth, CA). Homozygote *fatb-ko* plants were identified by PCR using the same primers used in the isolation of the *fatb-ko* mutant.

Real-Time PCR Quantification of mRNAs

Total RNA was prepared from leaf tissue of wild-type (Wassilewskija) and *fatb-ko* mutant Arabidopsis using the Qiagen Plant RNA Extraction Kit according to the instructions of the manufacturer. A 5-μg aliquot was used as a template for cDNA synthesis using the SuperScript First-Strand Synthesis system and oligo(dT) primers (Stratagene). Specific primers for the second and third exons of the *FATB* gene were designed with Primer Express software (PE-Applied Biosystems, Foster City, CA). The sequences of the forward and reverse primers were 5′-AATCATGTTAAGACTGCTGGATTGC-3′ and 5′-ATACCATTCTTTCCAGACTGACTGA-3′, respectively (Figure 1). Primers were verified by showing that the PCR product produced a single band after agarose gel electrophoresis. Real-time quantitative PCR analysis was performed according to the manufacturer's instructions (PE-Applied Biosystems). The reaction contained, in a final volume of 30 μL, 250 ng of reverse-transcribed total RNA, 1.5 μM of the forward and reverse primers, and 2× SYBR Green PCR Master Mix (PE-Applied Biosystems, Foster City, CA). All reactions were performed in triplicate. The relative amounts of all mRNAs were calculated using the comparative threshold cycle method as described in User Bulletin No. 2 from PE-Applied Biosystems. Arabidopsis eukaryotic protein synthesis initiation factor 4A1 (eIF4A1) mRNA was used as an internal control for variations in the amounts of mRNA. Levels of FATB mRNA were normalized to eIF4A1 mRNA levels and are presented as ratios between wild-type and *fatb-ko* mutant plants. The forward and reverse primers used to amplify eIF4A1 mRNA were 5′-CCAGAAGGCACACAGTTTGATGCA-3′ and 5′-AGACTGAGCCTGTTGAATCACATC-3′, respectively.

Scanning Electron Microscopy of Arabidopsis Seeds

Scanning electron microscopy images of mature Arabidopsis seeds were taken at the Center for Advanced Microscopy at Michigan State University with a JEOL JSM-6400V scanning electron microscope.

Fatty Acid Analysis of Glycerolipids from Different Tissues of Arabidopsis

Approximately 0.1 g fresh weight of tissue from 5-week-old Arabidopsis plants was heated at 90°C for 1 h in 0.3 mL of toluene and 1 mL of 10% (v/v) boron trichloride:methanol (Sigma) with heptadecanoic acid (17:0) as an internal standard. After acidification with aqueous acetic acid, fatty acid methyl esters were extracted two times with hexane and analyzed by GC with a flame ionization detector (GC-FID) on a DB-23 capillary column (J&W Scientific, Folsom, CA).

Individual Glycerolipid Analysis

One gram fresh weight of leaf tissue from 5-week-old Arabidopsis plants was ground in liquid nitrogen. Lipids were extracted in hexane-isopropanol, and glycerolipid classes were separated by thin layer chromatography on K6 silica plates (Whatman, Clifton, PA) impregnated with 0.15 M ammonium sulfate and activated for 3 h at 110°C (Kahn and Williams, 1977). The thin layer chromatography plates were developed three times with 91:30:8 (v/v/v) acetone:toluene:water, and lipids were detected after spraying with 0.2% (w/v) 2′-7′-dichlorofluorescein:methanol and viewing under UV light. Standards were used to identify the different glycerolipid classes. Lipids were eluted from the silica with chloroform:methanol, and fatty acid methyl esters were prepared and analyzed as described above.

Leaf Epicuticular Waxes

Approximately 3 g fresh weight of leaf tissue from 5-week-old Arabidopsis plants was used for epicuticular wax analysis. The tissue was dipped in chloroform for 30 s and then the following internal standards were added: *n*-octacosane at 20 μg/g fresh weight, and docosanoic acid and 1-tricosanol both at 10 μg/g fresh weight. All of the compounds were purchased from Sigma. After evaporation of the chloroform under nitrogen, the epicuticular waxes were silylated to convert free alcohols and carboxylic acids to their trimethylsilyl ethers and esters, respectively. The epicuticular waxes were heated at 110°C for 10 min in 100 μL of pyridine and 100 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Sigma). After cooling, the solvent was evaporated under nitrogen and the product was resuspended in 1:1 (v/v) heptane:toluene for GC analysis. GC conditions were as follows: an HP-5 capillary column (J&W Scientific; 30-m × 0.32-mm × 0.25-μm film thickness) with helium carrier gas at 2 mL/min was used; injection was in split mode; injector and FID detector temperatures were set to 360°C; and the oven temperature was programmed at 150°C for 3 min, followed by a 10°C/min ramp to 350°C, and then held for an additional 20 min at 350°C. GC–mass spectrometry analysis also was performed to identify components of the mixture.

