Modulating seed β-ketoacyl-acyl carrier protein synthase II level converts the composition of a temperate seed oil to that of a palm-like tropical oil

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Edited by George H. Lorimer, University of Maryland, College Park, MD, and approved January 22, 2007 (received for review December 14, 2006)

β-Ketoacyl-acyl carrier protein (ACP) synthase II (KASII) elongates 16:0-ACP to 18:0-ACP in the plastid, where it competes with three other enzymes at the first major branch point in fatty acid biosynthesis. Despite its key metabolic location, the influence of KASII in determining seed oil composition remains unclear, in part because the biochemical consequences of the *fab1-1* mutation were unresolved. Thus, *fab1-1*, and a newly identified knockout allele, *fab1-2*, were analyzed in the context of the hypothesis that modulating KASII activity is sufficient to convert the composition of a temperate seed oil into that of a palm-like tropical oil. No homozygous *fab1-2* individuals were identified in progeny of self-fertilized heterozygous *fab1-2* plants, ≈1/4 of which aborted before the torpedo stage, suggesting that *fab1-2* represents a complete loss of function and results in lethality when homozygous. Consistent with this hypothesis, homozygous *fab1-2* plants were identified when a *fab1-1* transgene was introduced, demonstrating that *fab1-1* encodes an active KASII. Strong seed-specific hairpin-RNAi reductions in *FAB1* expression resulted in abortion of ≈1/4 of the embryos in an apparent phenocopy of *fab1-2* homozygosity. In less severe *FAB1* hairpin-RNAi individuals, embryos developed normally and exhibited a 1:2:1 segregation ratio for palmitate accumulation. Thus, early embryo development appears sensitive to elevated 16:0, whereas at later stages, up to 53% of 16:0, i.e., a 7-fold increase over wild-type levels, is tolerated. These results resolve the role of KASII in seed metabolism and demonstrate that modulation of *Arabidopsis* KASII levels is sufficient to convert its temperate oilseed composition to that of a palm-like tropical oil.

condensing enzyme | fatty acid biosynthesis | metabolic engineering | plant oil

Temperate crops such as canola, soybean, and sunflower contain predominantly unsaturated 18-carbon fatty acids in their seed oils, whereas tropical oils such as palm oil contain higher proportions (≈50%) of 16-carbon saturated fatty acids (1). Whereas oils with high contents of long-chain or very-long-chain polyunsaturated fatty acids are desirable for many purposes including human nutrition, oils with highly saturated 16-carbon-chain-length fatty acids, including palm oil, can provide the starting materials for many industrial applications. It is well documented that different membrane fatty acid compositions, i.e., the ratio of saturated to unsaturated fatty acids, play key roles in adaptation to ambient temperatures (2); however, we reasoned that this need not be the case for seed oils. Thus, we hypothesized that it should be possible to mimic a palm-like oil composition in a temperate crop by genetic manipulation of oilseed biosynthesis.

The two-carbon elongation steps in fatty acid biosynthesis (3) are catalyzed by a small family of β-ketoacyl-acyl-carrier protein (ACP) synthases, commonly referred to as condensing enzymes of the KAS family (4). Three distinct plastidial KAS activities with characteristic chain-length specificities have been described. KASIII condenses acetyl-CoA with malonyl-ACP to form 4:0-ACP, KASI is responsible for the elongation of 4:0-ACP to 16:0-ACP, and KASII mediates the elongation of 16:0-ACP to 18:0-ACP.

16:0-ACP represents the first major branch point in fatty acid biosynthesis because it is the substrate for two major activities, KASII and the FATB thioesterase that releases 16:0 from 16:0-ACP. In addition, the 18:0-ACP desaturase (5) and the lysophosphatidic acid acyltransferase (6) can use 16:0-ACP as a substrate (Fig. 1).

Acyl-ACP thioesterases hydrolyze acyl-ACPs to release free fatty acids and thereby influence the level of palmitic acid accumulation. For example, overexpression of the *Arabidopsis* thioesterase gene *FATB1* in seeds results in a nearly 4-fold increase in seed 16:0 content (7), whereas disruption of *FATB1* accordingly decreases 16:0 and total saturated fatty acid accumulation (8). Acyl-ACP thioesterases have been the focus of several strategies to change fatty acid composition in oilseed crops (9–11), but none has effected substantial increases in 16:0 levels similar to those of palm oil.

The consequences of altering KASII activity are less well defined than those of FATB, in part because of the lack of a characterized knockout mutant in the *FAB1* gene, which encodes KASII. An ethyl methanesulfonate-derived mutant *fab1*, herein referred to as *fab1-1*, was identified as having increased 16:0 accumulation in leaves and seeds (12) as a result of reduced KASII activity (13), which favors flux toward the competing FATB and export of 16:0 from the plastid. The *fab1-1* mutation results from a point mutation conferring a L337F substitution in the active site of KASII (13), but the biochemical consequences of the *fab1-1* mutation are unclear because KASII forms insoluble aggregates when expressed in *Escherichia coli* (13). Although an equivalent mutation in an *E. coli* KASI enzyme resulted in an enzyme with no detectable activity (13), several lines of evidence suggest that *fab1-1* might encode a functional KASII enzyme: (*i*) extracts from *fab1-1* mutant lines retain 60% of WT KASII activity *in vitro* (14), and (*ii*) the isolation of only a single *fab1-1* allele, despite several independent mutant screens, suggests that complete loss-of-function mutations might not be viable. We hypothesized that by decreasing the level of KASII, elongation of 16-carbon fatty acids could be minimized. This approach required us to resolve the role of KASII in seed fatty acid metabolism and explore the plasticity of seed metabolism with regard to changes in KASII activity. A genetic approach involving the characterization of a T-DNA knockout

Author contributions: M.S.P. and H.T.N. contributed equally to this work; M.S.P., H.T.N., and J.S. designed research; M.S.P., H.T.N., I.H., and T.I. performed research; M.S.P., H.T.N., I.H., and J.S. analyzed data; and M.S.P., H.T.N., and J.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: ACP, acyl-carrier protein; T-DNA, portion of the Ti (tumor-inducing) plasmid that is transferred to plant cells.

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 www.pnas.org/cgi/doi/10.1073/pnas.0611141104

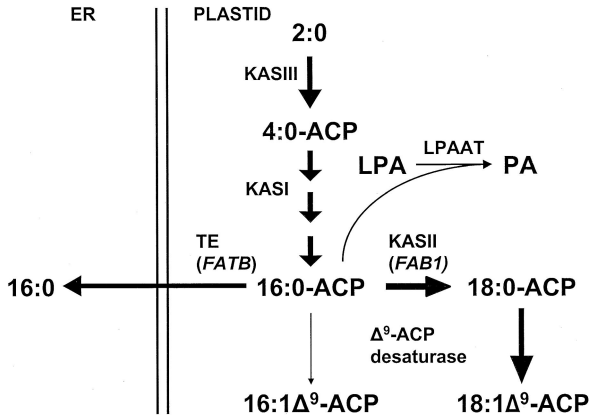

Fig. 1. Schematic diagram of fatty acid biosynthesis in *Arabidopsis*. Arrow widths reflect major and minor fluxes (29).

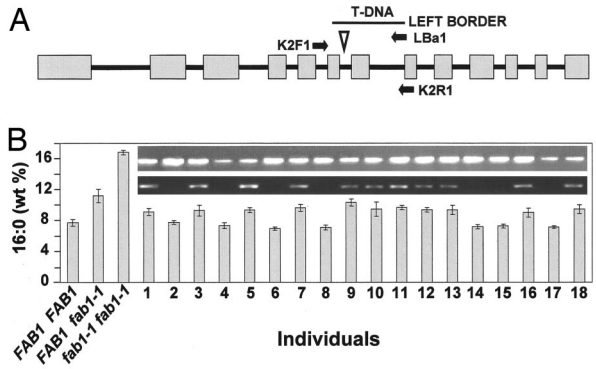

Fig. 2. Individuals homozygous for a T-DNA-disrupted allele of *FAB1* are not viable. (*A*) Genomic structure of the *fab1-2* allele of *FAB1*; gray boxes and black lines represent exons and introns, respectively. The open triangle depicts the position of the *fab1-2* T-DNA insertion. Horizontal arrows indicate the position of primers K2F, K2R1, and LBa1, which are used in combinations to amplify the *FAB1* or *fab1-2* alleles by using PCR. (*B*) Histogram depicting levels of 16:0 in seed pools from individuals of a population segregating for *fab1-2*. In comparison, 16:0 levels are presented for *FAB1* homozygotes, *FAB1/fab1-1* heterozygotes, and *fab1-1* homozygotes. (*Inset*) Ethidium bromide-stained gel indicating individual genotypes within a population segregating for *fab1-2*. Primers K2F1 and K2R1 were used to detect the presence of the wild-type *FAB1* allele (*Upper*), whereas primers K2F1 and TDNA left border-specific LBa1 were used in separate reactions to amplify the *fab1-2* allele (*Lower*).

and hairpin RNA-mediated reduction (RNAi) of KASII was used to test whether further reductions in KASII activity relative to those seen in the *fab1-1* mutant might be possible and potentially lead to further elevations in 16:0 content.

The results demonstrate that (*i*) individuals homozygous for a complete loss-of-function mutation in the *Arabidopsis FAB1* gene encoding KASII are not viable, (*ii*) the *fab1-1* mutant retains sufficient activity to restore viability to *Arabidopsis* lines homozygous for complete loss-of-function *FAB1* alleles, and (*iii*) seed-specific RNAi of *FAB1* can dramatically increase palmitic acid accumulation. These results support the hypothesis that manipulation of KASII activity is a potent regulator of seed oil composition and sufficient to convert the seed oil composition of a temperate plant to that of a palm-like oil.

Results

Arabidopsis seeds homozygous for the *fab1-1* allele accumulate ≈17% 16:0, approximately double that found in WT plants, demonstrating that KASII activity plays an important role in determining 16:0 levels in plants. However, physiological data previously reported showed that extracts from *fab1-1* plants retain ≈60% of their KASII activity (14). We therefore sought to resolve whether the *fab1-1* allele encodes a KASII with any residual activity. Direct biochemical analysis of recombinant *fab1-1*-encoded KASII enzyme produced in *E. coli* was precluded because the recombinant protein accumulates as inactive inclusion bodies (13). Thus, we initiated a genetic approach involving the identification and characterization of a T-DNA-disrupted *FAB1* allele.