Sphingoid Base Analysis

Approximately 1 g fresh weight of leaf tissue from 5-week-old Arabidopsis plants was heated at 110°C for 24 h with 4 mL of dioxane (Sigma)

plus 3.5 mL of 10% (w/v) aqueous $Ba(OH)_2$ (Sigma) (Sperling et al., 1998). D-*erythro*-Sphingosine (Matreya, Pleasant Gap, PA) at 25 μg/g fresh weight and heptadecanoic acid (Sigma) at 400 μg/g fresh weight were added as internal standards. After saponification, the sample was extracted with chloroform to obtain the sphingoid bases in the organic phase and fatty acids in the alkaline aqueous phase. Each phase was analyzed independently as described below.

The chloroform fraction containing the sphingoid bases was back-extracted with an equal volume of 0.4 M aqueous HCl. The acid aqueous phase (containing the protonated sphingoid bases) then was titrated with KOH to pH 10. The sphingoid bases were reextracted into chloroform and, after evaporation under nitrogen, resuspended in 1 mL of methanol. The sphingoid bases were oxidized to their corresponding aldehydes by stirring the sample with 100 μL of 0.2 M sodium periodate (Sigma) at room temperature for 1 h in the dark (Kojima et al., 1991). The aldehydes were recovered by hexane extraction and used directly for GC analysis. GC conditions were as follows: a HP-5 capillary column (30-m × 0.32-mm × 0.25-μm film thickness) with helium carrier gas at 2 mL/min was used; injection was in split mode; injector and FID detector temperatures were set to 250°C; and the oven temperature was programmed at 100°C for 3 min, followed by a 10°C/min ramp to 260°C, and then held for an additional 10 min at 260°C. GC–mass spectrometry analysis also was performed to identify components of the mixture.

The basic aqueous phase was acidified with HCl to pH < 4 and extracted twice with hexane to recover fatty acids. Fatty acids were transmethylated and analyzed by GC using the same protocol indicated above.

Monoglucosylceramide Analysis

Approximately 10 g of Arabidopsis leaf tissue was quenched with 50 mL of hot isopropanol and ground in a Polytron. The extract was filtered and the residue extracted with 25 mL of 2:1 (v/v) chloroform:methanol and refiltered. This residue was reextracted with 25 mL of 1:2 (v/v) chloroform:methanol and filtered again. All three filtrates were combined and evaporated to dryness on a rotary evaporator. Finally, the lipid fraction was dissolved in 5 mL of chloroform (lipid fraction in Table 5). The solvent-extracted residue was dried under vacuum (solvent-extracted residue in Table 5). The lipid fraction was subjected to a partial base transmethylation to convert most of the *O*-acyl glycerolipids to fatty acid methyl esters under conditions that leave the *N*-acyl groups intact. This was achieved by spinning the lipids (100 mg) in a vortex with 2 M KOH in methanol (0.6 mL) plus hexane (4 mL) for 2 min at room temperature. The reaction was quenched by adding 4 mL of 1 M aqueous acetic acid. The hexane phase was removed, and the acidified aqueous phase was extracted with 2:1 and then 1:2 (v/v) chloroform:methanol. The hexane and chloroform:methanol fractions were combined and evaporated to dryness under nitrogen. Lipids were analyzed by thin layer chromatography as described above for individual glycerolipid analysis. A band that comigrated with β-D-glucosyl ceramide standard (Matreya) was eluted from the silica with 2:1 and 1:2 (v/v) chloroform:methanol. The solvent was dried under nitrogen, and the monoglucosylceramide sample was cleaved by alkaline hydrolysis in 1 mL of dioxane (Sigma) and 1 mL of 10% (w/v) aqueous $Ba(OH)_2$ (Sigma) for 24 h at 110°C. Sphingoid bases were recovered and their aldehyde derivatives analyzed as indicated above for total sphingoid base analysis.

Acyl-ACP Thioesterase Activities

Assays for 16:0-ACP and 18:1-ACP hydrolysis were performed according to Eccleston and Ohlrogge (1998).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The accession numbers for the Arabidopsis proteins shown in Figure 5 and described in the text are as follows: acyl-ACP thioesterase B (FATB), At1g08510; acyl-ACP thioesterase A (FATA), At3g25110 and At4g13050; glycerol-3-phosphate acyltransferase (ACT1), At1g32200; 3-ketoacyl-ACP-synthase II (KAS-II), At1g74960; lysophosphatidate *sn*-2 acyltransferase (LPAAT), At4g30580; and eIF4A1, At3g13920.

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