Identification/Characterization of *fab1-2*: A T-DNA-Disrupted Allele of *FAB1*. A search of the Salk Institute Genomic Analysis Laboratory (SIGnAL) collection for T-DNA insertions in the *FAB1* gene locus (At1g74960) identified SALK_018337 as having an insertion in the sixth intron of *FAB1* (Fig. 2*A*). A small, segregating population was grown for the purpose of identifying an individual homozygous for this allele, and seeds from individual plants were subjected to GC-flame ionization detector analysis. With the assumption that this T-DNA-disrupted allele, hereafter referred to as *fab1-2*, would be at least as impaired as the original mutant allele *fab1-1*, approximately 1/4 of the individuals were expected to produce seeds with a 16:0 content equal to or greater than the ≈17% exhibited by *fab1-1* homozygotes. Surprisingly, none of the seed pools from 18 lines examined contained 16:0 levels as high as *fab1-1* homozygotes (Fig. 2*B*). Rather, seed pools from individual plants could roughly be categorized as containing seeds with WT levels (≈8% 16:0) or seeds with 16:0 levels elevated to levels found in *FAB1/fab1-1* heterozygotes (9–12% 16:0) (15). The observation of no seed pools with higher levels of 16:0 suggested either that the *fab1-2* allele was less impaired than the *fab1-1* allele, or more likely, given that there is a disruption of the *FAB1* gene, that homozygous *fab1-2* individuals were not viable. Under the latter scenario, seed pools with 16:0 levels resembling *FAB1/fab1-1* heterozygotes (15) would represent a 1:2 mixture of *FAB1/FAB1*:*FAB1/fab1-2* seeds. The observation that 7/18 of the seeds had WT levels of 16:0, whereas 11/18 had heterozygous-like levels, is consistent with the segregation expected for a T_2 generation of nonviable *fab1-2* homozygotes. The 18 individuals investigated showed a perfect correlation of genotype with phenotype; i.e., the seven lines identified with WT 16:0 levels were *FAB1* homozygotes, and the 11 lines containing elevated 16:0 levels were *FAB1/fab1-2* heterozygotes (Fig. 2*B*). Of 52 individuals analyzed from a population segregating for *fab1-2*, 19 were homozygous for FAB1 (one in three, $P < 0.05$), 33 were heterozygous, and none was homozygous for *fab1-2*. These results suggest that homozygosity for *fab1-2* is lethal, and that *fab1-2* is likely a bona fide KASII knockout. Because *fab1-1* homozygotes are viable and visibly indistinguishable from WT, a corollary of this hypothesis is that the original *fab1-1* allele encodes a functional, albeit compromised, KASII.

Because WT seeds and seeds segregating for *fab1-2* showed close to 100% germination, it is likely that *fab1-2* homozygosity results in embryo-lethality. To test this hypothesis, we dissected developing siliques from parental WT (i.e., *FAB1/FAB1*) and *fab1-1/fab1-1* homozygotes, and compared these to siliques from self-fertilized *FAB1/fab1-2* heterozygous plants (Fig. 3). Siliques from WT and *fab1-1* homozygotes contained uniform green developing ovules, whereas siliques on selfed *fab1-2* heterozygote plants contained 27/135 shrunken brown-colored ovules (one in four, $P < 0.05$; Fig. 3*C*). To determine whether these aborted ovules comprised the missing homozygous *fab1-2* individuals, we attempted to genotype them by using PCR. However, no discernable embryo could be identified in the aborted seeds when corresponding viable seeds contained torpedo stage embryos, implying abortion occurs before this stage.

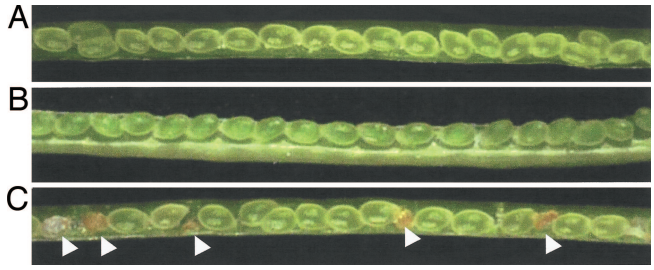

Fig. 3. Siliques containing seeds segregating for *fab1-2* contain aborted ovules. (*A*) Silique of parental genotype *FAB/FAB1* contain no aborted seeds. (*B*) Silique of parental genotype *fab1-1/fab1-1* contain no aborted seeds. (*C*) Siliques of *FAB1/fab1-2* heterozygotes contain several aborted seeds, as indicated by arrowheads.

***fab1-1* Encodes an Active KASII That Rescues Individuals Homozygous for *fab1-2*.** If, as our data suggests, *fab1-1* encoded a functional KASII enzyme, we reasoned that a transgenic copy of *fab1-1* might rescue homozygous *fab1-2* individuals. To test this hypothesis, a 5.5-kb genomic fragment spanning the *fab1-1* sequence (including ≈2 kbp of sequence upstream of the start codon) was transformed into plants segregating for the *fab1-2* allele. We identified 26 independent T_1 *fab1-1*-containing transformants by PCR-based screening. These 26 plants were subjected to PCR genotyping to distinguish between the *FAB1* and *fab1-2* alleles (Figs. 2*A* and 4*A*). Surprisingly, PCR using a *fab1-2* specific primer amplified fragments in only three of the 26 lines (Fig. 4*B Lower*), most likely due to the presence of *FAB1* homozygotes along with *FAB1/fab1-2* heterozygotes among the plants that were transformed. One of the three lines (line 3) was homozygous for *fab1-2* as deduced from the inability to amplify the *FAB1* allele from it and its T_2 progeny, all of which were thus homozygous for *fab1-2*. The two additional independent transformants (lines 23 and 26) also yielded progeny homozygous for *fab1-2* (data not shown). Because KASII activity in *Arabidopsis* is dose-dependent (12), seeds from *fab1-2* homozygotes containing a single copy of the genomic *fab1-1* fragment should have less KASII activity than a *fab1-1* homozygote, and therefore accumulate 16:0 above the ≈17% level characteristic of a *fab1-1* homozygote. However, all three lines displayed lower 16:0 levels than the *fab1-1* homozygote, suggesting that these lines either contain multiple copies of the *fab1-1* transgene, or the presence of the phaseolin promoter 5′ to the *fab1-1* native promoter resulted in overexpression of the transgenic *fab1-1* in seeds.

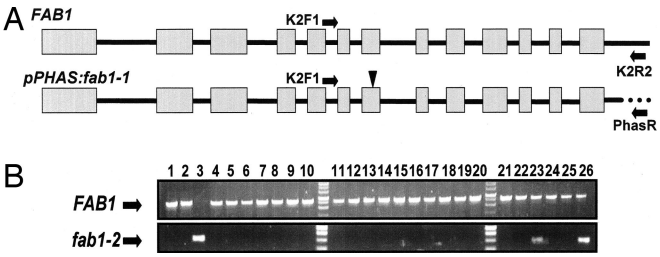

Fig. 4. Rescue of *fab1-2* homozygotes with a transgenic copy of *fab1-1*. (*A*) Schematic outlining the PCR strategy to distinguish between the *FAB1* and transgenic *fab1-1* alleles. The closed triangle indicates the position of the L337F point mutation of *fab1-1*. The dotted line represents phaseolin regulatory sequences of the transformation vector. *FAB1* and *fab1-1* alleles were distinguished based on the flanking sequences. (*B*) Ethidium bromide-stained gel indicating individual genotypes of a population segregating for *fab1-2*. Primers K2F1 and K2R2 were used to detect the presence of the wild-type *FAB1* allele (*Upper*), whereas primers K2F and TDNA left border-specific LBa1 were used in separate reactions to amplify the *fab1-2* allele (*Lower*) (for schematic see Fig. 2*A*).

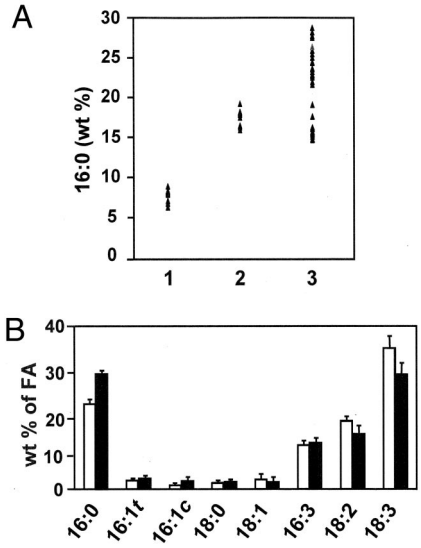

Fig. 5. Fatty acid accumulation in various genotypes at the *FAB1* locus. (*A*) 16:0 accumulation in individual seeds from plants homozygous for *FAB1* (column 1), homozygous for *fab1-1* (column 2), or heterozygous for *fab1-1/fab1-2* (column 3). $n = 10$ (for columns 1 and 2); and 20 (for column 3). (*B*) Leaf fatty acid composition of *fab1-1* homozygotes (open bars) and *fab1-1/fab1-2* heterozygotes (filled bars).

Consistent with the latter possibility, leaves of the line 3 plants had elevated 16:0 levels (23%) relative to WT (17%). Regardless, the identification of three independent lines in which embryo viability was restored by the introduction of *fab1-1* unambiguously demonstrates (*i*) that *fab1-1* encodes an active KASII enzyme, and (*ii*) that loss of viability associated with *fab1-2* results from disruption of *FAB1* rather than an unrelated linked mutation. To directly test whether a single copy of *fab1-1* is sufficient to maintain viability and, if so, to quantitate the resulting 16:0 phenotype, we created a *fab1-1/fab1-2* heterozygote by the genetic cross described below.

***fab1-1/fab1-2* Heterozygotes Have Lower KASII Activity than *fab1-1* Homozygotes.** A *fab1-1/fab1-2* heterozygote was created by fertilizing a *fab1-1* homozygote with pollen from a *FAB1/fab1-2* individual and its genotype was confirmed by PCR. The fatty acid contents of individual progeny of the *fab1-1/fab1-2* heterozygote are shown in Fig. 5*A* along with seeds from *fab1-1* and *FAB1* homozygotes for comparison. *FAB1* and *fab1-1* homozygotes contained ≈8.5 and ≈17.5% 16:0, respectively. Seeds produced by a *fab1-1/fab1-2* heterozygote, however, comprised two distinct classes with respect to 16:0 levels. Twelve of the seeds (one of three, $P < 0.05$) comprised a lower mean 16:0 of 16.1%, which is statistically indistinguishable from the *fab1-1* homozygote ($P < 0.05$). The other group (20/32) had a mean 16:0 level of 24.2%, significantly higher than the *fab1-1* homozygote level ($P < 0.01$), attributable to *fab1-1/fab1-2* heterozygous seed. The data are consistent with *fab1-1/fab1-2* heterozygotes having less KASII activity than homozygous *fab1-1* siblings. This experiment demonstrates that a single copy of *fab1-1* is sufficient for viability and that KASII activity can be decreased below the levels found in the *fab1-1* homozygote, resulting in increased 16:0 accumulation. The phenotypic consequences of the *fab1-1/fab1-2* genotype in leaves mirror those seen in seeds (Fig. 5).

Reduction of KASII Activity by Using Hairpin RNAi. According to data presented above, the KASII variant encoded by *fab1-1* is only mildly impaired in its activity. Therefore, it should be possible to decrease KASII levels beyond those seen in the homozygous

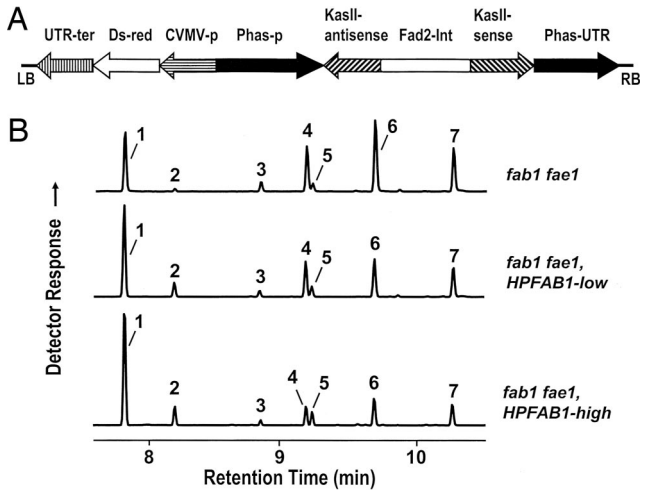

Fig. 6. Post-transcriptional gene silencing of *fab1-1* increases palmitic acid accumulation. (*A*) Schematic diagram of the pPHAS:*HPFAB1* construct under the control of the phaseolin promoter, (left border, LB; right border, RB). (*B*) Gas chromatograms of fatty acid methyl esters from single seed analysis: *fab1-1 fae1* double mutant seed; *fab1-1 fae1* T_1 seed expressing *HPFAB1* showing moderate increase in 16:0 accumulation (*HPFAB1*-low); and *fab-1-1 fae1* T_1 seed expressing *HPFAB1* showing strong increase in 16:0 accumulation (*HPFAB1*-high). Methyl esters are labeled: 16:0 (peak 1), 16:1 (peak 2), 18:0 (peak 3), 18:1Δ^9 (peak 4), 18:1Δ^{11} (peak 5), 18:2 (peak 6), and 18:3 (peak 7).

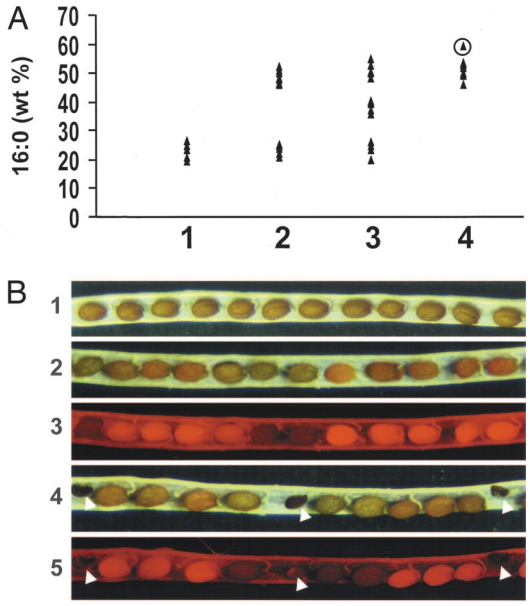

Fig. 7. *HPFAB1*-expressing T_2 lines that accumulate high levels of palmitic acid contain aborted ovules in the T_3 generation. (*A*) 16:0 accumulation in seeds of parental *fab1-1 fae1* background (column 1) or T_2 seeds of the *fab1-1 fae1* background expressing *HPFAB1* that results in either high (column 2) or low levels (column 3) of 16:0 as indicated. T_3 seeds from an *HPFAB1*-low line showing a single class of 16:0 phenotype (column 4); circled data point in (column 4) indicates an aberrant shrunken seed. (*B*) Silique of parental *fab1-1 fae1* background under white light (image 1). Silique of *HPFAB1*-low individuals segregating for *HPFAB1* under white light (image 2). Image 3 represents the silique depicted in image 2 visualized by using green light and red camera filters so as to detect DsRed fluorescence. Image 4 represents the silique of *HPFAB1*-high individuals segregating for *HPFAB1* under white light. Image 5 represents the silique depicted in image 4 visualized by using green light and red camera filters so as to detect DsRed fluorescence.

fab1-1 line and achieve corresponding increases in 16:0 accumulation. To test this hypothesis, we used intron-spliced hairpin RNAi (16) (Fig. 6*A*). pPHAS:*HPFAB1* was transformed into the *fab1-1 fae1* double mutant background, impaired in both plastidial and cytoplasmic 16- and 18-carbon elongation respectively; *fab1-1 fae1* has the highest seed 16:0 content (≈24%) described for a nontransgenic *Arabidopsis* genotype (Figs. 6*B* and 7) (15, 17). RNAi lines containing single loci of insertion were identified on the basis of a 3:1 segregation ratio of the visible DsRed marker in T_2 seed pools. The fatty acid compositions of individual T_2 seeds segregating for *HPFAB1* were determined (Figs. 6*B* and 7*A*). The 16:0 composition of *fab1-1 fae1* seeds transformed with *HPFAB1* showed some variation. Assuming that the T_1 individuals were hemizygous for *HPFAB1*, segregation should result in a 1:2:1 ratio of 16:0 levels. Interestingly, three classes of 16:0 accumulation were apparent in a fraction of the *HPFAB1* lines designated "*HPFAB1*-low" ($P < 0.05$), but only two classes were seen in other lines designated "*HPFAB1*-high" ($P < 0.05$). Segregation analysis indicated that progeny of *HPFAB1*-low lines hemizygous for *HPFAB1* contained, in addition to seeds hemizygous for *HPFAB1*, seeds either lacking or homozygous for *HPFAB1*; in contrast, the progeny of *HPFAB1*-high lines lacked seeds homozygous for *HPFAB1*.

The lack of individuals homozygous for *HPFAB1* in progeny of *HPFAB1*-high lines mirrors the absence of *fab1-2* homozygotes in the progeny of self-fertilized *FAB1*/*fab1-2* plants in which homozygous seeds failed to develop. We therefore examined siliques from hemizygous *HPFAB1*-high and *HPFAB1*-low lines for the presence of aborted seeds. Cosegregation of *HPFAB1* with the DsRed fluorescent marker allowed us to identify seeds lacking the *HPFAB1* transgene confirming that the chosen siliques contained segregating seeds. As expected, seeds from hemizygous *HPFAB1*-low plants contained no aborted ovules, were visually indistinguishable from *fab1-1 fae1* homozygous seeds, and showed the expected 1:2:1 segregation ratio ($P < 0.05$) (Fig. 7*B*). In contrast, hemizygous *HPFAB1*-high lines produced ≈1/4 aborted ovules and exhibited a 1:2 ratio ($P < 0.05$) (Fig. 7*B*). At ≈53% of the total fatty acids, the levels of 16:0 present in presumably heterozygous *HPFAB1*-high seeds were very similar to those of homozygous *HPFAB1*-low seeds. The genotype of a representative homozygous *HPFAB1*-low line was confirmed by analysis of T_3 seeds; all carried the DsRed marker and formed a single group equivalent in 16:0 to that of the heterozygous *HPFAB1*-high seeds (Fig. 7*A*). With the exception of one seed, the highest levels of 16:0 compatible with viability was ≈53%. The remaining homozygous seed was shrunken, deformed, and contained ≈59% of 16:0 when analyzed (Fig. 7*A*, see circled triangle). Table 1 shows the mean seed fatty acid compositions of WT, *fab1-1 fae1*, and *fab1-1 fae1 HPFAB1*-high

Table 1. Fatty acid composition of WT, *fab1–1 fae1*, and *fab1–1 fae1 HPFAB1*-high lines of *Arabidopsis*

	16:0	16:1	18:0	18:1Δ9	18:1Δ11	18:2	18:3	20:0	20:1	22:1	S/US
WT	9.2 ± 0.4	0.1 ± 0.06	3.6 ± 0.4	14.2 ± 2.2	1.7 ± 0.2	25.8 ± 0.9	19.5 ± 1.1	2.2 ± 0.3	21.3 ± 0.6	0.6 ± 0.3	0.18 ± 0.01
fab1-1 fae1	25 ± 1.4	1.6 ± 0.4	3.3 ± 0.6	19.1 ± 0.8	3.2 ± 0.1	26.9 ± 2.2	19.7 ± 1.8	0.4 ± 0.1	0	0.1 ± 0.1	0.41 ± 0.02
fab1-1 fae1-HPFAB1	51.9 ± 1.9	8.2 ± 0.9	2.1 ± 0.4	8.3 ± 0.9	5.5 ± 0.2	12.5 ± 1.5	10.7 ± 0.6	0.4 ± 0.3	0	0	1.2 ± 0.08

Means and standard deviations of three determinations are presented. S/US, saturated/unsaturated fatty acid ratio.

lines of *Arabidopsis* which show saturated/unsaturated fatty acid ratios of 0.18, 0.4, and 1.2, respectively.

Discussion

Despite its location at the first key branch point in fatty acid biosynthesis, the potential fates of 16:0-ACP and its metabolites have not been systematically explored. In this work, we focus on the role of KASII in determining the destiny of 16:0 by manipulating levels of the *FAB1* gene product. We report that modulation of KASII activity can result in substantial changes in seed storage fatty acid composition beyond those reported for the *fab1-1* mutant. In our strongest viable *HPFAB1* lines, 16:0 levels reach ≈53%, showing a >7-fold increase over levels seen in WT seeds and increasing the saturated/unsaturated ratio by 6.7-fold. These levels of 16:0 accumulation are also almost 3-fold increased over those of *Arabidopsis* seeds homozygous for *fab1-1*, the only mutant *FAB1* allele previously reported, which contains ≈16–18% of 16:0 in seed fatty acids (12). The 16:0 levels reached by our best *HPFAB1* lines are ≈3-fold increased over the ≈16% of 16:0 reported in a soybean line containing a mutation in *GmKAS IIA*, one of two isoforms of KASII expressed in seeds (18), and demonstrate that manipulation of KASII activity is sufficient to convert the seed oil composition of *Arabidopsis* from that of a temperate plant to that of a tropical palm-like oil. Thus, high seed palmitic acid accumulation can result from relatively minor changes in seed metabolism rather than by adaptation to differences in environmental conditions associated with temperate versus tropical growth.

Until the present study, the question as to whether *fab1-1* encoded an active KASII enzyme remained unresolved (13). Structural analysis previously revealed that the *fab1-1* point mutation (L337F) was adjacent to the enzyme's active site and that the corresponding mutation in *E. coli* KASI showed no detectable activity, suggesting that the KASII activity encoded by *fab1-1* might be severely impaired (13). The fact that homozygous *fab1-1* plants retained ≈60% of KASII activity relative to WT suggested that the mutant KASII enzyme encoded by *fab1-1* either retained activity, or another undefined gene product was able to extend 16-carbon fatty acids to the 18-carbon level (14). Ambiguity on the role of KASII persisted because purifying individual KAS activities to homogeneity from *Arabidopsis* is difficult, and heterologous expression of active plant KAS enzymes in *E. coli* has proven unsuccessful. Restoration of viability in plants homozygous for *fab1-2* upon the introduction of a 5-kb *Arabidopsis* genomic fragment comprising *fab1-1* (confirmed by analysis of *fab1-1*/*fab1-2* seed created by genetic crossing) provides direct evidence that *fab1-1* encodes a KASII enzyme that retains activity *in vivo* and that the T-DNA insertion in the *fab1-2* allele is responsible for the observed embryo-lethal phenotype, rather than a linked independent insertion.

Two lines of evidence indicate that KASII activity is necessary for successful embryo development. First, we were unable to identify viable plants homozygous for the *fab1-2* T-DNA disruption among the progeny of selfed *FAB1*/*fab1-2* heterozygotes, and inspection of siliques revealed ≈1/4 of the embryos to be aborted. Second, strong hairpin-RNAi of KASII phenocopied *fab1-2*, i.e., resulted in viable heterozygotes with highly elevated 16:0 levels (*HPFAB1*-high lines), that when allowed to self-fertilize, produced no viable seed homozygous for the hairpin RNAi transgene. Like siliques from self-fertilized FAB1/*fab1-2* heterozygotes, ≈1/4 of the progeny consisted of aborted embryos. In seeds from hairpin-RNAi experiments, the observed levels of 16:0 were increased to ≈53%, suggesting a lower limit of KASII activity below which seeds cannot develop. Lack of visible differences between WT and viable transgenic seeds within the same siliques suggests a sharp threshold for KASII-dependent development. Where viability is lost, i.e., in *fab1-2* homozygotes or with KASII expression strongly inhibited by hairpin RNAi, abortion occurs early in embryogenesis, i.e., before the torpedo stage. However, once this stage is passed, levels of 16:0 7-fold higher than those found in WT seeds are tolerated with no discernable effects on seed morphology or germination. The mechanism of embryo lethality remains to be defined; however, two possible explanations are envisaged: either a lack of membrane fluidity as a consequence of unusually high levels of palmitic acid, or a lack of 18-carbon fatty acids (or their metabolites). The former hypothesis could be tested by determining the ability of a 16:0-ACP desaturase enzyme (19) to rescue homozygous *fab1-2* individuals.

The present study also provides supporting evidence that $18{:}1\Delta^{11}$ is primarily synthesized via the prokaryotic pathway, i.e., through KASII, because $18{:}1\Delta^{11}$ levels did not increase in response to elevated $16{:}1\Delta^{9}$ in seeds expressing *HPFAB1* as they would be expected to do if $18{:}1\Delta^{11}$ elongation was mediated by one or more of the ≈20 cytoplasmic *Arabidopsis* β-ketoacyl-CoA synthase enzymes acting in the eukaryotic pathway (20–22).

WT *Arabidopsis* contains low levels of 16-carbon fatty acids in its seeds, and the data presented here are consistent with *FAB1* being the single locus responsible for most, if not all of the plastidial 16- to18-carbon elongation. However, there are many species of plants that contain high levels of 16-carbon seed fatty acids, e.g., *Doxantha*, but like *Arabidopsis* have predominantly 18-carbon fatty acids in their vegetative tissue. Differences such as these may reflect differential expression of distinct KASII isozymes in seeds and vegetative tissues, and consistent with this hypothesis, we have isolated three distinct KASII isoforms from *Doxantha* (M.S.P. and J.S., unpublished results). Results from expression of *HPFAB1* constructs in *Arabidopsis* demonstrate that reducing KASII expression in a tissue-specific fashion provides an effective strategy for increasing 16-carbon fatty acid accumulation. Furthermore, if decreases in *FAB1* dosage cause increases in 16:0 levels, it is possible that overexpression of *FAB1* will result in lower levels of 16:0 accumulation relative to WT individuals. Because 18:0 is more efficiently metabolized than 16:0, increasing KASII-dependent elongation of 16:0 may provide a useful strategy to limit the accumulation of saturated fatty acids in edible oils.

Materials and Methods

***Arabidopsis* Growth and Transformation.** *Arabidopsis* plants were grown in soil under continuous exposure to 300 microeinsteins of light in E7/2 controlled environment growth chambers (Conviron, Winnipeg, MB, Canada). Plants were transformed according to Clough and Bent (23) by using *Agrobacterium tumefaciens* strain GV3101. Individual T_1 seeds carrying the transgenes were identified by red fluorescence upon illumination with green LED light from an X5 flashlight (Inova, North Kingstown, RI) in conjunction with a Quantaray 25A red camera filter (Sigma Corp., Ronkonkoma, NY).

Genotyping. Genomic DNA was isolated from plants by using the Ultraclean Plant DNA Isolation Kit (Thomas Scientific, Swedesboro, NJ) for use in PCRs to genotype *Arabidopsis* individuals. Common forward primer K2F (5′-GATGTAATGCTCTGTGGTGG-3′) was used in PCR to identify the presence of each allele. Primer LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) was used as a reverse primer for the detection of *fab1-2*, K2R1 (5′-CCCATCACGTTTCCATCTCG-3′) and K2R2 (5′-GAAGAAGGCAAGTGGGGTGGTCAC-3′) were used as reverse primers for the identification of both *FAB1* and *fab1-1*, and PHASR (5′-GCATTTTAGTTCATACTGGTG-3′) was used as a reverse primer for the identification of transgenic *fab1-1*. *FAB1* (or *fab1-1*) homozygotes were identified by successfully amplifying *FAB1* (or *fab1-1*), but not *fab1-2* fragments from the genomic DNA samples. *fab1-2* homozygotes were identified by

the successful amplification of the *fab1-2*, but not *FAB1* (or endogenous *fab1-1*) fragments, from the genomic DNA samples.

Plasmid Construction. ***pGATE-DsRed-PHAS.*** pBBV-PHAS (17) sequences encoding phosphinothricin acetyl transferase were replaced with sequences encoding DsRed2 (Clontech, Mountain View, CA). In parallel, primers 5′-CATCCATCCATCCAGAGTAC-3′ (PHASF) and 5′-CTCGGAGGAGGCCATGGTTTCGATCCACTTTCTT-3′ were used in PCR to amplify a pBBV-PHAS fragment from the polylinker to the ATG of the gene encoding phosphinothricin acetyl transferase. These two fragments were then fused by PCR and the product was restricted with endonucleases BamHI and KpnI (New England Biolabs, Ipswich, MA) and ligated to a similarly restricted pBBV-PHAS fragment to produce pDsRed-PHAS. Gateway Vector Conversion cassette "B" (Invitrogen, Carlsbad, CA) was then cloned into the PmeI site of pDsRed-PHAS to create pGATE-DsRed-PHAS.

***pPHAS*:fab1-1.** Primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′ were used with *fab1-1* genomic DNA template in PCR to amplify a fragment spanning the *fab1-1* coding sequences, including >2 kb of 5′ regulatory sequences, which was cloned into pDONR221 by using Gateway BP Clonase II enzyme mix (Invitrogen). This *fab1-1* sequence was then transferred to pGATE-DsRed-PHAS by using Gateway LR Clonase II enzyme mix (Invitrogen) to create pPHAS:*fab1-1*.

***pPHAS*:HPFAB1.** Hairpin RNAi was used to decrease levels of KASII (16, 24). The sense, antisense and intron fragments were assembled in the plasmid vector pGEM-T-Easy (Promega) before cloning into the binary vector pDsRed-PHAS as a PacI-XhoI fragment. A 178-bp fragment from the 5′UTR of exon 1 of At1g74960 (*FAB1*), which is not homologous to any other *Arabidopsis* sequences encoding KASI or KASIII enzymes, was amplified from *Arabidopsis* genomic DNA by using oligonucleotides 5′-TTAATTAACGCATCGAAGCTCTCTGCACGC-3′ and 5′-GCTAGCGGCTTTGAGAAGAACCCAG-3′ and subsequently cloned into pGEM-T-Easy (Promega), such that the NheI site of the insert was adjacent to the PstI site of pGEM-T-Easy to create pGEM-T-Easy-HTM1. The first intron of *FAD2* (25) was then amplified by using oligonucleotides 5′-GCTAGCGTCAGCTCCATCTCCAGGTCC-3′ and 5′-GCTAGCGTTTCTGCAGAAAACCAAAAGC-3′, such that this fragment contained 17 bp of exon 1 and 4 bp of exon 2 to ensure the inclusion of the 5′ and 3′ splice site (26). This fragment was then cloned into the PstI/NheI site ofpGEM-T-Easy-HTM1 to create pGEM-T-Easy-HTM2. To complete the inverted repeat for the *FAB1* hairpin, the original 178 bp 5′ UTR fragment was amplified by using primers 5′-CTGCAGAAACCCGGGCATCGAAGCTCTCTGCACGC-3′ and 5′-GAGCTCCTCGAGGGCTTTGAGAAGAACCCAG-3′ and cloned into the SacI/PstI site of pGEM-T-Easy-HTM2 to yield pGEM-T-Easy-HTM3. The resulting *FAB1* hairpin sequence was excised from pGEM-T-Easy-HTM3 and inserted into pDs-Red-PHAS as a PacI/XhoI fragment to produce pPHAS:*HPFAB1* (Fig. 6*A*).

Fatty Acid Analysis. For single seed fatty acid analysis, fatty acid methyl esters were prepared by incubation with 0.2 M trimethylsulfonium hydroxide in methanol (26). Fatty acid methyl esters were analyzed by using either an HP6890 gas chromatograph-flame ionization detector (Agilent Technologies, Santa Clara, CA) or an HP5890 gas chromatograph-mass spectrometer (Hewlett–Packard, Palo Alto, CA) fitted with 60-m × 250-μm SP-2340 capillary columns (Supelco, Bellefonte, PA). The oven temperature was raised from 100°C to 240°C at a rate of 15°C min^{-1} with a flow rate of 1.1 ml min^{-1}. Mass spectrometry was performed with an HP5973 mass selective detector (Hewlett–Packard). Double-bond positions of monounsaturated fatty acid methyl esters were determined by using dimethyl disulfide derivatization (27).

Heterozygote Construction. Pollen from a heterozygous *FAB1*/*fab1-2* individual was used to fertilize a homozygous *fab1-1* individual. Because all progeny from this cross had to carry one *fab1-1* allele, *fab1-2*/*fab1-1* or *FAB1*/*fab1-1* heterozygotes were identified by the presence or absence of a *fab1-2* allele, respectively.

We dedicate this paper to the memory of Paul K. Stumpf. We thank Ed Whittle for advice and technical assistance and Minako Kaneda for technical assistance with imaging. This work was supported by the Office of Basic Energy Sciences of the U.S. Department of Energy, Dow, and Dow Agrosciences.

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